A product engineering approach to assess the reactivity of legume-based ingredients and its impact on the quality of processed foods such as cake

Svenja Krause

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Thèse de doctorat

NNT : 2021UPASB060

université PARIS-SACLAY

Evaluation de la réactivité d'ingrédients à base de légumineuses et de son impact sur la qualité des produits transformés de type cake dans une démarche d'ingénierie de produits

A product engineering approach to assess the reactivity of legume-based ingredients and its impact on the quality of processed foods such as cake

Thèse de doctorat de l'université Paris-Saclay

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Thèse présentée et soutenue à Paris-Saclay, le 16/12/2021, par

Svenja KRAUSE

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FOODENGINE

The present work was prepared within the framework of FOODENGINE, a ***MARIE CURIE-SKŁODOWSKA INNOVATIVE TRAINING NETWORK*** (ITN-ETN) funded by the European Commission under the Horizon 2020 Program. Over a period of three years, 13 early stage researchers (ESR) and I had the excellent opportunity to perform applied research with these common goals:

- Creation of low-waste/waste-free ways to transform raw materials from fruits, vegetables, legumes and algae and side streams thereof in high-quality food systems and/or multifunctional ingredients
- Monitoring, control and prediction of food quality as influenced by processing and storage by considering multiple quality attributes (physical stability, texture, flavor, digestion) as well as consumer preferences and acceptability

To achieve these objectives, FOODENGINE combined the interdisciplinary expertise and infrastructure of three highly-ranked European universities and research institutes (***KU LEUVEN***, Belgium; ***FOOD-UCPH***, Denmark; ***INRAE***, France), three large-turnover, multinational, R&D-based food companies (***DÖHLER***, Germany; ***CARGILL***, Belgium; ***UNILEVER***, the Netherlands), two medium-sized food companies (***GNT,*** Germany; ***GREENYARD***, Belgium) and an international market and consumer research company (***HAYSTACK***, Belgium) into a synergistic consortium to establish an international, interdisciplinary and intersectoral pioneering European food training program.

The presented project was prepared in close collaboration with the KU Leuven and Cargill in Belgium. During short-term stays as well as a six-month secondment (02.12.2020 – 06.06.2021), intersectoral research could be carried out, which complemented the experiments performed at my hosting organization INRAE.

Furthermore, intensive interactions between the ESR and the various academic and industrial partners was enabled through the organization of network-wide events that took place every six months at the various locations. These aimed at exchanging scientific and technological expertise, to give training in transferable skills and to stimulate interdisciplinary networking.

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COMMUNICATIONS & OUTREACH ACTIVITIES

PUBLICATIONS

Journal Article 1

Krause, S.; Keller, S.; Hashemi, A.; Descharles, N.; Bonazzi, C. & Rega, B. (**2021**). From flours to cakes: reactivity potential of pulse ingredients to generate volatile compounds impacting the quality of processed foods. *Food Chemistry*, 131379. https://doi.org/10.1016/j.foodchem.2021.131379

Conference Proceeding

Krause, S.; Hashemi, A.; Keller, S.; Bonazzi, C. & Rega, B. (**2021**). Odour compounds in thermo-mechanically processed food based on pulse ingredients. *In*: E. Guichard and J.L. Le Quéré (Eds), *Proceedings of the 16th Weurman Flavour Research Symposium*, https://doi.org/10.5281/zenodo.5513778

Journal Article 2

Krause, S.; Asamoah, E. A.; Moulin, G.; Bonazzi, C. & Rega, B. (**2021**). Lipid oxidation during the beating of cake batter containing yellow pea (*Pisum sativum* L.) flour. *LWT*, 112770. https://doi.org/10.1016/j.lwt.2021.112770

Journal Article 3

Krause, S.; Asamoah, E. A.; Huc-Mathis, D.; Moulin, G.; Jakobi, R.; Rega, B. & Bonazzi, C. (**2021**). Application of pea ingredients in baked products: Link between formulation, reactivity potential and physicochemical properties. *Accepted in Food Chemistry with modifications.*

Journal Article 4

Krause, S.; Debon, S.; Pälchen, K.; Jakobi, R.; Rega, B.; Bonazzi, C.; Grauwet, T. (**2021**). *In vitro* digestion of protein and starch in sponge cakes formulated with pea (*Pisum sativum* L.) ingredients. *Accepted in Food & Function with modifications.*

Journal Article 5

Martínez-Noguera, P.; Cosson, A.; Krause, S.; Bonazzi, C.; Saint-Eve, A. & Rega, B. (**2022**). The effect of formulation and processing conditions on the sensory qualities of pea-based sponge cakes and the odor-active compounds behind aroma changes. *Provisional title. In preparation for Food Chemistry*.

Popular Press Article

Manickam, I.*; Marínez-Noguera, P.*; Sharan, S.*; Krause, S.* (**2021**). The promise of pea and fava. *Published in the New Food Magazine*. https://www.newfoodmagazine.com/article/158620/pulse-power/
*Equal contribution of the authors.

CONFERENCES

35th International EFFoST Conference

Krause, S.; Asamoah, E.A.; Huc-Mathis, D.; Keller, S.; Moulin, G.; Berland, S.; Bonazzi, C.; Rega, B. Impact of pea ingredients (flour, protein isolate, starch) on structure and reactivity of bakery products. *Poster presentation*. Lausanne, Switzerland, 1-4 November 2021.

35th International EFFoST Conference

Krause, S.; Asamoah, E.A.; Keller, S.; Moulin, G.; Berland, S.; Descharles, N.; Bonazzi, C. & Rega, B. Ingredient mixing as a key processing step for lipid oxidation in cakes made with pea flour. *Poster presentation*. Lausanne, Switzerland, 1-4 November 2021.

35th International EFFoST Conference

Marínez-Noguera, P.; Cosson, A.; Krause, S.; Bonazzi, C.; Saint-Eve, A. & Rega, B. How formulation and processing conditions influence the sensory profiles of pea-based applications. *Poster presentation*. Lausanne, Switzerland, 1-4 November 2021.

Final FOODENGINE Conference

Krause, S.; Asamoah, E.A.; Keller, S.; Moulin, G.; Berland, S.; Descharles, N.; Bonazzi, C. & Rega, B. Impact of pea ingredients (flour, protein isolate, starch) on structure and reactivity of bakery products. *Poster presentation*. Leuven, Belgium, 29-30 September 2021. (2nd price for best poster presentation).

16th Weurman Flavour Research Symposium

Krause, S., Hashemi, A., Keller, S., Bonazzi, C. & Rega, B. Odour compounds in thermo-mechanically processed food based on pulse ingredients. *Oral presentation*. Online, 4-6 May 2021.

34th International EFFoST Conference

Krause, S., Hashemi, A., Bonazzi, C. & Rega, B. Application of pulse flours in baked products: Link between formulation, reactivity potential and physicochemical properties. *Oral presentation*. Online, 10-12 November 2020.

Journée des Doctorants

Krause, S.; Bonazzi, C. & Rega, B. Reaction potential of pulse ingredients in processed food and the effect on food quality. *Oral presentation*. Paris, France, 21-22 September 2020. (1st price for best oral presentation).

Internal Conferences

The progress of the work was presented every six months at FOODENGINE conferences, which took place at the KU Leuven (Leuven, Belgium, 04.-08.02.2019), Döhler (Darmstadt, Germany, 24.-28.06.2019), the University of Copenhagen (Copenhagen, Denmark, 27.-31.01.2020), Cargill (online, 02.-05.06.2020), INRAE (online, 01.-05.02.2021) and the KU Leuven (Leuven, Belgium, 29.-30.09.2021). Moreover, the work was presented at scientific seminars of the GéPro research team.

FOODENGINE OUTREACH ACTIVITIES

FOODENGINE E-Newsletter

Bahari, A.*; Henn, K.*; Krause, S.* Experience and learnings from attending an online scientific conference: More than just discussing science. https://foodengine.eu/newsletter/experience-and-learnings-from-attending-an-online-scientific-conference-more-than-just-discussing-science/
* Equal contribution of the authors

Bahari, A.*; Sharan, S.*; Teribia, N.*; Krause, S.* Testimonies of four FOODENGINE fellows: My journey so far as an ESR. https://foodengine.eu/newsletter/testimonies-of-four-foodengine-fellows/
* Equal contribution of the authors

FOODENGINE Movies

FOODENGINE – The fellows. https://www.youtube.com/watch?v=9MTnaxNEqHI&t=6s

FOODENGINE – The project. https://www.youtube.com/watch?v=rFObbPdZXoY&t=2s

Marie Skłodowska-Curie Actions Science is Wonderful! 2020 – Three-course meal from a can… can? https://www.youtube.com/watch?v=39qYA9pdPWk&t=3s

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LIST OF NOTATIONS

ASE	Accelerated solvent extraction
AUC	Area under the curve
BVA	Brabender viscoamylograph
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
CAGR	Compound annual growth rate
CD	Conjugated dienes
CLSM	Confocal laser scanning microscopy
CPF	Chickpea flour
CPB	Chickpea batter
CPC	Chickpea cake
DNS	3,5-Dinitrosalicylic acid
DP	Degree of polymerization
DS	Damaged starch
DSC	Differential scanning calorimeter
DTT	Dithiothreitol
EA	Emulsion activity
EC	Emulsifying capacity
ES	Emulsion stability
FA	Fatty acid
FAME	Fatty acid methyl ester
FC	Foaming capacity
FFA	Free fatty acids
FS	Foaming stability
GC-MS	Gas chromatography-Mass spectrometry
GI	Glycemic index
GPF	Green pea flour
GPB	Green pea batter
GPC	Green pea cake
H_2O	Water
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HI	Hydrolysis index
HPAEC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
HS-SPME	Headspace-solid phase microextraction
ISL	Internal starch lipids
$L^{\bullet}$	Alkyl radical
LDL	Low-density lipoprotein
LEF	Lentil flour
LEB	Lentil batter

LEC	Lentil cake
LGC	Least gelling concentration
LH	Fatty acid
LO•	Alkoxyl radical
LOO•	Peroxyl radical
LOOH	Hydroperoxide
LOX	Lipoxygenase
LPL	Lysophospholipids
LUF	Lupin flour
LUB	Lupin batter
LUC	Lupin cake
M	Molar
m/z	Mass-to-charge ratio
MaxF	Maximum mixing time after flour addition
MaxO	Maximum mixing time after oil addition
MinF	Minimum mixing time after flour addition
MinO	Minimum mixing time after oil addition
MS	Maize starch
MSB	Maize starch batter
MSC	Maize starch cake
MSPPIB	Maize starch + pea protein isolate batter
MSPPIC	Maize starch + pea protein isolate cake
MW	Molecular weight
NSL	Non-starch lipids
OHC	Oil holding capacity
OPA	*o*-Phthaldialdehyde
OT	Odor threshold
PAD	Pulsed amperometric detection
PC	Principle component
PCA	Principle component analysis
PF	Pea flour
PFB	Pea flour batter
PFC	Pea flour cake
PPI	Pea protein isolate
PS	Pea starch
PSB	Pea starch batter
PSC	Pea starch cake
PSPPIB	Pea starch + pea protein isolate batter
PSPPIC	Pea starch + pea protein isolate cake
PST	Potassium sodium tartrate tetrahydrate
PV	Peroxide value
RBP	Readily bioaccessible protein
$RBP_{hydrolyzed}$	Readily bioaccessible protein after acid hydrolysis of the TCA fraction
RDS	Rapidly digestible starch
RS	Resistant starch
RVA	Rapid visco analyzer

SDS	Slowly digestible starch
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SIM	Selected ion monitoring
SSF	Simulated salivary fluid
SSL	Starch surface lipids
TAG	Triacylglycerol
TCA	Trichloroacetic acid
TIC	Total ion current
UDMSO	Urea-dimethyl sulfoxide
UV	Ultraviolet
VOC	Volatile organic compound
WHC	Water holding capacity
WF	Wheat flour
W(F)B	Wheat batter
W(F)C	Wheat cake
YPF	Yellow pea flour
YPB	Yellow pea batter
YPC	Yellow pea cake
YPNF	Yellow pea flour processed and stored under nitrogen
YPNB	Yellow pea batter made with flour processed and stored under nitrogen
YPNC	Yellow pea cake made with flour processed and stored under nitrogen

SYNTHESE DE THESE (EN FRANÇAIS)

1 CONTEXTE ET OBJECTIFS DE LA RECHERCHE

Le changement climatique ainsi que la croissance démographique à l'échelle mondiale imposent des changements des modes de consommation qui posent de nouveaux défis à l'industrie alimentaire pour fournir des aliments nutritifs, sains et riches en protéines de haute qualité. Des stratégies pour une production alimentaire plus durable doivent être développées, incluant des recherches sur la valorisation de nouvelles alternatives de protéines végétales et des filières agroalimentaires.

Parmi les sources de protéines végétales, les légumineuses, comme les pois, pois chiches, lupins et lentilles, ont connu un essor considérable au cours des dernières années. Cela peut être attribué à leur teneur élevée en protéines de haute qualité, à leur faible indice glycémique, à leur disponibilité abondante, à leurs faibles coûts et à leur durabilité, si leur production est insérée dans des systèmes de productions agricoles plus durables (Asif *et al.*, 2013; Karaca *et al.*, 2011; Marinangeli *et al.*, 2009). Sur la base de leurs caractéristiques de composition, les légumineuses peuvent jouer un rôle important dans la prévention et la gestion d'un certain nombre de problèmes de santé tels que le diabète de type II, la gestion du poids et les troubles gastro-intestinaux (Boye *et al.*, 2010a; Polak *et al.*, 2015). Pour ces raisons, les ingrédients de légumineuses (farine, amidon, concentré de protéines et isolat de protéines) sont de plus en plus incorporés dans de nouveaux produits (Roland *et al.*, 2017; Sozer *et al.*, 2017). Le marché des aliments sans gluten, en particulier, connaît une croissance rapide, principalement due au diagnostic croissant de la maladie cœliaque, d'allergie au blé ou, surtout, de sensibilité au gluten non cœliaque (Gularte *et al.*, 2012; Laleg *et al.*, 2016; Skendi *et al.*, 2021).

Cependant, la substitution de matières premières traditionnelles comme le blé peut altérer la réactivité lors de la fabrication du produit et donc les différentes propriétés de l'aliment final, notamment l'arôme, la structure/texture et la digestibilité, en raison de leur composition chimique et de leurs caractéristiques fonctionnelles différentes. Les informations disponibles sur ces sujets étant encore très limitées, l'objectif de cette thèse était d'élucider les aspects précités lors du remplacement complet du blé par des ingrédients à base de légumineuses, dans un produit formulé et transformé classiquement consommé.

Le produit alimentaire choisi comme objet de cette recherche a été un gâteau de type génoise. Ce produit de boulangerie est un système intéressant pour étudier la relation entre la réactivité induite par la fabrication et la qualité**,** en raison de sa composition chimique complexe (Cepeda-Vázquez *et al.*, 2018; Maire *et al.*, 2013). Le produit est préparé à partir de divers ingrédients dans un processus en deux étapes de battage pour obtenir une mousse liquide (pâte) et de cuisson, au cours de laquelle se produisent des transferts couplés de chaleur et de matière, induisant des transformations intenses du produit (Maire *et al.*, 2013; Rega *et al.*, 2009). Celles-ci impliquent non seulement des modifications de structure, mais aussi des réactions chimiques qui peuvent donner naissance à divers composés volatils avec un impact notamment sur l'odeur, et donc l'acceptabilité du produit.

Ce travail de thèse a donc traité la question centrale de recherche qui a consisté à savoir comment l'utilisation d'ingrédients à base de légumineuses comme substituts du blé pouvait affecter la réactivité au cours des différentes étapes de fabrication d'une génoise et comment cela déterminait la qualité du

produit, à savoir l'arôme, la structure et la digestibilité des produits finaux, ainsi que l'apparition de composés néoformés contaminants. Au-delà de la formulation, nous nous sommes intéressés à comprendre comment le couplage formulation et procédé pouvait modifier cette réactivité et par conséquent les caractéristiques essentielles du produit. Pour répondre à ces questions, une démarche d'ingénierie de produit a été appliquée en utilisant les méthodes développées dans les différents laboratoires de recherche dans lesquels ce travail a été réalisé : UMR SayFood – AgroParisTech/INRAE (France), Laboratory of Food Technology – KU Leuven (Belgique) et Cargill – Centre de recherche de Vilvoorde (Belgique).

Les ingrédients utilisés comprenaient différentes farines de légumineuses disponibles dans le commerce (lentille, lupin, pois chiche, pois vert et pois jaune), ce qui a permis une compréhension globale des changements de qualité lorsqu'ils sont utilisés comme substituts du blé dans la fabrication de génoises. Par la suite, nous nous sommes intéressés plus particulièrement au pois car il s'agit de l'une des légumineuses les plus cultivées et les plus consommées dans le monde (Burger *et al.*, 2019; Joshi *et al.*, 2017; Rawal *et al.*, 2019) avec un taux de croissance annuel composé (TCAC) projeté de 12,7 % sur une période de prévision allant de 2021 à 2028 (Grand View Search, 2021). De plus, il présente l'une des plus faibles empreintes carbone, de bonnes fonctionnalités, un statut non transgénique et une allergénicité limitée, ce qui en fait une alternative prometteuse au blé, mais aussi au soja dans la fabrication de produits alternatifs (Lam *et al.*, 2018; Mertens *et al.*, 2012; Robinson *et al.*, 2021). Afin de pourvoir explorer le potentiel d'application de ces ingrédients à base de pois, les comportements de la protéine de pois purifiée et de l'amidon de pois ont été examinés au-delà de la farine de pois entière.

2 STRATEGIE DE RECHERCHE ET APPROCHE EXPERIMENTALE

Ce travail a été découpé en 5 études, dont 4 ont été soumis pour publication dans des revues scientifiques à comité de lecture.

L'ÉTUDE 1 visait à mieux comprendre les changements de qualité et de réactivité survenant au cours des différentes étapes de fabrication de la génoise lors de la substitution de la farine de blé par différentes farines de légumineuses (lentille, pois chiche, lupin, pois vert, pois jaune). Les questions de recherche étaient les suivantes :

- *Est-il possible de produire des génoises exclusivement à base de farines légumineuses à la place de celle de blé aux caractéristiques attractives ?*
- *Quelles réactions chimiques ont lieu à chaque étape de la fabrication du gâteau et selon quelle intensité ?*
- *Quels sont les paramètres responsables de ces réactions en conditions réelles de transformation ?*
- *Quels composés volatils sont générés, de quelles réactions sont-ils les marqueurs et comment pourraient-ils être liés à la qualité sensorielle du gâteau ?*

Afin de suivre les réactions spécifiques ayant lieu au cours des différentes étapes de développement du gâteau, le profil en composés organiques volatiles (COV) a été mesuré à la fois sur les farines, sur la pâte et sur le gâteau final. En raison de la forte susceptibilité des lipides des légumineuses à la dégradation oxydative, l'état oxydatif des produits a également été mesuré à chaque étape de production, en analysant des marqueurs intermédiaires de l'oxydation des lipides, à savoir les diènes

conjugués et les hydropéroxydes. Afin de séparer les gâteaux en fonction de leur profil en composés volatils, une analyse en composantes principales (ACP) a été réalisée pour associer certaines molécules à certains produits spécifiques et ainsi observer les tendances quant à l'effet potentiel des farines sur l'odeur des gâteaux. Outre cet aspect sensoriel, d'autres critères essentiels de la qualité des gâteaux ont également été évalués, comme la couleur et le gonflement, afin d'obtenir une compréhension globale du potentiel d'application des farines de légumineuses dans les gâteaux sans gluten.

L'ÉTUDE 2 visait à identifier, parmi le pool de COV extraits des gâteaux lors de de l'étude 1, les composés volatils clés ayant une forte probabilité de contribuer à la perception globale des odeurs. Une telle estimation des odeurs peut être réalisée par le calcul des valeurs d'activité olfactive de chaque molécule individuelle. Il permet de discriminer les différentes farines de légumineuses entre elle et avec celle de blé, et ainsi approfondir les connaissances sur l'importance de l'ingrédient dans la création de composés aromatiques.

Afin de développer de nouvelles approches pour améliorer la qualité sensorielle et globale des gâteaux à base de farines de pois (et de légumineuses en général), il est nécessaire d'avoir une meilleure compréhension des paramètres qui favorisent l'oxydation lors du battage de la pâte**,** et de l'importance des propriétés éventuelles de la structure du produit. La stratégie de ***l'ÉTUDE 3*** a donc été de modifier les temps de mélange après incorporation de farine et/ou de l'huile, car ce sont les deux étapes susceptibles des fournir la plupart des précurseurs et catalyseurs de l'oxydation des lipides. Une prolongation des deux temps de battage peut modifier le temps d'exposition de la lipoxygénase (LOX) contenue dans la farine aux acides gras insaturés, tout en modifiant en même temps la quantité d'air, et donc d'oxygène, incorporée dans la pâte. Ces questions de recherche étaient :

- *Quelle est l'importance du processus de battage de la pâte pour l'apparition de l'oxydation des lipides ?*
- *Comment la microstructure de la pâte change-t-elle lors de la modification du processus de battage ?*
- *Quelle corrélation peut être établie entre le degré d'oxydation des lipides et les propriétés structurales de la pâte ?*

Après une sélection minutieuse des temps de mélange maximum permettant de garder un volume global de produit relativement inchangé, un plan expérimental a été développé en allongeant les temps de battage signalés dans le protocole original. Nous avons également utilisé une huile strippée qui a été purifiée des antioxydants endogènes afin d'amplifier la réponse oxydative. Les pâtes ont ensuite été caractérisées d'une part en termes de réactivité, en analysant les acides gras, les diènes conjugués, les peroxydes et les COV, tel que décrit dans l'étude 1, et d'autre part en termes de propriétés de structure, en analysant la densité des pâtes et la répartition locale des ingrédients réactifs par microscopie confocale à balayage laser. Cette approche était importante pour clarifier l'influence de la structure sur le potentiel de réactivité au sein de produits complexes.

Comme alternatives à la farine de pois, des isolats protéiques de pois sont également disponibles sur le marché, qui se caractérisent par des propriétés fonctionnelles intéressantes. D'un point de vue de durabilité cependant, l'utilisation de telles fractions raffinées est moins favorable que l'utilisation de la farine entière, en raison de la consommation élevée en ressources (produits chimiques, eau, énergie)

exigée par de la purification (Lie-Piang *et al.*, 2021). Cependant, il a été rapporté que les isolats protéiques de pois contiennent moins de composés aromatiques, potentiellement négatifs en termes de perception, que la farine correspondante (Xu *et al.*, 2020). Néanmoins, l'importance de ces différences dans la perception sensorielle des aliments finaux n'a pas encore été clarifiée. De plus, l'effet du processus d'extraction sur la capacité de l'isolat protéique à former des composés volatils pendant le développement du produit par rapport à la farine de pois entière est toujours une question sans réponse. Pour ces raisons, ***I'ÉTUDE 4*** visait à évaluer le potentiel réactif de ces protéines de pois purifiées par rapport à la farine de pois et à la farine de blé et leur capacité à générer des composés volatils au cours de l'élaboration de la génoise. En plus de l'isolat protéique de pois, l'amidon de pois raffiné a également été utilisé pour explorer son potentiel d'application, car il est généralement considéré comme un sous-produit de l'isolement des protéines. Les questions de recherche étaient :

- *En quoi la farine de pois entière et des fractions de pois purifiées diffèrent-elles vis-à-vis des réactions ?*
- *Quels changements dans leur composition pourraient être responsables des différences possibles de leur potentiel de réactivité ?*
- *Quel est l'effet les divers ingrédients de pois sur la microstructure des pâtes ?*
- *Comment les propriétés structurelles des pâtes et leur potentiel de réactivité sont-ils corrélés ?*
- *Comment les différents ingrédients de pois affectent-ils les caractéristiques globales du gâteau ?*

Les isolats protéiques de pois ont été recombinés avec l'amidon de pois purifié dans le même rapport que celui de la farine de pois et leur influence sur la réactivité et la structure aux différentes étapes de la fabrication du gâteau a été étudiée. La contribution individuelle de chaque fraction à la formation de composés volatils a pu être évaluée par comparaison avec un produit préparé avec uniquement de l'amidon de pois en remplacement de la farine de blé, en supposant que les différences observées dans les profils de COV entre ce produit et le produit formulé avec l'isolat protéique de pois plus de l'amidon de pois étaient dus à la fraction protéique. Il a également été décidé d'utiliser de l'amidon de maïs purifié comme référence pour évaluer la réactivité de l'amidon de pois**,** en raison de son application courante dans l'industrie alimentaire ainsi que de sa réactivité chimique limitée (Bousquières *et al.*, 2017a). Comme dans l'étude 1, la libération des COV a été suivie dans les matières premières, les pâtes et les gâteaux finaux et un aperçu plus approfondi des processus d'oxydation a été obtenu grâce à l'analyse des diènes conjugués et des peroxydes. L'impact du processus de fractionnement de la farine sur la disponibilité des précurseurs pour participer aux réactions a été évalué par la quantification des acides gras, des sucres réducteurs et des acides aminés libres, ainsi que par la détermination de l'activité de la lipoxygénase dans les matières premières. En raison des différentes fonctionnalités couramment discutées des fractions purifiées par rapport à la farine entière, des modifications des propriétés de structure et de texture des produits**,** pouvant influencer la réactivité**,** étaient attendues. Les propriétés de structure des pâtes ont donc été mesurées, ainsi que leur densité, leur consistance et la distribution locale des ingrédients réactifs par microscopie confocale à balayage laser. De plus, la densité et la couleur des gâteaux ont été mesurées comme indicateurs de la qualité globale.

Les critères de qualité qui jouent un rôle important dans la décision d'achat des consommateurs sont non seulement l'apparence et l'arôme des produits, mais aussi leur valeur nutritionnelle. L'utilisation d'ingrédients à base de pois peut non seulement améliorer la teneur en protéines, mais également

réduire la teneur en amidon et l'index glycémique des produits. Cependant, il est nécessaire d'évaluer dans quelle mesure ces macronutriments peuvent être digérés afin d'avoir un réel bénéfice nutritionnel. L'objectif de ***I'ÉTUDE 5*** était donc d'étudier et de comparer la digestibilité *in vitro* des protéines et de l'amidon des différentes génoises produites dans l'étude 4. Les expériences ont été réalisées dans les installations de recherche de KU Leuven et de Cargill en Belgique au cours d'un détachement de six mois. Les questions de recherche comprenaient :

- *Dans quelle mesure les protéines et l'amidon du blé et du pois sont-ils bien digérés ?*
- *Les cinétiques de digestion des fractions de pois purifiées et de la farine de pois entière sont-elles différentes ?*
- *Quelles caractéristiques chimiques des différents ingrédients affectent la digestibilité ?*
- *La digestibilité est-elle influencée par les caractéristiques structurelles des génoises ?*

La digestion *in vitro* des génoises a été simulée à l'aide du protocole standardisé INFOGEST fourni dans la littérature. Pour évaluer la digestibilité des protéines et de l'amidon, les groupes aminés libérés et les sucres réducteurs ont été mesurés à l'aide de techniques spectrophotométriques courantes. De plus, une analyse chromatographique plus spécifique des produits individuels de dégradation de l'amidon a été réalisée, afin de mieux comprendre l'évolution de la digestion de l'amidon et de sélectionner les équations les plus appropriées pour modéliser les données expérimentales. De plus, les analyses quantitatives ont été complétées par une analyse microscopique des produits de digestion, susceptible de fournir des informations qualitatives sur l'avancement de la digestion. Afin de pouvoir mieux interpréter la digestibilité de l'amidon de pois, qui jusqu'à présent a rarement été discutée pour les aliments formulés complexes, des caractérisations supplémentaires ont été réalisées, c'est-à-dire la détermination des teneurs en amylose, en amidon endommagé et en amidon résistant. En plus de la composition chimique, l'influence éventuelle des propriétés de structure des gâteaux sur la dégradation enzymatique des macronutriments a été examinée. Pour cela, la morphologie des miettes de gâteau a été analysée par microscopie électronique à balayage.

3 RESULTATS ET DISCUSSION

3.1 LES LEGUMINEUSES PRESENTENT UNE PLUS GRANDE CAPACITE QUE LE BLE A GENERER DES COMPOSES VOLATILS INDUITS PAR LA TRANSFORMATION AVEC UN IMPACT PROBABLE SUR L'ODEUR ET LA SECURITE DU GATEAU (ETUDE 1 & 2)

La génération de COV a été suivie à chaque étape de la préparation des gâteaux (matières premières, pâtes, gâteaux) par une méthode quantitative HS-SPME/GC-MS (microextraction en phase solide en mode headspace couplée à la chromatographie en phase gazeuse avec détection par spectrométrie de masse) basée sur la dilution isotopique. Nous avons démontré que les farines de légumineuses entières avaient un potentiel réactif plus élevé que celle de blé. Lors de la préparation des pâtes à base de légumineuses, de nombreux marqueurs d'oxydation (alcools, aldéhydes, cétones) ont été formés (**Figure 50**), ce qui pourrait être attribué à une activité élevée de l'enzyme lipoxygénase et à des niveaux élevés de substrats acide linoléique et acide linolénique (**Tableau 25**). Les COV identifiés ont permis de conclure que le 13- et le 10-LOX étaient les principaux catalyseurs qui oxydent les acides gras pendant le battage de la pâte. Le potentiel de réactivité des farines suivait l'ordre : blé < lupin << pois chiche < lentille < pois vert < pois jaune < pois jaune produit sous azote. Ceci a pu être confirmé par l'analyse des intermédiaires de l'oxydation des lipides, c'est-à-dire les diènes conjugués et les

hydropéroxydes par des méthodes spectrophotométriques et titrimétriques (**Figure 52**). La présence de marqueurs de réaction thermique dans la farine de lupin (par exemple pyrazines et composés furaniques) a permis de supposer qu'un traitement thermique préalable avait eu lieu, conduisant à l'inactivation de la LOX, et à une capacité nettement réduite à former des COV par rapport aux autres farines de légumineuses. La farine de pois jaune, produite et stockée sous azote s'est révélée très sensible à l'oxydation, malgré les activités LOX et les profils d'acides gras similaires à ceux de la farine de pois jaune classique, suggérant une protection des précurseurs lors de la production, mais une réaction immédiate lors de l'exposition à l'oxygène atmosphérique lors de la fabrication du gâteau.

La cuisson déclenche ensuite la caramélisation et la réaction de Maillard, conduisant à la génération de pyrazines, de composés furaniques et d'aldéhydes de Strecker, en concentrations bien plus élevées dans les produits à base de légumineuses (**Figure 50**). À l'exception du lupin, les gâteaux formulés avec des légumineuses contenaient également des teneurs plus élevées en composés volatils provenant de l'oxydation des lipides que ceux de référence formulés avec du blé, dont la majorité était supposée s'être formée lors du battage de la pâte. En calculant les valeurs d'activité olfactive des composés volatils identifiés sur la base de leurs quantités et des valeurs seuils rapportées dans la littérature, il a été possible d'identifier les composants susceptibles d'avoir a priori une influence significative sur la qualité aromatique globale des gâteaux. Ceux-ci ont ensuite été analysés par une ACP, qui a révélé que les COV dérivés de l'oxydation, avec des notes olfactives « végétales », « vertes » plutôt désagréables (par exemple l'hexanal), étaient principalement liés aux gâteaux à base de lentilles, de pois chiches, de pois jaunes et surtout de farine de pois verts (**Figure 56**). De plus, les aldéhydes de Strecker (par exemple le 3-méthylbutanal à odeur maltée) sont supposés avoir une importance sensorielle substantielle dans tous les gâteaux, en particulier dans ceux à base de farines de blé ou de pois. Les gâteaux à base de farine de lentilles contenaient une quantité élevée de pyrazines (arôme terreux, de noisette), probablement en raison de la présence d'acides aminés libres en quantités élevées, parmi lesquelles la 2-éthyl-3,5-diméthylpyrazine semblait être un marqueur odorant important. Les gâteaux à base de farine de lupin étaient plus proches de ceux à base de blé que des autres gâteaux fabriqués avec des farines de légumineuses en termes de profil de COV.

3.2 LE DEGRE D'OXYDATION DEPEND DE LA DISTRIBUTION LOCALE ET DE LA TAILLE DES INGREDIENTS REACTIFS DANS LA PATE ET DU TEMPS D'EXPOSITION DE LA LOX A SES SUBSTRATS, DEUX PARAMETRES QUI SONT AFFECTES PAR LE PROCESSUS DE BATTAGE (ETUDE 3)

Cette conclusion a été obtenue en mesurant l'évolution de la concentration en marqueurs d'oxydation primaire (dienes conjugués, hydropéroxydes) et secondaire (COV) ainsi que la microstructure des pâtes contenant de la farine de pois jaune en fonction du temps de mélange de la pâte. En prolongeant le battement de la pâte après l'ajout de la farine de pois jaune, ni les quantités des hydropéroxydes intermédiaires (**Tableau 31**) ni celles de COV finaux (**Tableau 29**) n'ont changé de manière significative, ce qui pourrait s'expliquer par la répartition inégale et la forte proportion de grosses bulles d'air et de gouttelettes lipidiques dans la pâte, entravant leur probabilité de contact (**Figure 59**). Par contre, lorsque le temps de mélange après l'incorporation de l'huile de tournesol strippée a été augmenté, une concentration significativement plus élevée d'hydropéroxydes a été détectée. Cela indiquait une oxydation lipidique plus intense qui a été attribuée à une répartition plus homogène des lipides, des protéines et des bulles d'air dans la pâte, ainsi qu'à une réduction de la taille des bulles d'air et des gouttelettes lipidiques, synonymes d'une interface de réaction plus grande. Néanmoins, ce temps de

battage était insuffisant pour convertir les hydropéroxydes en COV. Ceci n'a été possible qu'en prolongeant séquentiellement les temps de mélange après l'ajout de la farine et de l'huile, et ce n'était pas seulement dû à l'homogénéité améliorée et à l'interface de réaction plus importante, mais aussi au temps d'exposition plus long de la LOX à ses substrats.

Il est intéressant de noter que la masse volumique de la pâte augmentait également avec le temps de battage (**Tableau 30**), suggérant une diminution du volume d'air inclus, et donc de la quantité d'oxygène nécessaire aux processus d'oxydation. Il semble donc que la quantité d'oxygène emprisonnée dans la pâte était non limitante vis à vis de l'oxydation des lipides. Ceci a été confirmé par l'analyse des masses volumiques des pâtes préparées avec d'autres farines de légumineuses : par exemple, la pâte fabriquée à partir de farine de pois jaune était aussi dense que la pâte fabriquée à partir de farine de lupin mais accumulait significativement plus de COV, et était inversement plus dense que la pâte fabriquée à partir de farine de pois verts, tout en contenant des concentrations similaires de COV (**Tableau 25, Figure 50**).

3.3 LE FRACTIONNEMENT DE LA FARINE DE POIS CONDUIT A DES INGREDIENTS DE POIS PURIFIES QUI SONT MOINS SENSIBLES A L'OXYDATION DES LIPIDES, MAIS PLUS SENSIBLES A LA REACTION DE MAILLARD PENDANT L'ELABORATION DU GATEAU (ETUDE 4)

Ce résultat a été obtenu en suivant la génération de COV par HS-SPME/GC-MS lors de la fabrication de génoises à base soit de farine de pois entière, soit d'un mélange binaire des constituants purifiés qui en dérivent (c'est-à-dire l'isolat protéique de pois et l'amidon de pois). La capacité réduite des fractions de pois dans la pâte composite à générer non seulement des marqueurs d'oxydation finaux (COV) mais aussi intermédiaires (dienes conjugués, hydropéroxydes) (**Figure 62, Figure 65**) pourrait être attribuée à l'inactivité de LOX, qui était probablement une conséquence de la purification des protéines (**Tableau 47**). Par rapport à celle fabriquée à partir de farine de blé cependant, cette pâte composite contenait des quantités plus élevées de COV liés à l'oxydation, ce qui pourrait être dû à la forte proportion d'acides linoléique et linolénique qui peuvent être facilement oxydés ainsi qu'à l'élimination des antioxydants pendant le processus de purification. Lorsqu'une pâte était préparée à base d'amidon de pois uniquement, pratiquement aucun marqueur d'oxydation n'était détecté, suggérant d'une part que les acides gras contenus dans la farine de pois restaient liés à l'isolat protéique et non à l'amidon après fractionnement (**Tableau 47**) et, d'autre part, que les acides gras de l'huile et de l'œuf étaient moins sensibles à l'auto-oxydation. La même conclusion a pu être apportée lorsque l'amidon de pois a été remplacé par de l'amidon de maïs purifié. Les deux pâtes étaient également caractérisées par des densités et consistances plus faibles (**Tableau 32**), confirmant ainsi que ces propriétés structurelles n'avaient pas d'influence évidente sur le degré d'oxydation.

Les différences considérables dans les quantités de composés volatils générés par oxydation observés, entre les pâtes faites avec de la farine de pois ou une combinaison de protéines de pois et d'amidon, étaient beaucoup moins prononcées après leur transformation en gâteaux (**Figure 62**). Néanmoins, il a été constaté que le profil de COV des gâteaux de farine de pois était encore déterminé par des marqueurs d'oxydation, alors que les gâteaux composites contenaient des niveaux plus élevés de molécules provenant de la dégradation des acides aminés et des sucres (c'est-à-dire les aldéhydes de Strecker, les composés furaniques et pyrazines) induite par la chaleur. C'était également le cas du gâteau formulé avec de l'amidon de pois seul. Apparemment, le fractionnement de la farine de pois

donne des ingrédients qui sont non seulement moins sensibles à l'oxydation des lipides, mais en même temps plus disponibles pour participer à la caramélisation et à la réaction de Maillard pendant la cuisson. Une ACP a mis en évidence que les marqueurs volatils liés à l'oxydation et aux notes olfactives « végétales » et « vertes » plutôt désagréables étaient plutôt caractéristiques des gâteaux à base de farine de pois, tandis que les pyrazines (notes de noisette, grillées) et, en particulier, les aldéhydes de Strecker (notes maltées) devaient avoir une contribution importante à l'arôme global des gâteaux d'amidon pur ou en mélange avec des protéines de pois (**Figure 66**). Quant à la référence blé, il est devenu évident que cette farine avait le potentiel de réaction le plus faible lors de la cuisson et donc un profil de COV moins complexe. Il est intéressant de noter que des profils de COV similaires ont été détectés lors du remplacement de l'amidon de pois par de l'amidon de maïs dans des produits à base d'amidon seul, ou en combinaison avec des protéines de pois. En plus, ces quatre formulations ont aussi conduit à la formation de furane, un composé néoformé indésirable, qui a été classé comme potentiellement cancérigène pour l'homme. Cela a corroboré l'hypothèse que les ingrédients de l'amidon purifié étaient plus susceptibles de participer aux réactions induites par la chaleur.

3.4 LES FARINES DE LEGUMINEUSES ET LES INGREDIENTS PURIFIES PRODUISENT DES GATEAUX AUX CARACTERISTIQUES ATTRAYANTES

La substitution complète de la farine de blé par différents ingrédients de légumineuses a conduit à des gâteaux attrayants comme en témoignent leurs volumes et leurs couleurs. Les gâteaux à base de farines de légumineuses étaient généralement caractérisés par des masses volumiques significativement plus faibles par rapport aux références à base de blé (**Figure 51**), ce qui a été attribué à leurs niveaux élevés de protéines (**Tableau 26**) pouvant exercer leurs importantes propriétés émulsifiantes et moussantes. Une exception était la farine de lupin, avec laquelle une diminution significative de la capacité de rétention des gaz a été observée, conduisant à un gâteau dense, probablement en raison de son rapport amidon/protéine élevé et de sa teneur élevée en fibres (**Tableau 26**). En revanche, l'utilisation de protéines de pois purifiées en combinaison avec de l'amidon de pois a donné des gâteaux avec des volumes plus importants que ceux obtenus avec de la farine de pois entière (**Figure 64**) et cela pourrait être attribué à l'absence de fibres (**Tableau 47**). Lorsque l'amidon de pois a été utilisé sans protéines supplémentaires, les gâteaux les plus volumineux ont été produits, en raison de la teneur élevée en amidon qui pouvait avoir augmenté la viscosité et donc la rétention d'air pendant la cuisson. De plus, nous pouvions imaginer que les protéines d'œuf développeraient tout leur potentiel émulsifiant et moussant en l'absence de protéines végétales. Il est intéressant de noter que le remplacement de l'amidon de pois par de l'amidon de maïs n'a été associé à aucun changement significatif de masse volumique, ce qui permet d'envisager que l'amidon de pois puisse être une alternative pour une utilisation dans les aliments.

D'autres tentatives pour élaborer des corrélations entre les propriétés de structure et la composition chimique ont été faites en utilisant des analyses supplémentaires sur des mies de gâteau, y compris des tests de compression et une analyse d'image de la structure cellulaire. Comme ils n'étaient que partiellement représentatifs, ils ne pouvaient être utilisés que pour décrire des tendances générales. Il est apparu que la masse volumique élevée du gâteau de blé était liée à une augmentation de la rigidité de la paroi cellulaire (**Tableau 35, Tableau 36**), probablement en raison de la forte proportion de protéines avec des acides aminés soufrés, qui peuvent former des ponts disulfures stables. Cette rigidité pouvait également avoir été responsable de la formation observée de grosses bulles d'air, avec des

parois cellulaires épaisses (**Figure 72**). En revanche, les mies à base de farine de pois ou d'une combinaison de protéines de pois et d'amidon avaient des alvéoles plus petites et des parois cellulaires plus minces. Cela pourrait être dû à la teneur accrue en protéines, avec une activité de surface potentiellement plus élevée. L'utilisation de farine de pois a également entraîné une augmentation de la fermeté de la mie, ce qui n'était pas le cas avec les ingrédients de pois purifiés et pourrait donc être dû à une teneur élevée en fibres absorbant l'eau et contribuant à la formation d'un réseau rigide.

De plus, il a été démontré que la coloration naturelle des ingrédients des légumineuses jouait un rôle dans la couleur finale des gâteaux. Alors que la couleur de la croûte restait comparable à la référence blé, les couleurs des mies pouvaient varier (**Figure 51, Figure 64**). Par exemple, les matières premières moins colorées telles que la farine de pois chiche, la farine de pois jaune et les fractions de pois n'ont pas induit de changement de couleur significatif, alors que la farine de pois vert, la farine de lentille et la farine de lupin pouvaient conduire à des teintes verdâtres, grisâtres et jaunâtres.

3.5 LA FARINE DE POIS ENTIERE ET LES INGREDIENTS DE POIS PURIFIES ONT MONTRE UNE SENSIBILITE SIMILAIRE A LA PROTEOLYSE ET A L'AMYLOLYSE AU COURS DE LA SIMULATION DE LA DIGESTION GASTRO-INTESTINALE *IN VITRO* (ETUDE 5)

La méthode INFOGEST est un bon outil pour mieux comprendre la digestibilité *in vitro* des protéines et de l'amidon dans des systèmes alimentaires complexes. Des cinétiques similaires de digestion des protéines, entre le gâteau à base de blé (référence) et les gâteaux fabriqués avec de la farine de pois ou une combinaison de protéines de pois et d'amidon de pois, ont pu être mesurées (**Tableau 52**) en quantifiant les groupes aminés et les petits peptides libérés tout au long de la digestion gastro-intestinale et correspondant à la fraction protéique facilement bioaccessible. Par hydrolyse acide de cette fraction, une conversion des petits peptides en acides aminés libres a pu être obtenue, ce qui a permis une meilleure comparaison entre les gâteaux. Il s'est avéré que la protéolyse était retardée dans les gâteaux contenant des protéines de pois (farine ou isolat) par rapport au blé de référence (**Figure 74**), ce qui pourrait éventuellement être lié au rapport enzyme/protéine plus faible ainsi qu'à la présence probable de facteurs antinutritionnels. Néanmoins, les concentrations de protéines facilement bioaccessibles étaient plus élevées dans les gâteaux à base de protéines de pois. De plus, les profils de digestion des protéines de pois contenues dans la farine ou dans l'isolat étaient comparables, ce qui implique que le processus de fractionnement n'altère pas la digestibilité des protéines.

En mettant en œuvre deux méthodes d'analyse basées respectivement sur la mesure spectrophotométrique des sucres réducteurs (méthode DNS) et la quantification chromatographique des produits individuels d'hydrolyse de l'amidon (méthode HPAEC-PAD), un comportement similaire de digestion de l'amidon biphasique a été détecté pour tous les gâteaux ce qui permis de le modéliser en combinant deux équations du premier ordre. Cette observation a été attribuée à la digestion immédiate de l'amidon complexe, dès l'ajout des enzymes amylolytiques, suivie de l'hydrolyse des produits de dégradation de l'amidon (par exemple le maltotriose) en maltose et glucose (**Figure 77**). L'amidon dans les gâteaux à base de farine de pois, d'amidon de pois ou d'amidon de pois combiné à des protéines de pois était moins sujet à l'amylolyse que celui de gâteaux à base de farine de blé, d'amidon de maïs ou d'amidon de maïs combiné aux protéines de pois (**Figure 76**). Ceci a été confirmé par l'observation en microscopie optique des produits de digestion, et la disparition des granules d'amidon dans les gâteaux à base de blé et de maïs, après un temps considérablement plus court (**Figure 75**). Les raisons possibles pourraient être la teneur plus élevée en amylose et en amidon résistant (**Tableau 38**), la

persistance de certaines structures granulaires après la cuisson (**Figure 76**) et les tailles des granules plus grandes dans les gâteaux à base de pois (**Figure 68**), qui pourraient avoir ralenti l'attaque enzymatique. De plus, la microscopie électronique à balayage a révélé un réseau protéine-amidon plus continu dans les gâteaux à base de pois par rapport aux gâteaux à base de blé et de maïs, suggérant une interaction enzyme-substrat facilitée dans ces derniers cas (**Figure 78**). En examinant de plus près les profils de digestibilité de l'amidon de pois sous sa forme purifiée ou en tant que partie de la farine entière, il a été constaté que les deux ingrédients présentaient une sensibilité similaire à l'amylolyse. On peut donc en déduire que le processus de fractionnement n'a pas conduit à une modification sensible de la digestibilité de l'amidon comme déjà signalé pour les protéines de pois.

4 PERSPECTIVES

Les résultats de cette thèse ont mis en évidence que le choix de l'ingrédient, ainsi que la façon dont il est produit et stocké, peut avoir un impact considérable sur le potentiel de réactivité au cours des différentes étapes de la fabrication d'un produit formulé et soumis à des procédés thermomécaniques, permettant ainsi de concevoir des produits aux attributs souhaités. Dans le travail présenté, des produits de type génoise ont été formulés avec une seule source d'ingrédients issus de légumineuses. Cependant, une approche alternative intéressante pourrait consister à combiner différentes farines de légumineuses entre elles en des proportions variables, car cela pourrait élargir les possibilités de création de profils aromatiques complexes, par exemple.

Pour identifier les composés volatils détectés qui déterminent les caractéristiques aromatiques des aliments finaux, il est conseillé d'identifier les composés aromatiques clés des gâteaux au moyen de méthodes sensorielles et olfactométriques. Ceci est particulièrement important parce que les farines de légumineuses ont été décrites comme étant plus sensibles à l'oxydation des lipides, conduisant à des substances volatiles qui ont été associées, dans la littérature, à des notes olfactives « végétales », « vertes » plutôt désagréables. Cependant, ces ingrédients de légumineuses avaient également un potentiel élevé pour générer des marqueurs thermiques par la réaction de Maillard et de caramélisation, dont beaucoup sont connus pour posséder de faibles seuils de détection olfactive. Il serait donc possible que ces molécules induites par la cuisson masquent les défauts d'arôme liés aux marqueurs d'oxydation. Les techniques qui pourraient permettre d'évaluer la qualité sensorielle des gâteaux à base de légumineuses seraient la chromatographie en phase gazeuse couplée à l'olfactométrie (GC-O), l'analyse sensorielle descriptive et d'autres méthodes sensorielles.

Cette approche serait également intéressante pour comparer les gâteaux cuits à partir de pâtes de pois jaunes préparées selon des temps de battage différents après l'ajout de farine et/ou d'huile. Cela permettrait de mieux comprendre si les différences dans le degré d'oxydation sont ensuite perçues dans les produits finaux. De plus, les différents temps de battage de la pâte pourraient être couplés à d'autres paramètres de procédé tels que le temps et la température de cuisson, afin de clarifier leur effet combiné sur l'apparition de composés volatils d'intérêt. Afin d'obtenir les premières réponses à ces questions, nous avons lancé un autre projet qui vise à comprendre l'importance sensorielle des COV mesurés dans des gâteaux obtenus en utilisant des temps de battage et de cuisson différents. Les méthodes appliquées comprennent l'analyse par chromatographie en phase gazeuse des composés odorants volatils combinée à des études sensorielles. Celles-ci seront ensuite complétées par d'autres techniques pour étudier la qualité globale des gâteaux élaborés, comme la couleur et la texture.

De plus, cette thèse a permis de montrer que les processus oxydatifs indésirables pouvaient être limités en utilisant des fractions protéiques purifiées de pois au lieu de la farine de pois entière pour faire des gâteaux. Cela a été attribué à l'inactivation de l'enzyme LOX lors de l'extraction des protéines. Cependant, étant donné que la purification des protéines de la farine est associée à une empreinte environnementale accrue, de nouvelles stratégies devraient être développées pour exploiter le potentiel d'application des farines de légumineuses entières. Cela peut inclure des recherches sur les moyens d'inactiver légèrement l'enzyme sans compromettre les fonctionnalités ainsi que les aspects nutritionnels et sensoriels. Une possibilité à tester serait le traitement à froid des matières premières susceptible induire une dénaturation des protéines (Helmick *et al.*, 2021) et donc une inactivation de LOX.

En ce qui concerne les fractions de pois, il a également été montré qu'il existe un potentiel plus élevé de formation de composés néoformés indésirables comme le furane. Ce composé pouvant se former par oxydation des lipides, réaction de Maillard et caramélisation. Pour cette raison, une question est de savoir quel est le risque pour la santé associé à l'utilisation de ces nouvelles matières premières dans des applications spécifiques incluant des traitements thermomécaniques. Cette étude a fourni un premier aperçu de la capacité des ingrédients à base de pois à générer des composés volatils néoformés par rapport au blé, en utilisant une approche globale et avec un focus sur les composés volatils. Cependant, afin de clarifier si ces molécules soulèvent réellement des problèmes de sécurité, des méthodes de quantification plus sensibles et adaptées aux différentes cibles devraient être mises en œuvre afin de déterminer avec précision les niveaux de composés néoformés indésirables formés dans ces types de produits. On peut envisager par exemple la méthode HS-trap proposée par Cepeda-Vázquez *et al.* (2019) pour la détermination du furane ou des méthodes d'UHPLC-MS pour la détermination de composés moins volatils tels que l'acrylamide et le HMF. De telles quantifications seraient utiles l'évaluation du risque lié à l'utilisation de ces nouveaux ingrédients.

Du point de vue de la texture, les travaux présentés permettent une appréhension globale des différences structurelles majeures entre les gâteaux à base de légumineuses et ceux à base de blé. Afin d'obtenir des informations plus approfondies, d'autres expériences doivent être effectuées. Par exemple, la microtomographie aux rayons X pourrait être utilisée pour évaluer la structure cellulaire du gâteau de manière non destructive. Enfin, les résultats instrumentaux obtenus devraient être complétés par des de mesures des attributs sensoriels tels que la sensation en bouche et la texture. Cela permettrait d'obtenir une vue globale sur l'applicabilité de nouveaux ingrédients de légumineuses dans les aliments. Des premiers tests ont permis de qualifier certaines différences entre la farine de pois entière et les fractions raffinées : les gâteaux formulés avec un mélange de protéines de pois et d'amidon de pois ont été décrits comme souples et aérés, alors que le gâteau de farine de pois entière était plutôt collant et humide (Martínez-Noguera *et al.*, 2021).

Un autre aspect très intéressant, lié à la composition et à la structure du gâteau et qui n'a pas été traité dans ce travail, concerne l'interaction des composés d'arôme avec la matrice du gâteau à l'échelle moléculaire. Il est connu que les substances odorantes peuvent interagir avec les constituants alimentaires, c'est-à-dire les protéines, les lipides et l'amidon. Ceci peut influencer leur libération dans l'espace gazeux et donc la perception sensorielle. Dans une étude sur les génoises à base de blé, Pozo-Bayon *et al.* (2008) ont pu montrer que certains composés odorants forment des complexes avec l'amylose contenu dans l'amidon et peuvent ainsi être piégés dans la matrice. La manière dont cette

interaction peut varier avec la formulation est donc une approche qui devrait être étudiée dans de futures expériences. Ceci est particulièrement intéressant à propos des ingrédients à base de pois car nous avons pu montrer dans cette thèse que ces produits forment moins de complexes amylose-lipides que les gâteaux à base de farine de blé ou d'amidon de maïs (**Tableau 38**), ce qui témoigne d'une affinité différente pour la formation de complexes, mais qui peut cependant être spécifique du ligand et debrait donc être élucidé.

En ce qui concerne les études de digestibilité, il faut tenir compte du fait que la méthode *in vitro* utilisée ne reflète pas pleinement les conditions physiologiques réelles. Ceci est dû au fait que les interactions avec l'hôte, la vidange progressive de l'estomac vers l'intestin grêle ainsi que les changements de pH, de concentration d'électrolytes et d'enzymes ne sont pas pris en compte. Afin de fournir des données plus pertinentes sur le plan physiologique, il serait intéressant d'utiliser des protocoles *in vitro* dynamiques tels que ceux décrits par Ménard *et al.* (2014) et Mulet-Cabero *et al.* (2020) qui prennent en compte les modifications de la valeur du pH, de la sécrétion digestive et des temps de transit gastrique et intestinal. Dans ce contexte, il convient d'envisager l'ajout d'une *α*-amylase en phase orale qui avait été exclue du protocole de digestion en raison du temps de séjour courts et d'une inactivation instantanée en phase gastrique (Pälchen *et al.*, 2021). Cependant, lors de l'application de méthodes de digestion *in vitro* semi-dynamiques, l'importance de l'*α*-amylase dans la phase orale doit être clarifiée, car son activité pourrait se poursuivre pendant la phase initiale de la digestion gastrique. Un autre aspect important à ne pas négliger est l'évolution de la viscosité du chyme au cours de la digestion, qui dépend de la composition du produit et peut ainsi influencer la catalyse enzymatique. Ceci devrait donc être pris en compte dans les expérimentations futures lors de la reformulation des produits. Une étape supplémentaire serait la réalisation d'études *in vivo* afin de confirmer les résultats rapportés dans ce travail et d'établir les effets métaboliques postprandiaux réels. De plus, l'évaluation de la bioaccessibilité de certains nutriments bénéfiques pour la santé pourrait être envisagée pour approfondir les différences entre les ingrédients à base de légumineuses et de céréales.

D'un point de vue global, la stratégie méthodologique poursuivie dans cette thèse pour lier la réactivité aux critères de qualité du produit était une approche d'ingénierie de formulation innovante précieuse pour évaluer l'applicabilité de nouveaux ingrédients dans des aliments formulés complexes. Il a pu être démontré que l'évaluation du potentiel de nouvelles matières premières en tant qu'ingrédients alimentaires fonctionnels ne devrait pas se limiter à l'analyse de leurs propriétés en tant que matières premières pures, mais devrait être étendue en combinaison avec d'autres ingrédients dans les systèmes alimentaires réels. De cette manière, les limitations importantes sur le produit peuvent être évaluées (sensorielles, texture, apparence) et des stratégies de traitement des matières premières (traitement thermique, traitement sous azote ou fractionnement de la farine) peuvent être évaluées.

On peut conclure qu'il existe un grand potentiel d'innovation lié à l'utilisation d'ingrédients à base de légumineuses pour confectionner des produits céréaliers de type génoise. Divers produits attrayants en termes d'aspect visuel, de structure, de digestibilité et de profil aromatique peuvent être envisagés à moyennant la définition du bon couple de la matière première/procédé.

PART I

GENERAL INTRODUCTION & RESEARCH OBJECTIVES

Global climate change along with a hastily growing world population and modified consumption patterns are posing permanent challenges to the food industry to provide nutritious, healthy and high-quality food rich in protein. Strategies toward a more sustainable production of foods need to be developed, including research on the valorization of new plant-based protein alternatives and agri-food side-streams.

Among the sources of plant-based proteins, grain legumes (from Latin *legere* = gather) have encountered considerable momentum during the last years (Pina-Pérez *et al.*, 2018). These nitrogen-fixing crops are defined as the edible fruits or seeds of pod-bearing plants belonging to the Fabaceae (or Leguminosae) family (Adebiyi *et al.*, 2011; Asif *et al.*, 2013; Pina-Pérez *et al.*, 2018). The harvested dried seeds that not primarily used for oil extraction are named ***PULSES***. Typical representatives comprise chickpeas (*Cicer arietinum*), lentils (*Lens culinaris*), lupins (*Lupinus* spp.) and peas (*Pisum* spp.) (Food and Agricultural Organization of the United Nations (FAO), 2016). Pulses are attractive commodities which fulfill consumer's requirements because of their abundant availability, low costs and high nutritional value (Asif *et al.*, 2013; Karaca *et al.*, 2011; Marinangeli *et al.*, 2009). They not only provide energy, dietary fiber, B vitamins and minerals but also possess high levels of protein with favorable amino acid composition as well as low levels of fat and a low glycemic index (Asif *et al.*, 2013; Klupšaitė *et al.*, 2015; Polak *et al.*, 2015; Sozer *et al.*, 2017). Based on these compositional characteristics, pulses play an important role in the prevention and management of a number of health conditions, including cardiovascular disease, cancer, diabetes type II, osteoporosis, gastrointestinal disorders, adrenal disease, weight management, hyperlipidemia and hypertension (Boye *et al.*, 2010a; Polak *et al.*, 2015). For these reasons, pulse ingredients (flour, starch, protein concentrate and protein isolate) are more and more incorporated in new products in recent years (Roland *et al.*, 2017; Sozer *et al.*, 2017). The market for gluten-free foods, in particular, is rapidly growing, which is majorly driven by the increasing diagnosis of celiac disease, wheat allergy or non-celiac gluten sensitivity (Gularte *et al.*, 2012; Laleg *et al.*, 2016; Skendi *et al.*, 2021).

However, the substitution of traditional raw materials like wheat can alter the reactivity during product making and thus the different properties of the food product, including ***AROMA***, ***STRUCTURE/TEXTURE*** and ***DIGESTIBILITY***, due to their different chemical composition and functional characteristics (**Figure 1**). Since the information available on these topics is still very limited, the aim of this thesis was to elucidate the aforementioned food aspects when fully replacing wheat with pulse-based ingredients.

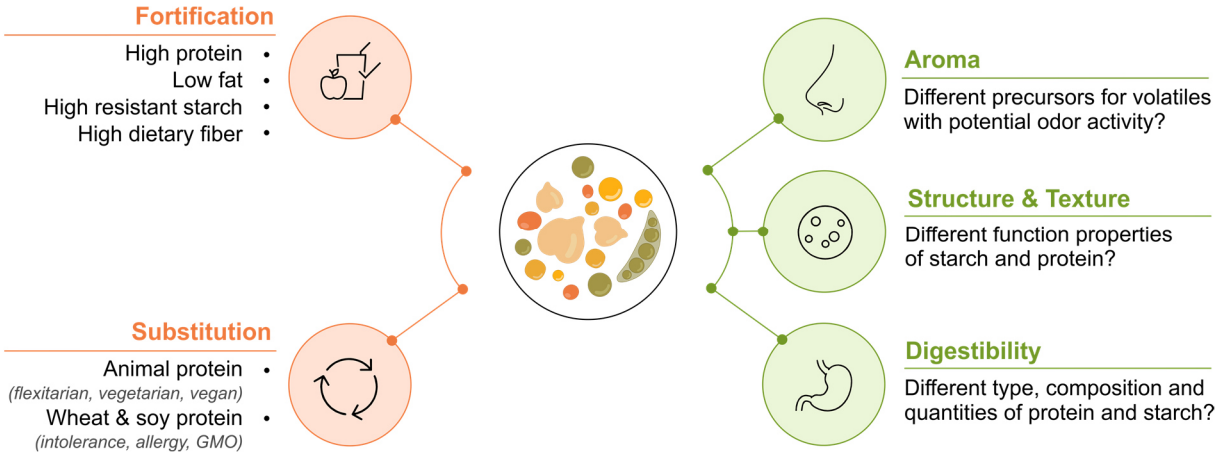

Figure 1. Reasons for the increasing interest in pulse ingredients and their effect on essential product criteria.

The food product chosen as the research object for this study was the ***SPONGE CAKE***. This baked good is an interesting system to investigate the relationship between reactivity and quality due to its complex chemical composition (Cepeda-Vázquez *et al.*, 2018; Maire *et al.*, 2013). The product is prepared from various ingredients in a two-step process of batter beating and baking, during which heat and mass transfer occur, which lead to intense product transformations (Maire *et al.*, 2013; Rega *et al.*, 2009). These involve not only structural modifications but also chemical reactions, which can give rise to diverse volatile compounds with potential odor impact.

The present thesis thus dealt with the central research question of how the use of pulse-base ingredients as alternatives to wheat can affect the ***REACTIVITY*** during the different steps of sponge cake development and how this determines ***PRODUCT QUALITY***, which is reflected in the aroma, structure and digestibility of the final cakes as well as the development of process-induced contaminants. Apart from formulation, it was of great interest to understand how the coupling of ***FORMULATION*** and ***PROCESS*** could modify this reactivity and consequently the aforementioned essential product criteria. To answer to these questions, an integrated enginomics approach was applied using state-of-the-art analytical methods developed at the different research facilities at which this work was performed:

(1) INRAE/AgroParisTech, Massy, France
(2) KU Leuven, Leuven, Belgium
(3) Cargill, Vilvoorde, Belgium

The ingredients used included a wide variety of different commercially available ***PULSE FLOURS*** (lentil, lupin, chickpea, green pea and yellow pea), which enabled a global understanding of the changes in quality when being used as alternatives to wheat. Thereafter, attention was turned to pea because it is one of the most cultivated and consumed pulse crops worldwide (Burger *et al.*, 2019; Joshi *et al.*, 2017; Rawal *et al.*, 2019) with an projected compound annual growth rate (CAGR) of 12.7% over a forecast period from 2021 to 2028 (Grand View Search, 2021). Moreover, it has one of the smallest carbon footprints, good functionalities, a non-transgenic status and limited allergenicity, which makes it a promising alternative to not only wheat but also soy in the manufacture of alternative products (Lam *et al.*, 2018; Mertens *et al.*, 2012; Robinson *et al.*, 2021). In addition to the whole ***PEA FLOUR***, the behaviors of ***PURIFIED PEA PROTEIN*** and ***PEA STARCH*** fractions were examined to exploit and compare the application potential of diverse pea ingredients.

The thesis is organized in six different parts (**Figure 2**). An extensive review of the literature data is provided in **Part II**, which itself is subdivided into three main chapters: *chapter 1* describes the current information about the chemical composition and functionalities of pea compared to cereals, *chapter 2* reviews the process of sponge cake development including knowledge about pulse-based cakes, and *chapter 3* gives insight into the chemical reactions occurring during processing, their occurrence in pea and relevance in odor generation. Subsequently, the strategy of research is presented in **Part III**, followed by a detailed section of the materials and methods implemented (**Part IV**). The data acquired throughout the study are discussed in **Part V**, which is structured in five chapters: the first two chapters cover the studies on different pulse flours, which evaluate the reactivity potential of various pulse flours to form volatile compounds (*chapter 1*) and develop a correlation between these molecules and their odor activity (*chapter 2*). The latter three chapters focus on pea and investigate the coupled effect of ingredients and process on reactivity during batter preparation (*chapter 3*), the correlation between

formulation, reactivity and structural properties throughout sponge cake development (*chapter 4*) as well as *in vitro* digestibility of protein and starch (*chapter 5*). Finally, the major outcomes of this dissertation and associated perspectives are discussed in **Part VI**.

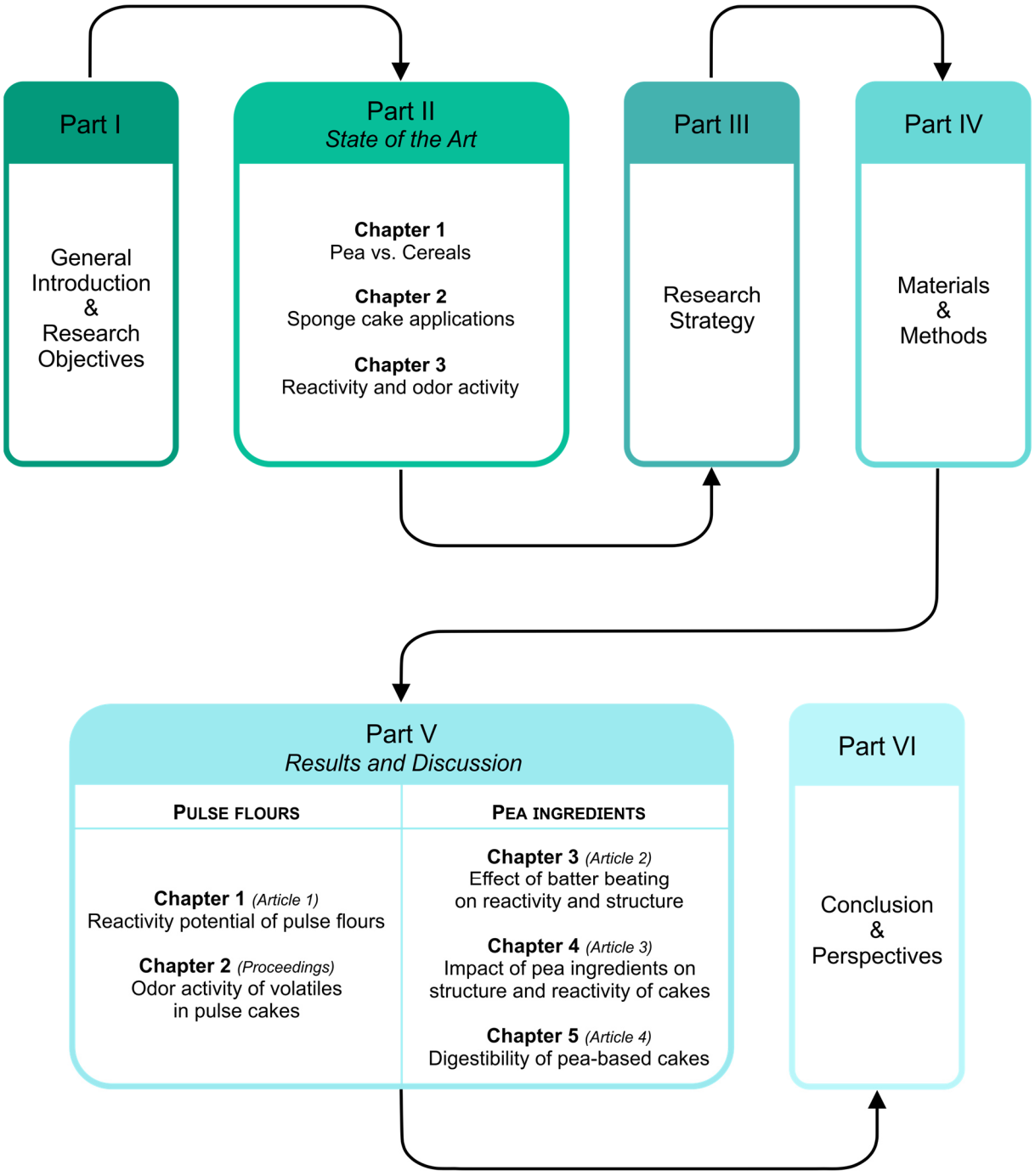

Figure 2. Schematic representation of the organization of the different parts of this dissertation.

PART II

STATE OF THE ART

CHAPTER 1 *PEA VS. CEREALS*

CHAPTER 2 *SPONGE CAKE APPLICATION*

CHAPTER 3 *REACTIVITY AND ODOR ACTIVITY*

CHAPTER 1

PEA VS. CEREALS

1 GENERAL DESCRIPTION

Peas (*Pisum sativum*) are cool season crops that have been cultivated for more than 10000 years (Robinson *et al.*, 2021). In 2019, approximately 7 million hectare of peas were harvested worldwide, which provided more than 14 million tons of peas, of which Canada took the largest share, followed by Russia, the USA, China and India (Boukid *et al.*, 2021; Rawal *et al.*, 2019; Robinson *et al.*, 2021).

The most cultivated botanical varieties of peas are garden peas (*Pisum sativum* L. var. *hortense*) and field peas (*Pisum sativum* L. var. *arvense*), which can develop three different cotyledon colors: yellow, yellow-green and green (Ratnayake *et al.*, 2002). Moreover, two phenotypes are distinguished, namely smooth pea and wrinkled pea, which differ in the morphology and characteristics of their starch granules (Ratnayake *et al.*, 2002).

In comparison to wheat flour, which is commonly used in baked goods, pea contain a remarkably higher level of proteins, while the proportion of starch is distinctively smaller (**Figure 3**). Detailed descriptions of the composition and functional properties of these major fractions as well as lipids is given in the following sections. These characteristics will be compared to wheat and maize due to their use as reference materials in the present study.

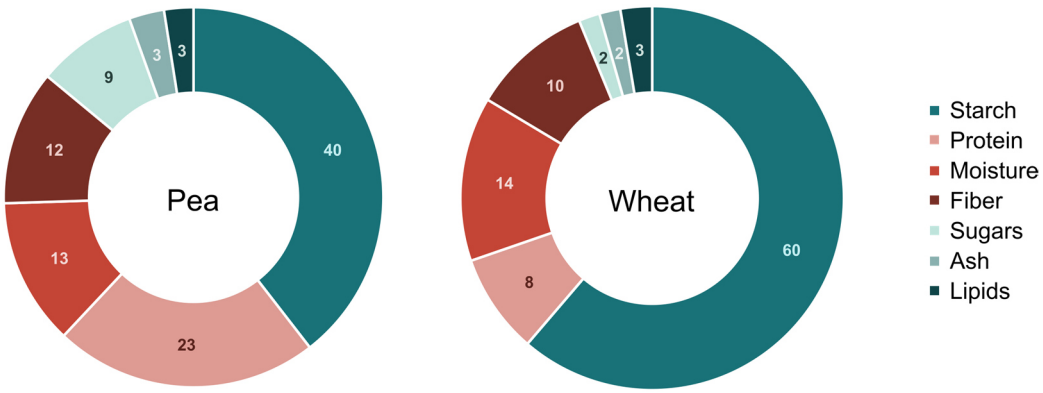

Figure 3. Average chemical composition [%] of pea seeds and wheat grains. Based on Hall *et al.* (2017), Carson *et al.* (2009), Lafiandra *et al.* (2014), Chung *et al.* (2009b) and Stone *et al.* (2009).

2 STARCH

Starch is the major constituent of pea carbohydrates, accounting for around 30 – 49% of the pea seed (Hall *et al.*, 2017). The macromolecule is primarily derived as a by-product from the extraction of pea proteins (Laleg *et al.*, 2016; Ratnayake *et al.*, 2002; Zhou *et al.*, 2019a). For this reason, it is considered as a relatively inexpensive source of biopolymer, which does not only provide energy but also serves as a versatile food ingredient, exerting numerous functional and nutritional benefits (Ratnayake *et al.*, 2002; Zhou *et al.*, 2019a; Zhou *et al.*, 2019b). In the following sections, different characteristics of pea starch are described and compared with the two cereal starches used as controls in the present dissertation: maize and wheat starch. As the information about the starch properties of pea are scarce, they are complemented by those available on other starches

2.1 GRANULE MORPHOLOGY

According to the literature, starch granules of pea typically exhibit different shapes, ranging from oval over spherical to irregular, with unimodal size distribution between 2 and 50 µm (**Table 1**) (Bertoft *et al.*, 1993; Hoover *et al.*, 2010; Ratnayake *et al.*, 2002). Whereas the starch fraction of smooth pea is mostly composed of simple granules, wrinkled pea starch also contains compound granules built from 3 to 10 joint simple granules (Colonna *et al.*, 1984; Ratnayake *et al.*, 2002). Scanning electron imaging revealed that the surfaces of both cultivars generally appear to be smooth, hence free of fissures or pores (Chung *et al.*, 2008; Ratnayake *et al.*, 2002; Zhou *et al.*, 2004). However, Zhou *et al.* (2004) observed indentations and hollows on single starch granules of wrinkled pea (**Figure 4**).

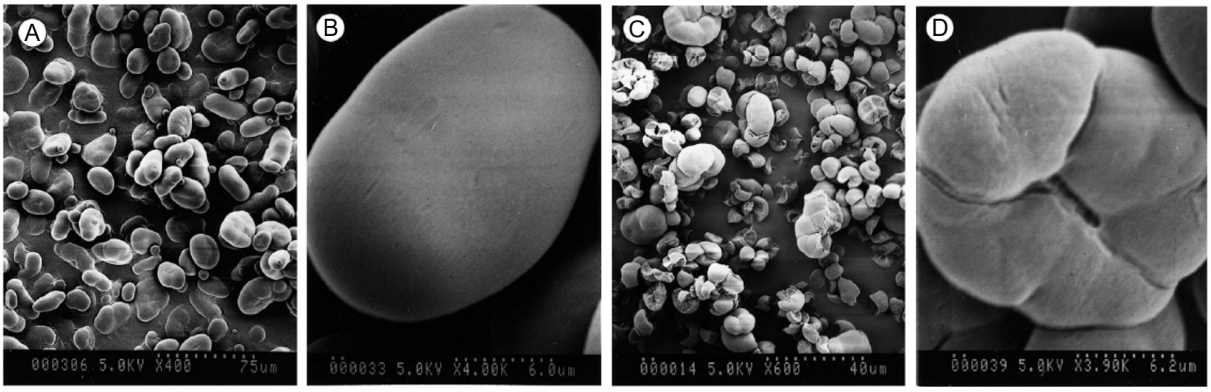

Figure 4. Scanning electron micrographs of native (A-B) smooth pea and (C-D) wrinkled pea (adapted from Zhou *et al.* (2004).

Table 1. Granular characteristics of starches from pea, wheat and maize.

	Pea [1-7]		Wheat [8-15]		Maize [4,8,9,11,13,15-19]
	Smooth	**Wrinkled**			
Shape	Oval, round, irregular	Round, irregular, compound	Lenticular (A-type)	Spherical (B-type)	Spherical, polyhedral
Size [µm]	2 – 50	10 – 40	10 – 35	2 – 10	2 – 30
Distribution	Unimodal	Unimodal	Bimodal		Unimodal
Amylose content[a] [%]	24 – 50	61 – 88	22 – 27		24 – 29
Amylose:Amylopectin ratio	1:1 – 1:3	2:1 – 7:1	1:3		1:3
Crystallinity[b] [%]	16 – 37	18	20 – 39		18 – 43
Crystalline pattern	C	B	A		A
B polymorph [%]	12 – 49	91 – 92			

[a] In many studies, the amylose content of starches have been determined by colorimetric methods without prior defatting, i.e. the indicated values are most often apparent amylose contents. Moreover, the iodine complexing ability of long-chain amylopectins has not been taken into account.

[b] Values strongly dependent on starch hydration and analysis technique (Buléon *et al.*, 1998).

[1] Hoover *et al.* (2010); [2] Ratnayake *et al.* (2002); [3] Bertoft *et al.* (1993); [4] Li *et al.* (2019); [5] Davydova *et al.* (1995); [6] Zhou *et al.* (2004); [7] Zhou *et al.* (2019b); [8] Tester *et al.* (2004); [9] Tester *et al.* (1994); [10] Shibanuma *et al.* (1994); [11] Jane (2009); [12] Yoo *et al.* (2002); [13] Buléon *et al.* (1998); [14] Kumar *et al.* (2016); [15] Morrison (1988); [16] Tam *et al.* (2004); [17] Naguleswaran *et al.* (2013); [18] Eckhoff *et al.* (2009); [19] Dhital *et al.* (2011)

In comparison to pea, maize starch granules are typically smaller in size, although a unimodal size distribution from 2 µm to 30 µm has been reported (Chen *et al.*, 2006; Mishra *et al.*, 2006; Tester *et al.*, 2004). Microscopic analysis of this material disclosed spherically to polyhedrally shaped granules with smooth surface or small pores (**Figure 5**) (Jane, 2009; Mishra *et al.*, 2006; Tester *et al.*, 2004). By contrast, a bimodal size distribution can be detected in wheat, composed of large type A granules with lenticular or disk shape (10 – 35 µm) and small type B granules with spherical appearance (2 – 10 µm) (Jane, 2009; Tester *et al.*, 2004). The surface of some type A granules might contain pores, which are absent in type B granules (BeMiller *et al.*, 2009).

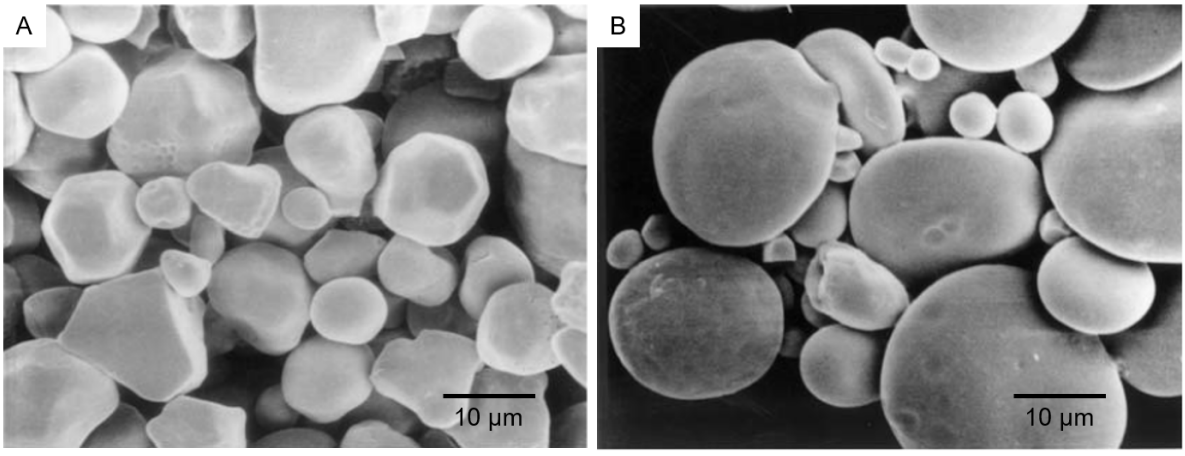

Figure 5. Scanning electron micrographs of (A) normal maize and (B) wheat (adapted from Jane *et al.* (1994)).

2.2 CHARACTERISTICS OF AMYLOSE AND AMYLOPECTIN

Starch is composed of the two *α*-glucans amylose and amylopectin, which differ in number and branching of their glucose units.

2.2.1 AMYLOSE

Amylose represents a relatively long polymer in which D-glucopyranosly residues are linked *via* *α*-(1→4) glucosidic bonds (Hoover *et al.*, 2010; Tester *et al.*, 2004). Although amylose is generally considered a linear molecule forming double helices, some branches might be present that can limit hydrolysis by β-amylase (Jane, 2009). Consequently, ***β-AMYLOLYSIS LIMITS*** of 79 – 87% have been determined for starches from pea, maize and wheat (**Table 2**) (Colonna *et al.*, 1984; Shibanuma *et al.*, 1994; Tester *et al.*, 2004).

In cereal starches, such as wheat and maize, the ***AMYLOSE CONTENT*** typically ranges from 22 – 29% (**Table 1**). By contrast, higher values have been reported for pea starches, amounting to 24 – 88% dependent on the pea seed phenotype (Hoover *et al.*, 2010). In general, wrinkled pea starch contains two to seven times more amylose than smooth pea starch (**Table 1**).

Table 2. Characteristics of pea, wheat and maize amyloses.

	Pea [1-4, 14]		Wheat [5-10,12]	Maize [3,9,11,14]
	Smooth	Wrinkled		
Molecular weight [g/mol]	1.7×10^6 – 2.4×10^6	1.3×10^6	1.9×10^5 – 3.9×10^6	1.1×10^5 – 4.9×10^5
Degree of polymerization	820 – 1400	1000 - 1300	830 – 1570	500 – 1000
Average chain length	340		135 – 270	305
Average chain number			4.8 – 6.5	2.9 – 3.1
β-Amylolysis limit [%]	82 – 87	85	79 – 85	82 – 84
Iodine binding affinity [g/100g]	18.8 – 19.2	18.8	19.0 – 19.9	20.0

[1] Hoover *et al.* (2010); [2] Ratnayake *et al.* (2002); [3] Li *et al.* (2019); [4] Colonna *et al.* (1984); [5] Mukerjea *et al.* (2010); [6] Ong et al. (1994); [7] Hanashiro (2015); [8] Shibanuma *et al.* (1994); [9] Maniñgat *et al.* (2009); [10] Tester *et al.* (2004); [11] Yoo *et al.* (2002); [12] Mua *et al.* (1997); [13] Zhang *et al.* (2017), [14] Ren *et al.* (2021)

The determination of the apparent amylose content is commonly based on the ability of the polymer to bind iodine within the helix to give blue complexes, the absorbance of which is measured by means of spectrophotometric methods (Morrison *et al.*, 1983). For pea, maize and wheat starches, similar ***IODINE BINDING AFFINITIES*** were found, which amount to 18.8 – 20.0 g iodine per 100 g amylose (**Table 2**) (Colonna *et al.*, 1984; Hoover *et al.*, 2010; Shibanuma *et al.*, 1994; Tester *et al.*, 2004). This iodine binding ability, however, is highly affected by the presence of internal starch lipids (Morrison, 1981) (detailed information in **§ 5.2 of Part II**). In case of lipid embedment in the amylose matrix, the iodine binding is considerably reduced. Any determination of the amylose content in the presence of starch lipids is thus giving the so-called ***APPARENT AMYLOSE CONTENT***. According to the literature, the percentage of amylose-lipid complexes in maize (3.8 – 6.7%) and wheat (4.4 – 6.8%) is generally higher compared to smooth pea (4.1%) but not wrinkled pea (9.6%) (Morrison *et al.*, 1983; Zhou *et al.*, 2004). In order to determine the absolute amylose content, also referred to as ***TOTAL AMYLOSE CONTENT***, the extraction of starch lipids is required. Morrison *et al.* (1983) proposed the use of ethanol, followed by the

colorimetric analysis of lipid-free amylose-iodide complexes. The values reported in the literature are often apparent amylose contents.

Apart from a quantitative perspective, amyloses can be characterized by their ***MOLECULAR WEIGHT*** (MW) and ***DEGREE OF POLYMERIZATION*** (DP) as indicated in **Table 2**. While both MW and DP are noticeably higher in pea amylose (1.7×10^6 – 2.4×10^6; 820 – 1400) compared to maize (1.1×10^5 – 4.9×10^5; 500 – 1000), both values appear to be included in the variability range of wheat (1.9×10^5 – 3.9×10^6; 830 – 1570) (Hoover *et al.*, 2010; Mukerjea *et al.*, 2010; Ong *et al.*, 1994; Ratnayake *et al.*, 2002; Ren *et al.*, 2021; Shibanuma *et al.*, 1994; Tester *et al.*, 2004).

2.2.2 AMYLOPECTIN

Amylopectin is characterized by a much larger structure built from *α*-(1→4) linkages (95%) with *α*-(1→6) branch points (5%) (Tester *et al.*, 2004). The polymer is composed of a complex mixture of branch chains categorized into A-, B- and C-chains dependent on their chain length, which are on average considerably shorter compared to amylose (**Table 3**) (Tester *et al.*, 2004). A-chains possess the smallest DP and are located at the exterior part of the starch (Tester *et al.*, 2004). These chains are not branched themselves but bound *via* their reducing end (i.e. *α*-(1→6) linkage) to B-chains or the single C-chain present in the polymer (Hizukuri, 1986; Jane, 2009). By contrast, B-chains are linked to other B-chains or the C-chain through their reducing residues and are branched by A- or B-chains (Hizukuri, 1986; Jane, 2009). The C-chain represents the backbone of the amylopectin as it carries the sole reducing end of the polymer (Jane, 2009; Tester *et al.*, 2004). The branching points of the diverse chains are not randomly distributed but clustered as illustrated in **Figure 6** (Jane, 2009).

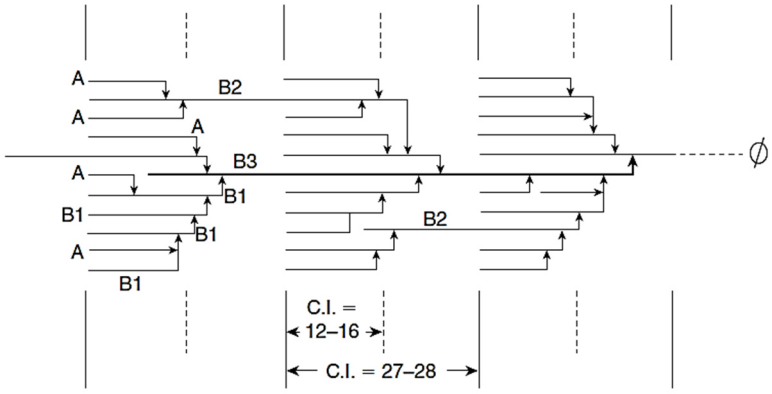

Figure 6. Cluster model of amylopectin proposed by Hizukuri (1986) and adapted by Jane (2009). The chain carrying the reducing end (ø) is the C-chain.

Regions in which short linear chains group are supposed to possess higher crystallinity than those where the chains are interlinked (Jane, 2009). As opposed to A-chains, which can only participate in one chain cluster, B-chains can be part of several clusters (Jane, 2009). For this reason, Hizukuri (1986) proposed to subdivide the B-chains into B_1, B_2, B_3 and B_4 chains, based on their ability to participate in one, two, three or four chain clusters (Tester *et al.*, 2004). Typically, the DP of these chains are 6-12 (A), 13-24 (B_1), 25-36 (B_2) and >37 (B_3) (Hanashiro *et al.*, 1996).

This large and complex structure of amylopectin leads to high ***MOLECULAR WEIGHTS*** in the range of 1.9×10^7 – 9.8×10^7 for pea and maize, however are exceeded by wheat (3.1×10^8) (**Table 3**). Moreover, amylopectins are characterized by high ***DEGREES OF POLYMERIZATION***, which, for instance in maize, can reach values of 15900 (Takeda *et al.*, 2003). Based on the multiple branching of amylopectin, the hydrolysis by β-amylase is highly limited, yielding ***β-AMYLOLYSIS LIMITS*** of 56-61% for pea, wheat and maize starches (**Table 3**).

Table 3. Characteristics of pea, wheat and maize amylopectins.

	Pea [1-5]		**Wheat** [6-12,14,15]	**Maize** [3,10-11,13,15]
	Smooth	**Wrinkled**		
Molecular weight [g/mol]	8.1×10^7	1.9×10^7	3.1×10^8	7.0×10^7 – 9.8×10^7
Branch-chain length distribution				
DP 6 – 12 (A) [%]	14.7 – 25.4	-	16.0 – 27.2	17.9 – 27.0
DP 13 – 24 (B1) [%]	56.1 – 60.4	-	41.7 – 50.2	47.9 – 51.5
DP 25 – 36 (B2) [%]	13.1 – 16.0	-	14.4 – 18.6	12.1 – 14.9
DP >37 (B3) [%]	8.8 – 9.8	-	13.0 – 21.7	9.3 – 19.3
Average chain length	17 – 24	34	23 – 26	20
β-Amylolysis limit [%]	57	61	56 - 59	60
Iodine affinity [g/100g]	0.5 – 1.3	1.7 – 5.3	0.7 – 1.1	1.1 – 1.3

[1] Hoover *et al.* (2010); [2] Ratnayake *et al.* (2002); [3] Li *et al.* (2019); [4] Colonna *et al.* (1984); [5] Chung *et al.* (2008); [6] Mukerjea *et al.* (2010); [7] Ong et al. (1994); [8] Hanashiro (2015); [9] Shibanuma *et al.* (1994); [10] Maniñgat *et al.* (2009); [11] Tester *et al.* (2004); [12] Yoo *et al.* (2002); [13] Mua *et al.* (1997); [14] Zhang *et al.* (2017), [14] Ren *et al.* (2021)

2.3 STARCH STRUCTURE AND POLYMORPHISM

Starch is considered to be a semi-crystalline structure composed of alternating amorphous and crystalline domains arranged in concentric rings of 100 – 400 nm thickness (Pérez *et al.*, 2009; Thakur *et al.*, 2019). This radial organization can be visualized as a dark birefringence pattern, the so-called Maltese cross, under the polarizing microscope. Typically, the short linear chains of amylopectin situated at the exterior part of the polymer form double helices, leading to crystalline segments (Pérez *et al.*, 2009; Tester *et al.*, 2004). On the other hand, the amorphous layers consist of the branching points of longer amylopectin chains as well as amylose, the latter of which appears to be interspersed among the amylopectin molecules (**Figure 7**) (Bertoft, 2017; Jane, 2006).

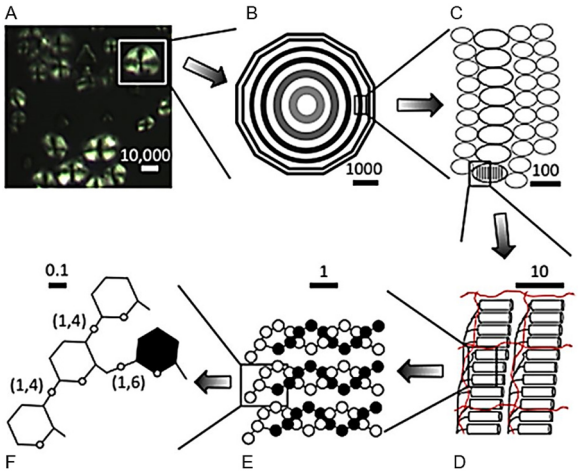

Figure 7. Schematic illustration of the semi-crystalline structure of starch (adapted from Bertoft (2017)). (A) Under polarized light, starch granules show a Maltese cross, which indicates the radial direction of amylose and amylopectin. (B) These polymers form growth rings of alternating amorphous and crystalline layers. (C & D) The crystalline domain is formed by double helices of amylopectin (cylinders), whereas the amorphous domain is formed by branched segments of amylopectin (black lines). Amylose molecules are interspersed among the amylopectin (red lines). (E & F) The amylopectin double helices are composed of two polyglucosyl chains, in which the glucosyl units are linked via α-(1,4)- and α-(1,6)-linkages.

Dependent on the amylopectin branch chain length, three types of starch polymorphs can be distinguished, the average branch chain-length distribution of which can be seen in **Figure 8** (Hoover *et al.*, 2010; Jane, 2006). ***A-TYPE STARCH*** contains densely packed amylopectin of short chain lengths, ***B-TYPE STARCH*** loosely packed amylopectin of longer chain lengths which extend into two or more clusters and ***C-TYPE STARCH*** can be considered a mixture of A- and B-type polymorphic structures.

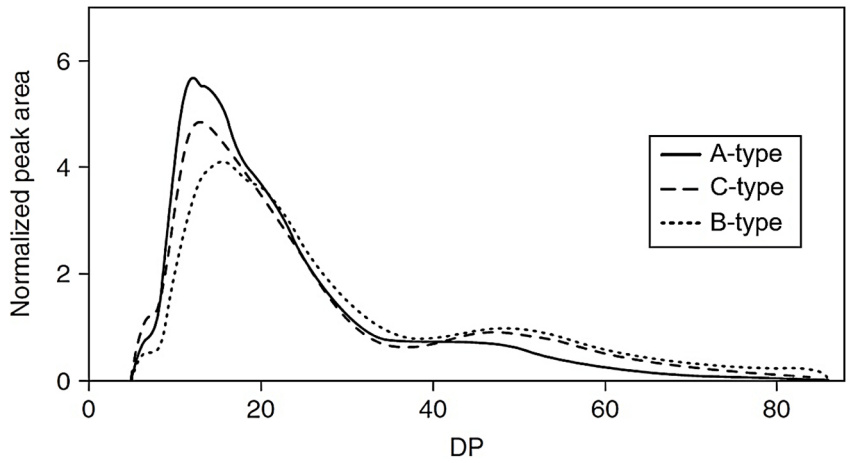

Figure 8. Average chain length distribution of amylopectins isolated from the starches of A-, B- and C-type polymorphism (adapted from Jane (2006)).

In cereal starches like maize and wheat, amylopectin typically crystallizes as A-type starches, whereas the C-type pattern is dominating in pulse starches such as smooth pea (**Table 1**) (Hoover *et al.*, 2010). In the latter case, the A-polymorph (51 – 88%) surrounds the centrally arranged B-polymorph (12 – 49%)

(Hoover *et al.*, 2010). Wrinkled pea starch, however, represents an exception among the pulse starches as it is considered as B-type starch (tuber-type) due to a high percentage of B-polymorph of around 91 – 92% (Hoover *et al.*, 2010). This indicates poor structural organization and a low degree of crystallinity (**Table 1**) (Colonna *et al.*, 1984).

2.4 FUNCTIONAL PROPERTIES

2.4.1 GRANULAR SWELLING AND GELATINIZATION

Heating starch in excess of water leads to the swelling of granules due to the thermally induced disruption of internal hydrogen bonds, which subsequently interact with the water molecules to form new hydrogen bonds (Ratnayake *et al.*, 2002). As a consequence, the polymer's double helices uncoil and dissociate, resulting in the loss of the crystalline order and thus birefringence as well as amylose leaching (Hoover *et al.*, 2010). This order-disorder phase transition is denoted as gelatinization (Hoover *et al.*, 2010).

The extent of ***GRANULAR SWELLING*** can be expressed either as swelling power, which measures both inter- and intragranular water, or as swelling factor, which measures only intergranular water (Hoover *et al.*, 2010). In pulse starches, the imbibing of water is typically induced around 60 °C and increases dramatically as the temperature rises (Chung *et al.*, 2008; Hoover *et al.*, 2010). For instance, Chung *et al.* (2008) reported swelling powers of pea flour in the range of 4.6 g swollen flour/g dry flour (60 °C) to 11.8 g swollen flour/g dry flour (90 °C). By contrast, Tester *et al.* (1990) noticed that lower temperatures of 40 – 50 °C were sufficient to initiate granular swelling in maize and wheat. From this, it can be concluded that a higher thermal energy is needed to loosen the structure of pulse starches compared to cereal starches and this might be attributed to their elevated content of amylose, which are aligned in parallel and form strong hydrogen bonds (Hoover *et al.*, 2010; Hoover *et al.*, 1991). This assumption is corroborated by the determination of relatively lower swelling powers of wrinkled pea starch, which is characterized by considerably higher amylose contents than smooth pea starch (Ratnayake *et al.*, 2002). In addition, this starch contains high levels of amylose-lipid complexes, which are known to restrict granular swelling and solubilization (Ai *et al.*, 2013).

Similarly, ***GELATINIZATION*** occurs over a temperature range characteristic of the starch investigated and is commonly determined by means of a differential scanning calorimeter (DSC) (Hoover *et al.*, 2010). The parameters thus obtained comprise onset (T_O), peak (T_P) and conclusion (T_C) temperatures as well as enthalpy (ΔH) of starch gelatinization. The latter reflects the amount of energy needed to lose the double helical structure and overall crystallinity (Hoover *et al.*, 2010). **Table 4** lists typical values of the aforementioned parameters reported for pea, wheat and maize starches. As described for the granular swelling, gelatinization is likewise affected by the amylose content, distribution of amylopectin chains and amylose-lipid complexes (Jayakody *et al.*, 2007). Moreover, gelatinization temperatures of flours are generally 4 – 15 °C higher than those of starch derived thereof, presumably due to the presence of other compounds, such as ash, lipids, proteins and dietary fibers, which could act as physical barriers against heat and moisture transfer (Wang *et al.*, 2020).

Table 4. Gelatinization and pasting characteristics of pea, wheat and maize starches.

	Pea [1-4,9-12]		Wheat [5-8]	Maize [3,11]
	Smooth	Wrinkled		
Onset temperature T_O [°C]	55 – 64	117	55 – 61	60 – 66
Peak temperature T_P [°C]	62 – 71	133	59 – 66	70 – 70
Conclusion temperature T_C [°C]	73 – 85	138	64 – 70	82 – 84
Gelatinization enthalpy ΔH [J/g]	10 – 14	3	8 – 11	11 – 15
Retrogradation [%]	37 – 50		15 – 27	32
Pasting temperature [°C]	53 – 80		85 – 91	84

[1] Zhou *et al.* (2004); [2] Hoover *et al.* (2010); [3] Li *et al.* (2019); [4] Chung *et al.* (2008); [5] Yoo *et al.* (2002); [6] Tam *et al.* (2004); [7] Zhang *et al.* (2017); [8] Kumar *et al.* (2016); [9] Sandhu *et al.* (2008); [10] Huang *et al.* (2007); [11] Tester (1997)

2.4.2 PASTING PROPERTIES AND RETROGRADATION

After gelatinization has occurred, continuous heating of the starch suspension leads to further swelling and leaching of polysaccharides, resulting in a considerable viscosity increase (Hoover *et al.*, 2010). This change in the viscosity can be monitored using a Rapid Visco Analyzer (RVA) or Brabender Viscoamylograph (BVA) at constant shearing and specific temperature program. Several parameters can be determined from the pasting curve obtained (**Figure 9**), including ***PASTING TEMPERATURE*** (minimal temperature at which an increase in viscosity is noticeable), ***PEAK VISCOSITY*** (maximal viscosity during heating, indicating the water-holding capacity or swelling power of starch), ***TROUGH OR HOLD VISCOSITY*** (viscosity at the end of heating), ***BREAKDOWN*** (difference between peak and hold viscosity, indicating the degree of disintegration caused by shearing and heating), ***FINAL VISCOSITY*** (maximal viscosity at the end of cooling) and ***SETBACK*** (difference between final and peak viscosity, indicating the retrogradation potential during cooling) (Rincón-Londoño *et al.*, 2016; Shafie *et al.*, 2016). These values are often expressed with different units, for which reason their direct comparison remains a difficult task.

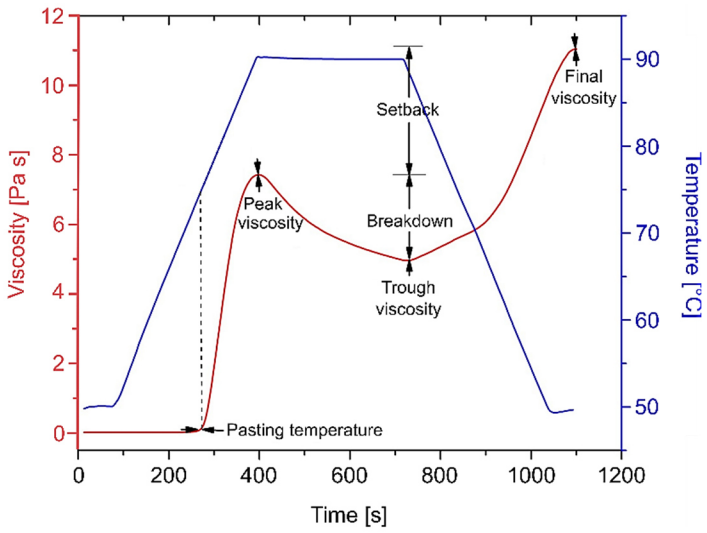

Figure 9. Pasting profile and conventional definitions used in the analysis of pasting properties (adapted from Rincón-Londoño *et al.* (2016)).

The pasting curve of pea starch is typically characterized by pasting temperatures in the range of 52.5 – 79.5 °C, which are exceeded by those of maize and wheat starches (>84 °C) (**Table 4**), attributable to the elevated content of amylose-lipid complexes in the cereals that limit granular swelling (Ai *et al.*, 2013). Moreover, pea starch displays a comparatively lower peak viscosity, which might be a consequence of its elevated amylose content, and thus low portion of amylopectin (Li *et al.*, 2019). While swelling and pasting are mainly properties of amylopectin, amylose is rather considered to be a diluent, which limits granular swelling owing to the development of strong internal hydrogen bonds (Hoover *et al.*, 2010; Hoover *et al.*, 1991; Tester *et al.*, 1990).

The percentual dominance of amylose in pea starch furthermore seems to affect the trough viscosity, which has been observed to deviate only slightly from the peak viscosity, indicating the absence of breakdown (Hoover *et al.*, 2010; Li *et al.*, 2019). By contrast, considerable drops in the viscosity appear in pasting curves of cereal starches, such as wheat and maize, which are correlated to a pronounced breakdown (Li *et al.*, 2019; Yoo *et al.*, 2002). This emphasizes the remarkable potential of amylose to maintain the integrity of the swollen granules under shear, leading to a higher resistance to disintegration in pea starch.

Lastly, the high levels of amylose in pea starch equips the pulse with the improved ability to re-associate during cooling, hence contributing to the formation of a rigid network and high viscosity (Li *et al.*, 2019). In fact, cooling of the gelatinized paste is typically accompanied by the formation of molecular interactions, i.e. hydrogen bonding, within the starch polymers, which lead to a partially ordered structure (Hoover *et al.*, 2010; Miles *et al.*, 1985; Wang *et al.*, 2015). This phenomenon is denoted as ***RETROGRADATION***, to which mainly amylose contributes. According to Miles *et al.* (1985), amylose rapidly forms strong interchain associations due to its linearity, many of which resist heating to 100 °C. At starch concentrations above 6%, a gel is formed, which is characterized by high elasticity and strength against deformation (Goesaert *et al.*, 2005; Wang *et al.*, 2015). Nevertheless, re-crystallization of amylopectin occurs as well, however over a longer storage period of several days or weeks (Goesaert *et al.*, 2005; Wang *et al.*, 2015). This long-term development of crystallinity is often responsible for the staling of bakery products (Wang *et al.*, 2015). These amylopectin crystallites melt during subsequent heating to 100 °C (Miles *et al.*, 1985). A schematic representation of the pasting and retrogradation processes are illustrated in **Figure 10**.

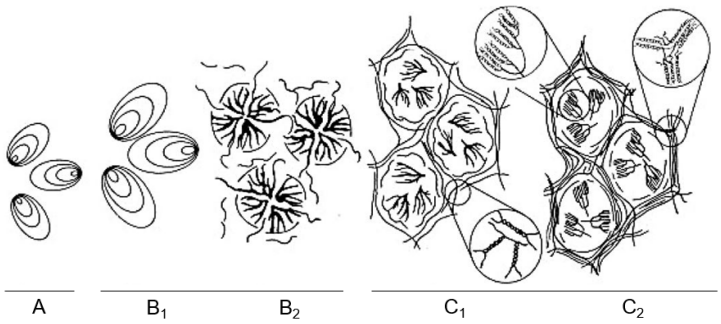

Figure 10. Schematic representation of the pasting and retrogradation processes occurring during heating and cooling of starch in excess water (adapted from Goesaert *et al.* (2005)). (A) Native starch granule; (B) Gelatinization upon heating associated with (B_1) swelling and (B_2) amylose leaching; (C) Retrogradation upon cooling, leading to the formation of (C_1) an amylose network and (C_2) ordered or crystalline amylopectin molecules.

Apart from the increase in firmness, retrogradation is correlated with the exudation of water (syneresis) as well as the appearance of X-ray diffraction patters of the B-type (Hoover *et al.*, 2010; Miles *et al.*, 1985). The retrogradation can be determined using many techniques and devices, such as rheology, texturizer, DSC, turbidimetry, syneresis, X-ray diffraction, nuclear magnetic resonance, Raman spectroscopy and Fourier transform infra-red spectroscopy (Hoover *et al.*, 2010). Retrogradation values reported in the literature therefore rather help to identify tendencies than absolute differences between different products. In general, it appears that pulse starches tend to retrograde stronger than cereal starches (**Table 4**), which might be assigned to their higher amylose content and/or molecular structure (Hoover *et al.*, 2010; Li *et al.*, 2019).

2.5 RAPIDLY DIGESTIBLE, SLOWLY DIGESTIBLE AND RESISTANT STARCH

After consumption, starch is digested in the gastrointestinal tract by a mixture of enzymes, comprising amylase, sucrose isomaltase and glucoamylase (Bede *et al.*, 2021). Depending on the rate of hydrolysis as well as the absorption in the gastrointestinal tract, starch can be classified into three fractions (Chung *et al.*, 2009a).

(i) ***RAPIDLY DIGESTIBLE STARCH*** (RDS) is mainly composed of amorphous and dispersed starch that is immediately hydrolyzed into glucose, and thus leads to an instant increase in blood glucose level (Jeong *et al.*, 2019).

(ii) ***SLOWLY DIGESTIBLE STARCH*** (SD) is mainly rigid amorphous and imperfect crystalline starch that is completely digested, however releasing glucose at a slower rate into the blood compared to RDS, hence improving satiety and diabetes management (Chung *et al.*, 2009a; Jeong *et al.*, 2019).

(iii) ***RESISTANT STARCH*** (RS) is mainly crystalline starch that remains resistant to enzymatic hydrolysis in the small intestine and is fermented in the colon of the large intestine by gut microflora (Bede *et al.*, 2021; Englyst *et al.*, 1990; Hoover *et al.*, 2003; Jeong *et al.*, 2019; Mason, 2009; Robinson *et al.*, 2021).

Based on the mentioned digestion characteristics, resistant starch is considered a type of dietary fiber with beneficial physiological and functional properties, which will be discussed in the following paragraphs.

2.5.1 TYPES OF RESISTANT STARCH

Five types of RS can be distinguished. ***RS1*** is physically inaccessible to enzymes because of its entrapment in structures like intact cells in grains, seeds and tubers, which cannot be degraded due to the lack of cell wall degrading enzymes in the gastrointestinal tract (Bede *et al.*, 2021; Hoover *et al.*, 2003; Raigond *et al.*, 2015). ***RS2*** are native and uncooked starch granules, which resist enzymatic hydrolysis owing to a high degree of crystallinity or amylose content, leading to a compact structure (Raigond *et al.*, 2015). ***RS3*** is retrograded non-granular starch, mainly retrograded amylose, formed during cooling of gelatinized starch by polymer chain reassociation (Ashwar *et al.*, 2016; Homayouni *et al.*, 2014; Raigond *et al.*, 2015). ***RS4*** is chemically modified starch developed in the laboratory by substitution, conversion or cross-linking, the artificial bonds of which impede enzymatic attack (Ashwar

et al., 2016; Bede *et al.*, 2021). ***RS5*** are amylose-lipid complexes and resistant maltodextrins naturally present in the raw materials (Bede *et al.*, 2021; Bello-Perez *et al.*, 2020).

2.5.2 FUNCTIONAL AND HEALTH PROPERTIES OF RESISTANT STARCH

RS is known to have attractive physicochemical properties, including swelling, viscosity increase, gelling and water-binding capacity (Raigond *et al.*, 2015). In comparison to traditional fibers, RS might improve structure, tenderness, flavor and color of food products, such as baked goods (Ashwar *et al.*, 2016; Bede *et al.*, 2021).

Apart from their functional properties, the high resistance of RS to enzymatic hydrolysis in the small intestine as well as fermentation in the colon makes them attractive food ingredients from a health point of view. The health benefits associated with RS include weight management, hypercholesterolemic and hyperglycemia control, colonic cancer prevention, fat accumulation inhibition, reduced bile stone formation and improved mineral absorption (Ashwar *et al.*, 2016; Bede *et al.*, 2021).

2.5.3 CONTENTS OF RESISTANT STARCH

It is well known that starches with B-type polymorph possess higher resistance to enzymatic attack than A-type polymorphs (Ashwar *et al.*, 2016; Raigond *et al.*, 2015). The latter type mainly contains amylopectin with short branch chains, which are limited to form stable double helices, and thus can be easily hydrolyzed by enzymes (Jeong *et al.*, 2019). By contrast, longer amylopectin chains as present in B-type starch are able to crystallize upon double helix formation, leading to an enlarged crystal lattice, which is resistant to depolymerization (Jeong *et al.*, 2019). Consistently, legumes containing C-type starch, which combine A- and B-type structures, are less vulnerable to degradation compared to A-type starch of maize and wheat (Bede *et al.*, 2021). Moreover, a higher amylose content is considered to contribute to RS formation, attributable to the organization of long amylose chains into double helices (Ashwar *et al.*, 2016).

In pea seeds, the content of RS was found the vary between 10% and 15% (Chung *et al.*, 2008; Goñi *et al.*, 1996; Li *et al.*, 2019). These values were considerably enhanced in purified pea starch, amounting to 41 – 85% (Jeong *et al.*, 2019). By contrast, wheat flour typically contains less than 1% resistant starch (Goñi *et al.*, 1996).

2.6 DAMAGED STARCH

During flour preparation, the starch contained in the seeds and grains is subjected to shear, collision and friction forces, which can result in granule damage (Wang *et al.*, 2020). The major factors impacting the degree of damage include raw materials hardness, starch composition, flour coarseness and milling conditions (Wang *et al.*, 2020). According to Xu *et al.* (2018), waxy wheat flour lacking amylose contained higher contents of damaged starch (DS) contents than wild wheat at similar hardness. From this, it can be concluded that amorphous amylose might be less susceptible to mechanical disruption due to its higher elasticity compared to the fragile semi-crystalline amylopectin (Wang *et al.*, 2020). Moreover, Liu *et al.* (2016) reported higher damaged starch levels with reducing particle size of whole wheat flours.

Different methods for DS analysis are available, which include the determination of iodine absorption. As discussed in **§ 2.2 of Part II**, the helical conformation of amylose chains allows the complexation of

iodine. With increasing DS, these amylose structures are easily accessible, resulting in an increased iodine absorption (Monnet *et al.*, 2019).

2.6.1 EFFECT ON STRUCTURE

By contrast to native and intact starch, the ***GRANULAR STRUCTURE*** of DS might be rough, distorted and deformed with possible fissures, cracks, pores or grooves on the surface (Wang *et al.*, 2020). Furthermore, the ***CRYSTALLINITY*** of DS is typically affected, although the polymorphic type mostly remains unchanged (Wang *et al.*, 2020). On molecular level, reductions in the average ***MOLECULAR WEIGHT*** of maize starch have been reported as a consequence of starch damage and could be attributed to the successive conversion of amylopectin into amylose (Shi *et al.*, 2015). According to Wu *et al.* (2018), however, such distinct alteration in molecular weight were not monitored in wheat starch at varying degrees of DS. Nevertheless, the authors reported a shift of the ***CHAIN LENGTH DISTRIBUTION*** towards shorter chains, which indicated fracturing of the amylopectin molecules (Wu *et al.*, 2018). On basis of these findings, it can be concluded that the source of the starch and its particular composition plays an important role in its susceptibility to mechanical damage.

2.6.2 EFFECT ON FUNCTIONALITY

Due to the degradation of starch polymers during damage, being associated with a disruption of crystalline regions, less energy is required to gelatinize DS, which typically leads to a decrease of the onset ***GELATINIZATION TEMPERATURE*** (Shi *et al.*, 2016; Wang *et al.*, 2020). However, high degrees of SD might result in an increase in the gelatinization temperature, attributable to the aggregation of starch granules, which impede granule swelling (Wang *et al.*, 2020).

According to Barrera *et al.* (2013) and Liu *et al.* (2017), a high degree of DS is accompanied with lower ***PEAK VISCOSITY*** and ***PEAK TEMPERATURE*** of the starch solution. This can be assigned not only to the simplified permeability and diffusion of water through the fractured surface of the granules but also to the decrease of crystallinity, which presumably results in a reduced thermal stability of starch (Wang *et al.*, 2020). Although DS granules exhibit a high ability to hydrate and swell, they are more prone to be deformed during heating and shearing. This leads to a reduced volume fraction of swollen granules and an elevated level of unbound water, both leading to a lower peak viscosity (Barrera *et al.*, 2013). The authors also stated that more DS led to a decreased ***BREAKDOWN*** and ***SETBACK*** (Barrera *et al.*, 2013).

These characteristics have been found to affect the quality of bakery products. Kang *et al.* (2015) reported an increase in the batter hardness during gluten-free bread development with increasing DS content, which could be assigned to the elevated ***WATER HOLDING CAPACITY***. Moreover, León *et al.* (2006) observed an increasing bread crumb hardness with enhanced level of DS, attributable to a higher degree of retrogradation.

2.6.3 EFFECT ON ENZYMATIC DIGESTIBILITY

According to the general consensus in the literature, an increasing DS content is accompanied by a greater degree of enzymatic digestibility (Dhital *et al.*, 2010a; Yu *et al.*, 2015). Both the presence of irregularities in the damaged granule as well as the increase in the amorphous, and thus more flexible domain, pave the way for enzymes to penetrate (Almeida *et al.*, 2019; Lv *et al.*, 2019). In addition, DS

granules tend to be less associated with macromolecules, such as proteins, which could act as protective layer against enzymatic attack (Angelidis *et al.*, 2016).

2.6.4 EFFECT ON REACTIVITY

DS typically contains a higher amount of reducing sugars which are freely available and might undergo changes *via* Maillard reaction during baking, leading to the browning of the products (Wang *et al.*, 2020). Furthermore, a positive correlation between the degree of DS and acrylamide content has been reported in bread (Wang *et al.*, 2017b).

3 PROTEINS

3.1 CONTENT AND COMPOSITION

The protein content in pea ranges between 14% and 31%, and is thus approximately double the amount found in cereals, such as wheat, where it typically averages around 8% (**Figure 3**). Pea proteins exhibit a favorable amino acid composition due to their high levels of lysine, which complements and balances the profile in cereals (**Table 5**) (Asif *et al.*, 2013).

The main protein fraction of peas are storage proteins, accumulating during seed development in membrane-bound organelles of cotyledonary parenchyma cells named protein bodies (Foschia *et al.*, 2017). According to Osborne (1907), these proteins are typically divided into albumins, globulins, prolamins and glutelins based on their solubility. An overview of the percentage distribution of mentioned fractions as well as their sizes in wheat and pea is given in **Figure 11**.

In pea, salt-extractable globulins account for the majority of proteins (44 – 70%), followed by water-soluble albumins (15 – 25%), alkali-soluble glutelins (1 – 11%) and ethanol-soluble prolamins (0 – 5%) (Foschia *et al.*, 2017; Hall *et al.*, 2017). By contrast, a mirror-inverted profile of Osborne classes has been documented for wheat, the protein of which consists mainly of glutelins (22 – 50%) and prolamins (35 – 80%), while albumins and globulins are only marginally present (Eliasson *et al.*, 1993; Feillet, 2000; Foschia *et al.*, 2017).

In wheat, glutelins are named glutenins and the prolamins are named gliadins, which combined are referred to as gluten. Glutenins have MWs in the range of 12 – 130 kDa and participate in the formation of disulfide bonds, which can result in structures with MWs ranging into the millions (Damodaran, 2017). Gliadins, on the other hand, can be divided into four groups of *α*-, *β*-, *γ*- and *ω*-gliadins, the MWs of which vary between 30 and 80 kDa (Damodaran, 2017). As opposed to glutenins, these polymers do not interact with other proteins but solely form intramolecular disulfide bonds (Damodaran, 2017).

Due to the predominance of globulins and albumins in pea, they are discussed in detail in the following sections.

Table 5. Amino acid composition of pea and wheat.

	Pea [1] [g/100 g protein]	Wheat [2-4] [g/100 g protein]
Essential amino acids		
Histidine	2.6 – 3.2	2.2 – 3.9
Isoleucine	4.6 – 5.4	2.5 – 4.5
Leucine	7.0 – 8.1	4.9 – 7.6
Lysine	7.2 – 8.2	2.0 – 3.3
Methionine + Cysteine	2.8 – 4.0	2.6 – 3.9
Phenylalanine + Tyrosine	7.1 – 8.8	5.9 – 7.6
Threonine	3.5 – 4.1	2.4 – 3.5
Tryptophan	0.7 – 1.3	0.9 – 1.1
Valine	4.8 – 5.6	3.4 – 6.6
Non-essential amino acids		
Alanine	4.2 – 4.9	2.5 – 5.2
Arginine	6.7 – 9.8	1.3 – 4.8
Aspartic acid	11.1 – 12.5	2.8 – 5.5
Glutamic acid	15.2 – 17.6	24.8 – 37.1
Glycine	4.1 – 4.7	2.4 – 6.7
Proline	4.0 – 4.5	6.8 – 14.3
Serine	3.6 – 4.6	2.8 – 5.5

[1] Wang *et al.* (2004); [2] Ewart (1967); [3] Jood *et al.* (1995); [4] Jiang *et al.* (2008)

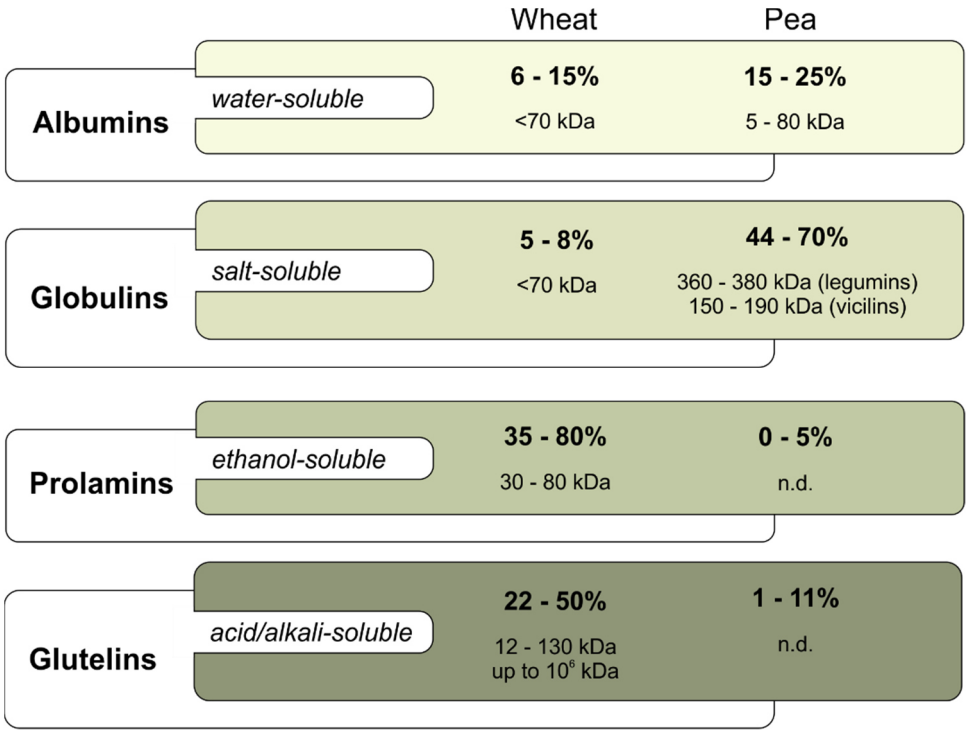

Figure 11. Proportions of Osborne protein fractions and their sizes identified in wheat and pea. Values are based on Boye *et al.* (2010a), Damodaran (2017), Eliasson *et al.* (1993), Feillet (2000) Foschia *et al.* (2017), Hall *et al.* (2017), Tiwari *et al.* (2011) and Veraverbeke *et al.* (2002). n.d. = not determined.

3.1.1 PEA GLOBULINS

Globulins are rich in arginine, phenylalanine, leucine and isoleucine (Swanson, 1990). Furthermore, they possess a rigid structure, which can be assigned to the presence of disulfide bonds and hydrophobic interactions, which limit their solubility in aqueous media (Oomah *et al.*, 2011; Singh, 2017). Two classes of globulins have been identified in pea, which are differentiated by their sedimentation coefficients in ***LEGUMINS*** (11S) and ***VICILINS*** (7S) (Croy *et al.*, 1980). The legumin fraction is characterized by a hexameric quaternary structure composed of three pairs of disulfide-bonded acidic (40 kDa) and basic (20 kDa) subunits, leading to a total molecular weight around 360 – 380 kDa (Boye *et al.*, 2010a; Oomah *et al.*, 2011). Vicilins, on the other hand, possess a trimeric structure with molecular weights of 150 – 190 kDa (Boye *et al.*, 2010a; Oomah *et al.*, 2011). The ratio of legumin to vicilin was found to vary between 0.6 and 3.7 for smooth pea cultivars, whereas higher ratios of up to 6.2 were identified in wrinkled peas (Gueguen *et al.*, 1988). Aside from these, Croy *et al.* (1980) identified a third storage protein named ***CONVICILIN*** with a molecular mass of 290 kDa, which contains little carbohydrate and has a subunit of 71 kDa (Boye *et al.*, 2010a; Foschia *et al.*, 2017). However, the consideration of this structure as separate globulin was countermanded by O'Kane *et al.* (2004), who proposed to consider convicilin as a subunit of vicilin.

3.1.2 PEA ALBUMINS

Compared to globulins, albumins are characterized by a high content of sulfur-containing amino acids, such as cysteine and methionine, as well as tryptophan, lysine and threonine (Swanson, 1990). This fraction generally includes enzymes, proteases, amylase inhibitors and lectins with hydrophilic surfaces and relatively low molecular weights ranging between 5 kDa and 80 kDa, hence enabling their water solubility (Boye *et al.*, 2010a; Oomah *et al.*, 2011). Some of these are considered anti-nutritive factors. Among over 1000 different structures, two major albumins have been reported for pea, which account for 34% of the total albumin fraction present (Schroeder, 1984). While the first possesses polypeptides of 22 – 25 kDa, the second is formed from polypeptides of 6 – 8 kDa (Boye *et al.*, 2010a; Schroeder, 1984).

The differences in protein and amino acid composition between wheat and pea can affect the proteins' functional properties, which will be described in detail in the following sections.

3.2 FUNCTIONAL PROPERTIES

3.2.1 SOLUBILITY

Solubility of proteins is based on the interaction of hydrophilic amino acids on the surface of proteins with surrounding water, which is strongly affected by the system conditions, such as the ***PH VALUE*** (Boye *et al.*, 2010a; Foschia *et al.*, 2017). In the case of pea proteins, an inverted bell-shaped solubility curve can be observed (**Figure 12**), with highest values at both acidic (pH 1 – 3) and alkaline conditions (pH 7 – 10) and lowest values near the isoelectric point (pH 4 – 6) (Boye *et al.*, 2010b; Tiwari *et al.*, 2011). In the latter pH range, proteins carry a zero net charge, implying minimal ionic hydration and electric repulsion between proteins, which lead to protein precipitation (Lam *et al.*, 2018; Tiwari *et al.*, 2011). Interestingly, the solubility curve of wheat gluten exhibits a similar U-shape, but its minimum is shifted toward higher pH values (pH 6 – 8) (Mimouni *et al.*, 1994). This emphasizes the different amino

acid composition of wheat gluten and pea protein, which will affect their further functional properties, such as emulsifying and foaming.

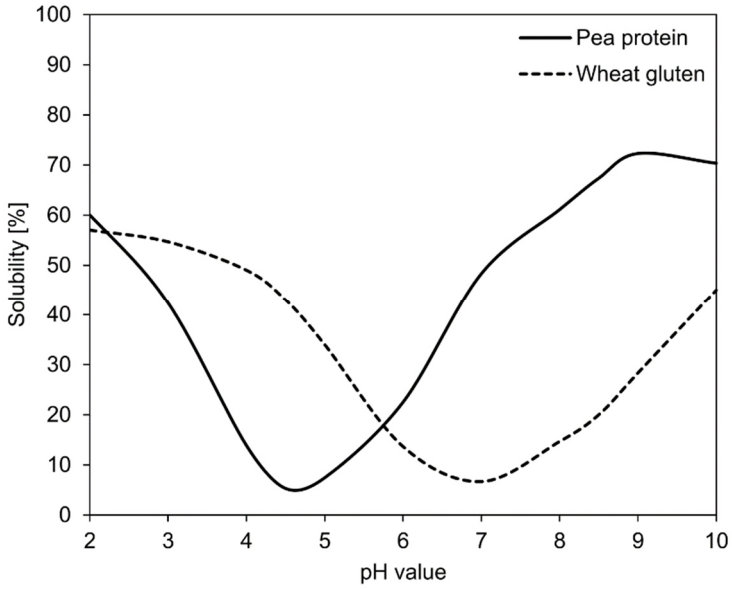

Figure 12. pH-dependent solubility of pea proteins and wheat gluten. The graph on pea protein solubility represents the mean of the results reported by Boye *et al.* (2010b), Shand *et al.* (2007), Tanger *et al.* (2021) and Vose (1980). The graph on wheat gluten represents the mean of the results reported by Kong *et al.* (2007), Mimouni *et al.* (1994) and Wang *et al.* (2006).

The solubility of proteins is likewise influenced by the ***IONIC STRENGTH*** of the medium. In the case of pea proteins, an increase in the salt concentration has been shown to improve solubility at the isoelectric point, presumably due to the dominance of globulins with high salt solubility (Lam *et al.*, 2018; Tanger *et al.*, 2021). At the same time, the presence of salts results in a decrease in the solubility in the acidic and alkaline range (Tanger *et al.*, 2021). This can be attributed to the shielding of protein charges, which leads to a reduction of electrostatic repulsion and thus to protein aggregation (Lam *et al.*, 2018). Moreover, ***THERMAL TREATMENT*** is considered to be an important factor that can alter protein solubility. Bogahawaththa *et al.* (2019) observed a visible decrease in pea protein solubility after heating for 2.8 min at 121 °C. A similar tendency was recorded by Wang *et al.* (2006), who studied the solubility of wheat gluten after heating for 20 min at temperatures varying from 50 °C to 90 °C. Such findings can be explained by the denaturation of proteins induced at temperatures above 40 °C, causing the polymer to unfold and expose hydrophobic groups (Bogahawaththa *et al.*, 2019; Damodaran, 2017). These nonpolar residues can participate in hydrophobic and covalent interactions, which promotes protein precipitation (Bogahawaththa *et al.*, 2019).

3.2.2 WATER HOLDING CAPACITY

Water holding capacity (WHC) is defined as the amount of water that can be absorbed per gram protein ingredient during the application of forces, pressing, centrifugation or heating (Boye *et al.*, 2010a; Zayas, 1997e). Alternatively, it can be denoted as the ability of protein material to retain water against gravity

(Lam *et al.*, 2018). The noun is likewise expressed as water binding capacity, water absorption capacity and hydration capacity (Lam *et al.*, 2018).

WHC is an essential characteristic of proteins in structured food products, such as baked goods or meat analogues, due to the prevention of water loss (syneresis), which helps to maintain mouthfeel, texture and flavor binding (Daba *et al.*, 2021; Vatansever *et al.*, 2020; Zayas, 1997e). Lower WHC have been observed for wheat gluten (0.7 – 1.6 g/g) in comparison to pea proteins (1.9 – 4.8 g/g), which might be attributable to their different ratio of hydrophobic to hydrophilic residues (protein concentration in both cases ≥70%) (Boye *et al.*, 2010b; Daba *et al.*, 2021; Deng *et al.*, 2016; Fernández-Quintela *et al.*, 1997; Foschia *et al.*, 2017; Fuhrmeister *et al.*, 2003; Lam *et al.*, 2018; Naczk *et al.*, 1986; Shevkani *et al.*, 2015; Sosulski *et al.*, 1987; Stone *et al.*, 2015; Withana-Gamage *et al.*, 2011).

Apart from the amino acid composition and conformation of proteins, several other factors have been identified to impact WHC. In logical agreement with the solubility behavior of proteins as a function of the ***PH VALUE***, lowest WHC can be measured at the isoelectric point at which the electrical repulsion between the proteins is minimal (Tiwari *et al.*, 2011; Zayas, 1997e). Furthermore, ***THERMAL TREATMENT*** can generally improve water absorption owing to the dissociation of proteins and exposure of hydrophilic groups (Han *et al.*, 1990; Lam *et al.*, 2018; Swanson, 1990). Consistently, Sosulski *et al.* (1987) reported increments in the WHC of pea proteins with increasing severity of heat processing (2.5 g/g at 20 °C and 3.0 g/g at 70 °C for isolated pea proteins). In addition, an elevated ***IONIC STRENGTH*** might trigger the competition of present ions with protein for available water, thus resulting in protein dehydration (Zayas, 1997e). Nevertheless, Naczk *et al.* (1986) noticed constant WHCs for wheat gluten and pea proteins over a salt concentration range from 0 M to 1 M. Moreover, the WHC appears to be determined by the purity of the protein material, which can be correlated with the absolute ***PROTEIN CONTENT***. According to Sosulski *et al.* (1987), pea protein isolate (80% protein) exhibits a higher ability to bind water (2.5 g/g) than pea protein concentrate (47% protein, 1.1 g/g) and pea flour (25% protein, 0.8 g/g) at ambient temperature.

3.2.3 OIL HOLDING CAPACITY

Oil holding capacity (OHC), also referred to as oil absorption capacity, is defined as the amount of oil/fat absorbed per weight of protein ingredient by non-polar amino acids (Foschia *et al.*, 2017; Lam *et al.*, 2018; Zayas, 1997d). OHC plays a crucial role in products like baked goods, meat replacers and soups as it relates to the emulsifying capacity of proteins, and thus affects the food's sensory properties, including texture, mouthfeel and flavor retention (Kaur *et al.*, 2007b; Lam *et al.*, 2018; Shevkani *et al.*, 2015; Vatansever *et al.*, 2020; Withana-Gamage *et al.*, 2011).

The OHC for pea proteins (concentration ≥70%) reported in the literature vary markedly (0.9 – 7.2 g/g), which can be assigned to the strong dependence of the oil binding on the protein extraction method applied (Boye *et al.*, 2010b; Daba *et al.*, 2021; Fernández-Quintela *et al.*, 1997; Fuhrmeister *et al.*, 2003; Lam *et al.*, 2018; Naczk *et al.*, 1986; Shevkani *et al.*, 2015; Sosulski *et al.*, 1987; Stone *et al.*, 2015; Withana-Gamage *et al.*, 2011). Nevertheless, pea proteins generally exhibit similar or higher OHC than wheat gluten (1.0 – 1.7 g/g), presumably due to the presence of numerous non-polar amino acids (Deng *et al.*, 2016; Foschia *et al.*, 2017; Naczk *et al.*, 1986).

In addition to the protein characteristics (e.g. amino acid composition, surface hydrophobicity) and method of preparation, the capacity of proteins to retain oil can be altered by the ***PROTEIN CONTENT***. As indicated by Sosulski *et al.* (1987), higher OHC are obtained with isolated pea proteins (80% protein, 1.0 g/g), followed by pea concentrates (47% protein, 0.6 g/g) and pea flour (25% protein, 0.4 g/g). By contrast, the same authors observed no adverse effect of ***THERMAL TREATMENT*** at 70 °C for 10 min on OHC of pea proteins (Sosulski *et al.*, 1987). However, more intense heat processing might favor oil binding as proteins denature and expose the hydrophobic groups from the interior of the molecule (Abbey *et al.*, 1987).

3.2.4 FOAMING CAPACITY AND FOAM STABILITY

Foams are composed of an aqueous continuous phase in which air is dispersed (Damodaran, 2017). These systems are thermodynamically unstable owing to the high free energy at the air-water interface (Lam *et al.*, 2018). In the absence of surface-active molecules, the gas cells tend to reduce the interfacial tension by fusion to form bigger bubbles. Solubilized proteins can counteract these destabilization mechanisms by accumulation at the interface to lower the surface tension. Upon adsorption, the proteins unfold and direct their hydrophilic groups towards the water and hydrophobic residues towards the air, thereby forming a protective continuous film (**Figure 13**) (Zayas, 1997b). High molecular flexibility equips the protein with the ability to diffuse rapidly to the gas-liquid interface and prevent foam collapse.

The most frequently used indices to measure foaming properties are foaming capacity (FC) and foam stability (FS) (Foschia *et al.*, 2017). FC represents the relative volume increase of a protein solution resulting from air inclusion, whereas FS indicates the ability of the system to retain air bubbles during stress (Kiosseoglou *et al.*, 2011; Lam *et al.*, 2018). Both are key parameters in food applications such as cakes, mousses, muffins, gluten-free breads and beverages (Boye *et al.*, 2010a; Foschia *et al.*, 2017).

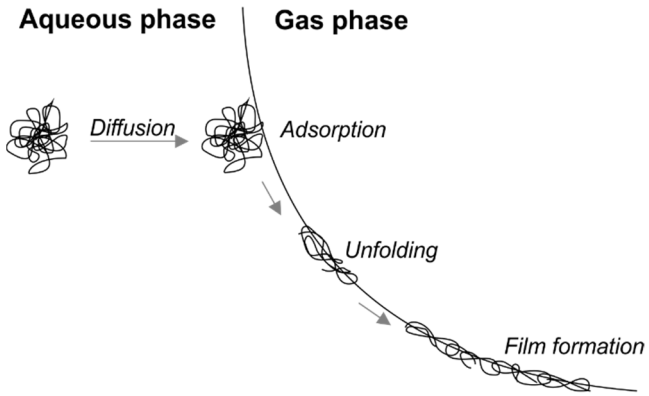

Figure 13. Schematic representation of air-liquid interface stabilization by proteins. The native protein adsorbs at the interface, unfolds and directs the polar groups towards the water and hydrophobic residues towards the air. Interaction with other proteins leads to the formation of a stable, continuous film.

Comparison of available FC and FS data for pea proteins and wheat gluten appears to be a difficult task based on the application of different methods with varying whipping times (typically 2 – 6 min), aging periods (typically 30 – 60 min) and sample concentrations (3 – 10%). Nevertheless, **Table 6** lists some values reported in the literature. From this overview, it emerges that wheat gluten leads to considerably lower FC (29 – 70%) and FS (25 – 38%) at pH 7 compared to pea proteins (87 – 429% and 40 – 100%,

respectively). The latter, however, exhibit lower FC and FS compared to egg white, for which values of 472% and 100%, respectively, have been measured by Fuhrmeister *et al.* (2003). This indicates high flexibility of egg white proteins as well as pea proteins in comparison to wheat gluten, potentially due to different molecular weights and percentage of inflexible disulfide bonds.

Table 6. Foaming properties of wheat gluten and pea proteins (protein content ≥70%). Measurements were carried out at pH 7.

Protein source	Foaming capacity [%]	Foam stability [%]	Whipping time [min]	Concentration of suspension [%]	Aging time [min]	Reference
Wheat	29	25	5	5	60	Wang *et al.* (2006)
Wheat	70	38	5	10	60	Zhang *et al.* (2011)
Pea	351 – 429	40 – 100	5	7	60	Fuhrmeister *et al.* (2003)
Pea	n.d.	50 – 67	6.5	3	60	Sosulski *et al.* (1987)
Pea	133 – 263	53 – 78	5	1	30	Stone *et al.* (2015)
Pea	87 – 132	94 – 96	2	1	30	Shevkani *et al.* (2015)

n.d. = not determined

Foaming properties are furthermore dictated by the ***TOTAL HYDROPHOBICITY*** of the proteins. Kato *et al.* (1983) established a positive correlation between average protein hydrophobicity and foam stability. This implies, that the ability of proteins to denature and unmask their non-polar groups at the air-water interface is of greater importance than the surface hydrophobicity of the native protein. In addition, foamability might be improved by partial denaturation of proteins through moderate ***THERMAL TREATMENT*** due to the exposure of hydrophobic residues (Lam *et al.*, 2018; Pozani *et al.*, 2002; Zayas, 1997b). However, excessive heating can cause a decline in FC and FS, which can be ascribed to protein coagulation and aggregation (Abbey *et al.*, 1987; Zayas, 1997b). Such adverse changes have been observed for pea proteins that were processed at 80 – 100 °C for 30 min (Chao *et al.*, 2018) or 90 °C for 20 min (Ma *et al.*, 2011). Apart from temperature, the ***PH VALUE*** of the medium appears to be a relevant factor influencing foaming properties. Several authors reported that FS is the highest at the proteins' isoelectric point owing to minimal electrostatic repulsion, which maximizes protein-protein interactions at the interface (Lam *et al.*, 2018; Zayas, 1997b). However, contradicting results have been reported by Adebiyi *et al.* (2011) on pea proteins, which exhibited higher FC and FS at pH 7 and pH 9 compared to pH 4. In agreement with these findings, Shevkani *et al.* (2015) likewise detected lowest foaming properties of pea proteins near the isoelectric point, whereas highest values were measured in the acidic and alkaline regions. This higher foamability in the latter pH ranges might be a consequence of the enhanced protein solubility, which promotes protein refolding around the air bubbles. Furthermore, an increase in foam formation and stability can be achieved by a higher ***PROTEIN CONTENT***. According to Aluko *et al.* (2009), isolated pea protein possess a significantly higher capability to entrap air compared to whole pea flours. Moreover, FC visibly increased with increasing concentration of isolated proteins from 10 to 50 mg/mL (Aluko *et al.*, 2009). This might be due to the formation of thick viscous films which act as physical barriers between air bubbles. Nonetheless, when the sample amount was further increased to 100 mg/mL, the foaming ability was considerably reduced, which might suggest limited protein solubility (Aluko *et al.*, 2009). The authors additionally emphasized the relevance of

PARTICLE SIZE in foaming, with smaller particles causing higher FC (Aluko *et al.*, 2009). Eventually, the presence of other molecules like ***SUGARS*** and ***SALTS*** can improve foamability, which can be linked to an increase in lamella fluid viscosity and an improved protein solubility, respectively (Lam *et al.*, 2018).

3.2.5 EMULSIFYING CAPACITY AND EMULSION STABILITY

Food emulsions are composed of immiscible liquids, mainly oil and water, which are commonly prepared by homogenization. During this process, one liquid is dispersed as small droplets in the other, which leads to either oil-in-water (e.g. milk) or water-in-oil emulsions (e.g. butter). Similar to foams, the presence of numerous small droplets is energetically unfavorable owing to the high surface free energy. In order to obtain a thermodynamically stable system, the dispersed phase hence strives to reduce this free energy, which can lead to several destabilization phenomena, and thus to an emulsion break (Damodaran, 2005; Lam *et al.*, 2018; McClements *et al.*, 2017):

Creaming	Reversible rise of oil droplets to the top against gravity, which is driven by density differences
Flocculation	Reversible or irreversible aggregation of oil droplets to a flock due to the prevalence of attractive forces
Coalescence	Irreversible fusion of two or more oil droplets to a single larger structure, resulting from the rupture of the thin liquid film separating the droplets
Partial coalescence	Irreversible aggregation of partially crystallized oil droplets
Ostwald ripening	Irreversible diffusion of small oil droplets to large ones, owing to a higher Laplace pressure in small droplets

Similar to foams, these physical changes can be counteracted by proteins. These diffuse to the oil-water interface and orient their hydrophobic groups to the oil and hydrophilic groups to the water phase, thereby forming a gel-like film to achieve the state of lowest free energy (Damodaran, 2005; Zayas, 1997a).

The emulsifying properties of proteins are typically expressed as emulsion activity (EA), emulsifying capacity (EC) and emulsion stability (ES). EA defines the interfacial area stabilized per gram protein, EC the amount of oil emulsified per gram protein and ES the ability of the system to resist physical separation (Lam *et al.*, 2018; Zayas, 1997a). These parameters can be determined applying different techniques, which complicates comparison of in the literature available information. The calculation of EA is often based on the spectrophotometric analysis of the emulsion turbidity (used by Barać et al. (2011), Boye *et al.* (2010b), Fuhrmeister *et al.* (2003), Karaca *et al.* (2011) and Wang *et al.* (2006)). EC is typically analyzed by conductometric measuring of the maximal point before the oil-in-water emulsion is inverted into a water-in-oil emulsion, which results in a drop of conductivity (used by Karaca *et al.* (2011) and Stone *et al.* (2015)). ES can be determined by monitoring (a) the time to reach half the initial turbidity of the emulsion (used by Barać et al. (2011), Boye *et al.* (2010b), Karaca *et al.* (2011), Wang *et al.* (2006) and Withana-Gamage *et al.* (2011)), (b) the percentage ratio of the initial oil droplet size to that after 30 min (used by Aluko *et al.* (2009) and Chao *et al.* (2018)), (c) the percentage volume of separated aqueous phase after a drainage period of 30 min (used by Stone *et al.* (2015)) or (d) the drainage constant obtained by kinetic studies of the creaming phenomenon (used by Mimouni *et al.* (1994)).

Table 7 gives an overview of the emulsifying properties of wheat gluten and pea proteins reported by several authors. Direct comparison of the EA and ES values for both commodities reveals pea proteins as superior emulsifiers, which has been attributed to their different content and composition of globulins (Foschia *et al.*, 2017).

Table 7. Emulsifying properties of wheat gluten and pea proteins (concentration ≥70%). Values were determined at pH 7.

Protein source	Emulsifying activity	Emulsifying capacity	Emulsion stability			Reference
	[m^2/g]	[g/g]	[min]	[%]	[1/s]	
Wheat	-	n.d.	-	-	n.d.	Mimouni *et al.* (1994)
Wheat	5	-	9	-	-	Wang *et al.* (2006)
Pea	-	-	-	100	-	Aluko *et al.* (2009)
Pea	-	-	-	~64 – 84	-	Chao *et al.* (2018)
Pea	-	188 – 244	-	97 – 100	-	Stone *et al.* (2015)
Pea	10 – 27	-	-	-	-	Fuhrmeister *et al.* (2003)
Pea	43	478 – 485	11 – 12	-	-	Karaca *et al.* (2011)
Pea	-	-	~18	-	-	Withana-Gamage *et al.* (2011)
Pea	5	-	~18	-	-	Boye *et al.* (2010b)
Pea	~65	-	~28	-	-	Barać *et al.* (2011)

n.d. = not detectable

In this context, the particular importance of the ***MOLECULAR FLEXIBILITY*** of proteins needs to be emphasized as a decisive characteristic positively affecting the protein's ability to rapidly unfold at the oil-water interface (Damodaran, 2005). As described in **§ 3.1 of Part II**, wheat gluten contains a great number of disulfide bridges, presumably leading to a higher rigidity and compact structure compared to pea proteins, which thus might hinder the molecule to undergo conformational changes. Consequently, the interfacial tension can only be reduced to a limited extent, yielding a poorly stabilized emulsion (Damodaran, 2005). In addition, the ***SURFACE HYDROPHOBICITY*** of proteins plays a governing role as it reflects the affinity to adsorb to the oil-water interface (Damodaran, 2005). According to the literature, the surface hydrophobicity of wheat gluten is considerably lower compared to pea proteins (35 – 57 vs. 78 – 832), which implies reduced potential of wheat gluten to anchor to the interface (Karaca *et al.*, 2011; Shevkani *et al.*, 2015; Wang *et al.*, 2006; Zhang, 2012).

Apart from structural properties, the environment can alter the emulsifying abilities of proteins, including the ***PH VALUE***. Following the general consensus in the literature, proteins exhibit higher EC, EA and ES at pH values above or below the isoelectric point, which can be ascribed to their improved solubility as well as increased surface charge, and thus electrostatic repulsion (Adebiyi *et al.*, 2011; Barać *et al.*, 2011; Chao *et al.*, 2018; Karaca *et al.*, 2011; Lam *et al.*, 2018; Liang *et al.*, 2013; Wang *et al.*, 2006). The parameters listed in **Table 7** were determined at pH 7, which reflects typical properties of common foods. However, this value corresponds to the isoelectric point of wheat gluten, and thus represents system conditions at which these proteins possess lowest emulsifying properties as opposed to pea proteins (Adebiyi *et al.*, 2011; Wang *et al.*, 2006). Moreover, an increase in the ***IONIC STRENGTH*** can lower the ability of proteins to stabilize emulsions owing to the weakening of electrostatic repulsion

between the molecules (Lam *et al.*, 2018). As a consequence, the attractive forces between proteins adsorbed to different oil droplets are strengthened, causing the dispersed phase to come into close proximity (Lam *et al.*, 2018). By contrast, moderate ***THERMAL TREATMENT*** can ameliorate the surface activity of proteins, attributable to the partial denaturation and thus exposure of hydrophobic residues previously buried in the interior of the molecule (Damodaran, 2005; Kato *et al.*, 1980). In this regard, Ma *et al.* (2011) discussed beneficial effects of roasting (80 °C, 1 min) and boiling (90 °C, 20 min) on EA of pea flour, which can be explained by the dissociation of globular proteins and alignment of non-polar groups towards the dispersed phase. In addition, Peng *et al.* (2016) observed smaller oil droplet sizes of emulsions stabilized by thermally treated pea proteins (95 °C, 30 min) compared to the untreated isolate. These findings were confirmed by Chao *et al.* (2018), who detected a reduction in oil droplet size after heat processing of pea proteins (50 – 100 °C, 30 min). However, the authors also described adverse effects on ES, probably due to the aggregation of proteins, leading to their insolubility. This indicates that the heating conditions in combination with further factors, such as ingredient and medium composition, can alter the emulsifying properties of proteins in both positive or negative directions. One influencing parameter might be the ***PROTEIN CONTENT*** as higher amounts can stimulate the development of stable multilayers at the oil-water interface (Lam *et al.*, 2018; Zayas, 1997a). This was reflected in the smaller oil droplet sizes of emulsions made with pea protein isolate (82% protein, ~20 µm) compared to those based on pea flours (≤10% protein content, 30 – 100 µm) as reported by Aluko *et al.* (2009). Nevertheless, the authors further asserted that the presence of non-protein compounds, such as starch, can support emulsion stability by acting as bulky barriers, which prevent oil droplet coalescence (Aluko *et al.*, 2009). Another important parameter affecting emulsification is the ***PARTICLE SIZE***, with smaller particles improving emulsion formation owing to their homogenous dispersion and larger surface area (Aluko *et al.*, 2009; Chao *et al.*, 2018).

3.2.6 GELATION

One possibility to induce gelation is by thermal treatment. Upon heating of a protein solution above the protein denaturation temperature, the macromolecules can partially unfold and form a three-dimensional network by crosslinking of exposed uncoiled polypeptides as well as interactions with the surrounding solvent (Shand *et al.*, 2007; Zayas, 1997c). Consequently, the developed gel can be considered a matrix built from interconnected unit structures embedded within a continuous liquid phase (Foegeding, 1989; Lam *et al.*, 2018). Attractive and repulsive forces between the proteins need to be balanced in order to avoid aggregation and thus precipitation (Zayas, 1997c). Typical denaturation temperatures of pea proteins and wheat gluten are listed in **Table 8**. Apart from water, the gels can entrap lipids, flavors, pigments and other food constituents, which are relevant during the development of food products (Lam *et al.*, 2018; Shand *et al.*, 2007).

Table 8. Denaturation temperature and least gelation concentration of pea proteins and wheat gluten (protein concentration ≥70%).

	Pea [1-6,9]	**Wheat** [7,8]
Denaturation temperature [°C]	83 – 86	50 – 85
Least gelation concentration [% w/v]	12 – 18	22

[1] Boye *et al.* (2010b); [2] Shevkani *et al.* (2015); [3] Shand *et al.* (2007); [4] Arntfield *et al.* (1981); [5] Bora *et al.* (1994); [6] Withana-Gamage *et al.* (2011); [7] Wang *et al.* (2007); [8] León *et al.* (2003); [9] Fernández-Quintela *et al.* (1997)

The ability of proteins to form a self-supporting gel is commonly expressed as least gelation concentration (LGC), signifying the minimal amount necessary to induce gelation (Kiosseoglou *et al.*, 2011). At this concentration, the gel resists sliding along the walls of a test tube in an inverted position (Moure *et al.*, 2006). Isolated pea proteins appear to have lower LGC than wheat gluten (**Table 8**), indicating superior capacity to form a gel.

In general, a ***PROTEIN CONTENT*** in excess of the critical LGC results in gels with high strength because more water becomes tightly bound and the number of crosslinks between proteins increases (Boye *et al.*, 2010b; Withana-Gamage *et al.*, 2011; Zayas, 1997c). It could be therefore imagined that pea flours develop stronger gels than wheat flour due to their typically elevated protein content (**Figure 3**). Nevertheless, it has been noted that apart from the protein content, the interaction of the macromolecules with ***FURTHER INGREDIENTS***, such as lipids and carbohydrates, exerts influence on the gelling properties (Kaur *et al.*, 2007a; Raikos *et al.*, 2014). In this context, Adebowale *et al.* (2004) argued that the presence of carbohydrates can reduce the thermodynamic affinity of proteins for water, hence intensifying protein-protein interactions. The considerably higher carbohydrate contents in wheat flour compared to pea flour might thus explain the similar LGC values of 12% (w/v) reported by Raikos *et al.* (2014). In addition, the ***IONIC STRENGTH*** of the medium appears to alter the gelation capacity of proteins. With the addition of salt, the denaturation temperature of proteins rises, which might be a consequence of reinforced intramolecular hydrophobic associations due to the charge-shielding effects of the salt (Adebowale *et al.*, 2004; Mession *et al.*, 2013; Shand *et al.*, 2007). However, reverse effects might be obtained at high salt concentrations due to limited protein unfolding and salting-out (Adebowale *et al.*, 2004; Mession *et al.*, 2013). The gelling abilities of proteins can further be modified by the ***PH VALUE*** of the system. Typically, pH values above or below the isoelectric point introduce net charges in the protein, which intensify electrostatic repulsion between proteins and thus protein unfolding (Meng *et al.*, 2001). Nevertheless, in the strong alkaline range, the repulsive forces might outbalance the attractive forces, which might restrict protein-protein interactions and thus weaken the gel strength (Elofsson *et al.*, 1997; Shand *et al.*, 2007). Interestingly, Tanger *et al.* (2021) measured increased stiffness of gels based on pea proteins at their isoelectric point (pH 4.5) compared to pH 7 and 9, which indicated the inclusion of protein aggregates as active fillers in the network.

4 ISOLATION OF PROTEIN AND STARCH

The use of purified proteins and starch from pea is an attractive approach in the food industry in order to benefit from their functional and nutritional properties. In general, the isolation of both fractions from the whole flour ingredient can be performed under wet or dry conditions. While dry fractionation yields protein-rich flours or protein concentrates with protein contents of 40 – 75%, wet extraction produce protein concentrates or protein isolates with protein contents of more than 80% (Boye *et al.*, 2010a; Kiosseoglou *et al.*, 2011).

4.1 DRY FRACTIONATION

Dry fractionation denotes a separation technique that consists of two major steps. Firstly, the pea seeds (whole or dehulled) are milled into a fine flour, which results in the physical disentanglement of small

protein bodies from large starch granules (Boye *et al.*, 2010a; Pelgrom *et al.*, 2013a; Pelgrom *et al.*, 2013b). Secondly, the flour is transferred into an ***AIR CLASSIFIER*** and separated by means of a spiral air stream into a light and fine protein-rich fraction as well as a heavy and coarse starch-rich fraction based on their size, shape and density (Boye *et al.*, 2010a; Pelgrom *et al.*, 2013b). The working principle of the air classifier is illustrated in **Figure 14**.

Nevertheless, the separation of both fractions is incomplete as some protein bodies remain attached to the starch granules even after repeated air classification (Boye *et al.*, 2010a). In general, the separation efficiency can be improved by milling the seeds to particles with sizes of less than 40 µm, which typically correspond to whole cells or parts of cells (Pelgrom *et al.*, 2013b). Moreover, the classifier wheel speed can be increased, however might concurrently lead to ***STARCH DAMAGE*** (Pelgrom *et al.*, 2013b).

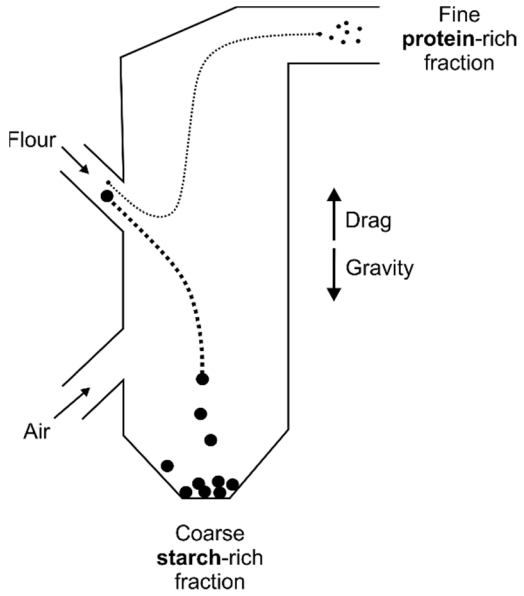

Figure 14. Principle of air classification (adapted from Shapiro *et al.* (2005)).

Tyler *et al.* (1981) reported protein contents of 56 – 59% in the fine fraction after a first air classification step of peas, which contained only a little amount of starch (2 – 3%) (**Table 9**). The coarse fraction, on the other hand, was rich in starch (73 – 73%) and contained around 12% protein. After re-milling and air classification of this latter starch-rich fraction, a second fine fraction containing 46 – 52% protein and 4 – 7% starch was obtained. This slight increase in the amount of starch compared to the first classification step was attributed to the damage of starch occurring during re-milling (Tyler *et al.*, 1981). The corresponding coarse fraction comprised mainly starch (82 – 86%) and minor amounts of protein (5 – 6%).

Table 9. Protein and starch contents of pin-milled pea flour and air-classified fractions (according to Tyler *et al.* (1981)).

Classification step	Air classified material	Protein-rich fraction		Starch-rich fraction	
		Protein [%]	Starch [%]	Starch [%]	Protein [%]
1	Flour	56 – 59	2 – 3	72 – 73	12
2	Starch-rich fraction	46 – 52	4 – 7	82 – 86	5 – 6

4.2 WET FRACTIONATION

In addition to dry separation, several approaches can be applied in order to isolate proteins and starch from pea under wet conditions. These techniques typically require the milling of the seeds (hulled or dehulled) into flour and subsequent dispersion in water (Boye *et al.*, 2010a).

ALKALINE EXTRACTION is the most widely used wet fractionation technique, the principle of which is based on the maximal solubility of pea proteins in the alkaline range (see **§ 3.2 of Part II** and **Figure 12**). Accordingly, the pH value of the flour suspension is adjusted to pH ≥ 8, which is typically maintained during 0.5 – 3 h (Boye *et al.*, 2010a). Occasionally the medium temperature is elevated to 55 – 65 °C, which might favor protein solubilization (Boye *et al.*, 2010a; Patras *et al.*, 2011). Alternatively, the pH of the medium can be lowered to < 4 to perform ***ACID EXTRACTION*** by taking advantage of the high solubility of pea proteins at acidic conditions (**Figure 12**) (Boye *et al.*, 2010a; Patras *et al.*, 2011). In each case, the generated extracts are subjected to filtration or centrifugation, which aims at the removal of insoluble materials, i.e. fiber and starch (Boye *et al.*, 2010a; Patras *et al.*, 2011). A third fractionation approach represents salt extraction, also referred to as ***MICELLIZATION***, however is less frequently used (Boye *et al.*, 2010a; Patras *et al.*, 2011).

Typically, the proteins are recovered from the filtrate obtained after alkaline or acid extraction by ***ISOELECTRIC PRECIPITATION*** which involves the pH adjustment to 4 – 5 to induce protein aggregation (Boye *et al.*, 2010a; Patras *et al.*, 2011). Thereafter, the precipitate is separated from the supernatant by centrifugation and the remaining salts removed by washing (Boye *et al.*, 2010a; Patras *et al.*, 2011). Instead of isoelectric precipitation, proteins can be concentrated after extraction by ***ULTRAFILTRATION*** using a membrane with adequate molecular weight cut-off (Boye *et al.*, 2010a). This technique produces protein isolates composed of both albumin and globulin fractions unlike isoelectric precipitation with which solely globulins are recovered (Boye *et al.*, 2010a). However, the major drawbacks of ultrafiltration are the low flow rate and risk of membrane plugging with increasing protein concentration (Gueguen, 1983). Alternatively, protein purification can be achieved by ***CRYO-PRECIPITATION***, which denotes the cooling of the solution to temperatures around 4 °C (Boye *et al.*, 2010a).

In order to ensure storage stability, the isolates generated *via* the different methods are typically dehydrated by freeze or spray drying (Boye *et al.*, 2010a; Patras *et al.*, 2011).

The most often applied combination of alkaline extraction and isoelectric precipitation is visualized in **Figure 15**.

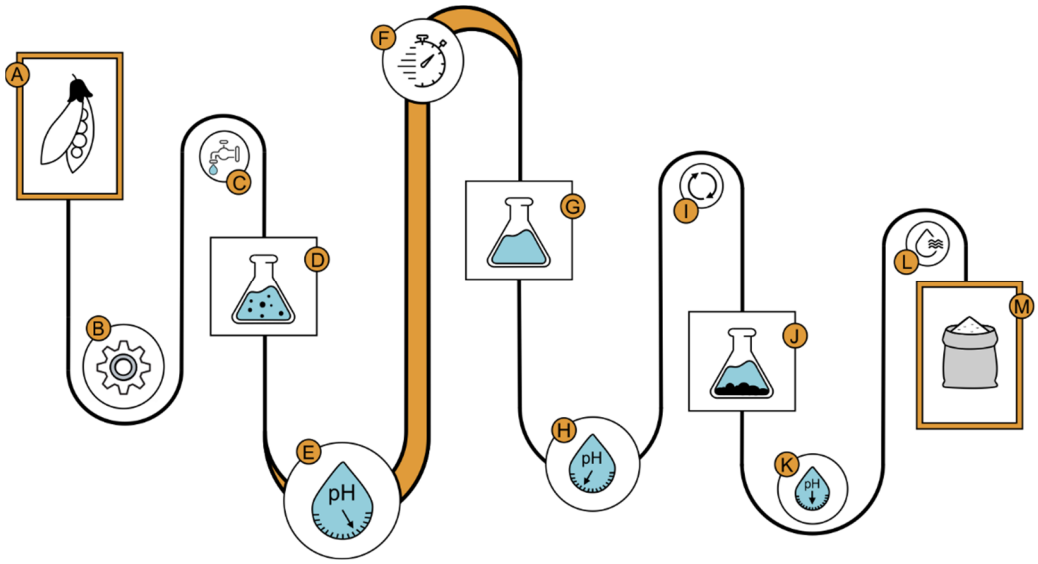

Figure 15. Schematic illustration of alkaline protein extraction and isoelectric precipitation. (A+B) Pea seeds are milled into flour. (C+D) The flour is suspended in water and (E) the pH value adjusted to ≥8 at which pea proteins exhibit maximal solubility. (F) The solution is kept for 0.5 – 3 h and might be heated to 55 – 65 °C in order to improve protein solubility. (G+H) The pH value of the protein solution is adjusted to the isoelectric point (pH 4 – 5) and (I+J) centrifuged to obtain the protein precipitate. (K+L+M) Afterwards, it is neutralized, washed from salts and dried, yielding the protein isolate.

5 LIPIDS

5.1 CONTENT AND COMPOSITION

The ***FAT CONTENT*** of peas is generally lower than 4% (Hall *et al.*, 2017). Phospholipids account for the majority of total lipids (52 – 62%), followed by triacylglycerols (26 – 40%) (**Table 10**) (Hoover *et al.*, 1988; Yoshida *et al.*, 2007). By contrast, glycolipids, diacylglycerols, monoacylglycerols, free fatty acids and sterol esters are only marginally present (1 – 4% each) (Hoover *et al.*, 1988; Yoshida *et al.*, 2007). A similar picture emerges with regard to wheat. This raw material is characterized by a maximal lipid content of 4%, to which mainly phospholipids (38 – 39%), triacylglycerols (27 – 47%) and glycolipids (13 – 19%) contribute (**Table 10**) (Chung *et al.*, 2009b). As for peas, diacylglycerols, monoacylglycerols, free fatty acids and sterol esters form only minor fractions (Chung *et al.*, 2009b).

In both commodities, the ***FATTY ACID PROFILE*** is dominated by linoleic acid, typically amounting to 48 – 56% in pea and 58 – 62% in wheat (**Table 10**) (Hall *et al.*, 2017; Liu, 2011; Oomah *et al.*, 2011; Paucean *et al.*, 2018). This C_{18}-fatty acid contains two unsaturated double bonds at the positions 9 and 12 of the carbon chain, hence being considered an omega-6 fatty acid. Oleic acid accounts for the second largest proportion of fatty acids in peas (17 – 28%), while relatively lower levels are found in wheat (12 – 13%) (Hall *et al.*, 2017; Liu, 2011; Oomah *et al.*, 2011; Paucean *et al.*, 2018). Owing to the presence of one double bond at position 9 of the carbon chain, this C_{18}-fatty acid is categorized as omega-9 fatty acid. Moreover, pea contains approximately three to four times more *α*-linolenic acid than wheat (9 – 12% vs. 3 – 4%) (Hall *et al.*, 2017; Liu, 2011; Oomah *et al.*, 2011; Paucean *et al.*, 2018), an essential C_{18}-fatty acid with three double bonds at the positions 9, 12 and 15 of the carbon chain, which is also referred to as omega-3 fatty acid. In addition, both raw materials comprise the saturated fatty acids palmitic and

stearic acid with 16 and 18 carbon units, respectively, the sum of which is greater in wheat compared to pea (20 – 22% vs. 14 – 17%) (**Table 10**). This indicates visibly higher amounts of unsaturated fatty acids in pea than wheat.

Table 10. Lipid and fatty acid composition of pea and wheat.

	Pea [1,2,6,7]	**Wheat** [3-5]
Lipid content [%]	1 – 4	2 – 4
Lipid composition [%]		
Free fatty acids	1 – 3	3 – 9
Monoacylglycerols	2 – 4	2 – 3
Diacylglycerols	1 – 4	2 – 3
Triacylglycerols	26 – 40	27 – 47
Sterol esters	1 – 3	2 – 3
Phospholipids	52 – 62	38 – 39
Glycolipids	3 – 4	13 – 19
Fatty acid composition [%]		
C16:0 (Palmitic acid)	11 – 13	19 – 21
C18:0 (Stearic acid)	3 – 4	1
C18:1 (Oleic acid)	17 – 28	12 – 13
C18:2 (Linoleic acid)	48 – 56	58 – 62
C18:3 (*α*-Linolenic acid)	9 – 12	3 – 4
Sum saturated fatty acids	14 – 17	20 – 22
Sum unsaturated fatty acids	74 – 96	73 – 79

[1] Hall *et al.* (2017); [2] Oomah *et al.* (2011); [3] Liu (2011); [4] Chung *et al.* (2009b); [5] Paucean *et al.* (2018); [6] Hoover *et al.* (1988); [7] Yoshida *et al.* (2007)

5.2 STARCH AND NON-STARCH LIPIDS

Lipids in flours can be categorized according to their location into starch and non-starch lipids. Despite their low concentration, starch lipids are important contributors to the functional properties of starch (see **§ 2.2 of Part II**).

5.2.1 NON-STARCH LIPIDS

Non-starch lipids (NSL) comprise a complex mixture of lipids, which are dispersed throughout the endosperm (Chung *et al.*, 2009b). They can be classified into free and bound flour lipids (Chung *et al.*, 2009b). While free lipids are typically extracted with non-polar solvents, such as hexane, extraction of bound lipids requires more polar solvents, such as water-saturated butanol (Chung *et al.*, 2009b).

In the case of wheat, 1.1 – 2.6% NSL are present in the flour, which corresponds to approximately 60% of the total lipids (Chung *et al.*, 2009b). The free lipids fraction of the NSL, which amounts to 0.9 – 1.8%, is primarily composed of non-polar lipids like triacyclglycerols and free fatty acids (Chung *et al.*, 2009b). By contrast, bound lipids (0.2 – 0.9%) are mostly composed of polar lipids, such as galactolipids and phospholipids (Chung *et al.*, 2009b). Unfortunately, the literature is lacking information on the NSL content in pea flour.

5.2.2 STARCH LIPIDS

In the early 80's, Morrison (1981) proposed a classification of cereal starch lipids into internal starch lipids and starch surface lipids. ***INTERNAL STARCH LIPIDS*** (ISL), also referred to as true starch lipids, are lipids located within the starch granule and are exclusively composed of monoacyl lipids, including lysophospholipids (LPL) and free fatty acids (FFA) (Buléon *et al.*, 1998; Morrison, 1981; Morrison *et al.*, 1984). The content in wheat typically ranges between 0.7% and 1.2%, which corresponds to approximately 40% of the total lipids (Chung *et al.*, 2009b). For pea, contents of 0.2 – 0.5% have been reported (Ratnayake *et al.*, 2001; Zhou *et al.*, 2004). While wheat starch almost solely contains LPL, maize starch is rich in FFA and has only minor proportions of LPL (Buléon *et al.*, 1998). Such information is not available on pea starch. The ISL can either build complexes inside the helices of amylose or are trapped between amylose and amylopectin chains (Hoover *et al.*, 1988; Morrison, 1981). ISL are typically extracted with polar solvents. ***STARCH SURFACE LIPIDS*** (SSL), on the other hand, specify lipids that are attached to the starch granule surface (Hargin *et al.*, 1980; Morrison, 1988; Rocha *et al.*, 2012; Zhang *et al.*, 2019). These SSL are assumed to derive from the non-starch lipid fraction of the flour and adsorb at the surface during the starch isolation process (Hargin *et al.*, 1980; Liukkonen *et al.*, 1992; Morrison *et al.*, 1984). Consistently, SSL are considered NSL which are associated with the starch granule surface. Unlike internal starch lipids, SSL can be easily extracted with low-polarity solvents, such as butanol, chloroform and ether (Kar *et al.*, 2005; Maniñgat *et al.*, 1980). According to the literature, the SSL content in pea starches is minimal, amounting to 0.02 – 0.05% (Ratnayake *et al.*, 2001; Zhou *et al.*, 2004).

5.3 FUNCTIONAL PROPERTIES

Like proteins, the amphiphilic nature of certain lipids, including phospholipids, mono- and diacylglycerols, equips them with the ability to diffuse to the air-water and oil-water interfaces and reduce the interfacial tension (Damodaran, 2005). By contrast to proteins, however, no conformational change occurs upon adsorption, suggesting that the ***SURFACE ACTIVITY*** is largely determined by the rate of diffusion. Although this high mobility allows the low molecular weight surfactants to align rapidly at interfaces, the generated systems are less stable against coalescence (Bos *et al.*, 2001). This can be attributed to a lack of steric repulsion as obtained with protein-based emulsions and foams (Bos *et al.*, 2001). Moreover, unfolded proteins are able to stabilize the dispersed phase through multiple segments, thereby forming viscoelastic films, which are rather liquid-like in the case of lipids (Bos *et al.*, 2001). From this, it can be concluded that lipids appear relevant stabilizers during the initial stage of emulsion or foam generation, whereas long-time stability might be rather a characteristic exerted by proteins.

CHAPTER 2

SPONGE CAKE APPLICATION

For centuries, bakery products have been a basic part of a balanced human diet (Smith *et al.*, 2004; Villarino *et al.*, 2016). Among the huge variety of baked goods, cakes are widely consumed across the civilized world (Cepeda-Vázquez *et al.*, 2019; Godefroidt *et al.*, 2019; Pycarelle *et al.*, 2019). Typically, cake recipes are composed of wheat flour, sugar, eggs and, optionally, fat (Godefroidt *et al.*, 2019; Pycarelle *et al.*, 2019). Depending on the ingredients, their ratios and the manufacturing method, cakes are classified into either batter-type (e.g. layer and pound cake), foam-type (e.g. angel food and sponge cake) or chiffon cakes (Godefroidt *et al.*, 2019). Whereas representatives of the batter-type category contain a significant amount of fat leading to the formation of an oil-in-water emulsion, foam-type cakes are prepared with only little if any fat so as to their batter can be considered a liquid foam (Godefroidt *et al.*, 2019; Wilderjans *et al.*, 2013). A combination of both styles is defined as chiffon cake (Godefroidt *et al.*, 2019).

Foam-type cakes, also referred to as unshortened-style cakes, can be divided into angel food and sponge cakes, differing in the type of egg used: unlike sponge cakes, which contain whole egg, angel food cakes are solely made with egg white (Conforti, 2006; Godefroidt *et al.*, 2019). Sponge cakes are characterized by a fine and porous structure, which allows the absorption of different fillings, such as jams and syrups, hence making it widely applicable and popular among the consumers (Assad Bustillos *et al.*, 2020c; Pycarelle *et al.*, 2019). For this reason, sponge cakes were selected as research object of this study. They are moreover composed of multiple ingredients of different chemical compositions that can trigger certain reactions which affect food safety and quality. Accordingly, the composition and functionalities of these ingredients during the different steps of sponge cake production applied in this thesis are explained in the following sections.

6 INGREDIENTS

6.1 EGGS

Hen eggs are made up of two fractions which are separated by a vitelline membrane and protected by a strong shell: egg white and egg yolk (Kiosseoglou *et al.*, 2006). Apart from water, the major constituents of the whole eggs are proteins (13%) and lipids (11%), however also minor amounts of carbohydrates (1%) and minerals are present (Godefroidt *et al.*, 2019; Li-Chan *et al.*, 2008).

Approximately two third of the liquid egg comprise ***EGG WHITE*** (albumen), which can be described as protein system of ovomucin fibers (4%, 5500 – 8300 kDa) in an aqueous solution of circa 40 globular proteins, including ovalbumin (54%, 45 kDa), ovotransferrin (12%, 76 kDa), ovomucoid (11%, 28 kDa), ovoglobulins (8%, 30 – 45 kDa) and lysozyme (3%, 14 kDa) (**Figure 16**) (Allais *et al.*, 2006; Kiosseoglou *et al.*, 2006; Li-Chan *et al.*, 2008). The total protein content in fresh albumen accounts for 10 – 15% (Deleu *et al.*, 2015; Lai *et al.*, 2006).

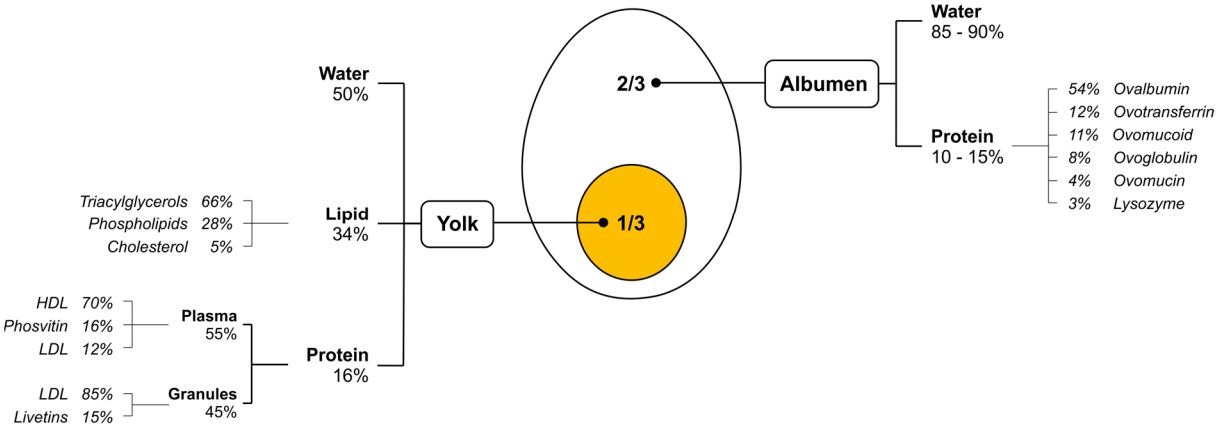

Figure 16. Schematic representation of the chemical composition of eggs based on Anton *et al.* (2003), Kiosseoglou *et al.* (2006), Deleu *et al.* (2017) and Nau *et al.* (2010)

The other third of the egg magma is ***EGG YOLK*** that is considered a suspension of non-soluble protein aggregates, the so-called granules (20%), in yellow plasma (80%) (Anton, 2013; Strixner *et al.*, 2014). Unlike egg white, which is practically free of lipids, egg yolk is not only rich in proteins (16%) but also lipids (34%) (**Figure 16**) (Godefroidt *et al.*, 2019; Kiosseoglou *et al.*, 2006). The lipid fraction is mainly composed of triacylglycerols (66%), phospholipids (28%) and cholesterol (5%) and is almost exclusively found in the yolk plasma (Deleu *et al.*, 2017; Kiosseoglou *et al.*, 2006). According to the literature, the fatty acid composition of egg yolk highly depends on the feed for the laying hen, however follows typically the order: oleic acid (36 – 47%) > palmitic acid (24 – 26%) > linoleic acid (12 – 19%) > stearic acid (8 – 13%) (Hammershøj *et al.*, 2016; Réhault-Godbert *et al.*, 2019). Compared to the yolk lipids, the yolk proteins are evenly distributed between granules (45%) and plasma (55%), although their composition varies (Anton, 2013; Nau *et al.*, 2010).

Typically, granules are circular complexes constituted of both high-density lipoproteins (HDL, 70%, 400 kDa) linked by phosvitin (16%, 35 kDa) *via* phosphocalcic bridges and low-density lipoproteins (LDL, 16%, 3300 – 10300 kDa) (Anton, 2013; Deleu *et al.*, 2017; Kiosseoglou *et al.*, 2006; Mine, 2008; Nau *et*

al., 2010; Strixner *et al.*, 2014). LDLs are defined as lipids of triacylglycerol and cholesteryl esters surrounded by proteins, phospholipids and cholesterol (**Figure 17**) (Anton, 2013; Kiosseoglou *et al.*, 2006). By contrast, the proteins mainly associated with the plasma are LDL (85%) and water-soluble livetins (15%) (Anton, 2013; Deleu *et al.*, 2017; Kiosseoglou *et al.*, 2006; Nau *et al.*, 2010; Strixner *et al.*, 2014). On a quantitative basis, LDLs are the most abundant proteins in egg yolk (70%), followed by HDLs (16%), livetin (10%) and phosvitin (4%) (Deleu *et al.*, 2017).

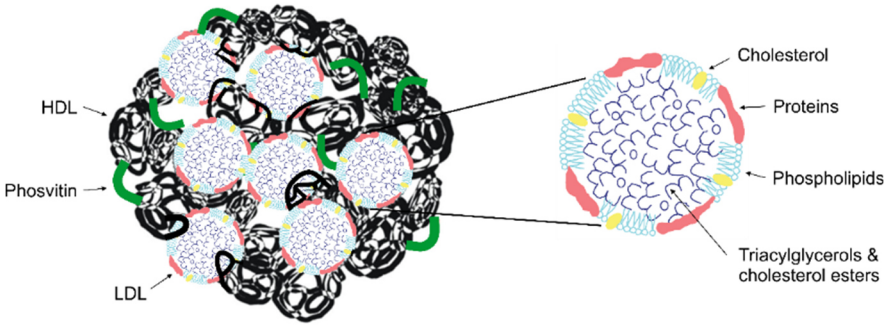

Figure 17. Schematic representation of a granule from egg yolk (modified and combined illustrations from Anton (2013) and Strixner *et al.* (2014)).

6.2 VEGETABLE OIL

The main constituents of vegetable oils are triacylglycerols (TAGs), which account for up to 97% of the total oil (Chernova *et al.*, 2018). These TAGs are composed of glycerol esterified at the hydroxyl residues with three fatty acids. A huge variety of fatty acids exists, owing to a high variability of the carbon chain length and the degree of saturation. These parameters determine the oil's physical and chemical properties, including melting point and stability to oxidative deterioration, both of which in general decrease with the number of double bonds.

SUNFLOWER OIL is a vegetable oil characterized by high concentrations of the polyunsaturated linoleic acid (C18:2, 58 – 62%) and the monounsaturated oleic acid (C18:1, 28 – 34%). In addition, approximately 8 – 15% saturated fatty acids, including palmitic (C16:0) and stearic acid (C18:0) are present (Chernova *et al.*, 2018; Garcés *et al.*, 2009; Orsavova *et al.*, 2015).

6.3 SUGAR

Sugars are polyhydroxyaldehydes or polyhydoxyketones as well as some derivatives thereof, which can be divided into monosaccharides, oligosaccharides or polysaccharides. The latter two are differentiated according to their number of monosaccharides residues. Whereas the polymerization of two to ten monosaccharides leads to the formation of oligosaccharides, polysaccharides consist of *n* monosaccharides, with *n* being greater than 10.

SUCROSE is a typical representative of the oligosaccharide group, which is composed of the monosaccharides fructose and glucose linked by an *α*-(1,2) glycosidic bond. The non-reducing disaccharide is a sugar commonly used in cake making.

6.4 FLOUR

Wheat (*Triticum aestivum* L.) is the traditional cereal used in bakery products. In novel approaches, however, wheat flour is increasingly replaced with alternative plant-based sources, such as pulse ingredients. The composition of both commodities is described in detail in **Chapter 1 of Part II**.

7 BATTER PREPARATION

7.1 PROCESS STEPS

The springy texture of sponge cakes is controlled by the egg's ability to entrap air in the continuous phase and generate a stable foam (Assad Bustillos *et al.*, 2020c; Conforti, 2006; Pycarelle *et al.*, 2019; Ureta *et al.*, 2016). The batter mixing is performed in a two-step process in which whipping of egg and sugar into a thick, pale foam is followed by gently folding in of flour and oil (Hao *et al.*, 2016; Pycarelle *et al.*, 2019; Wilderjans *et al.*, 2013). This enables not only the complete hydration, dispersion and functionalization of all ingredients but also the incorporation and integrity of multitudinous gas cells in the continuous phase (Conforti, 2006; Orthoefer, 2008; Rodríguez-García *et al.*, 2014). These properties, especially the amount, size and stability of incorporated air cells, are important prerequisites for the obtainment of high quality cakes with proper texture since no further gas cells are formed during baking (Campbell *et al.*, 1999; Conforti, 2006; Lau *et al.*, 2005; Orthoefer, 2008; Rodríguez-García *et al.*, 2014).

7.2 DYNAMICS OF AERATION

The air occlusion in the continuous phase is a dynamic phenomenon of concurrently operating air bubble entrainment and release (Massey *et al.*, 2001). During the first mixing phase, the rate of gas embedment predominates that of disentrainment until a maximal gas hold-up is reached (**Figure 18**) (Massey *et al.*, 2001). The rate of aeration is thereby negatively correlated with the whisk speed (Massey *et al.*, 2001). With proceeding whipping, the turbulence induced by the whisk entails the breakup of larger air bubbles into smaller cells as well as the diffusion of surface-active compounds to the air-water interface to stabilize the gas cells (Godefroidt *et al.*, 2019; Massey *et al.*, 2001). These surfactants are proteins and lipids naturally occurring in the egg and flour (see **§ 3.2.4**, **§ 3.2.5** and **§ 5.3 of Part II**) (Godefroidt *et al.*, 2019). As soon as the maximal gas hold-up is exceeded, bubble stabilization is deficient due to the tight packing of small gas cells (Massey *et al.*, 2001). The main destabilization mechanisms acting in the unstable colloidal system are gravitational drainage, disproportionation and coalescence (Alavi *et al.*, 2020). However, in rather high viscosity batters, which were beaten for short times, drainage and cell disproportionation are expected to be of less significance (Allais *et al.*, 2006). The bubble coalescence depends on the breaking strength of the inter-bubble lamellae, which decreases with reducing bubble sizes and increasing surface tension (Allais *et al.*, 2006; Lau *et al.*, 2005). As a consequence, gas hold-up progressively declines and asymptotically approaches a plateau at which air incorporation and release from the continuous phase are balanced (Massey *et al.*, 2001).

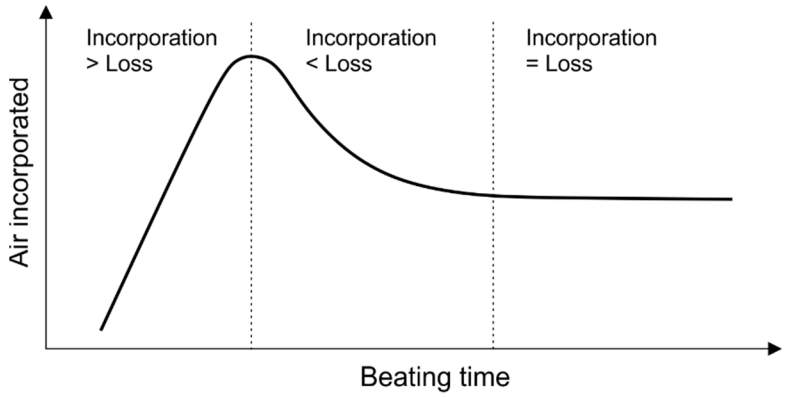

Figure 18. Schematic illustration of the dynamics of aeration as a function of batter beating time (according to Massey *et al.* (2001)).

7.3 FUNCTIONAL PROPERTIES OF INGREDIENTS

7.3.1 EGG

The major portion of air is entrapped into the batter during the whipping of the egg. In order to avoid destabilization, these gas cells need to be stabilized.

The ***GAS CELL STABILIZATION*** of ***EGG PROTEINS*** in aerated food systems is attributable to their various fractions with diverging molecular weights and structural flexibilities (Kiosseoglou *et al.*, 2006). In general, foam generation is promoted by the unfolding and reorientation of the proteins upon diffusion and interfacial accumulation. Thereby, the molecules' hydrophilic and hydrophobic regions orient themselves towards the polar water phase and non-polar gas phase, respectively. This alignment is not only essential to lower the interfacial tension to reach a thermodynamically favorable situation but also to form a continuous film at the bubble surface, thus resulting in a stable foam (Godefroidt *et al.*, 2019; Hasenhuettl, 2008; Zayas, 1997b). Among the egg proteins, albumen proteins, and in particular ovalbumins and ovoglobulins, are considered exceptional foaming agents (Kiosseoglou *et al.*, 2006; Lechevalier *et al.*, 2005). Upon interfacial adsorption and unfolding, egg white proteins expose their sulfhydryl groups, which can interlink through disulfide bridges, resulting in the formation of a viscoelastic film (Godefroidt *et al.*, 2019; Kiosseoglou *et al.*, 2006). However, these globular proteins are characterized by a rigid molecular structure (Kiosseoglou *et al.*, 2006). Consistently, their rate to undergo conformational changes at the interface is slow, leading to the retention of a certain proportion of their secondary and tertiary structure (Godefroidt *et al.*, 2019; Kiosseoglou *et al.*, 2006). The high molecular weight molecules exhibit low lateral diffusivities, and hence, their stabilization mechanism is mainly based on the formation of a strong layer surrounding the air cells (Allais *et al.*, 2006; Godefroidt *et al.*, 2019). Yolk proteins, on the other hand, possess low conformational stability, which entails their rapid surface denaturation in the presence of an air-water interface (Godefroidt *et al.*, 2019; Kaur *et al.*, 2007b; Kiosseoglou *et al.*, 2006). Proteins of the highly abundant LDLs exhibit a labile and flexible nature, which allows fast adsorption and subsequent unfolding at the interface (Kiosseoglou *et al.*, 2006). In addition, they might interact with the surface-active phospholipids released from the LDL micelles, thus forming an interfacial film which stabilizes against coagulation (Kiosseoglou *et al.*, 2006; Wilderjans *et al.*, 2013).

Upon unfolding of ***EGG YOLK LOW-DENSITY LIPOPROTEINS*** at the air-water interface during batter whipping, parts of the lipid core of the micelles can contribute to the layer buildup at the bubble surface whereas neutral lipids coalescence with oil droplets or accumulate at the interface (Dauphas *et al.*, 2006; Godefroidt *et al.*, 2019). The former phenomenon has been described for phospholipids, which are known as amphiphilic compounds that direct in the presence of an interface their hydrophobic hydrocarbon chains towards the gas phase and the hydrophilic phosphate head group towards the water phase. Since phospholipids are constituents of LDL micelles, they can contribute to the stabilization of the protein foam by the formation of a fluid-like film, in particular due to their high mobility, and hence rapid diffusion to the interface (Godefroidt *et al.*, 2019). Nonetheless, this direct adsorption is suggested to appear only in a small amount (Anton *et al.*, 2003). In addition, bubble stabilization by lipids with low molecular weight has been reported and can be explained by the Gibbs-Marangoni effect (Allais *et al.*, 2006). This phenomenon occurs in case of local thinning of the lamella between gas cells, which is antagonized by the lipids by diffusion into the depleted area and simultaneous sweeping in of liquid, leading to the re-thickening of the lamella and thus stabilization of the air bubbles (Allais *et al.*, 2006).

7.3.2 SUCROSE

The literature reports an elevated stability of aerated protein solutions in the presence of sucrose, assignable to an increase in the continuous ***PHASE VISCOSITY***, which lowers the drainage rate (Lau *et al.*, 2005; Yang *et al.*, 2010). On the other hand, however, this high solution viscosity restricts the level of air incorporation owing to a slower diffusion rate of proteins toward the interface (Alavi *et al.*, 2020; Lau *et al.*, 2005; Yang *et al.*, 2010). This results in a reduced foamability of the protein solution and is reflected in a reduced overrun (Lau *et al.*, 2005; Yang *et al.*, 2010). Interestingly, this lower overrun is positively correlated with the average size of incorporated air bubbles that can be explained by improved stabilization in more viscous batters (Lau *et al.*, 2005; Yang *et al.*, 2010). Moreover, the polyhydroxy compound can enhance the conformational stability of proteins, which at the same time decelerates protein unfolding and thus delays the ability of interfacial stabilization (Godefroidt *et al.*, 2019).

7.3.3 FLOUR

As soon as the flour is added, the ***PROTEINS*** and ***LIPIDS*** contained therein can diffuse to the air-water interface and exert their ***SURFACE ACTIVITIES*** (**§ 3.2.4 of Part II; § 5.3 of Part II**). As discussed in **§ 3.2.4 of Part II**, wheat proteins possess considerably lower foamabilities compared to pea proteins at typical food pH conditions, which might be attributed to their limited solubility and different molecular flexibility.

However, the presence of lipids along proteins can result in the competition of both components at the gas-liquid interface (Godefroidt *et al.*, 2019). The accompanied undesirable effects are reflected in the rupture of the stabilizing protein film and subsequent collapse of air bubbles (Zayas, 1997b). The obtained foam therefore exhibits less foam stability as compared to those generated by the individual surface-active compounds (Wilde, 2000). Consequently, fat-rich pulses are linked to limited foaming properties, as communicated for chickpea (Han *et al.*, 1990; Toews *et al.*, 2013) and soy (Toews *et al.*, 2013; Traina *et al.*, 1994; Yasumatsu *et al.*, 1972).

In addition, a beneficial contribution to foamability could possibly be exerted by ***STARCH GRANULES*** acting as ***PHYSICAL BARRIERS*** between air bubbles, which in turn retard gas cell coalescence and

Ostwald ripening (Godefroidt *et al.*, 2019; Saari *et al.*, 2016). The polysaccharide also increases ***BATTER VISCOSITY***, thereby probably improving foam stability (Zhang *et al.*, 2015b). The same could be concluded for other flour ingredients, such as proteins and fibers.

7.3.4 SUNFLOWER OIL

Unlike egg yolk, sunflower oil consists practically exclusively of triacylglycerols which do not possess any surface activity (Garcés *et al.*, 2009). Moreover, the addition of oil typically triggers a reduced batter viscosity, attributable to a lubrication effect of uniformly dispersed fat particles (Prakash *et al.*, 2001; Sakiyan *et al.*, 2004).

8 CAKE BAKING

The typical evolution of the moisture content as well as the temperature inside and on the surface of sponge cakes baked at 170 °C is illustrated in **Figure 19** (adapted from Fehaili *et al.* (2010)). Within short time, the temperature on the surface of the product can reach values above 100 °C, leading to a temperature gradient inside the batter, which results in a conductive heat transport and a rise of the core temperature. As a consequence, the melting of fat crystals is initiated, which might lead to a reduction in batter ***VISCOSITY***, although the lipid content in sponge cakes is generally low. In wheat-based batter-type cakes, such reduction in batter viscosity was observed at temperatures varying between 20 – 50 °C (Gaines *et al.*, 1982; Wilderjans *et al.*, 2008).

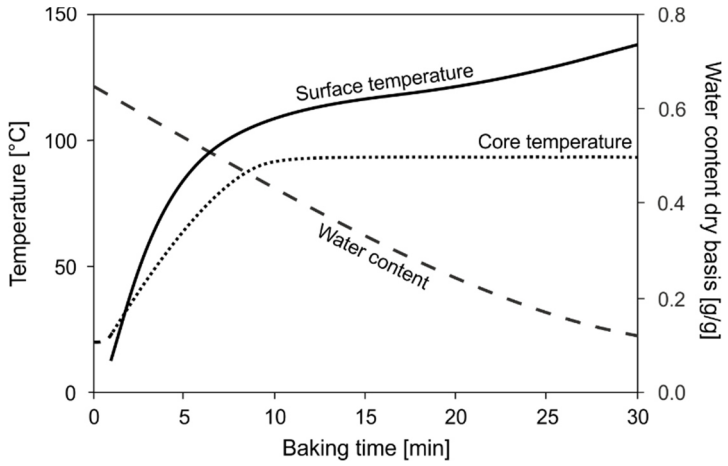

Figure 19. Evolution of water content as well as temperature inside and on the surface of sponge cakes baked at 170 °C (adapted from Fehaili *et al.* (2010)).

Moreover, this steep temperature increase stimulates the growth of gas cells due to the expansion of incorporated air, evaporation of water as well as decrease of gas solubility (Godefroidt *et al.*, 2019). Since no further air bubbles are generated during the baking process, this expansion depends on the amount air entrapped in the batter as well as on its distribution within the matrix. With the evaporation of water, the product is successively dehydrated (**Figure 19**) and causes a ***DIMINUTION OF THE SAMPLE WEIGHT*** by typically 5 – 14% (Baik *et al.*, 2003; Baik *et al.*, 1999; Gómez *et al.*, 2012; Sato *et al.*, 1987;

Zanoni *et al.*, 1994). Under consideration of this moisture loss, the ***EXPANSION*** of the batter can be determined as the ratio of final cake volume to initial batter volume, which correlates with augmentations of 1.5 – 1.8 times in the case of wheat and 1.8 – 2.0 in the case of pea, lentil and chickpea (de la Hera *et al.*, 2012; Gómez *et al.*, 2012; Gómez *et al.*, 2008). This value can be affected by the initial viscosity of the batter. While highly viscous products might restrict gas cell expansion, too low viscosities might insufficiently retain air bubbles (Sahi *et al.*, 2003). According to Lostie *et al.* (2002), expansion of wheat-based sponge cake batters primarily occurs in the temperature range 45 – 85 °C. As soon as the temperature in the core of the product exceeds 85 °C, batter expansion is decelerated while water evaporation continues (Lostie *et al.*, 2002). This might be attributed not only to the opening of the cake internal structure but also to the stiffening of the structure (Lostie *et al.*, 2002).

The development of such solid crumb and crust occurs towards the end of the first baking phase. As the diffusion of liquid water from the core is lower compared to the evaporation of water at the surface, the latter gradually dries, leading to the successive formation of a ***CRUST*** (Lostie *et al.*, 2002). Concurrently, the ***STRUCTURE SETTING*** of the crumb is induced, which correlates with a remarkable increase in ***VISCOSITY***, attributable to both protein denaturation and starch gelatinization (Donovan, 1977; Gaines *et al.*, 1982; Wilderjans *et al.*, 2008). The heat-induced loss of the three-dimensional structure of proteins takes places at different temperatures dependent on the protein origin. In the literature, typical denaturation temperatures in the range 60 – 85 °C, 50 – 85 °C and 83 – 86 °C have been reported for proteins originating from egg, wheat and pea, respectively (Donovan, 1977; Kiosseoglou *et al.*, 2005; León *et al.*, 2003; Shevkani *et al.*, 2015; Wang *et al.*, 2007). Upon coagulation, the proteins unfold and gradually aggregate into a network *via* electrostatic and hydrophobic interactions, Van der Waals forces as well as hydrogen and disulfide bonds (Mine, 1995; Wilderjans *et al.*, 2008; Zayas, 1997c). Further details are described in **§ 3.2.6 of Part II**. Egg proteins, in particular, appear to be susceptible to crosslinking owing to a considerably higher content of sulfhydryl groups than wheat gluten (approximately seven times), which are able to build covalent disulfide bonds and thus connect neighboring peptides (Wilderjans *et al.*, 2010; Wilderjans *et al.*, 2013). Among these, the most abundant protein in egg albumen, ovalbumin, plays a crucial role based on the presence of four free sulfhydryl groups (Kiosseoglou *et al.*, 2006; Wilderjans *et al.*, 2013). Moreover, Kiosseoglou *et al.* (2006) and Dewaest *et al.* (2017) suggested interactions between egg and wheat proteins above 90 °C to create a mixed protein matrix. The developed protein network can be considered “mortar” surrounding starch granules (“bricks”) contained in the flour, which themselves are prone to undergo molecular changes as a consequence of the thermal treatment (Donovan, 1977). As discussed in **§ 2.4.1 of Part II**, heating of starch in excess water leads to granular swelling followed by the uncoiling and dissociation of the polymer’s double helices. This phenomenon is denoted as starch gelatinization, which is initiated at temperatures of 55 – 66 °C for wheat, pea and maize starches (see **Table 4**). Continuous heating causes the starch polysaccharides to leach out of the granule and contribute to the viscosity increase of the system. In the presence of sucrose, however, both protein denaturation and starch gelatinization appear to be delayed, which can be attributed to the ability of the polyhydroxy component to bind water, which is therefore less freely available for proteins and starch (Beleia *et al.*, 1996; Donovan, 1977; Spies *et al.*, 1982). For instance, Donovan (1977) detected increments of 13 °C and 30 °C for egg protein denaturation and wheat starch gelatinization, respectively, at a sucrose concentration of 50%. Apart from sucrose-water interactions, also direct interactions between sucrose and amylose in the

amorphous region of the starch granule were reported, which could restrict the polymer's flexibility, thereby retarding starch gelatinization (Gonera *et al.*, 2002). Nevertheless, once starch swelling commences, the swelling rate is higher compared to solutions in the absence of sucrose and leads to granules with larger diameters (Bean *et al.*, 1978). In addition, sucrose might stabilize proteins against thermal unfolding not only through interactions with surrounding water but also through strengthening of hydrophobic interactions within the protein molecule (Semenova *et al.*, 2002; Wilderjans *et al.*, 2013). At moderate sucrose levels, the described consequences can positively affect cake volume and porosity because the dough has more time to expand. Elevated sucrose concentrations, on the other hand, might entail the release of entrapped air cells and thus the collapse of the structure.

9 STUDIES ON PULSE-BASED CAKES

Several studies have been performed on the full or partial replacement of wheat flour by pulse ingredients in order to evaluate their effect on the structural properties of the developed batters and associated cakes.

9.1 BATTER CHARACTERISTICS

9.1.1 DENSITY

In general, the batter density increased when introducing pulse ingredients as full or partial wheat substitutes during sponge cake development, reflecting less air incorporation (**Table 11**) (Assad Bustillos *et al.*, 2020c; de la Hera *et al.*, 2012; Gómez *et al.*, 2012; Gómez *et al.*, 2008). Opposite results were obtained with layer cake batters, which were characterized by higher volumes with increasing level of pulse flours (de la Hera *et al.*, 2012; Gómez *et al.*, 2012; Gómez *et al.*, 2008). Similarly, Monnet *et al.* (2020) determined more voluminous pound cake batters on the basis of pea flour compared to those made with wheat flour. The three cake products mainly differ in their oil content, which directly determines the proportion of egg incorporated. While the sponge cake formulations contained 37% egg, remarkably lower portions were used in the pound (25%) and layer cake recipes (15%). However, the percentage of flour remained practically constant in all samples (around 28%). This implies that the air-liquid interfaces in the sponge cake batters were largely stabilized by the highly prevalent egg proteins with important surface activities (see **§ 7.3.1 of Part II**) and that the addition of protein-rich pulse flours presumably led to the competition of egg and pulse proteins for the surface, thereby impairing their foamabilities. By contrast, the incorporation of high-protein pulse flours into the layer and pound cake batters with lower egg protein contents might allow the pulse proteins to exert their foaming properties and enhance gas cell stabilization, being accompanied by the entrapment of more air. In addition, the particle size of the flour seems to affect the batter characteristics. According to Gómez *et al.* (2010) and de la Hera *et al.* (2012), wheat and lentil flours, respectively, with finer particle sizes produced batters with smaller air bubbles, which were evenly distributed throughout the matrix and resulted in higher batter densities.

9.1.2 VISCOSITY

The batter viscosity generally increased with the addition of pulse ingredients, attributable to an increasing protein-to-starch ratio (de la Hera *et al.*, 2012; Gómez *et al.*, 2012). Additionally, it could be

imagined that fibers contributed to this increase in viscosity, which are typically present at higher concentrations in pulses compared to wheat.

Table 11. Characteristics of cake batters made with wheat flour or pulse ingredients.

Pulse	Level	Density [g/cm^3]		Viscosity [Pa·s]		Reference
	[%]	Wheat	Pulse	Wheat	Pulse	
Sponge cakes						
Chickpea flour	100	0.51	0.57 – 0.62	n.d.	n.d.	Gómez *et al.* (2008)
Lentil flour	100	0.34	0.39 – 0.50	15	18 – 23	de la Hera *et al.* (2012)
Pea flour	100	0.47	0.50	3	2	Gómez *et al.* (2012)
Layer cakes						
Chickpea flour	100	1.05	0.94 – 0.98	n.d.	n.d.	Gómez *et al.* (2008)
Lentil flour	100	0.99	0.89 – 0.94	9	18 – 24	de la Hera *et al.* (2012)
Pea flour	100	1.16	0.97	3	3	Gómez *et al.* (2012)
Pound cakes						
Pea flour	40	0.98 – 0.99	0.96 – 0.97	n.d.	n.d.	Monnet *et al.* (2020)

n.d. = not determined

9.2 CAKE CHARACTERISTICS

9.2.1 DENSITY

According to the literature, the cake density generally augments as the level of pulse flour increases in both layer and sponge cakes (**Table 11**) (de la Hera *et al.*, 2012; Gómez *et al.*, 2012; Gómez *et al.*, 2008; Singh *et al.*, 2015). It was assumed that with increasing protein content, the amount of starch gradually decreases, which is relevant for the structuration of the crumb (see **§ 8 of Part II**). No correlation between the batter density and cake volume could be elaborated (de la Hera *et al.*, 2012).

An important factor impacting cake volume is starch gelatinization. If gelatinization occurs at low temperatures, the expansion of the batter might be limited and could result in a high-density cake (Gómez *et al.*, 2008). However, elevated gelatinization temperatures might retard the setting of the structure, hence enabling entrapped air to escape from the batter, thereby leading to a high-density cake (Singh *et al.*, 2015). Moreover, de la Hera *et al.* (2012) postulated that flours with smaller particle sizes produced cakes with higher volumes, which is attributable to a more homogenous distribution of air bubbles during batter preparation that could be better retained during baking.

9.2.2 TEXTURE

Apart from density, researchers likewise evaluated the effect of pulse flours on cake texture. As shown in **Table 12**, the products containing pulse ingredients appeared to be firmer compared to wheat, which was inversely proportional to the cake volume (de la Hera *et al.*, 2012; Gómez *et al.*, 2012; Gómez *et al.*, 2008). Contrariwise, pulse flour addition led to a decrease in cake cohesiveness, which defines the rate at which food disintegrates under mechanical action (de la Hera *et al.*, 2012; Gómez *et al.*, 2012; Gómez *et al.*, 2008). Similarly, the springiness of the layer cakes decreased, implying restricted recovery of the food shape between the end of the first bite and the beginning of the second bite (de la Hera *et al.*, 2012; Gómez *et al.*, 2012; Gómez *et al.*, 2008).

Table 12. Characteristics of cakes made with wheat flour or pulse ingredients.

Pulse	Level [%]	Density [g/cm³]		Firmness [N]		Springiness		Cohesiveness		Reference
		Wheat	Pulse	Wheat	Pulse	Wheat	Pulse	Wheat	Pulse	
Sponge cakes										
Chickpea flour	100	0.28	0.28 – 0.31	3.6	5.7 – 8.7	0.9	0.9	0.8	0.6 – 0.7	Gómez *et al.* (2008)
Lentil flour	100	0.24	0.19 – 0.28	3.8	4.5 – 8.3	0.9	0.9	0.7	0.5 – 0.6	de la Hera *et al.* (2012)
Pea flour	100	0.23	0.23	3.3	5.1	0.9	0.9	0.7	0.5	Gómez *et al.* (2012)
Layer cakes										
Chickpea flour	100	0.46	0.46 – 0.57	4.4	5.2 – 7.1	0.9	0.7 – 0.8	0.7	0.5 – 0.6	Gómez *et al.* (2008)
Lentil flour	100	0.31	0.36 – 0.41	2.4	3.1 – 5.8	0.9	0.7	0.6	0.4	de la Hera *et al.* (2012)
Pea flour	100	0.31	0.32	3.9	8.9	0.9	0.7	0.6	0.4	Gómez *et al.* (2012)
Pound cakes										
Pea flour	40	0.29 – 0.30	0.30 – 0.31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Monnet *et al.* (2020)

n.d. = not determined

9.2.3 COLOR

Furthermore, pulse flours might also alter the color properties of the cake crumb based on their inherent color (de la Hera *et al.*, 2012; Gómez *et al.*, 2008; Singh *et al.*, 2015). In addition, these ingredients might intensify the browning of the cake crust during baking, which might be explained by their higher contents of proteins providing amino acids that can undergo Maillard reaction (Gómez *et al.*, 2008; Singh *et al.*, 2015). The exact phenomena will be discussed along with further chemical reactions likely to occur during sponge cake development in the following chapter.

CHAPTER 3

REACTIVITY AND ODOR ACTIVITY

One of the most important attributes of food influencing consumer acceptance or rejection is flavor, which combines gustative, olfactory and trigeminal sensations (Kilcast, 2013; Thompson, 2010). Taste describes the perception of non-volatile substances like saponins and phenolic acids, carried by saliva to the receptors on the tongue and other oral surfaces (Kilcast, 2013; Roland *et al.*, 2017). By contrast, volatile aroma compounds are the chemical stimuli responsible for food odor (Jeleń *et al.*, 2017; Reineccius *et al.*, 2013). These molecules are transmitted *via* inhaled air either through orthonasal or retronasal pathways to the olfactory receptors of the nasal cavity (Kilcast, 2013; Reineccius *et al.*, 2013). While the orthonasal pathway describes a direct travel of the odorant substances through the nostrils, the retronasal pathway comprises the indirect route during exhaling through the nasopharynx (Reineccius *et al.*, 2013; Siebert, 2013). A prerequisite for the detection of aroma compounds is their presence in concentrations higher than a specific odor threshold, which is typically in the mg/L to pg/L range (Jeleń et al., 2017). Furthermore, flavor might encompass trigeminal perceptions, such as cooling and burning, which can be triggered by volatiles or dissolved chemicals (Kilcast, 2013). Typical examples include carbonic acid in fizzy drinks as well as pungent thiocyanates in mustard (Kilcast, 2013).

The present dissertation will solely focus on odorous compounds, in particular their occurrence in pea raw materials as well as their possible generation during product development through lipid oxidation, Maillard reaction and caramelization.

10 ODOR ACTIVITY AND MATRIX EFFECTS

Odorant volatile organic compounds, commonly abbreviated to VOCs, comprise a large variety of molecules with low molecular mass (<300 Da), which belong to different chemical families, including alcohols, aldehydes, ketones, acids, terpenes as well as sulfurous, nitrogenous and furanic compounds (Jeleń *et al.*, 2017). To detect these VOCs, they must occur in concentrations higher than their specific ***ODOR THRESHOLD***. This value is determined by the structural properties of the molecule, i.e. chain length, stereochemistry, degree of saturation, position of double bonds and position of functional groups (Jeleń *et al.*, 2017).

In order to assess the aroma impact of such structures on food products, their ***ODOR ACTIVITY VALUE*** is often discussed, which is defined as the ratio of the molecule's concentration to its odor threshold (Jeleń *et al.*, 2017). Accordingly, compounds with high odor thresholds might be of sensory importance if they accumulate at high concentrations, whereas odorants with low odor thresholds are likely to be perceived already at trace amounts.

Nevertheless, the perception of odor-active compounds depends on their ***INTERACTIONS WITH FOOD CONSTITUENTS***, which affects the odorant's partitioning between the matrix and gas phase (Jeleń *et al.*, 2017; Paravisini *et al.*, 2017; Wang *et al.*, 2017a). Food products are complex systems which contain lipids, proteins and carbohydrates, among other ingredients. An estimation of the distribution of volatiles between the oil and water phase can be achieved by calculation of the so-called log *P* value, which corresponds to the logarithm of their octanol-water partition coefficients (Paravisini *et al.*, 2017). Consistently, hydrophilic compounds with log $P < 1$ exhibit high water solubilities, while those with log $P > 1$ solubilize better in oil. Food systems with high lipid contents might thus lead to a greater retention of hydrophobic molecules with log $P > 1$ (Paravisini *et al.*, 2017). In addition, carbohydrates like cyclodextrins and starch can interact with aroma molecules by formation of inclusion complexes in which the odorants are entrapped (Paravisini *et al.*, 2017; Wang *et al.*, 2017a). Effects of mono- and disaccharides on flavor release have likewise been reported. With increasing amount of these sugars, the water activity of the medium gradually decreases, which lowers the solubility of hydrophilic volatiles and thus stimulates flavor release (Paravisini *et al.*, 2017; Wang *et al.*, 2017a). Simultaneously, the dehydration results in a more hydrophobic environment fortifying the retention of more hydrophobic molecules. Steric effects related to the sugar-indcued viscosity increase might also reduce aroma liberation (Paravisini *et al.*, 2017). Apart from lipids and carbohydrates, also proteins are capable of binding volatiles (e.g. aldehydes) through their amino acid side chains *via* hydrophobic interactions, hydrogen and ionic bonds, van der Waals forces and covalent linkages (Wang *et al.*, 2017a). Such matrix effects might be beneficial in products where process-induced off-flavors are generated, however might rather be undesirable in products with added aroma compounds.

11 LIPID OXIDATION

Lipid oxidation is one of the major causes of aroma quality deterioration of food during production or storage. This chemical reaction signifies the degradation of fatty acids in the presence of oxygen, leading to the formation of multitudinous odor-active volatiles.

Dependent on availability of catalysts, lipid oxidation takes place through three different pathways, namely autoxidation, photooxidation or enzyme-catalyzed oxidation. The principles of these are visualized in **Figure 20** and will be thoroughly described in the following sections.

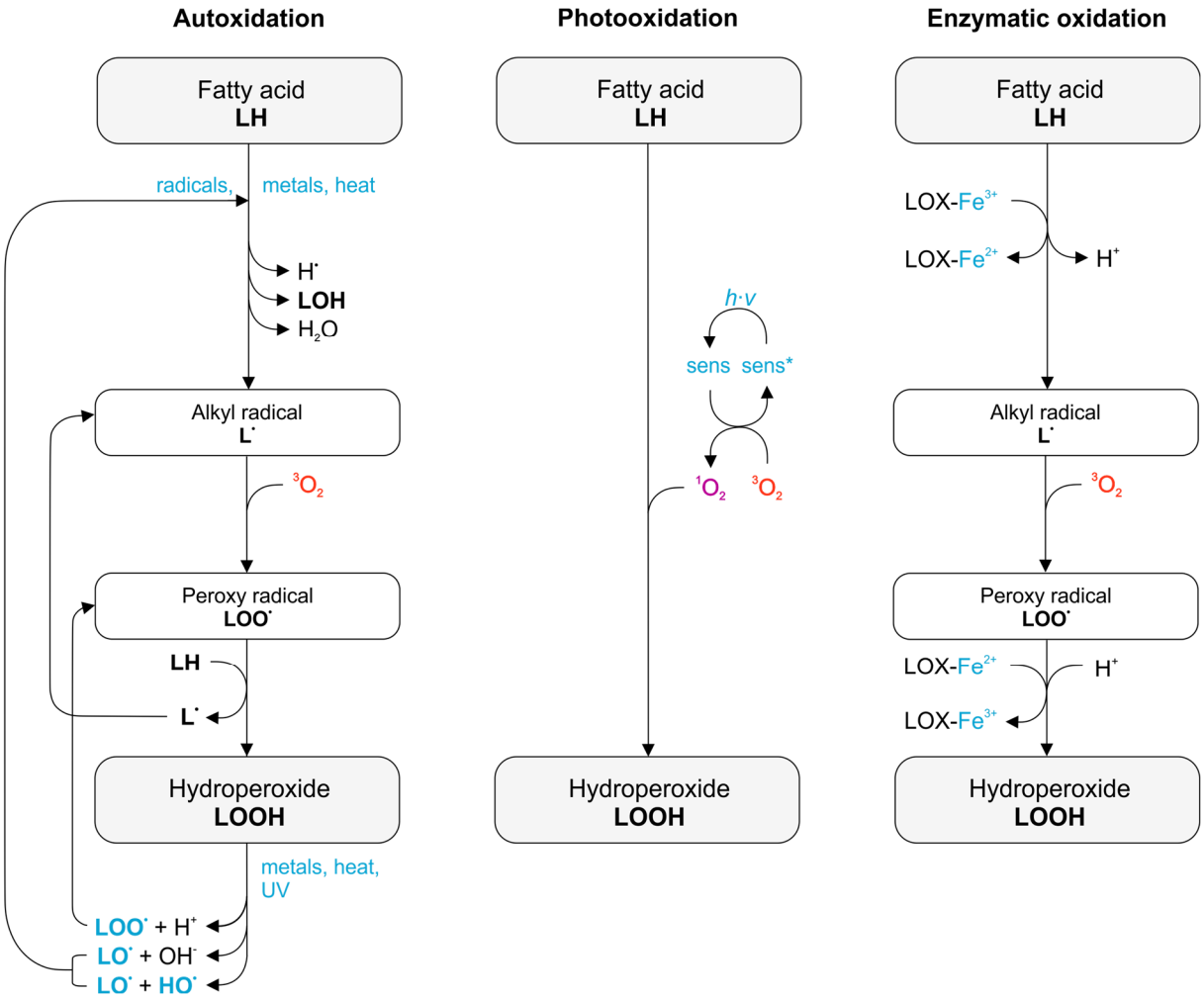

Figure 20. Reaction scheme of the principle lipid oxidation pathways autoxidation, photooxidation and enzyme-catalyzed oxidation. Catalysts are marked in blue, triplet oxygen ($^3O_2$) in orange and singlet oxygen ($^1O_2$) in pink. Radicals are indicated by a dot (•). The enzyme lipoxygenase is abbreviated to "LOX" and photosensitizers to "sens", with their activated form being indicated as "sens*". Information is based on Kamal-Eldin *et al.* (2003) and Schaich (2013).

11.1 AUTOXIDATION

Autoxidation denotes the autocatalytic process of oxidative fatty acid breakdown by a free radical chain reaction mechanism through three phases: initiation, propagation and termination (Kamal-Eldin *et al.*, 2003; Schaich, 2013).

11.1.1 INITIATION

As indicated in **Figure 20**, the oxidative phenomenon is initiated by the abstraction of hydrogen from the fatty acid (LH) to create a resonance-stabilized alkyl radical ($L^{\bullet}$) (**Eq. 1**) (Schaich, 2013):

$$LH \rightarrow L^{\bullet} + H^{\bullet} \quad (1)$$

Despite numerous studies on lipid oxidation, the exact mechanism responsible for the formation of the first alkyl radical during autoxidation remains an area of controversy (Kamal-Eldin *et al.*, 2003). Some researchers suggested that trace amounts of ***METAL IONS***, such as iron and copper, can act as catalysts (Kamal-Eldin *et al.*, 2003). Moreover, ***ULTRAVIOLET (UV) LIGHT*** and ***ELEVATED TEMPERATURES*** can also be considered as initiators (Schaich, 2013).

How easily hydrogen is abstracted from the fatty acid depends on its structure. The lowest energy input is needed for hydrogens in methylene groups of 1,4-pentadiene systems as present in polyunsaturated fatty acids like linoleic and linolenic acid (Belitz *et al.*, 2009). This can be ascribed to the effective stabilization of the generated alkyl radical by electron delocalization over five adjacent carbon atoms (Belitz *et al.*, 2009). Accordingly, the energy input needed is comparatively higher for monounsaturated fatty acids (e.g. oleic acid) due to resonance stabilization over only three carbon atoms, whereas even higher values are needed for saturated fatty acids (Belitz *et al.*, 2009). Therefore, considerably higher oxidation rates have been identified for polyunsaturated fatty acids compared to monounsaturated and saturated fatty acids as shown in **Table 13**.

Table 13. Relative oxidation rates of fatty acids at 25 °C (according to Belitz *et al.* (2009)).

Fatty acid	Relative oxidation rate
Stearic acid (C18:0)	1
Oleic acid (C18:1)	100
Linoleic acid (C18:2)	1200
Linolenic acid (C18:3)	2500

11.1.2 PROPAGATION

FORMATION OF HYDROPEROXIDES

The conversion of fatty acids into the corresponding alkyl radicals is an important prerequisite for the subsequent reaction with atmospheric oxygen, which otherwise would not take place owing to the special arrangement of valence electrons in molecular oxygen. This compound contains two unpaired electrons with same spin occupying separate orbitals, hence leading to a biradical in triplet state (Kim *et al.*, 2008). Direct reaction with fatty acids would violate the exclusion principle of Pauli, describing that orbitals cannot contain electrons with same spin (Zhou *et al.*, 2013). By contrast, this so-called ***TRIPLET***

OXYGEN ($^3O_2$) can rapidly react with fatty acid radicals under formation of peroxyl radicals ($LOO^\bullet$) (**Eq. 2**):

$$L^\bullet + {}^3O_2 \rightarrow LOO^\bullet \qquad \textbf{2}$$

This step is characterized by a high reaction rate constant of approximately 3×10^8 M^{-1} s^{-1} (Kamal-Eldin *et al.*, 2003). By contrast, the direct reaction of fatty acids with triplet oxygen is correlated with a rate constant of 6×10^{-11} M^{-1} s^{-1} (Kamal-Eldin *et al.*, 2003).

Thereafter, the peroxyl radical is transformed into the corresponding hydroperoxide (LOOH) by abstracting a hydrogen from a second unsaturated fatty acid (**Eq. 3**). The alkyl radical thereby released can react again with molecular oxygen as described in **Eq. 2**.

$$LOO^\bullet + LH \rightarrow LOOH + L^\bullet \qquad \textbf{3}$$

Depending on the number of double bonds of a fatty acid, different hydroperoxides can be formed. In the case of ***OLEIC ACID*** (C18:1), the hydrogen can be removed from the allylic methylene group on either C8 or C11, giving rise to the four hydroperoxides 8-LOOH (27%), 9-LOOH (23%) 10-LOOH (23%) and 11-LOOH (27%) (**Figure 21**) (Belitz *et al.*, 2009; Frankel, 2012c).

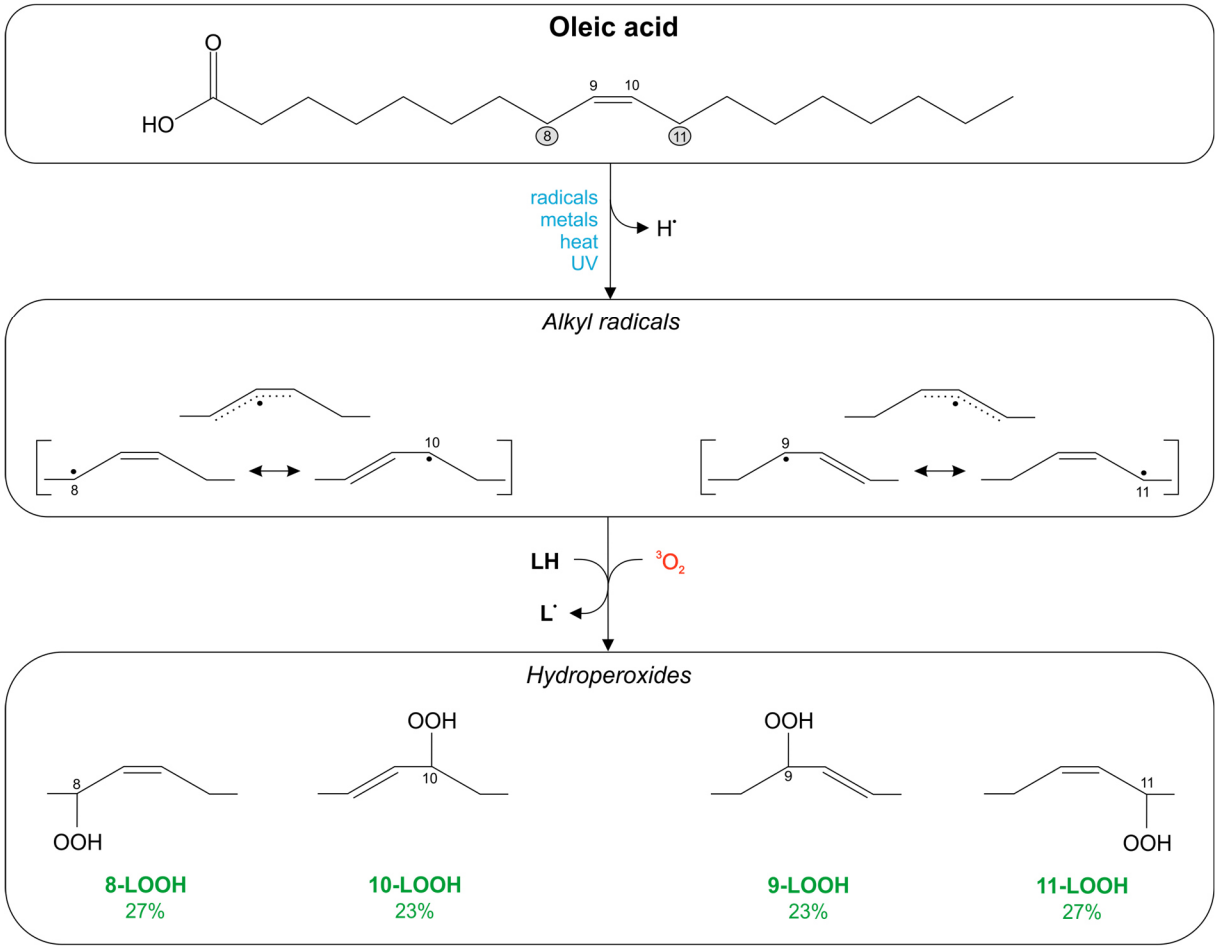

Figure 21. Autoxidation of oleic acid (based on Belitz *et al.* (2009) and Frankel (2012c)). Primary oxidation products are 8-hydroperoxyoctadec-9-enoic acid (8-LOOH), 10-hydroperoxyoctadec-8-enoic acid (10-LOOH), 9-hydroperoxyoctadec-10-enoic acid (9-LOOH) and 11-hydroperoxyoctadec-9-enoic acid (11-LOOH). The simplified reaction scheme does not consider the possible configurations of the double bonds.

By contrast, only two major hydroperoxides are formed from ***LINOLEIC ACID*** (C18:2) by abstraction of hydrogen from the active bis-allylic methylene group on C11 and stabilization of the pentadienyl radical through formation of 9-LOOH (47%) and 13-LOOH (50%) (**Figure 22**) (Belitz *et al.*, 2009; Frankel, 2012c). These hydroperoxides possess conjugated double bonds, which can absorb in the ultraviolet range around 235 nm (Belitz *et al.*, 2009). In addition, traces (3 – 4%) of 8-LOOH, 10-LOOH, 12-LOOH and 14-LOOH can be found, which originate from the removal of hydrogen from the mono-allylic groups on C8 and C14 (Belitz *et al.*, 2009).

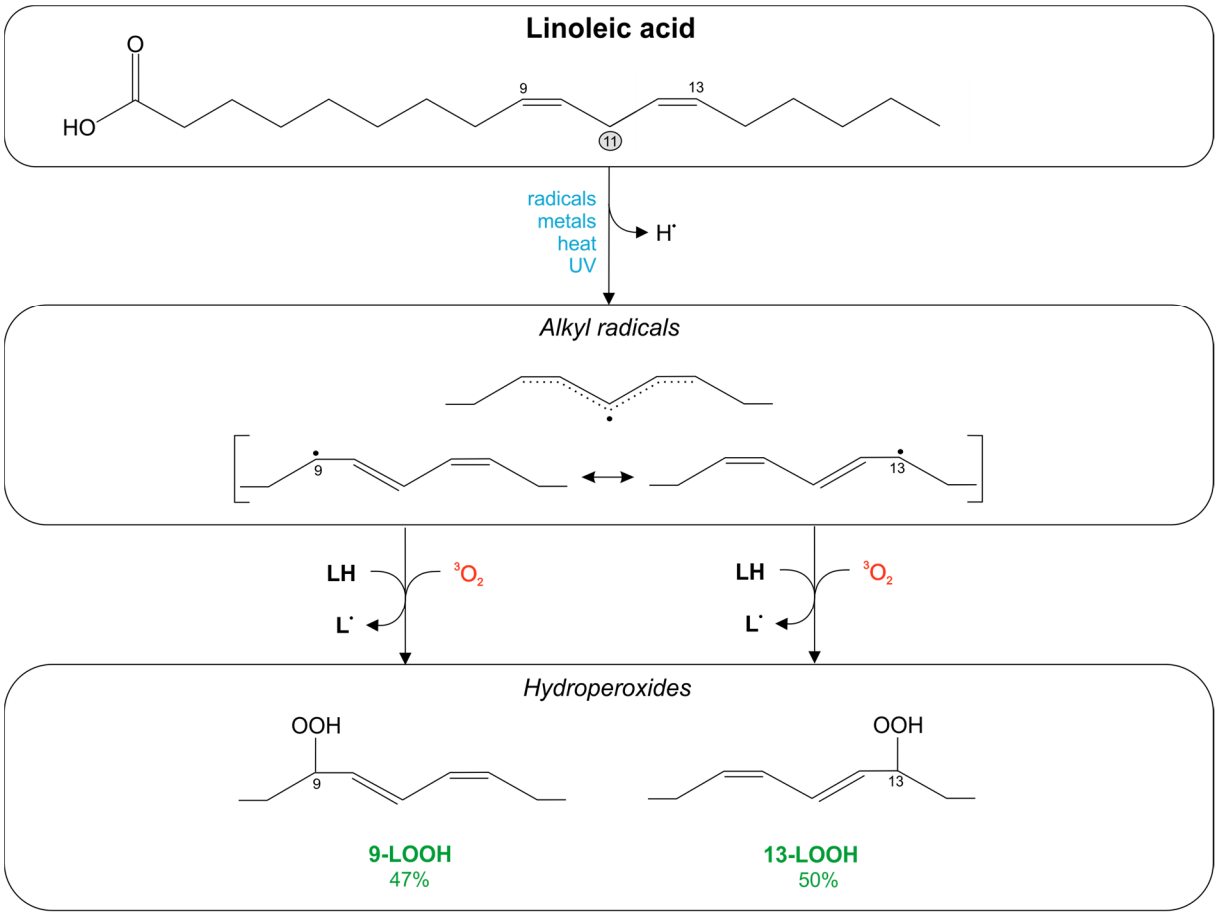

Figure 22. Autoxidation of linoleic acid (based on Belitz *et al.* (2009) and Frankel (2012c)). Primary oxidation products are 9-hydroperoxyoctadeca-10,12-dienoic acid (9-LOOH) and 13-hydroperoxyoctadeca-9,11-dienoic acid (13-LOOH). The simplified reaction scheme does not consider the possible configurations of the double bonds.

Due to the presence of two bis-allylic methylene groups in ***LINOLENIC ACID*** (C18:3), two pentadienyl radicals can be produced, from which four main hydroperoxides are formed during the autoxidation of this compound. While the removal of hydrogen from C11 leads to the formation of 9-LOOH (31%) and 13-LOOH (12%), the abstraction from C14 leads to the release of 12-LOOH (11%) and 16-LOOH (46%) (**Figure 23**) (Belitz *et al.*, 2009; Frankel, 2012c). The percentage minority of 12- and 13-LOOH can be explained by the high reactivity of their radicals, which can rapidly undergo 1,3- and 1,5-cyclizations and react with molecular oxygen to generate hydroperoxide epidioxides (examples are shown in **Figure 23**).

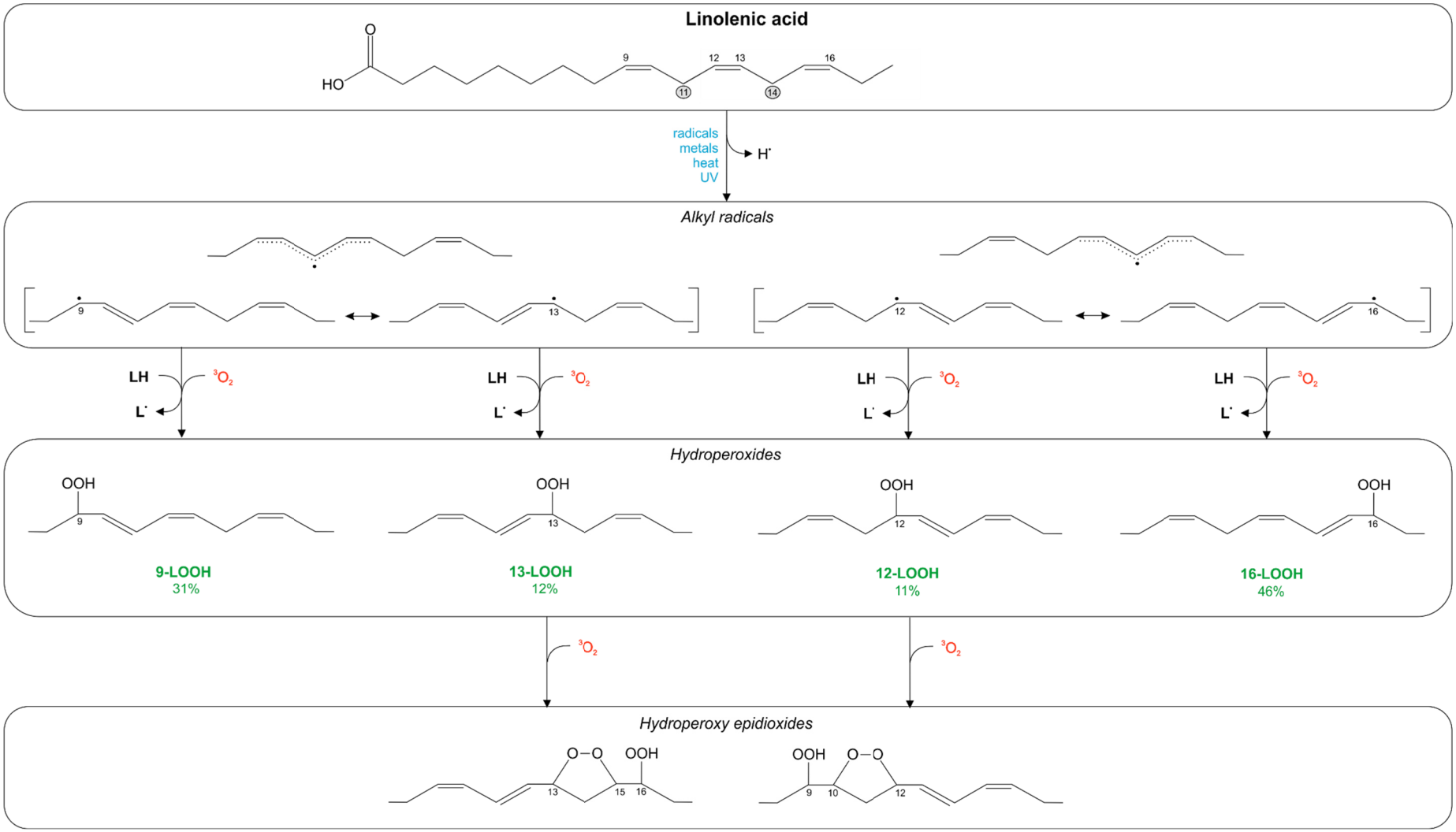

Figure 23. Autoxidation of linolenic acid (based on Belitz *et al.* (2009) and Frankel (2012c)). Primary oxidation products are 9-hydroperoxyoctadeca-10,12,15-trienoic acid (9-LOOH), 12-hydroperoxyoctadeca-9,13,15-trienoic acid (12-LOOH), 13-hydroperoxyoctadeca-9,11,15-trienoic acid (13-LOOH) and 16-hydroperoxyoctadeca-9,12,14-trienoic acid (16-LOOH). The simplified reaction scheme does not consider the possible configurations of the double bonds.

DECOMPOSITION OF HYDROPEROXIDES

In the absence of metal ions and UV light, the presence of antioxidants and at low temperatures, the produced hydroperoxides possess relatively high stability (Kamal-Eldin *et al.*, 2003). However, opposite conditions can lead to the decomposition of hydroperoxides under formation of peroxyl ($LOO^{\bullet}$), alkoxyl ($LO^{\bullet}$) and hydroxyl ($HO^{\bullet}$) radicals (**Eq. 4 - 6**, **Figure 20**):

$$LOOH \rightarrow LOO^{\bullet} + H^{+} \quad (\mathbf{4})$$

$$LOOH \rightarrow LO^{\bullet} + HO^{\bullet} \quad (\mathbf{5})$$

$$LOOH \rightarrow LO^{\bullet} + HO^{-} \quad (\mathbf{6})$$

The generated radicals can re-initiate autoxidation of fatty acids according to **Eq. 3 and Eq. 7 - 8**, which hence proceeds in an autocatalytic and greatly accelerated manner:

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (\mathbf{3})$$

$$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet} \quad (\mathbf{7})$$

$$HO^{\bullet} + LH \rightarrow H_2O + L^{\bullet} \quad (\mathbf{8})$$

Nevertheless, the radicals can abstract hydrogen also from newly formed hydroperoxides instead of fatty acids, thereby simultaneously forming further highly reactive radicals (**Eq. 9 – 10**):

$$LO^{\bullet} + L'OOH \rightarrow LOH + L'OO^{\bullet} \quad (\mathbf{9})$$

$$LOO^{\bullet} + L'OOH \rightarrow LOOH + L'OO^{\bullet} \quad (\mathbf{10})$$

11.1.3 TERMINATION

With increasing concentration of radicals, the probability of their collision is increasing, which leads to the termination of the chain reaction. Possible interactions include (Belitz *et al.*, 2009; Frankel, 2012a):

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow stable\ products \quad (\mathbf{11})$$

$$L^{\bullet} + L^{\bullet} \rightarrow stable\ products \quad (\mathbf{12})$$

$$L^{\bullet} + LOO^{\bullet} \rightarrow stable\ products \quad (\mathbf{13})$$

$$L^{\bullet} + LO^{\bullet} \rightarrow stable\ products \quad (\mathbf{14})$$

Alternatively, termination can involve antioxidants, such as tocopherol, which donates a hydrogen to the respective radicals to create inactive products (Belitz *et al.*, 2009). The created antioxidant radical can further scavenge free radicals through direct collision (Belitz *et al.*, 2009).

11.2 PHOTOOXIDATION

Photooxidation denotes the light-induced oxidation of unsaturated fatty acids. This reaction takes place in the presence of ***PHOTOSENSITIZERS***, such as chlorophylls, pheophytins, riboflavins and heme proteins (e.g. catalase and peroxidase), which are capable of absorbing light energy and transferring it to ground state triplet oxygen (**Figure 20**) (Belitz *et al.*, 2009; Velasco *et al.*, 2010). This leads to the formation of ***SINGLET OXYGEN*** ($^1O_2$) in which the previously unpaired electrons pair in a common orbital (see **§ 11.1**). In this excited state, oxygen is highly electrophilic and thus rapidly reacts with electron-rich compounds containing double bonds like unsaturated fatty acids (Kim *et al.*, 2008). The reaction rate is approximately 1500 times higher compared to triplet oxygen (Frankel, 2012d). In the course of this

reaction, oxygen is incorporated at both sides of the double bond, hence leading to two hydroperoxides. Unlike autoxidation, the oxidation rates of oleic, linoleic and linolenic acid differ only slightly, implying that double bonds in polyunsaturated fatty acids behave as isolated alkene units (Belitz *et al.*, 2009).

The hydroperoxide compositions obtained from autoxidation and photooxidation are indicated in **Table 14**. It is generally suggested that once radicals have been formed by the reaction of fatty acids with singlet oxygen, the free-radical autoxidation reaction is initiated (Kim *et al.*, 2008). Alternatively to this pathway, light-activated photosensitizers can also directly react with fatty acids under formation of radicals, which afterwards induce autoxidation (Belitz *et al.*, 2009).

Table 14. Hydroperoxides formed by autoxidation and photooxidation of unsaturated fatty acids (according to Belitz *et al.* (2009)).

Fatty acid	Hydroperoxide	Double bond position	Proportion [%]	
			Autoxidation	Photooxidation
Oleic acid	8	9	27	-
(C18:1)	9	10	23	48
	10	8	23	52
	11	9	27	-
Linoleic acid	8	9,12	1.5	-
(C18:2)	9	10,12	46.5	32
	10	8,12	0.5	17
	12	9,13	0.5	17
	13	9,11	49.5	34
	14	9,12	1.5	-
Linolenic acid	9	10,12,15	31	23
(C18:3)	10	8,12,15	-	13
	12	9,13,15	11	12
	13	9,11,15	12	14
	15	9,12,16	-	13
	16	9,12,14	46	25

11.3 LIPOXYGENASE-CATALYZED OXIDATION

Lipoxygenases (linoleic acid oxygen oxidoreductase, EC 1.13.11.12, LOX) are iron-containing enzymes naturally occurring in various plants, animals and microorganisms that catalyze the oxidation of polyunsaturated fatty acids into conjugated hydroperoxides in the presence of oxygen (Belitz *et al.*, 2009; Rackis *et al.*, 1979; Szymanowska *et al.*, 2009). Solely fatty acids with characteristic ***CIS,CIS-1,4-PENTADIENE SYSTEM*** as in linoleic and linolenic acid are adequate substrates of LOX (Belitz *et al.*, 2009). These are often freely available in the raw materials, however might also be released from glycerides by the action of lipolytic enzymes (Velasco *et al.*, 2010). Oleic acid is not oxidized.

LOX-mediated oxidation takes place readily after the injury of plant tissue, e.g. as a consequence of harvesting or grinding, owing to the disruption of the compartmentalization of cells, which allows the enzyme and its substrates to come in close proximity. In the native enzyme, the active center of LOX contains iron in Fe^{2+} form, which needs to be oxidized to Fe^{3+} in order to initiate the oxidative breakdown

of polyunsaturated fatty acids (Robinson *et al.*, 1995). Such activation of the enzyme is achieved by its product, the hydroperoxide (Robinson *et al.*, 1995). After activation, the second step involves the removal of hydrogen from the methylene group of the acid's pentadiene system to create an alkyl radical (L•), which is rearranged into a conjugated diene system (Belitz *et al.*, 2009). Thereafter, molecular oxygen is introduced under formation of a peroxyl radical (LOO•), which is transformed into the corresponding hydroperoxide (LOOH) upon hydrogen uptake and finally released from the enzyme (Belitz *et al.*, 2009). A typical scheme of the lipoxygenase catalysis is presented in **Figure 24**.

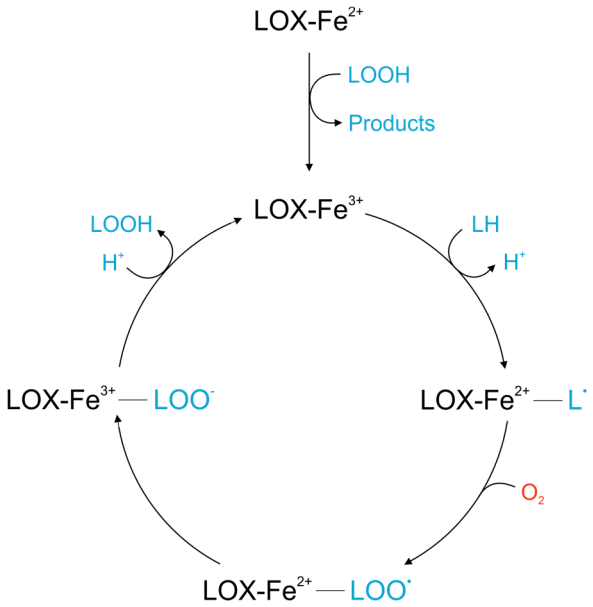

Figure 24. Proposed mechanism of lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids (according to Belitz *et al.* (2009)).LH = fatty acid, L• = alkyl radical, LOO• = peroxyl radical and LOOH = hydroperoxide.

11.3.1 LIPOXYGENASES IN PEA

Pea might contain three, four or five ***ISOENZYMES*** of LOX, which differ in their amino acid sequence but catalyze the same chemical reaction (Fischer *et al.*, 2020; Shannon, 1968). According to a study of Eriksson (1967), approximately 80% of the total enzyme of green peas were located in the outer part of the cotyledon, whereas only 12% were present in the inner part of the cotyledon and 5 – 8% in the skin. However, highest enzyme activities were determined in the inner part of the cotyledon, followed by the outer part and the skin (Eriksson, 1967).

Szymanowska *et al.* (2009) ascertained, that the main substrate of pea LOX is ***LINOLEIC ACID*** with which a relative activity of 100% was obtained, whereas the value was considerably lower with linolenic acid (40%). The insertion of oxygen into these fatty acids is ***REGIO- AND STEREOSPECIFIC***. In the case of pea, LOX with abilities to include oxygen at the positions 9, 10 and 13 of the carbon chain have been identified, leading to the formation of 9-, 10- and 13-hydroperoxides (Fischer *et al.*, 2020). As typical for all enzymes, these pea LOX also exhibit specific ***PH OPTIMA*** at which the catalytic activity is highest. Of the five in total identified pea LOX isoenzymes, one has a pH optimum above 8 and three around pH 6 – 7 (Fischer *et al.*, 2020). Fischer *et al.* (2020) assumed that the latter three possess high

TEMPERATURE SENSITIVITY, whereas the former is rather heat-stable (Fischer *et al.*, 2020). An overview of the pea LOX properties is given in **Table 15**.

Table 15. Properties of pea lipoxygenase isoenzymes(according to Fischer *et al.* (2020)

Isoenzyme	pH optimum	Temperature sensitivity	Hydroperoxide
LOX-1	>8	Heat-stable	13
LOX-2	6 – 7	Heat-labile	13
LOX-3	6 – 7	Heat-labile	9
LOX-4	Unknown	Unknown	unknown
LOX-5	6 – 7	Heat-labile	10

Apart from thermal inactivation, pea LOX can be inhibited by phenolic compounds as reported by Szymanowska *et al.* (2009). Among the polyphenols studied, the authors yielded highest inhibition (58%) with caffeic acid at highest concentration of 2 mM (Szymanowska *et al.*, 2009).

11.4 VOLATILE LIPID OXIDATION MARKERS

Metal ions as well as elevated temperatures can catalyze the decomposition of hydroperoxides formed *via* enzymatic and non-enzymatic lipid oxidation (**§ 11.1 – 11.3**) into alkoxyl radicals ($LO^{\bullet}$) according to **Eq. 5** and **Eq. 6** (Belitz *et al.*, 2009; Frankel, 2012b). In the course of autoxidation, these can subsequently re-initiate the radical chain reaction by abstraction of hydrogen from fatty acids or hydroperoxides (**Eq. 7**, **Eq. 9**). Concurrently, the alkoxy radicals can be homolytically cleaved under formation of volatile compounds following a mechanism referred to as ***β-SCISSION***. Two fragmentation paths have been discussed: path A involves the cleavage of the C-C bond on the side of the oxygen-bearing carbon atom, while path B involves the cleavage of the C-C bond between the double bond on the oxygen-bearing carbon atom (Kochhar, 1996). The latter mechanism is energetically favored due to the formation of intermediate resonance-stabilized “oxoene” or “oxo-diene” molecules (Belitz *et al.*, 2009). The volatiles formed typically belong to the chemical classes of alcohols, aldehydes and ketones, the majority of which are known to possess pronounced odor-activity. Further enzymes may be involved in the development of such molecules, including alcohol dehydrogenases, hydroperoxide lyases and hydroperoxide isomerases (Duy Bao *et al.*, 2013; Springett, 1996). **Table 16** summarizes/proposes potential origins of multiple molecules reported in the literature, i.e. fatty acid hydroperoxide and scission path, as well as their associated odors and threshold values.

Table 16. Volatile organic compounds originating from oleic, linoleic and linolenic acid oxidation. Their corresponding hydroperoxide (LOOH), scission path, associated odor and odor threshold are indicated.

Compound	LOOH	Path	Odor	Threshold in water [µL/L]	Reference
Oleic acid (C18:1)					
1-Heptanol	11	B	Green, bean, oxidized	330 – 520	1,10,11,13,14,24,27
1-Octanol	10	B	Earthy, moldy, vegetable	110 – 875	10,11,13,14,23,24,27
Heptanal	11	B	Fatty, rancid, citrus, malty	3 – 5	1,2,7,10,11,23,27
Octanal	10 / 11	B / A	Citrus, flowery, fatty, soapy	0.7 – 5	1,2,6,10,23,27
Nonanal	9 / 10	B / A	Citrus, soapy, tallowy	1	1,2,6,10,23,27
Decanal	8	B	Orange peel, citrus	2 – 7	1,2,6,10,23,27
2-Decenal	9	A	Metallic	0.4	1,2,6,10,23,27
2-Undecenal	8	A	Fruity	n.d.	1,6,10,27
Linoleic acid (C18:2)					
1-Butanol	14	B	Fruity, solvent, oxidized	500 – 7500	1,10,11,12,23
1-Pentanol	13 / 14	B / A	Balsamic, fruity, fusel-like sweet	4000 – 4500	1,6,7, 10,11,12,23,27
1-Hexanol	13	A	Green, bean, flowery, woody	2500	1,2,5,6,10,11,23,24
1-Heptanol	n.d.	n.d.	Green, bean, oxidized	330 – 520	1,10,11,24
1-Octen-3-ol	10	B	Mushroom, musty	1	1,3,4,10,23,25
2-Octen-1-ol	10	B	Green, vegetable	50 – 840	1,3,4,10,25
Butanal	14	B	Malty	9	10,23
Pentanal	13 / 14	B / A	Pungent, acrid, bitter almond	12 – 18	1,6,7,10,23,27
Hexanal	12 / 13	B / A	Green, grassy, leafy, tallowy	4.5 – 5	1,2,5,6,10,23,24,27
Heptanal	n.d.	n.d.	Fatty, rancid, citrus, malty	3 – 5	1,10,23
Octanal	n.d.	n.d.	Citrus, flowery, fatty, soapy	0.7 – 5	1,10,23,25
2-Heptenal	12	A	Green, fatty, cream	0.8 – 13	1,5,10,23
2-Octenal	9 / 10	B / B	Nutty, fatty, roasted	3	1,2,4,5,10,23,25
2-Nonenal	9 / 10	B / A	Fatty, green, tallowy	0.08 – 6	1,2,5,10,23,25
2,4-Nonadienal	9	B	Deep fat-fried	0.01 – 0.05	1,2,10,25
2,4-Decadienal	9	A	Deep fat-fried	0.03 – 0.5	1,2,5,10,23,25,27
3-Nonenal	9 / 10	B / A	Green, melon	n.d.	1,5,26,27
1-Octen-3-one	10	B	Mushroom, moldy, metallic, fatty	0.02 – 0.1	1,4,10,11,25
3-Pentanone	n.d.	n.d.	Acetone	n.d.	8,9
Linolenic acid (C18:3)					
Ethanol	16	B	Alcoholic	16000	13,25
1-Penten-3-ol	13	B	Burnt, butter, grass, green	400 – 3000	1,10,16,17,24
2-Penten-1-ol	13	B	Green	n.d.	10,13,27
2-Hexen-1-ol	12 / 13	B / A	Green, sweet wine	100 – 6700	1,10,15,24
3-Hexen-1-ol	12 / 13	B / A	Grassy, green	100 – 1550	6,10,15,24
Acetaldehyde	16	B	Fruity	15	10,11,13,23
Propanal	16	A	Malty, fruity, pungent	10	1,6,7,10,11,23,27
2-Pentenal	13	B	Pungent, apple	n.d.	6,7,11,13,27
2-Hexenal	12 / 13	B / A	Green, leafy, fatty	17 – 100	1,2,7,10,11,15,23,24
2-Heptenal	n.d.	n.d.	Green, fatty, cream	0.8 – 13	1,7,10,23
2,4-Heptadienal	12	A	Fatty, green	49	1,2,6,7,11,20,24,27
2,5-Octadienal	10	B	n.d.	n.d.	7
3,6-Nonadienal	9 / 10	B / A	Fatty, green	0.05	6,13,19,27
2,4,7-Decatrienal	9	A	Fatty, pungent	n.d.	1,6,7,13,27
3-Hexenal	12 / 13	B / A	Cut beans	1 – 3	1,6,7,15,18,21,27
1-Penten-3-one	13	B	Sharp, fishy, oily, painty	1	1,11,15,16,17
1,5-Octadien-3-one	10	B	Green, geranium-like	0.001	1,7,10
3,5-Octadien-2-one	n.d.	n.d.	Fatty, fruity	150	1,7,11,12

n.d. = not documented

[1] Kochhar (1996); [2] Zhang *et al.* (2015a), [3] Kakumyan *et al.* (2009), [4] Kermasha *et al.* (2002), [5] Cerny (2007); [6] Frankel (2012b); [7] Belitz et al. (2009); [8] Sánchez-Ortiz *et al.* (2013); [9] Sakouhi *et al.* (2016a); [10] Pico *et al.* (2015); [11] Murray *et al.* (1976); [12] Buttery *et al.* (1998); [13] Hsieh *et al.* (1989); [14] Przybylski *et al.* (1995b); [15] Luning *et al.* (1995); [16] Salch *et al.* (1995); [17] Gardner *et al.* (1996); [18] Ullrich *et al.* (1988); [19] Maarse *et al.* (1996); [20] Burdock (2009); [21] Saxby (1996); [22] Hammer *et al.* (2013); [23] Buttery *et al.* (1988); [24] Qian *et al.* (2005); [25] Zhang *et al.* (2018); [26] Yajima *et al.* (1985); [27] Schaich (2013)

12 MAILLARD REACTION

Apart from lipid oxidation, odorous volatile compounds can emerge during thermal processing of raw ingredients or foods like cake, bread, coffee and meat from heat-induced degradation of nutrients (Parliment, 1999). In this context, the Maillard reaction represents an important chemical pathway, which has been named after the physicist and chemist Louis Camille Maillard (1878 – 1936) (Tamanna *et al.*, 2015). The reaction denotes the interaction between reducing sugars, such as glucose and fructose, and $–NH_2$ carrying compounds like amino acids, peptides and proteins (Maire, Rega, Cuvelier, Soto, & Giampaoli, 2013; Roland *et al.*, 2017). The thermally-induced process leads not only to the formation of brown melanoidins but also to a range of volatile compounds with low threshold values and key importance in the flavor development of food (Ma *et al.*, 2016; Reineccius, 2006). Therefore, Maillard reaction is commonly referred to as non-enzymatic browning reaction.

12.1 PRIMARY PHASE

12.1.1 FORMATION OF AMADORI AND HEYNS COMPOUNDS

In a first step, a reducing sugar is nucleophilic attacked by an amino compound at the carbonyl function, which leads to the formation of an imine (Belitz *et al.*, 2009; Cerny, 2007; Hodge, 1953). This so-called Schiff base can be rearranged *via* 1,2-eneaminols into an aminoketone (Amadori compound) or an aminoaldose (Heyns compound) (Belitz *et al.*, 2009). While the former arises from aldose sugars like glucose, the latter is formed in the presence of ketose sugars like fructose (Cerny, 2007) (**Figure 25**).

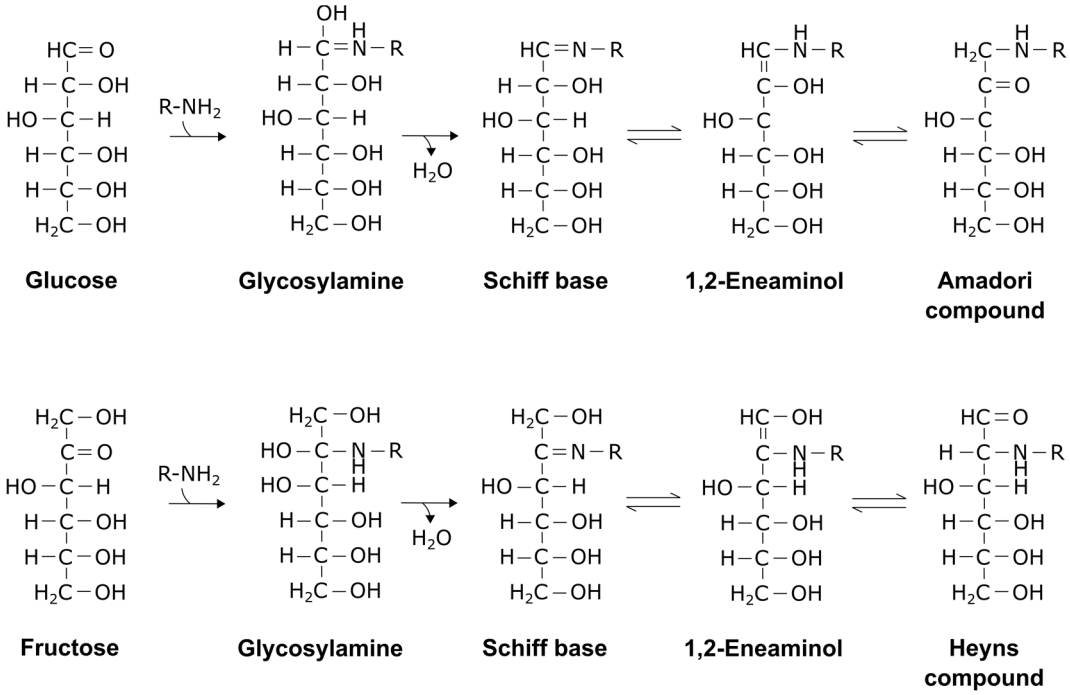

Figure 25. Formation of Amadori and Heyns compounds from glucose and fructose, respectively, during the initial stage of Maillard reaction (according to Belitz *et al.* (2009)).

12.1.2 FORMATION OF DEOXYOSONES

The generated N-glycosides have limited stability and are therefore easily transformed into α-dicarbonyl compounds, including 1-, 3- and 4-deoxyosones, as depicted in **Figure 26** (Belitz *et al.*, 2009). The reaction mechanism involves the enolization of the Amadori compound into either 1,2-eneaminol or 2,3-eneaminol, which subsequently give rise to the corresponding deoxyosones after elimination of water and/or the amino group.

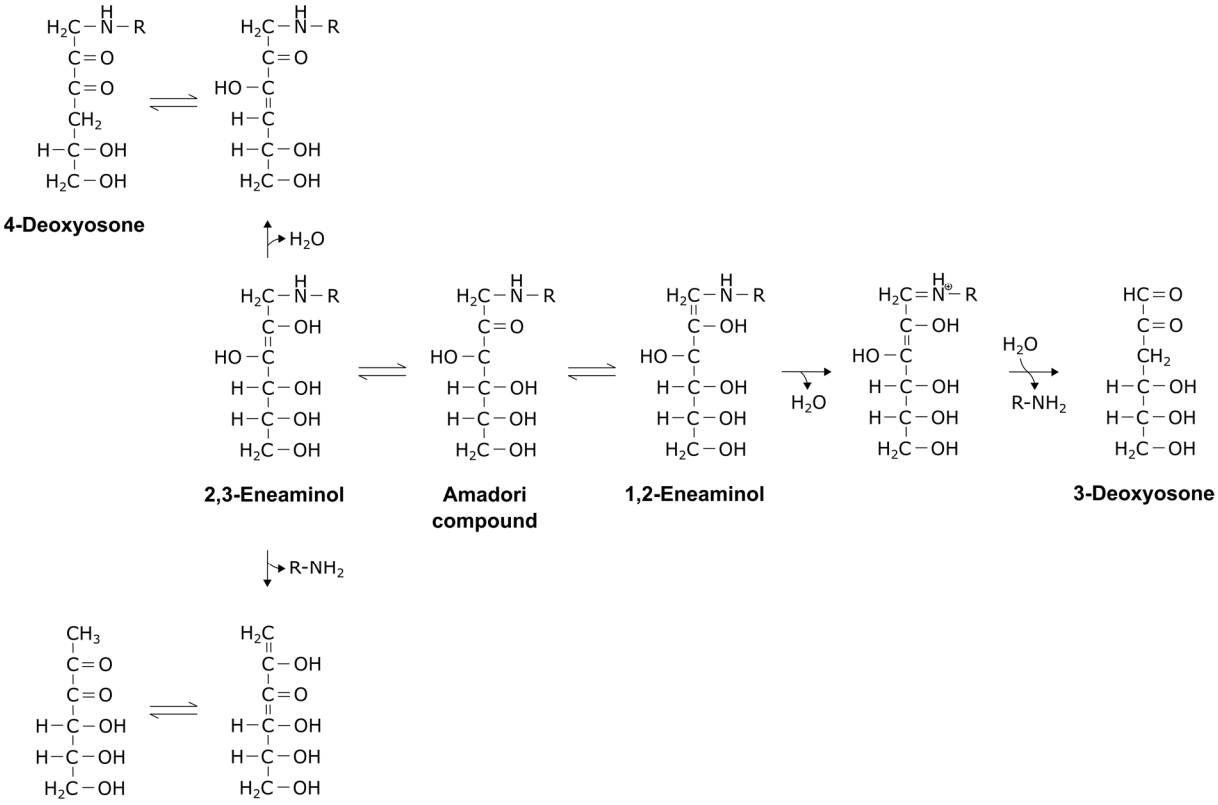

Figure 26. Formation of 1-, 3- and 4-deoxyosones from Amadori compound during Maillard reaction. (adapted from Belitz *et al.* (2009)).

12.2 SECONDARY PHASE

The produced 1-, 3- and 4-deoxyosones are highly reactive compounds, which are likely to undergo further reactions, resulting in the release of a wide variety of molecules. Moreover, they can be fragmented into shorter α-dicarbonyl compounds, which likewise possess high reactivity. For instance, 1- and 3-deoxyosone can undergo retroaldolisation either directly, which gives rise to glyceraldehyde and pyruvaldehyde (also referred to as methylglyoxal or 2-oxopropanal) or after enolization, which leads to glycolaldehyde that can be oxidized to glyoxal (Nursten, 2005; Yaylayan *et al.*, 1999) (**Figure 27**).

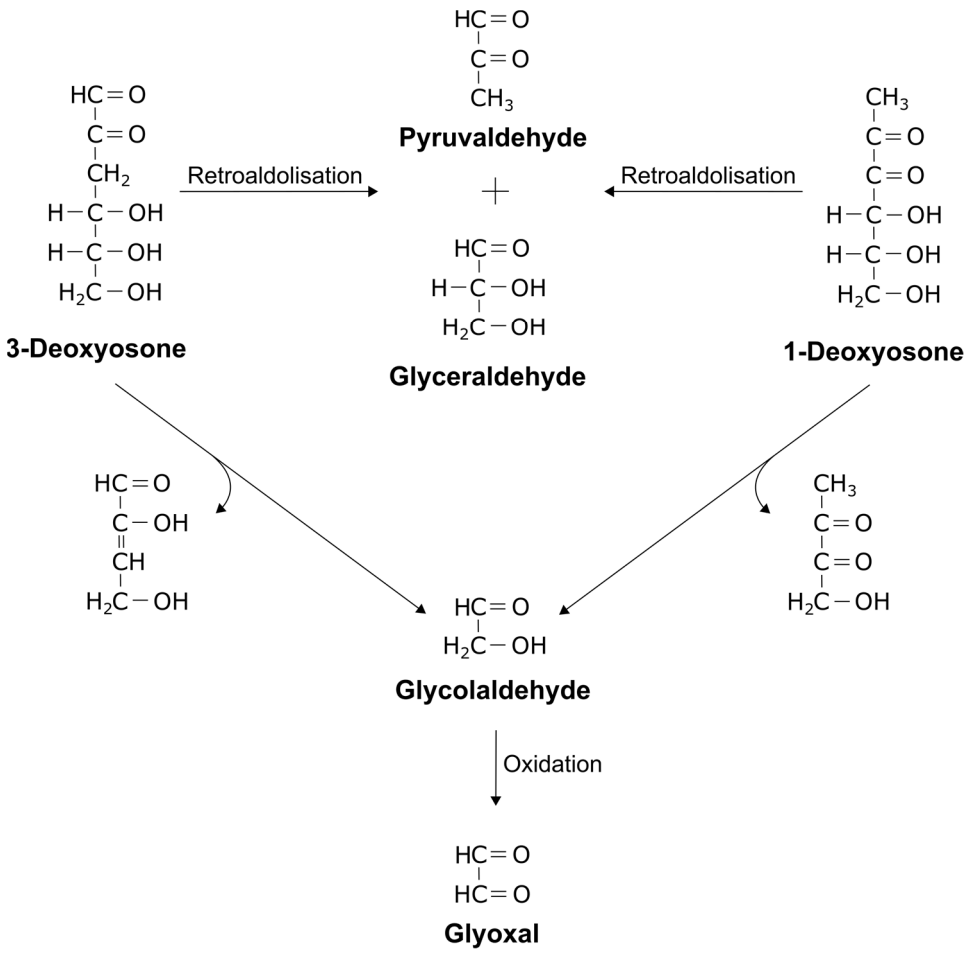

Figure 27. Fragmentation of 1- and 3-deoxyosones into short α-dicarbonyl compounds (adapted from Nursten (2005)).

Some of the generated short α-dicarbonyl compounds can derive from alternative pathways. For example, glyoxal could likewise originate from glucose upon dehydration and retro-aldol cleavage (Yaylayan *et al.*, 2000) and pyruvaldehyde from Amadori products through retro-aldol cleavage (Yaylayan *et al.*, 2000). The wide variety of α-dicarbonyl compounds created can participate in subsequent reactions, which might yield molecules with pronounced odor activity. Some of these pathways will be described in the following sections.

12.2.1 SPECIFIC KETONES

2,3-Butanedione (diacetyl), 2,3-pentanedione, 1-hydroxy-2-propanone (hydroxyacetone or acetol) and 3-hydroxy-2-butanone (acetoin) are volatiles with characteristics sweet and buttery odors that are commonly detected in bakery products (Cepeda-Vázquez *et al.*, 2019; Maire *et al.*, 2013; Pico *et al.*, 2015). According to Yaylayan *et al.* (1999) and Yaylayan *et al.* (2000), 2,3-butanedione might be either directly formed from glucose upon dehydration and retro-aldol cleavage or from pyruvaldehyde, followed by chain elongation by one carbon atom coming from amino acids like glycine. The latter pathway can be similarly applied to describe the production of 2,3-pentanedione, however requires the incorporation of two carbon units into pyruvaldehyde, which, for instance, could be donated by alanine (Yaylayan *et al.*, 2000). In addition, 2,3-butanedione can be reduced under formation of 3-hydroxy-2-butanone

(Yaylayan *et al.*, 2000). The authors further suggested that both dehydrated Amadori product and isomerized 1-deoxyosone could be precursors for 1-hydroxy-2-propanone upon retro-aldol cleavage (Yaylayan *et al.*, 2000). An overview of the reaction pathways is schematically illustrated in **Figure 28**.

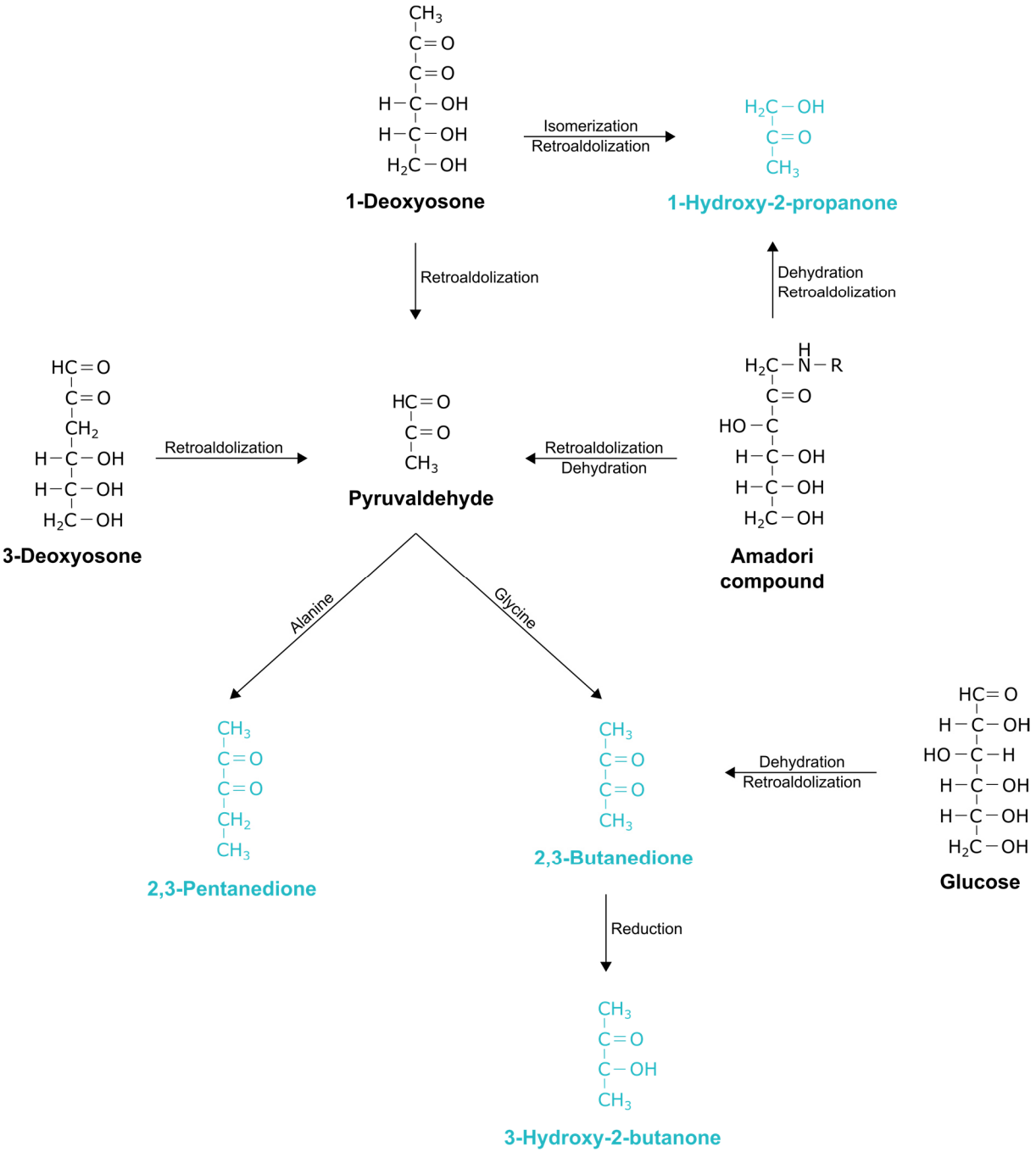

Figure 28. Proposed generation pathways of 2,3-butanedione, 2,3-pentanedione, 1-hydroxy-2-propanone and 3-hydroxy-2-butanone (based on Yaylayan *et al.* (2000), Yaylayan *et al.* (1999) and Nursten (2005)).

12.2.2 STRECKER ALDEHYDES AND ALKYLPYRAZINES

Strecker degradation denotes the reaction of amino acids with α-dicarbonyl compounds, such as deoxyosones, pyruvaldehyde, glyoxal, 2,3-butanedione and 2,3-pentanedione (see previous sections). The phenomenon typically occurs at the high prevalence of amino acids and under drastic reaction conditions like strongly elevated temperatures (Belitz *et al.*, 2009). The widely accepted reaction mechanism commences with the nucleophilic addition of the amino group of the amino acid to one of the carbonyl groups of the deoxyosone. The generated condensation product undergoes dehydration and decarboxylation, leading to an amino alcohol after water addition, which decomposes upon release of a ***STRECKER ALDEHYDE*** (**Figure 29**) (Belitz *et al.*, 2009).

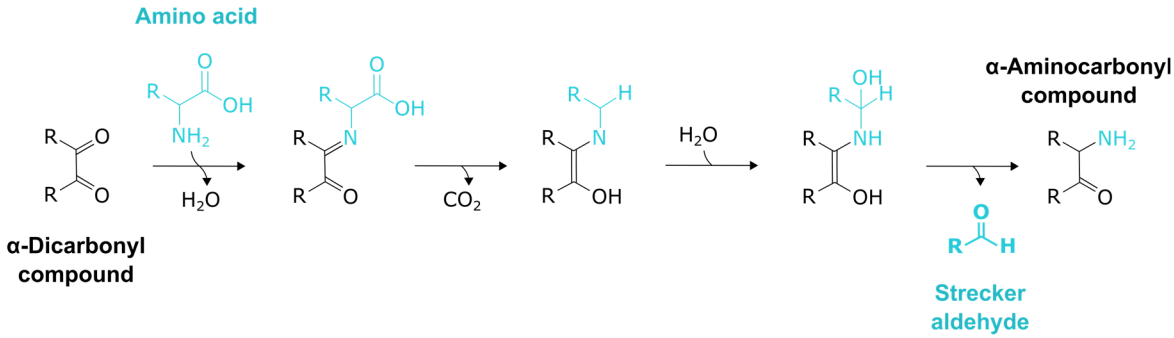

Figure 29. Principle of Strecker degradation (according to Belitz *et al.* (2009)).

The generated Strecker aldehydes contain one carbon atom less than the amino acid from which they derived. These highly volatile compounds exhibit great odor potential, for which reason they are of crucial sensory importance in diverse thermally treated food products like cakes and breads (Pico *et al.*, 2015; Rega *et al.*, 2009). An overview of common Strecker aldehydes, their origin, associated odor as well as odor threshold in water is given in **Table 17**.

Table 17. Strecker aldehydes as well as their origin, associated odor and odor threshold in water (according to Belitz *et al.* (2009)).

Strecker aldehyde	Origin	Odor	Threshold [µg/L]
Formaldehyde	Glycine	Mouse-urine, ester-like	50000
Acetaldehyde	Alanine	Sharp, penetrating, fruity	10
Phenylacetaldehyde	Phenylalanine	Flowery, honey-like	4
2-Methylpropanal	Valine	Malty	1
2-Methylbutanal	Isoleucine	Malty	4
3-Methylbutanal	Leucine	Malty	0.2
Methional	Methionine	Potatoes, boiled	0.2

Apart from Strecker aldehydes, the Strecker degradation gives rise to α-aminocarbonyl compounds (**Figure 29**). Two α-aminocarbonyl compounds can react under formation of dihydropyrazine, which in turn can be substituted (e.g. by aldehydes) to create ***ALKYLPYRAZINES*** (Adams *et al.*, 2008; Belitz *et al.*, 2009). Since the carbon skeleton of the α-aminocarbonyl compound generated during Strecker

degradation entirely derives from the α-dicarbonyl compound, it can be assumed that pyrazine formation is rather independent of the nature of the amino acid. However, the reactivity of the amino acids might vary depending on the side chain characteristics, which might affect the extent to which pyrazines are generated. Amino acids with an additional amino function (i.e. lysine and arginine) might possess higher reactivity due to facilitated nucleophilic addition to the α-dicarbonyl compound (Adams *et al.*, 2008). Furthermore, amino acids with no or short side chains (e.g. glycine) could favor the condensation with α-dicarbonyl compounds, attributable to their high flexibility (Adams *et al.*, 2008). By contrast, proline with a secondary amino function might be rather less reactive (Adams *et al.*, 2008). Nevertheless, such assumptions might be valid in simple model solutions, however might not be fully applicable to complex systems due to matrix effects (Hwang *et al.*, 1995).

Contrary to this theory, however, recent studies have suggested that pyrazines can be produced by incorporation of carbon atoms from amino acids (Chu *et al.*, 2011; Guerra *et al.*, 2012). This implies alternative pathways of pyrazines formation apart from Strecker degradation and thus underlines the high complexity of the not yet fully understood Maillard reaction.

12.2.3 FURANIC COMPOUNDS

Furanic compounds are another class of molecules that can be generated in the course of Maillard reaction. Typical representatives include furfural, 2-furanmethanol, furan and 5-hydroxymethylfurfural.

According to the literature, ***FURFURAL*** (or 2-furalaldehyde) can be generated from either pentoses or glucose (Belitz *et al.*, 2009; Hu *et al.*, 2018; Srivastava *et al.*, 2018). The latter can take place through isomerization/enolization, cyclization, decarboxylation and dehydration steps, during which fructose and 3-deoxyosone are formed as intermediates (Hu *et al.*, 2018). Alternatively, Yaylayan *et al.* (2000) suggested glucuronic acid, an oxidation product of glucose, as potential precursor that could undergo decarboxylation, dehydration and cyclization reactions to form furfural. More recently, Delatour *et al.* (2020) discussed another pathway, which involves the oxidation of 2-furanmethanol to furfural during heat treatment of food, such as coffee roasting.

2-FURANMETHANOL (commonly referred to as furfuryl alcohol) itself might originate under pyrolytic conditions from glucuronic acid after decarboxylation (Yaylayan *et al.*, 2000). In addition to glucuronic acid, the oxidation of glucose can produce gluconic acid, which was found to also give rise to 2-furanmethanol upon decarboxylation, dehydration and cyclization (Yaylayan *et al.*, 2000). Alternatively, glyceraldehyde deriving from deoxyosones as described in **Figure 27** might yield 2-furanmethanol, however the exact mechanism could not be elucidated (Yaylayan *et al.*, 2000).

An overview of possible generation pathways of furfural and 2-furanmethanol discussed in the literature is given in **Figure 30**.

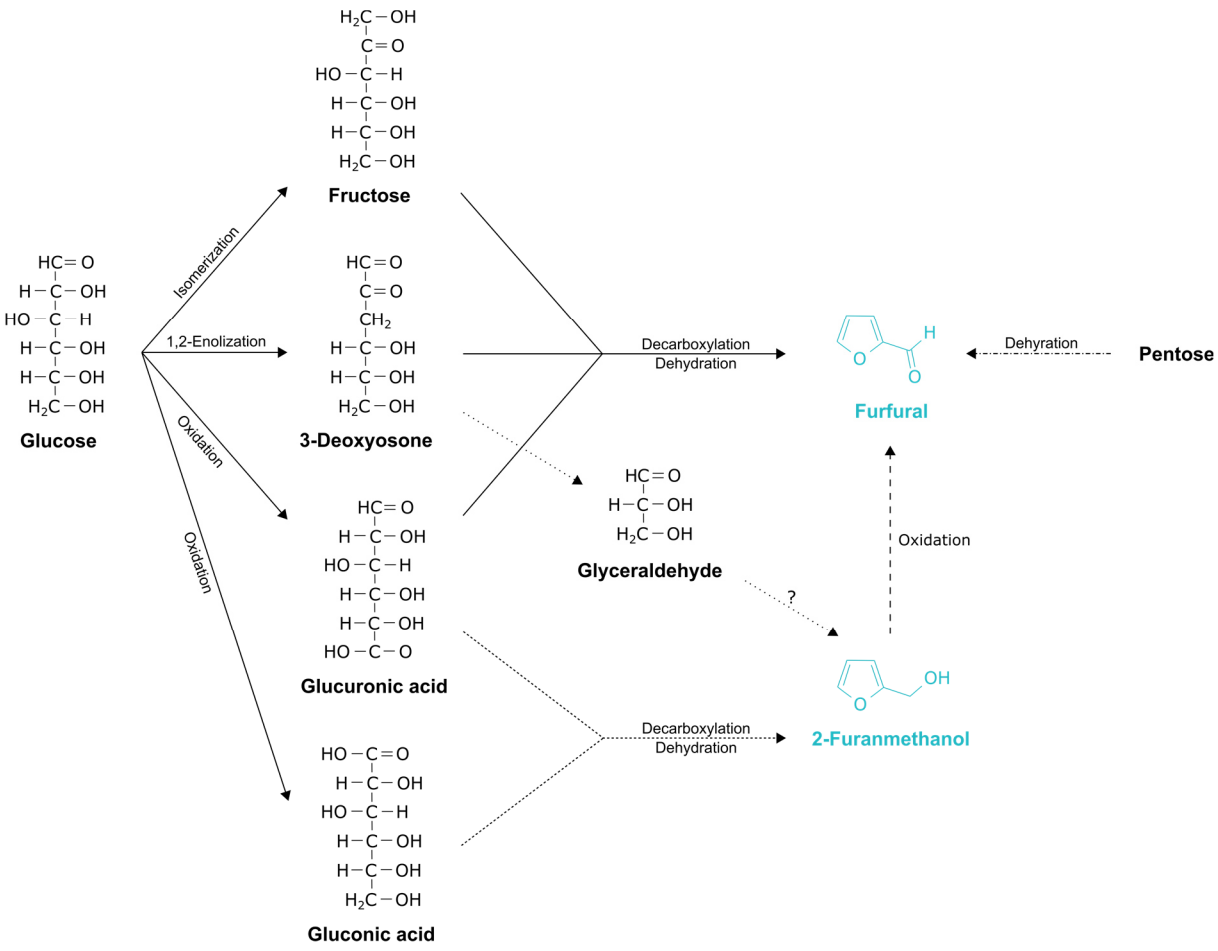

Figure 30. Proposed generation pathways of furfural and 2-furanmethanol (based on Yaylayan *et al.* (2000), Delatour *et al.* (2020), Srivastava *et al.* (2018) and Hu *et al.* (2018)).

Another common furanic compound that can be released during Maillard reaction is ***FURAN*** (Belitz *et al.*, 2009; Perez Locas *et al.*, 2004; Srivastava *et al.*, 2018). One widely accepted pathway of furan formation involves the 2,3-enolization of glucose into 1-deoxyosone, which is converted into furan after decarboxylation, dehydration and reduction steps (Perez Locas *et al.*, 2004). A second possibility commences with the dehydration of glucose, followed by retro-aldol cleavage and further dehydration (Perez Locas *et al.*, 2004). Thirdly, glucose might be transformed into 3-deoxyosone *via* 1,2-enolization and subsequently cleaved, oxidized and decarboxylated to create furan (Perez Locas *et al.*, 2004). A concrete reaction scheme can be accessed from Perez Locas *et al.* (2004). Apart from these, however, furan generation from polyunsaturated fatty acids, carotenoids or ascorbic acid has been postulated as well (Perez Locas *et al.*, 2004; Yaylayan, 2006), however might be less relevant pathways during sponge cake development (Cepeda-Vázquez *et al.*, 2019).

The mentioned molecules are considered aromatic compounds with characteristic burnt and caramel-like scents (Cepeda-Vázquez *et al.*, 2018; Garvey *et al.*, 2021; Pico *et al.*, 2015). Apart from their odor activity, some generated compounds are additionally assumed to be carcinogenic or mutagenic, and thus are of great health-concern (Cepeda-Vázquez *et al.*, 2018; Yaylayan, 2006). In this context, furan is an important process-induced contaminant with potential toxic properties and thus has been categorized as “possibly carcinogenic to humans” (Group 2B) by the International Agency for Research on Cancer (IARC, 1995).

Safety concerns have also been raised for ***5-HYDROXYMETHYLFURFURAL*** (HMF), which is formed from 3-deoxyosone *via* 3,4-dideoxyosone through dehydration and cyclization (Belitz *et al.*, 2009). When consumed beyond the prescribed dietary limit, HMF is considered to be cytotoxic to humans and possesses a potential mutagenic and genotoxic activity (Choudhary *et al.*, 2020).

13 CARAMELIZATION

In addition to Maillard reaction, heat-driven sugar degradation can arise through another reaction pathway defined as caramelization, yielding both typical caramel flavors and dark pigments (Parker, 2015). This reaction takes place in the absence of amino groups and typically requires more drastic conditions compared to Maillard reaction, i.e. highly elevated temperatures as well as an acidic or alkaline environment (Belitz *et al.*, 2009).

Key markers of caramelization include α-dicarbonyl compounds, in particular deoxyosones, which are also of major importance during Maillard reaction. As opposed to the latter, however, these deoxyosones are formed by enolization and dehydration of sugars without participation of amino groups (Belitz *et al.*, 2009). Nevertheless, they give rise to the same volatile compounds as discussed for Maillard reaction, such as furanic compounds and specific ketones (**Figure 27**, **Figure 28** and **Figure 30**).

Apart from volatile compounds with potential odor activity, both caramelization and Maillard reaction produce pigments which give the thermally treated products their characteristic brownish color. Those pigments containing nitrogen (around 3-4%) are denoted as melanoidins (Belitz *et al.*, 2009; Nursten, 2005). To this day, information about their exact structure and generation pathways is still scarce. Browning in bakery products is normally initiated at water activities ≤0.6 and temperatures ≥120 °C (Purlis *et al.*, 2009; Srivastava *et al.*, 2018).

14 VOLATILES IDENTIFIED IN PEA INGREDIENTS

Pea ingredients might contain volatile compounds, which can be either inherent to the plant or formed during harvesting, processing and storage (Roland *et al.*, 2017). In addition, the heterogenicity of volatiles can be affected by the plant cultivar, crop year, growing environment, and stage of maturity (Azarnia *et al.*, 2011b; Reineccius *et al.*, 2013).

14.1 INHERENT COMPOUNDS

Peas are known to contain a limited number of volatile compounds with potential odor activity. Among these, ***3-ALKYL-2-METHOXYPYRAZINES*** have been widely reported, which carry either isopropyl, *sec*-butyl, isobutyl or 5/6-methyl-3-isopropyl as alkyl group (Jakobsen *et al.*, 1998; Murray *et al.*, 1975). Owing to their low odor threshold values, these compounds are considered chief compounds responsible for the typical pea pod and bell pepper notes of peas (Jakobsen *et al.*, 1998; Murray *et al.*, 1975; Roland *et al.*, 2017). According to Murray *et al.* (1975), 3-alkyl-2-methoxypyrazines mainly accumulate in pea shells and are much less present in the seeds.

In addition, peas typically contain ***MONOTERPENES***, such as limonene, *α*-pinene, *β*-pinene, 3-carene, *β*-myrcene, sabinene, linalool, *β*-ocimene, *β*-cyclocitral, *α*- and *β*-ionone, which might be products of endogenous isoprenoid biosynthesis or carotenoid degradation (Jakobsen *et al.*, 1998; Murray *et al.*, 1976). Murray *et al.* (1976) also detected cineole in green peas and assumed that it was absorbed from the soil.

14.2 COMPOUNDS FROM MICROBIAL ACTIVITY

Microbial spoilage can be an alternative source of volatile compounds in peas. Commonly detected structures comprise ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol and 2-phenylethanol as well as their corresponding aldehydes and acids, which are produced from alanine, valine, leucine, isoleucine and phenylalanine through the Ehrlich pathway (Murat *et al.*, 2013; Murray *et al.*, 1976). The aroma associated with these compounds has been described as ***ALCOHOLIC***, ***MALTY*** and ***FLOWERY*** (Pico *et al.*, 2015).

14.3 PROCESS-INDUCED COMPOUNDS

The chemical composition of peas makes them vulnerable to breakdown reactions that might take place during harvesting, milling, processing or storage (Roland *et al.*, 2017; Sessa *et al.*, 1977). Typical processing techniques involve germination, soaking, heat-treatment and fermentation of the raw ingredients. These aim to improve the (i) nutritional quality of peas by decreasing the presence of antinutritive factors like flatulence-causing oligosaccharides, trypsin inhibitors, phytates and alkaloids through leaching in water and enzymatic degradation and (ii) functional properties of their major pea constituents (Abu-Ghannam *et al.*, 2021; Carbonaro, 2011; Emkani *et al.*, 2021; Ma *et al.*, 2011; Roland *et al.*, 2017; Shi *et al.*, 2021; Wang *et al.*, 2003).

However, the applied methods can injure the tissue of the seeds, which allows the generation of a wide variety of different volatile compounds (Rackis *et al.*, 1979; Sessa *et al.*, 1977; Xu *et al.*, 2019). This can be attributed to the decompartmentalization of cells, leading to the liberation of previously enclosed substances, which on the one hand are more susceptible to degradation due to environmental triggers and on the other hand can also enter into reactions with one another, such as enzyme-substrate interactions.

In the case of peas (and other pulses), this physical cell disruption can release enzymes like lipoxygenase and alcohol dehydrogenase, which mainly act on highly prevalent unsaturated fatty acids to create molecules with unpleasant green, beany and grassy aroma (Lee *et al.*, 1958; Rackis *et al.*, 1979; Roland *et al.*, 2017; Sessa *et al.*, 1977; Siddiqi *et al.*, 1956; Wagenknecht *et al.*, 1956). By means of chromatographic techniques, the major contributors to these sensations have been identified to be low-molecular weight ***ALCOHOLS***, ***ALDEHYDES*** and ***KETONES*** (Murray *et al.*, 1968; Ralls *et al.*, 1965; Whitfield *et al.*, 1966). Those, that have been recognized as strong sources of ***GREEN VEGETABLE, LEAFY ODORS*** include especially hexanal and 1-hexanol, but also 1-penten-3-ol, 2-penten-1-ol, 3-hexen-1-ol and 2,4-heptadienal (Jakobsen *et al.*, 1998; Murat *et al.*, 2012; Murray *et al.*, 1968; Murray *et al.*, 1976). Apart from these, multiple volatiles with characteristic ***FATTY*** (2-octenal, 3,5-ocatdien-2-one, 2,4-nonadienal, 2,4-decadienal), ***MUSHROOM*** (1-octen-3-ol, 3-octanone) and ***FRUITY*** odors (octanal, 2-nonanone) have been identified as well (Jakobsen *et al.*, 1998; Murat *et al.*, 2012; Murray *et*

al., 1968; Murray *et al.*, 1976). According to Xu *et al.* (2019), the formation of these compounds increases with longer germination times due to ongoing oxidative processes.

Apart from enzymatic pathways, the described odorants can also be formed through non-enzymatic autooxidation triggered by light, metals or heat (Murat *et al.*, 2013; Rackis *et al.*, 1979; Sessa *et al.*, 1977). As demonstrated by Bi *et al.* (2020) and Ma *et al.* (2016), elevated temperatures can also lead to the development of other volatiles with potential odor activities, including pyrazines, Strecker aldehydes and furanic compounds.

In addition, storage conditions can strongly affect off-flavor generation as reported by (Azarnia *et al.*, 2011a). The authors documented higher total areas of volatile compounds kept at elevated temperatures (22 °C and 37 °C) compared to those stored in a chilled atmosphere (4 °C), which was mainly assigned to high amounts of aldehydes, such as hexanal with characteristic green, grassy off-odor (Azarnia *et al.*, 2011a).

PART III

RESEARCH STRATEGY

Based on the literature research performed and described in **Part II** of this thesis, pulses such as peas are attractive raw materials that not only provide a particularly high nutritional quality, but also possess promising functionalities, which might yield products like cakes with favorable characteristics.

First successes in the use of pulse ingredients in cakes to develop gluten-free baked goods have already been achieved, which were measured on the basis of the textural and color properties of the products generated (see **§ 9 of Part II**).

Nevertheless, there is still a large gap in knowledge regarding the reactivity potential of these raw materials to form volatile organic compounds with potential odor activities during product development. As numerous studies revealed, the chemical composition of the legume ingredients and the presence of the enzyme lipoxygenase favor the occurrence of oxidative phenomena, which can lead to the release of a wide variety of different molecules typically considered as off-flavors with green-beany odors (see **§ 14 of Part II**). Most of these studies, however, deal with the ability of pulses to form volatiles during processing of the raw material, such as grinding and germination, among others. Although these preparation techniques might result in the adhesion of a certain number of unpleasant odors to the pulse ingredients, little is known about the relevance of these volatiles in the final food. Furthermore, the potential of pulses to undergo reactions while manufacturing food products has not been elucidated yet. In particular, the generation and/or mitigation of odor-active and process-induced compounds as a function of both product formulation and process as well as their correlation with structural properties of the food have not been addressed. A very interesting question related to this concerns the behavior of minimally processed whole pulse flours in comparison to the refined proteins and starches derived thereof. The latter two are disadvantaged from a sustainability point of view due to the higher footprint. However, their higher degree of purity could be related to different functionalities and susceptibilities to undergo changes *via* diverse reactions during product development, and thus modify the quality-determining aspects of the pulse-based food. Apart from the odor and structure perspective, these could also influence their digestibility behaviors and thus the nutritive quality of the product, which is of decisive importance for the consumer and has so far only been rarely discussed and evaluated in the literature.

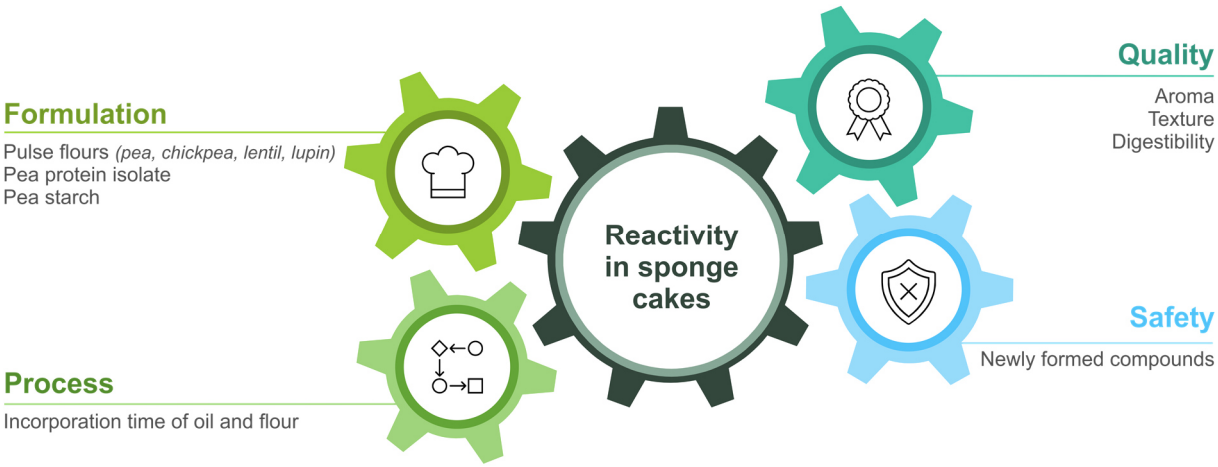

Figure 31. Research strategy of the present work.

In order to clarify these various unanswered questions, the present work was structured in five studies. ***STUDY 1*** aimed to gain a better understanding of the changes in quality and reactivity occurring during the individual steps of sponge cake development when substituting wheat flour with different pulse flours (lentil, chickpea, lupin, green pea, yellow pea). The research questions included:

- *Is it possible to produce purely pulse-based cakes with attractive characteristics?*
- *Which chemical reactions take place at each step of cake production and to which extent?*
- *Which are the triggers for these reactions in real food application conditions?*
- *Which volatiles are generated and how might they relate to the sensory cake quality?*

In order to evaluate the global alterations to cake quality when using pulse flours as an alternative to wheat, two indicators were selected: color and density. These parameters are important product criteria that can be used to quickly assess whether the cakes generated might be considered high-quality foods or are more likely to be rejected by the consumer. Subsequently, diverse reaction markers and their potential precursors at each step of the making were investigated in order to draw conclusions about the reactions taking place as well as their extent. Earlier studies performed by Maire *et al.* (2013) and Cepeda-Vázquez *et al.* (2018) could demonstrate that the production of sponge cakes based on wheat flour led to the generation of volatile organic compounds (VOCs) *via* lipid oxidation, Maillard reaction and caramelization. Accordingly, it was assumed that these reactions would occur in the present formulations as well, for which reason the concentrations of relevant precursors, namely fatty acids, reducing sugars and free amino acids, were examined in the flours. Moreover, the profile of inherent VOCs in these raw materials was determined, which provided information about the starting conditions and thus allowed to better identify changes in the VOC profile and quantities occurring in the batters and cakes. Optimal VOC determination was ensured by developing a repeatable method based on headspace-solid phase microextraction coupled with gas chromatographic analysis (**§ 6.2 of Part IV**). In addition, the frequently discussed high susceptibility of pulses to oxidation prompted us to implement further methods, which helped to gain insight into the changes in the oxidative status from the raw materials to the batters to the cakes. For this purpose, the strategy proposed by Maire *et al.* (2013) was applied and deepened with the analysis of the intermediate oxidation products, i.e. hydroperoxides and conjugated dienes, in addition to the final markers, i.e. VOCs, and initial levels of free fatty acids. In order to ascertain the most relevant factors impacting oxidation, not only the typical precursors but also the activity of the enzyme lipoxygenase as well as the density of the batters as a measure of the amount of air incorporated were determined. The methodological approach is illustrated in **Figure 32**.

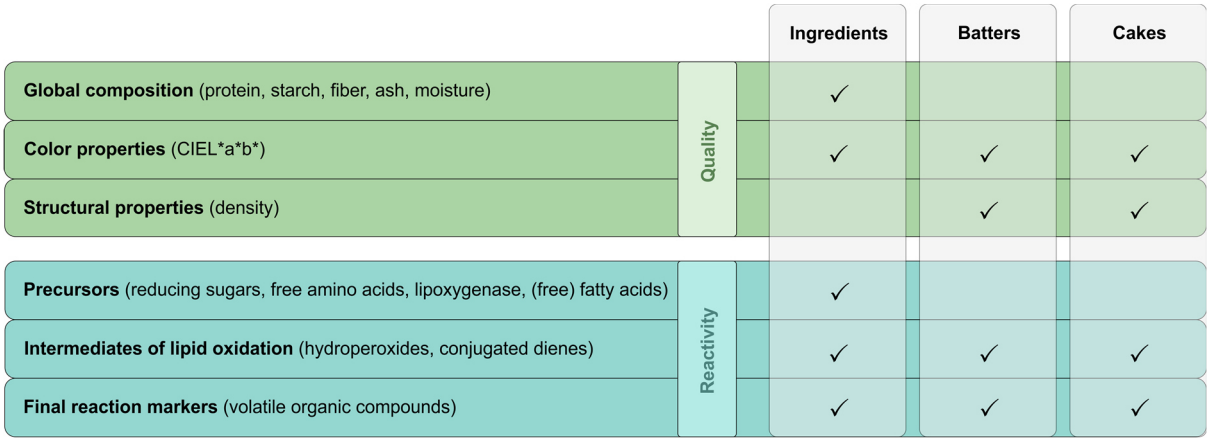

Figure 32. Methodological approach of study 1.

Subsequently, ***STUDY 2*** aimed to identify of the pool of VOCs extracted from the cakes of study 1 those key volatiles with high probability to contribute to the overall odor perception. Such estimation of the potent odors can be achieved by calculating the odor activity values of each individual molecule. It enables to discriminate the different pulse-based cakes from each other and from wheat, and thus to expand the knowledge on the importance of the ingredient in the creation of aroma compounds.

Thereafter, attention was turned to pea because the previous studies 1 and 2 revealed pea (green and yellow) as the raw material with strongest potential to produce VOCs during sponge cake development among the pulse flours investigated. Yellow pea, in particular, was characterized by a pronounced ability to participate in oxidative reactions during batter beating, which led to a large number of volatiles typically associated with green, grassy and fatty odors.

In order to develop new approaches to improve the sensory and overall quality of cakes based on pea flours (and pulse flours in general), better understanding of both the critical process steps that promote oxidation during batter beating and of the relevant impact of structural batter properties on reactivity needs to be gained. The strategy of ***STUDY 3*** was therefore to modify the mixing times after the incorporation of flour and/or oil, as these are both relevant ingredients that provide most of the precursors and catalysts for lipid oxidation. An extension of both beating times may alter the exposure time of lipoxygenase contained in the flour to unsaturated fatty acids, while at the same time changing the amount of air, and thus oxygen, embedded into the batter. These research questions were:

- *Which relevance has the batter beating process to the occurrence of lipid oxidation?*
- *How does the microstructure of the batter changes upon modification of the beating process?*
- *Which correlation can be elaborated between the degree of lipid oxidation and the structural properties of the batter?*

A prerequisite was the selection of maximum mixing times after flour and oil incorporation, which were sufficiently long to modify air entrapment and exposure time of enzymes to their substrates while at the same time avoiding collapse of the batter structure (see **§ 3.2 of Part IV**). The chosen maximal limits were then crossed with the minimal boundaries reported in the original protocol to generate four batters. For this, stripped oil was used, which was purified from endogenous antioxidations in order to magnify the oxidative response. The batters were then characterized on the one hand in terms of reactivity by analyzing fatty acids, conjugated dienes, hydroperoxides and VOCs as described in study 1 and on the other hand in terms of their structural properties by analyzing batter density and the local distribution of reactive ingredients using confocal laser scanning microscopy (CLSM). The methodological approach is illustrated in **Figure 33**.

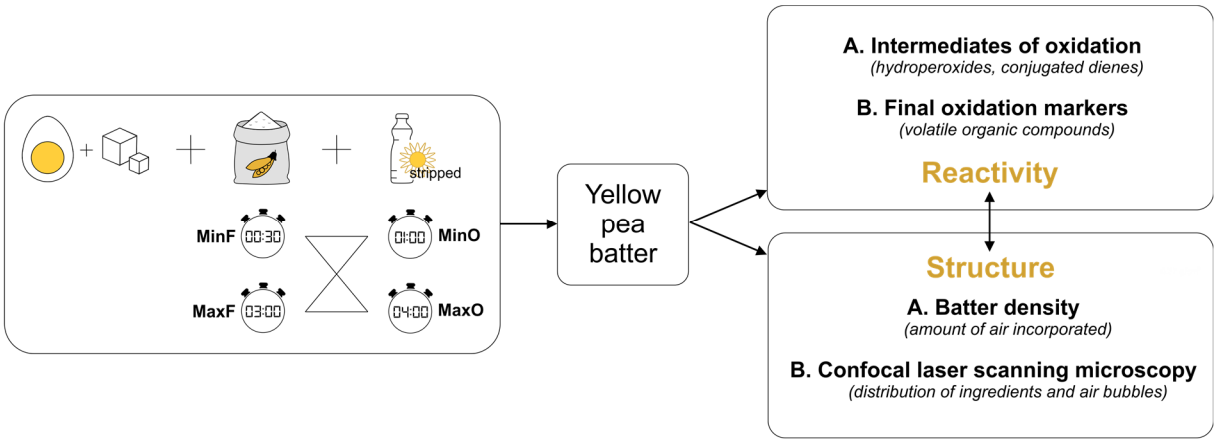

Figure 33. Methodological approach of study 3.

As an alternative to pea flour, pea protein isolates are also available on the market, which are characterized by attractive functional properties (see **§ 3.2 of Part II**). From a sustainability point of view, however, the use of such refined fractions is less favorable than the whole flour owing to the elevated requirement of resources (chemicals, water, energy) during purification (Lie-Piang *et al.*, 2021). Isolated pea proteins, however, have been reported to contain less aroma compounds than the corresponding flour (Xu *et al.*, 2020). Nevertheless, how significant these differences are in the final foods has not yet been clarified. Furthermore, the effect of the extraction process on the ability of the protein isolate to form volatiles during product development in comparison to the whole pea flour is still an unanswered question. For these reasons, ***STUDY 4*** aimed to evaluate the reactivity potential of these purified pea proteins compared to both pea flour and wheat flour to generate volatiles in the course of sponge cake elaboration. In addition to pea protein isolate, refined pea starch was also used to exploit its applicability as it is typically considered a by-product of protein isolation. The research questions were:

- *How do the abilities of whole pea flour and purified pea fractions to undergo reactions differ?*
- *Which changes in their composition could be responsible for the possible differences in their reactivity potential?*
- *Which effect do the diverse pea ingredients have on the microstructure of the batters?*
- *How do the structural properties of the batters and their reactivity potential correlate?*
- *How do the different pea ingredients affect global cake characteristics?*

To answer to these questions, it was decided to recombine the isolated pea proteins with the purified pea starch in the same ratio as present in the pea flour and to investigate their influence on the reactivity and structure at the different steps of cake making. The individual contribution of each fraction to the formation of volatile components could be assessed by the preparation of a pure pea starch product, since the noticed differences in the VOC profiles between this product and the product composed of both isolate pea proteins and pea starch presumably originated from the protein isolate. It was also decided that purified maize starch should be used as a reference to pea starch due to its common application in food industry as well as limited chemical reactivity (Bousquières *et al.*, 2017a). The formulation strategy is illustrated in **Figure 34**. Similar to study 1, the release of VOCs was followed from the raw materials to the batters to the final cakes and deeper insight into the oxidative processes was obtained through the analysis of conjugated dienes and hydroperoxides. Whether the flour fractionation process had an impact on the availability of precursors to participate in reactions was

assessed by quantifying fatty acids, reducing sugars and free amino acids as well as by determining the lipoxygenase activity in the raw materials. Due to the commonly discussed different functionalities of the purified fractions in comparison to the whole flour, altered structural and textural properties of the generated products were expected, which might influence reactivity. The structural properties of the batters were therefore additionally examined, including their density, consistency and the local distribution of reactive ingredients (CLSM). Moreover, the global quality of the developed cakes was compared by measuring their density and color properties.

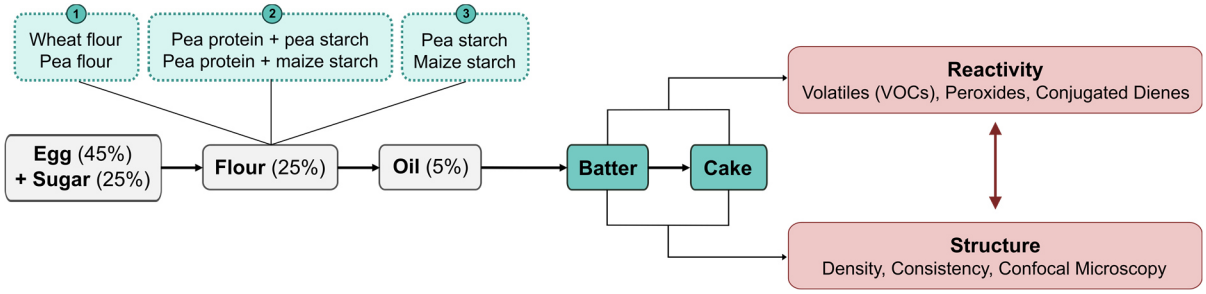

Figure 34. Formulation and analysis strategy of study 4.

Another important quality criterion that plays a role in the purchase decision of consumers is not only the appearance and aroma of the developed pea-based products, but also their nutritional value. The use of pea ingredients may not only enhance the protein level but at the same time also reduce the starch level of the products. However, it is necessary to assess how well these macronutrients can be digested in order to have a real nutritional benefit. The aim of ***STUDY 5*** therefore was to investigate and compare the *in vitro* protein and starch digestibility of the different sponge cakes produced in study 4. The protocol of choice was the INFOGEST *in vitro* static digestion method, which can be used to simulate nutrient digestion during the typical *in vivo* oral, gastric and intestinal phases using a simple, reproducible, robust and cost-effective approach. The experiments were performed at the research facilities of KU Leuven and Cargill in Belgium during a 6-month secondment. The research questions included:

- *How well are proteins and starch from wheat and pea digested?*
- *How do the digestion kinetics of purified pea fractions and whole pea flour differ?*
- *Which chemical characteristics of the different ingredients affect digestibility?*
- *How do structural characteristics of the sponge cakes influence digestibility?*

To assess the digestibility of proteins and starch, the released amino groups and reducing sugars were determined using common spectrophotometric techniques. In addition, a more detailed chromatographic analysis of the individual starch degradation products was carried out in order to gain a deeper insight into the evolution of starch digestion and thus to select the most suitable equations for data modeling. Moreover, the quantitative analyses were complemented by microscopic analysis of the digestion pellets, which could provide qualitative information about the progress of digestion. In order to be able to better interpret the digestibility of pea starch, which up to now has only rarely been discussed in complex foods, additional characterizations were performed, which included the determination of the contents of amylose, damaged starch and resistant starch. In addition to different chemical compositions that could influence the enzymatic breakdown of the nutrients, an influence of the structural properties

of the cakes was also conceivable. For this reason, the morphology of the cake crumb was captured using scanning electron microscopy.

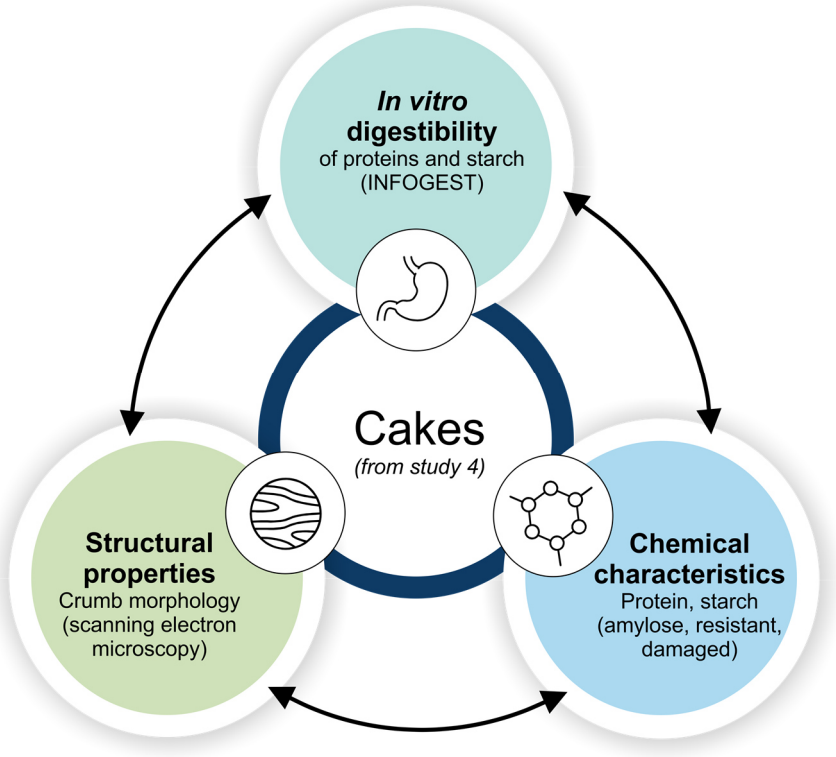

Figure 35. Analysis strategy of study 5.

PART IV

MATERIALS & METHODS

1 INGREDIENT SUPPLIERS

An overview of the ingredients used and their corresponding suppliers is given in **Table 18**.

Table 18. Raw ingredients, their suppliers and studies, in which they were used.

Raw material	Supplier	Study used in
Wheat flour (T55)	Grands Moulins de Paris, Ivry-sur-Seine, France	1
Wheat flour (T65)	Axiane Meunerie, Olivet, France	2/3/4/5
Green pea flour	Moulin Meckert-Diemer, Krautwiller, France	1
Lentil flour	Celnat, Saint-Germain Laprade, France	1
Lupin flour	Moulin Meckert-Diemer, Krautwiller, France	1
Chickpea flour	Celnat, Saint-Germain Laprade, France	1
Yellow pea flour (I)	Improve, Dury, France	1
Yellow pea flour (N_2)	Improve, Dury, France	1
Yellow pea flour (II)	Cargill, Vilvoorde, Belgium	2/3/4/5
Yellow pea protein isolate	Cargill, Vilvoorde, Belgium	2/3/4/5
Yellow pea starch	Cargill, Vilvoorde, Belgium	2/3/4/5
Maize starch	Cargill, Vilvoorde, Belgium	2/3/4/5
Egg (whole, liquid, pasteurized)	Ovoteam, Locmine, France	1/2/3/4/5
Sucrose	Tereos, Lille France	1/2/3/4/5
Sunflower oil	Lesieur, Asnières-sur-Seine, France	1/2/3/4/5

2 STORAGE CONDITIONS

All flours and fractions were vacuum packed in PA/PE bags and stored at -20 °C until use. The eggs were heat-sealed in PA/PE bags and stored at -20 °C. Sucrose was stored at ambient temperature. Sunflower oil was filled in aluminum-wrapped glass bottles (30 mL) and stored under nitrogen at -20 °C. Immediately after preparation, batters were stored in glass bottles under nitrogen and cakes in hermetically closed glass jars at -20 °C.

3 BATTER AND CAKE DEVELOPMENT

3.1 FEASIBILITY OF COMPLETE WHEAT REPLACEMENT

The use of novel ingredients as substitutes for traditional wheat flour might alter the product's viscoelastic properties. Therefore, it was important to test the technological feasibility of a 100% exchange of wheat by different pulse ingredients. For this purpose, pulse-based sponge cakes were generated without adapting the protocol, in order to ensure the same energy input and air exposure during batter whipping as well as thermal treatment during baking.

The sponge cake development protocol applied was based on Cepeda-Vázquez *et al.* (2017) without the addition of salt. Accordingly, whole pasteurized liquid eggs were thawed overnight at 2 °C and weighed into the bowl of a KitchenAid Artisan 5KSM150 stand mixer (St. Joseph, Michigan, USA). The sucrose was added and both ingredients whipped together for 10 min at maximum speed (10/10) using a vertical whisk. Thereafter, pre-weighed non-sifted flour was gradually added within 1.5 min at minimum speed (1/10) and the mass blended for 30 s at the same speed. In a final step, sunflower oil was gently added within 15 s at minimum speed (1/10) and the batter beaten for 60 s at the same speed. A total of 500 g batter was obtained and filled in 21 aluminum molds (20 ± 0.5 g each, 9.8 cm x 6.2 cm x 3.3 cm). All batters were baked at the same time at 170 °C for 25 min (level of convection: 25 Hz) in a pre-heated insulated convection oven (Bongard, Wolfisheim, France), which was designed to guarantee thermal homogeneity (Fehaili *et al.*, 2010).

The use of some pulse ingredients (e.g. lupin flour) led to an increase in batter viscosity compared to wheat. Nevertheless, whipping and filling was doable, thus excluding the necessity of wheat-pulse flour blends. The cakes obtained using lupin flour were, however, remarkably denser than the reference, which complicated the volume determination by means of the VolScan apparatus (see **§ 5.2.2 of Part IV**). For this reason, the sponge cake recipe was slightly adapted for all formulations and the total ***AMOUNT OF BATTER INCREASED*** from 20 g to 25 g per aluminum mold.

3.2 MODIFICATION OF BATTER BEATING PROCESS

Deeper understanding of the relevance of individual ingredient and process steps to the promotion of oxidative reactions and thus VOC generation during pea batter making can be gained by modification of the whipping protocol. Due to the known presence of relevant precursors or catalysts for lipid oxidation in flour and oil, it was of interest to extend the mixing times after the incorporation of each ingredient. A preliminary test was carried out to select the maximal beating times which could favor lipid oxidation but at the same time did not lead to extreme changes in the batter density and thus highly different structural properties. Accordingly, pea flour was added to the egg-sugar foam and the batter density measured every 30 s over a period of 3 min. Afterwards, the oil was added and sampling performed in the same manner over a period of 4 min. The yielded density values as a function of time are shown in **Figure 36**.

It can be seen that the density of the egg-sugar foam increased upon flour addition. Slightly more air got lost when the flour incorporation was extended by 30 s, which however did not further change even with prolonged whipping of up to 3 min. Once the sunflower oil was incorporated, a second increment in batter density was visible, which remained nearly constant over the whole extended oil beating period of 4 min. On basis of these results, it appeared appropriate to select maximal beating times of 3 min

and 4 min after flour and oil incorporation, respectively. The set-up for this experiment was thus composed of the following combinations of mixing times:

(i) 0.5 min flour + 1 min oil (MinF – MinO, original protocol),
(ii) 3 min flour + 1 min oil (MaxF – MinO),
(iii) 0.5 min flour + 4 min oil (MinF – MaxO),
(iv) 3 min flour + 4 min oil (MaxF – MaxO).

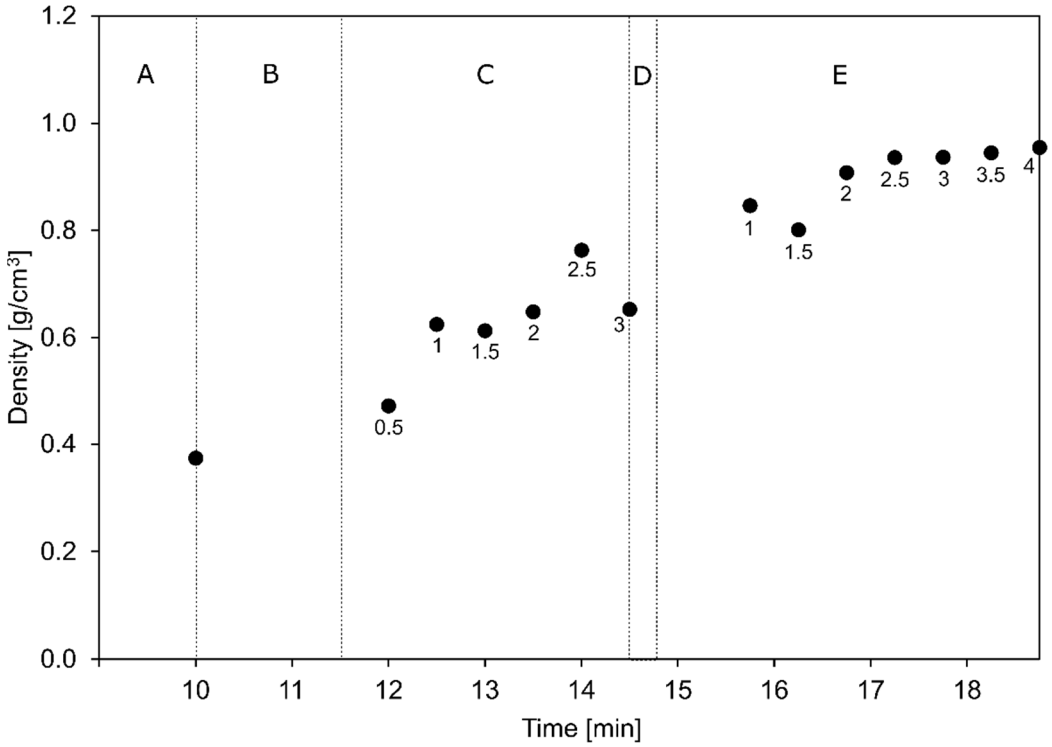

Figure 36. Batter density as a function of batter beating time. The batter beating process involves (A) whipping of egg and sugar for 10 min, (B) incorporation of flour within 1.5 min, (C) beating of batter after flour addition for 3 min with density measurement every 30 s, (D) incorporation of oil within 15 s and (E) beating of batter after oil addition for 4 min with density measurement every 30 s.

3.3 REPEATABILITY OF BATTER AND CAKE PRODUCTION

To investigate the data variability between individual batter preparations and cake baking trials of the same flour type, three batches of lentil batters as well as lentil cakes were produced and analyzed in terms of selected analysis. The results are shown in **Figure 37**. It emerges, that no significant differences between the three independent batter or cake preparations were visible. Therefore, it was assumed that the elaboration of one batter or batch of cakes was sufficient for upcoming studies.

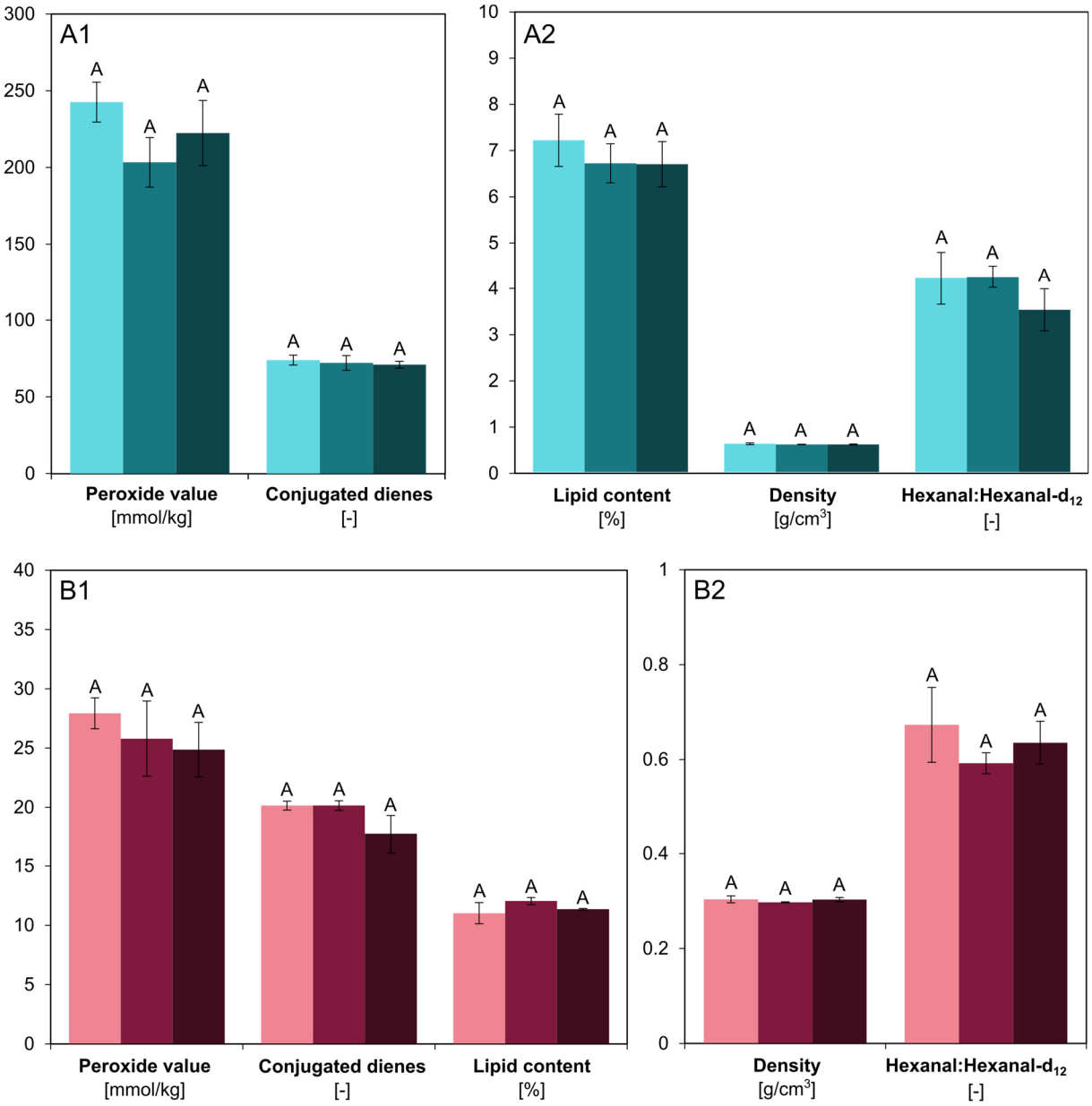

Figure 37. Repeatability of three independent batter (A1, A2) and cake (B1, B2) preparations based on lentil flour, (three repetitions per product) evaluated in terms of selected analyses (lipid extraction, peroxide value, conjugated dienes, density and ratio of inherent hexanal to internal standard hexanal-d_{12}). Different small letters indicate significantly different means ($p < 0.05$).

4 CHEMICAL CHARACTERISTICS

Table 19 lists the methods applied to analyze the chemical composition of flours, batters, cakes. An overview of the chemical composition is given in **Table 54** in the appendix.

Table 19. Methods applied to analyze the chemical composition of eggs, flours/fractions, batters and cakes.

Parameter	Method	Egg	Study pulse flours			Study pea fractions			Location
			Flour	Batter	Cake	Flour	Batter	Cake	
Dry matter	Weighing before & after drying for 24 h at 105 °C	X	X		X				Massy
	Moisture analyzer					X		X	Cargill
Lipid	Accelerated solvent extraction	X	X	X	X	X	X	X	Massy
Protein	Kjeldahl method (NF EN ISO 5983-2)	X	X			X		X	Improve
Free amino acids	ISO 13903:2005 and ISO 13904:2016	X	X			X			Improve
Starch	EC 152/2009		X						Improve
	Enzymatic hydrolysis and HPLC analysis					X		X	Cargill
Damaged starch	Amperometric SDmatic method (AACC 76-33.01)					X			Massy
Resistant starch	AOAC 2002.02							X	Improve
Amylose	Spectrophotometry					X		X	Cargill
Sugars	Ion chromatography and pulsed amperometry	X	X			X			Improve
	Ion chromatography and pulsed amperometry							X	Cargill
Fiber	AOAC 985.29		X			X			Improve
Ash	Dry combustion during 12 hours at 550 °C	X	X			X			Improve

4.1 LYOPHILIZATION OF EGGS

In order to determine the chemical composition of eggs, lyophilization of the eggs had to be carried out. For this purpose, eggs were pre-frozen in thin plates at -20 °C. Thereafter, 900 g frozen eggs were broken into small pieces and placed without overlapping inside glass or plastic containers of low height. The containers were put into the freeze dryer (Alpha 2-4 LDplus freeze dryer, Christ, Osterode am Harz, Germany) and the water sublimated during 52 hours. 214 g freeze-dried egg powder was obtained, which was immediately packed in aluminum bags and stored in a desiccator at 4 °C to avoid absorption of air humidity and chemical alterations.

4.2 DRY MATTER

Moisture content of eggs, flours and cakes of the pulse flour study was determined by differential weighing before and after drying of 3 – 5 g sample for 24 hours at 105 °C in a ventilated oven (Memmert, Germany) and cooling to ambient temperature in a desiccator.

Moisture content of flours and cakes of the pea fraction study was determined thermogravimetrically using a moisture analyser MA30 (Sartorius, Göttingen, Germany).

4.3 CARBOHYDRATES

4.3.1 STARCH CONTENT

The starch content of flours, fractions and cakes of the pea fraction study was determined by adapting the method described by Morrison *et al.* (1983). Flours, fractions and cakes were dispersed in UDMSO (90% v/v dimethyl sulfoxide, 10% v/v 6 M urea) to reach approximately 8 mg anhydrous starch per mL UDMSO. After stirring at room temperature at 200 rpm for one week, the samples were centrifuged (2000 *g*, 10 min) and 1 mL supernatant mixed with 4 mL sodium acetate buffer (0.2 M, pH 4.5) containing amyloglucosidase. Subsequently, starch was ***ENZYMATICALLY HYDROLYZED*** at 55 °C during 72 h. To ensure that the presence of UDMSO did not hinder enzymatic activity, a control sample, which contained maltodextrin in UDMSO, was also digested.

The released glucose was determined by ***HIGH PERFORMED LIQUID CHROMATOGRAPHY*** (HPLC) using a 717plus autosampler, 515 HPLC pump, column heater and 2410 refractive index detector (Waters, Antwerp, Belgium). The digested samples were filtered through 0.45 µm Minisart NML filters and diluted (1/50 v/v) with purified water. 20 µL of dilution were injected on two Aminex HPX-87C columns connected in series (300 × 7.8 mm) and protected by a micro-guard de-ashing cartridge (30 × 4.6 mm) (BioRad, Temse, Belgium). Isocratic elution of glucose was performed at a flow rate of 0.5 mL/min using degassed purified water. The column was operated at 75 °C. Glucose was quantified using an external calibration curve of a glucose standard (**Figure 38**) and converted into starch equivalents by multiplication by 0.9.

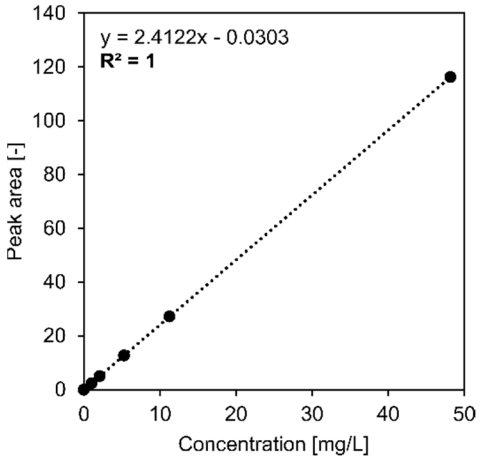

Figure 38. Glucose calibration curve used to determine the starch content of ingredients and cakes.

4.3.2 DAMAGED STARCH CONTENT

As discussed in **§ 2.6 of Part II**, the presence of damaged starch can affect the functional properties of the starch, its susceptibility to enzymatic digestion and availability for chemical reactions. Therefore, the damaged starch content of the ingredients used for the pea fraction studies was analyzed using the SDmatic method reported by Monnet *et al.* (2019).

The reagent solution was prepared by dissolving 1.5 g citric acid with 3 g potassium iodide in 120 mL water. Thereafter, one drop of sodium thiosulfate solution (0.1 M) was added and the solution placed in the SDmatic equipment (Chopin Technologies, Villeneuve-la-Garenne, France). Prior addition of the ingredient, the temperature of the solution was raised to 35 °C and free iodine produced by the electrodes, leading to a yellowish tint of the reagent solution. Once the flour was added, the color of changed to blue and the electrical current dropped due to the binding of iodine to the damaged starch granules. This change was greater the more the starch was damaged. It was expressed as percentage iodine absorption (AI) [%], which was then translated in the proportion of damaged starch [%] using the following equations for wheat and pulses, respectively:

$$SD_{wheat} = 0.02436 \times AI^2 - 3.904 \times AI + 156.5 \quad (\mathbf{15})$$

$$SD_{pulses} = 0.03398 \times AI^2 - 5.861 \times AI + 253.1 \quad (\mathbf{16})$$

The equation for wheat was likewise used for determination of damaged starch in maize starch due to their similar starch composition (amylose content, amylose-lipid complexes).

4.3.3 AMYLOSE CONTENT

Similar to damaged starch, the proportion of amylose as well as amylose-lipid complexes in starch can affect its functional properties as well as proneness to enzymatic degradation (see **§ 2 of Part II**). For this reason, both apparent amylose (with complexed lipids) and total amylose contents (without complexed lipids) of ingredients and cakes of the pea fraction study were analyzed adapting the iodine reagent method described by Morrison *et al.* (1983).

Accordingly, ingredients and cakes were dispersed in 50 mL UDMSO (90% v/v dimethyl sulfoxide, 10% v/v 6 M urea) to reach an anhydrous starch content of 0.4-0.5 g. After stirring for one week at ambient temperature (200 rpm) and centrifugation (2000 *g*, 10 min), 1 mL supernatant was either directly transferred into a 100 mL volumetric flask (apparent amylose) or firstly diluted with 9 mL ethanol, centrifuged (2000 *g*, 10 min) and the pellet resuspended in 1 mL UDMSO before transfer (total amylose). Thereafter, the following three steps were carried out within 60 s: (i) addition of approximately 95 mL purified water, (ii) addition of 2 mL aqueous solution containing 0.1% iodine and 1% potassium iodide, (iii) filling up to 100 mL with purified water and (iv) mixed by repeated inversion. After exactly 15 min (starting from the addition of water), the absorbance was recorded in 1 cm quartz cuvettes at 635 nm against diluted iodine reagent (1/50 v/v) using a Lambda 650 UV/Vis Spectrophotometer (PerkinElmer, Zaventem, Belgium). The measured absorbances for both apparent and total amylose were then used to calculate their respective Blue Value, which was expressed as absorbance of 10 mg anhydrous starch in 100 mL iodine reagent at 635 nm and 20 °C (**Eq. 17**). The correction factor of 0.0078 proposed by Morrison *et al.* (1983) was used to convert the Blue Value measured at a certain temperature into the Blue Value at 20 °C. This temperature-corrected Blue Value was then converted into the apparent or total amylose content using the regression equation suggested by the authors (**Eq. 18**). The content of complexed lipids was calculated from the difference of total and apparent amylose (**Eq. 19**).

$$Blue\ Value = \frac{A \times 10\ mg\ \times V_{UDMSO}}{m_{starch-db}} + (T - 20\ °C) \times 0.0078 \quad (17)$$

$$Amylose\ content\ [\%] = (28.414 \times Blue\ Value) - 6.218 \quad (18)$$

$$Complexed\ lipids\ [\%] = Total\ amylose\ [\%] - Apparent\ amylose\ [\%] \quad (19)$$

where A is the absorbance at 635 nm [-], V_{UDMSO} is the volume of UDMSO solution used [mL], $m_{starch-db}$ is the amount of starch contained in the dry sample used [mg] and T is the temperature during measurement [°C].

4.3.4 DIETARY FIBER CONTENT

Dietary fiber of the raw ingredients was determined by Improve (Dury, France) using the AOAC 985.29 enzymatic-gravimetric standard method, which is based on the removal of proteins and starch by enzymatic digestion, followed by fiber recovery *via* precipitation, filtration and washing.

4.3.5 SUGAR COMPOSITION

The analysis of sugars in the raw materials and final cakes had various strategic goals. At the level of the raw materials, the presence of high levels of reducing sugars (e.g. glucose, fructose) could imply a high potential to undergo thermal reactions like Maillard reaction and caramelization during cake development, leading to the generation of odorous volatiles. At the level of the cakes, the occurrence of certain sugars in high amounts could indicate thermal hydrolysis of starch (e.g. maltose, maltotriose), which would need to be considered when interpreting starch digestibility.

The profiling and quantification of sugars contained in the raw materials (pulse flours, pea fractions) was carried out by Improve (Dury, France) using a non-specified method based on ion chromatography coupled with pulsed amperometric detection.

By contrast, sugar analysis of cakes was performed internally. Two different solvents were tested to extract the sugars from the cakes: UDMSO and purified water. In the first case, UDMSO-cake dispersions were prepared as described in **§ 4.3.1 of Part IV**. In the latter case, cakes were suspended in water at ratios as described for the digestion experiments (1.25 g/10 mL) and either vortexed for 60 s or mixed with an ultraturrax for 2 min at 5200 rpm. After centrifugation of all samples (3500 *g*, 15 min), the supernatants were recovered and filtered through 0.45 µm Minisart filters. They were diluted in purified water and analyzed according to Vennard *et al.* (2020) with slight modifications. 25 µL of diluted sample were injected on a Dionex ICS-5000 ion chromatograph system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an AS-AP autosampler, a dual pump, a CarboPac PA20 analytical column (3 × 150 mm) protected by a CarboPac PA20 guard column (3 × 30 mm), and an electrochemical detector. Separation of the sugars was performed at 30 °C and a flow rate of 0.5 mL/min using 600 mM NaOH, 300 mM NaOH and purified water as eluents A, B and C, respectively. The gradient program was set as follows: 0% A, 3.3% B and 96.7% C isocratic (13 min); 0% A, 3.3% to 66.6% B and 96.7% to 33.3% C (12 min); 0% A, 66.6% B and 33.3% C isocratic (2 min); 0% to 100% A, 66.6% to 0% B, 33.3% to 0% C (0.1 min); 100% A, 0% B and 0% C isocratic (2.9 min); 100% to 0% A, 0% to 3.3% B and 0% to 96.7% C (0.1 min). Sugars were detected using a gold working electrode and an AgCl reference electrode applying the quadrupole potential waveform of E1 = +0.1 V (400 ms), E2 = -2.0 V (20 ms), E3 = +0.6 V (10 ms) and E4 = -0.1 V (70 ms). The quantities of fructose, glucose, maltose and maltotriose were determined by means of external calibration curves of the respective standards (**Figure 39**).

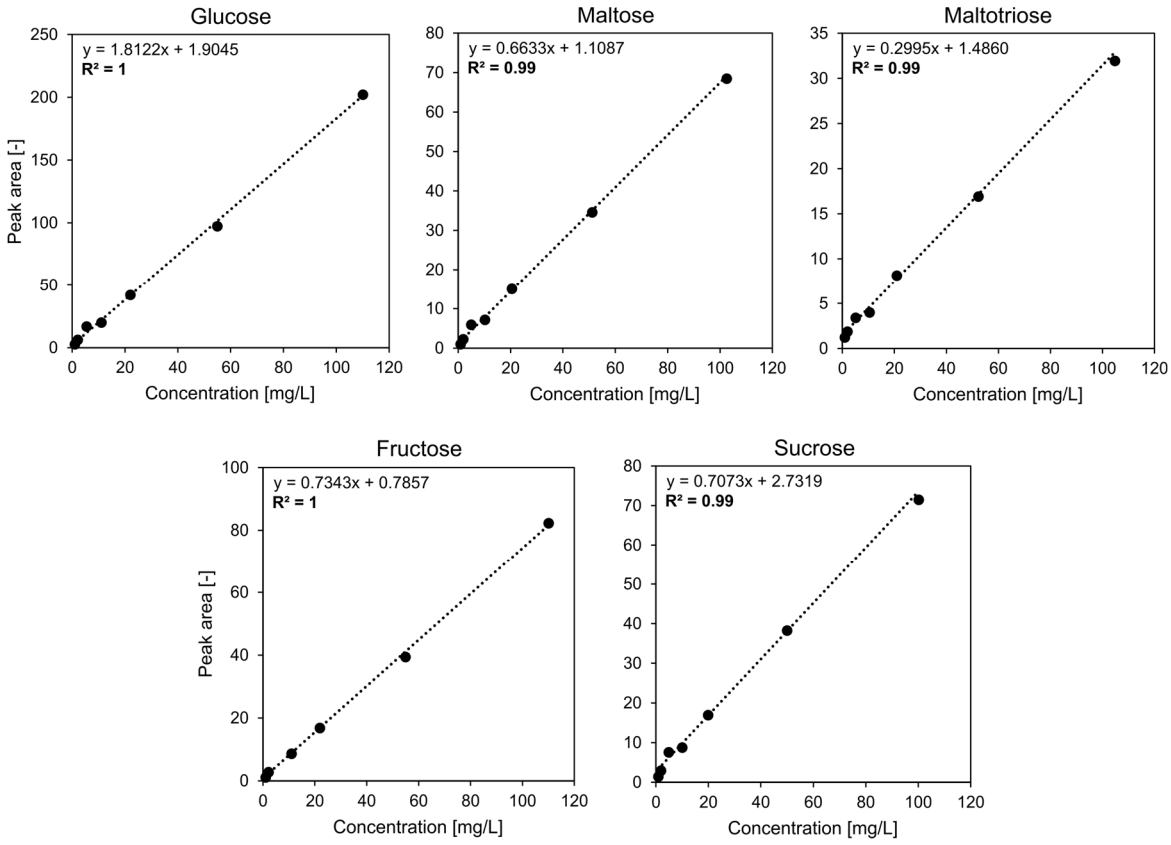

Figure 39. Calibration curves of glucose, maltose, maltotriose, fructose and sucrose used to quantify sugars in sponge cakes.

4.4 LIPIDS

4.4.1 LIPID EXTRACTION

Accelerated solvent extraction (ASE) is a common technique to rapidly extract lipids from oil-rich materials using appropriate solvents at elevated temperatures and pressure.

Due to the expected low fat content of the flours, the extraction conditions had to be optimized to increase the oil extraction yield. The extraction temperature is one of the crucial parameters that affects oil recovery, attributable to its impact on the viscosity of the oil. Therefore, a preliminary test was performed and the fatty matter of pea flour extracted using different extraction temperatures (ambient temperature, 40 °C and 70 °C). As visible from **Figure 40A**, the oil yields were non-significantly different between the different temperatures. However, the lowest standard deviation and thus highest repeatability was achieved with 40 °C, which thus was considered the most suitable temperature. Nevertheless, elevated temperatures might induce thermal degradation of fatty acid and thus release of primary and secondary oxidation markers (see details in **§ 6.2 of Part IV** and **§ 6.3 of Part IV**). To assess whether the extraction temperature led to adulterations of the oxidative status of the oil, the occurrence of primary oxidation markers, namely conjugated dienes, was analyzed. Detailed description of the method is given in **§ 6.3 of Part IV**. As can be seen from **Figure 40A**, the specific absorbance of conjugated dienes in oil extracted at 40 °C was lower compared to that obtained at ambient temperature and non-significantly different from that at 70 °C. Moreover, the standard deviation of analysis was remarkably smaller. This confirmed that 40 °C was an adequate temperature to extract high amounts of oil without modifying the sample's oxidation status.

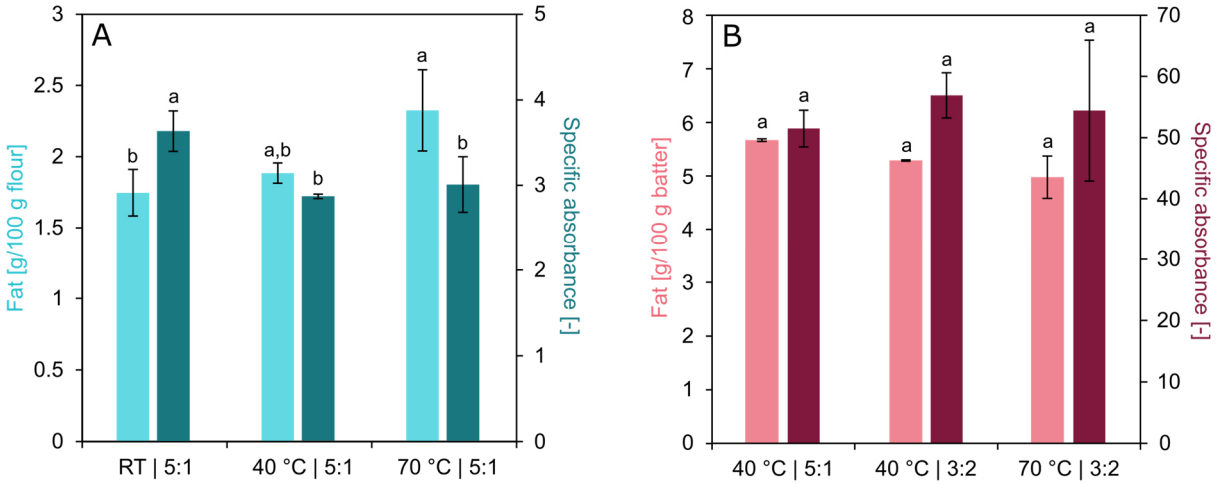

Figure 40. Effect of extraction temperature and heptane-to-isopropanol solvent ratio on the amount of extracted oil and specific absorbance of conjugated dienes from pea flour (A, *n* = 3) and pea batter (B, *n* = 2). Different small letters indicate significantly different means ($p < 0.05$). RT = room temperature.

Apart from pea flour, the effect of temperature on oil extraction yield was likewise evaluated for pea batters owing to their high structural complexity, which might complicate extractability. Based on the results obtained for pea flours, extraction at room temperature was not carried out. In addition to temperature, two different heptane-to-isopropanol ratios (v/v) of 3/2 and 5/1 were tested due to their potential impact on oil solubilization. As indicated in **Figure 40B**, neither the solvent ratio nor the

extraction temperature had a significant influence on the oil extraction yield. Moreover, both conditions did not affect the specific absorbance of conjugated dienes, which suggests that no adulterating oxidative reactions were triggered at any time. Similar to the flours, however, the data variability was greater when extraction was performed at 70 °C than at 40 °C, for which reason an ***EXTRACTION TEMPERATURE OF 40 °C*** was selected for any upcoming experiment. Furthermore, a ***HEPTANE-TO-ISOPROPANOL RATIO OF 5/1 (v/v)*** was selected due to its slightly higher oil extraction yield and lower specific absorbance of conjugated dienes compared to those obtained at a ratio of 3/2 (v/v). In addition, repeatable results were obtained for pea flour with this ratio.

Prior extraction, the samples are typically mixed with an inert filler, such as diatomaceous earth like Celite® 545, that absorbs water and hinders sample compaction due to particle aggregation during the extraction process. For the diverse food matrices studied, different ratios of Celite® 545 were thus selected depending on their moisture content. Accordingly, the final method involved the mixing of sample with Celite® 545 in ratios (w/w) of 1/1, 3/2, 10/9 and 14/5 for eggs, flours, batters and cakes, respectively, followed by the extraction of oil at 40 °C with heptane/isopropanol (5/1 v/v) for 25 min using a Dionex ASE 350 (Thermo Fisher Scientific, Waltham, MA, USA). The solvents were evaporated at 40 °C under vacuum, the yield determined by differential weighing before extraction and after evaporation and the oil stored under nitrogen at -20 °C until analysis.

4.4.2 FATTY ACID COMPOSITION

The analysis of the fatty acids in the raw materials yields important information about the presence of saturated and unsaturated fatty acids and thus vulnerability to oxidative breakdown.

The commonly applied method involves the conversion of fatty acids into their corresponding methyl esters, also referred to as FAMEs, yielding molecules with high volatility, which simplifies their analysis by ***GAS CHROMATOGRAPHIC*** techniques. In the present work, the method described by Roman *et al.* (2013) was performed with slight modifications. Approximately 30 mg of oil was weighed into snap cap vials, dissolved in 1 mL petroleum ether containing 3 mg of the internal standard heptadecanoic acid and gently mixed for 10 s. Base-catalyzed transesterification was performed by addition of 200 µL sodium hydroxide in methanol (2 M) and subsequent incubation of the tubes in a water bath at 52 °C while constantly being agitated (40 s). The reaction was stopped by addition of 400 µL of HCl in methanol (1 M). Samples were allowed to stand for 2 min for phase separation before the upper phase was transferred into 2 mL GC vials with integrated 0.2 mL glass micro-insert and stored at -20 °C until gas chromatographic analysis.

Elution of FAMEs was carried out on a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Supelco SP-2560 GC column specifically designed to separate FAMEs (100 m × 250 µm × 0.2 µm, Merck, Darmstadt, Germany). The conditions selected were based on the protocol reported by Supelco, with slight modifications. 1 µL of sample was injected at an inlet temperature of 250 °C and a split flow of 448 mL/min. To improve repeatability, the following conditions were optimized: 5× pre-injection washing with petroleum ether, 10× sample strokes, 0.3 µL/s sample pullup speed, 1 s delay bubble elimination, 1 µL/s bubble elimination pullup speed and 100 µL/min sample injection speed. Helium was used as carrier gas at a flow rate of 1.20 mL/min. The FAMEs were separated by increasing the initial oven temperature of 100 °C, which was held for 4 min, to 200 °C at a rate of 25 °C/min. After a holding time of 8 min, the temperature was raised again to 250 °C at a rate of

5 °C/min, which was maintained during 12 min. Mass spectra (*m/z* 40-400) were detected in the electron impact ionization mode at 250 °C using an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific). The peaks were identified by comparison of their retention times with those of a commercially obtained Supelco FAME standard mixture containing 37 compounds (Merck) subjected to the same procedure. Relative FAME quantities were calculated using the internal standard heptanoic acid. Repeatability of the GC performance was evaluated by analyzing three times the same vial of methyl esterified sunflower oil. Repeatability of the esterification process was evaluated by analyzing three independent methyl esterified sunflower oil samples. The results are given in **Table 20**. The determined fatty acid profile of the sunflower oil is in good agreement with that reported in the literature (see **§ 6.2 of Part II**).

Table 20. Repeatability results of FAME analysis (mean ± SD, *n* = 3).

Repeatability	Standard	C16:0	C18:0	C18:1	C18:2
Concentration [g/100 g fat]					
Esterification	9.6 ± 0.3	4.4 ± 0.1	2.5 ± 0.2	25.2 ± 0.4	48.1 ± 1.4
GC analysis	9.4 ± 0.0	4.6 ± 0.0	2.7 ± 0.0	25.8 ± 0.3	47.0 ± 0.5
Profile [%]					
Esterification		5.5 ± 0.2	3.1 ± 0.3	31.4 ± 0.7	60.0 ± 1.1
GC analysis		5.7 ± 0.0	3.1 ± 0.5	31.4 ± 1.6	59.7 ± 2.0

4.5 PROTEINS

4.5.1 PROTEIN CONTENT

Protein content of the eggs, flours, fractions and cakes was determined by Improve (Dury, France) using the official ***KJELDAHL METHOD*** (NF EN ISO 5983-2), which comprises the following steps: (i) sulfuric acid-catalyzed proteolysis at high temperatures, (ii) distillation with sodium hydroxide, (iii) recovery of released ammonia in hydrochloride acid containing an indicator, (iv) colorimetric titration with sodium hydroxide to the equivalence point at which the color of the solution changes and (v) calculation of the protein content from the required volume of sodium hydroxide to reach the equivalence point using a nitrogen-to-protein conversion factor of 5.7 for wheat based-products and 6.25 for pea- and maize-based products.

4.5.2 FREE AMINO ACID CONTENT

Free amino acids are reactive constituents of ingredients which can undergo Maillard reaction and thus contribute to the generation of volatile compounds. For this reason, the free amino acids in the raw materials were analyzed externally by Improve (Dury, France) according to ISO 13903:2005 and ISO 13904:2016. The determination is generally based on the extraction of free amino acids with dilute hydrochloric acid, separation by ***ION EXCHANGE CHROMATOGRAPHY***, reaction with ninhydrin and photometric detection at 570 nm.

5 STRUCTURAL AND RELATED PROPERTIES

5.1 MICROSCOPIC ANALYSES

5.1.1 STARCH GRANULE MORPHOLOGY

In order to compare the morphological characteristics of starch granules contained in the used ingredients with those reported in the literature, the different flours/fractions selected for the pea fraction study were suspended in water and visualized under the ***LIGHT MICROSCOPE*** (Olympus BX51, Olympus, Tokyo, Japan). As this analysis only aimed at giving some qualitative information about the general characteristics of the raw ingredients, the applied protocol was not optimized.

5.1.2 DISTRIBUTION OF INGREDIENTS IN BATTERS AND CAKES

The use of novel ingredients with specific composition and functionalities can lead to a different organization and distribution of macromolecules in food matrices like cakes compared to traditional raw materials like wheat, which might affect not only textural properties but also digestibility and reactivity. Therefore, it was of great interest to gain deeper understanding of the partitioning of lipids, proteins and starch as well as air bubbles entrapped during batter preparation in batters and cakes based on both wheat and pulse ingredients.

CONFOCAL LASER SCANNING MICROSCOPY (CLSM) is a suitable technique to capture 3D images of complex food samples at high resolution. Light with specific wavelength is emitted by the light source and converged onto one point of the sample by means of a pinhole. This results in the reduction of scattered light from the environment and thus enhancement of the contrast. The fluorescence emitted by the sample is focused by the detector pinhole aperture and produces a clear and colored image. By scanning the sample from point to point, a 3D image can be reconstructed. Moreover, the use of different excitation wavelengths aids to visualize different sample components, which produce fluorescence of varying wavelengths.

In the present study, two markers were selected to stain lipids in red and proteins in green: Bodipy 665/676 and DyLight 488, respectively. Both dyes were mixed in similar volumes (1/1 v/v) and 3 µL used to stain 1 g batter or a center piece of cake (1 × 1 × 1 cm^3). After excitation at 488 nm (green) and 638 nm (red) by means of two lasers, the emitted fluorescence was detected in two channels of the Leica TC2 SP8 confocal laser scanning microscope (Wetzlar, Germany) in the wavelength ranges of 497-601 nm and 643-765 nm. Images were acquired at both 10× and 40× magnification using a scan speed of 400 Hz, 70.7 µm pinhole and xyz scan mode.

5.1.3 CAKE CRUMB MORPHOLOGY

SCANNING ELECTRON MICROSCOPY (SEM) is a useful technique to capture the microstructure of cakes that provides information about the topography and porosity of the crumb as well as organization of ingredients. Basically, an electron beam is accelerated towards the sample and produces different signals as it hits the surface. These include backscattered electrons, among others, which are reflected from the sample after elastic interaction. After collection by an appropriate detector, a micrograph with large depth of field and strong shadowing effect is obtained. Due to the absorption of electrons by the non-conductive sample, charging can occur, which might lead to distortion of the image. In order to

avoid such charging effects, the samples can be either coated with conductive materials (e.g. gold) or analyzed under low vacuum, the latter of which can be attributed to the neutralizing effect of gas molecules.

The latter was applied to image the crumb of cakes prepared for the pea fraction study. For this, a center piece (0.8 cm × 0.8 cm × 0.3 cm) was mounted on a metal stub and placed inside a TM4000 Plus (Hitachi, Maidenhead, UK). The surface of the cake crumb was scanned in the charge-up reduction mode by an electron beam accelerated with 15 kV. Back-scattered electron images were collected at 100× magnification.

5.2 MESOSCOPIC AND MACROSCOPIC ANALYSES

5.2.1 PASTING PROPERTIES

Knowledge about the pasting properties of starch can give insight into the structuration behavior during baking (see **§ 2.4.2 of Part II**). Therefore, the pasting properties of the ingredients used for the pea fraction studies were analyzed by means of a Rapid Visco Analyzer RVA 4500 (Perten Instruments, New South Wales, Australia).

Flours and starches were suspended in purified water to reach a concentration of 9% w/w (30 g total weight). This concentration was likewise used in the literature for the analysis of the pasting properties of pea starch (Ratnayake *et al.*, 2001). The pasting profiles were recorded using the following temperature program: (i) 40 °C isothermal for 2 min, (ii) heating to 90 °C at 5 °C/min, (iii) 90 °C isothermal for 5 min, (iv) cooling to 20 °C at 2.5 °C/min, and (v) 20 °C isothermal for 5 min.

5.2.2 BATTER AND CAKE DENSITY

Density is an important measure that can be used to describe the amount of air incorporated during batter whipping (Monnet *et al.*, 2020). Due to the fragility of the batters, density determination had to be performed immediately after preparation in a manner that limited gas cell destruction. A first attempt to use containers of known volumes was, however, not successful as the high stickiness of the product quickly led to the inclusion of void spaces and thus to an incorrect volume. Syringes of 10 mL appeared to be an attractive alternative, which allowed slowly drawing up of the freshly prepared batter without creating void spaces. However, to limit gas cell destruction, the inlet area of the syringe had to be increased by cutting of the tip of the syringe. Thereafter, the volume of the ***OPEN-MOUTH SYRINGES*** was measured in quadruplicates by filling with distilled water and subsequent weighing. Batter density was then determined by filling the syringes completely, weighing and calculation of the ratio of the batter mass to the pre-determined syringe volume.

Similar to the batter density, the density of cakes provides information about the amount of air conserved during baking. It can be measured using a ***VOLSCAN PROFILER*** (Stable Micro Systems, Surrey, UK), which scans vertically the contours of the product by means of a laser from the top to the bottom at 2 mm intervals while it constantly rotates. The software automatically determines the cake volume from the numerous data points acquired, which can be related to the mass of the cake to determine the cake density.

5.2.3 BATTER CONSISTENCY

The consistency of batters reflects their resistance to flow, which is impacted by the composition and structuration of the matrix. While batters with low consistencies might only poorly retain air bubbles during baking, too high consistencies might hinder heat-induced expansion (Fiszman *et al.*, 2013). In addition, the transfer of heat and moisture might be affected, which in turn could alter chemical reactivity.

In order to evaluate the consistencies of the batters of the pea fraction study, the method proposed by Monnet *et al.* (2020) was performed with modifications. Batter was freshly prepared and filled in 40 mL pots. A 20 mm aluminum plate was attached to a TA-XT2i ***TEXTURE ANALYZER*** equipped with 5 kg load cell (Stable Micro Systems, Surrey, UK) and calibrated. Thereafter, the measurement was started by slowly inserting the probe into the batter at a speed of 2 mm/s until a distance of 20 mm was reached. The resistive force to compression [N] was plotted against the compression distance [mm] and the batter consistency determined from the average force over the constant part at the end of the curve.

5.2.4 CELLULAR CRUMB STRUCTURE

Analysis of the cellular structure of the cake crumb can be achieved by ***IMAGE ANALYSIS*** using mathematical morphology. It allows to conclude about the size of cells and the thickness of cell walls and thus about the openness and homogeneity of the cakes studied. This method is based on the transformation of pixel of a grey image by dilatation and erosion steps using a structuring element of defined size. During the dilation operation, the centered pixel of the structuring element is replaced by the brightest pixel of the structuring element. Consequently, the dilatation operation reveals information about the resistance of dark objects to brightening. This evidences the disappearance speed of dark objects and corresponds to the cells. By contrast, during erosion, the centered pixel of the structuring element is replaced by the darkest pixel of the structuring element, leading to a darkening of the image. This allows to gain insight in the resistance of bright objects to darkening and consequently in the disappearance speed of bright objects, which is linked to the cell walls. Several dilation and erosion steps are successively applied, thereby gradually inducing changes in the sum of grey levels of the image. These grey levels range from 0 (black) to 255 (white). Images with high proportion of bright pixels thus have a higher sum of grey levels, which is also referred to as image volume *V*. With increasing number of dilations, an increase in the image volume can be monitored, whereas the opposite can be observed with growing number of erosion steps. In order to quantify the variation in this image volume from one operation step *i* to another, its derivative *g* is calculated and normalized by the difference between the volume of the original image (V_0) and the image after the last operation step (V_f) (**Eq**. **20**).

$$g_i = \left| \frac{V_i - V_{i+1}}{V_0 - V_f} \right| \quad (\mathbf{20})$$

The plot of *g* as a function of the size of the structuring element yields a granulometric curve of the change of the sum of grey levels. The size *z* of the structuring element after each operation step *i* is calculated using the size *s* of the pixel according to **Eq. 21**.

$$z = (2 \cdot s + 1) \cdot i \quad (\mathbf{21})$$

This granulometric curve of the change of the sum of grey levels (“erosion-dilation curve”) are then used for principal component analysis.

Before performing image analysis, the images of the cake crumbs had to be captured. For this, the cakes were cut horizontally and the crumb particles gently removed by the aid of a brush. The bottom half of the cake was positioned on the center of a flatbed scanner (HP Scanjet G3110, Hewlett-Packard Development Co, Canada) and covered with a black box to obtain a good contrast between the black background and the bright crumb. As suggested by Dewaest *et al.* (2018), full color images were captured in tif format without automatic color adjustment at a resolution of 600 dpi, so as to 1 pixel = $(42)^2$ μm^2 (size s = 42 µm). The color images were then converted into monochromatic images of 256 grey levels. A binary mask of each image was obtained by choosing a grey level of 10 to extract the cake from the background. The crust was removed by erosion of the cake mask using a squared structuring element of size 40 (= 81 x 81 pixels = 3.4 x 3.4 mm). This means that the color of the middle pixel of the squared structuring element was replaced by the darkest color appearing in the whole structuring element. Subsequently, 40 erosion and dilatation steps were performed using a squared structuring element of the size 3 × 3 pixels as reported by Dewaest *et al.* (2018). This allowed to observe elements ranging from 0.13 mm to 3.4 mm.

5.2.5 STIFFNESS OF CAKE CRUMB AND CELL WALLS

In order to discriminate the textural properties of the different cakes, the analysis of their response to mechanical stress appears to be a promising approach as it has been discussed in the literature (Attenburrow *et al.*, 1989; Dewaest *et al.*, 2018; Monnet *et al.*, 2020). Several authors suggested the use uniaxial compression for aerated food like cakes, which provides information about the stiffness of the crumb at small deformations as well as the stiffness of cell walls at large deformations (Attenburrow *et al.*, 1989; Gibson *et al.*, 1997; Monnet *et al.*, 2020).

The mechanical properties of the aerated crumb of the cakes of the pea fraction study were thus analyzed by performance of a uniaxial compression test using a TAHD$^+$ ***TEXTURE ANALYZER*** equipped with 750 kg load cell (Stable Micro Systems, Surrey, UK). In order to yield comparable results, the dimensions of the investigated cake samples had to be identical. This was achieved by designing and 3D-printing two cutting boards with which a squared center piece of the cake crumb with the dimensions of 3 cm × 3 cm × 1 cm was obtained. The cutting procedure is illustrated in (**Figure 41**). A bread knife was used.

The obtained cake pieces were then compressed to 90% of their initial height at a speed of 2 mm/s using an aluminum probe with 10 cm diameter. The resulting force-versus-distance curves were converted into stress-versus-strain curves using the sample dimensions. This curve could be used to determine the Young modulus of the crumb (measure of crumb stiffness) as well as the Young modulus of the cell walls (measure of cell wall stiffness). While the former corresponded to the initial slope of the curve, the latter corresponded to the slope at the end of compression.

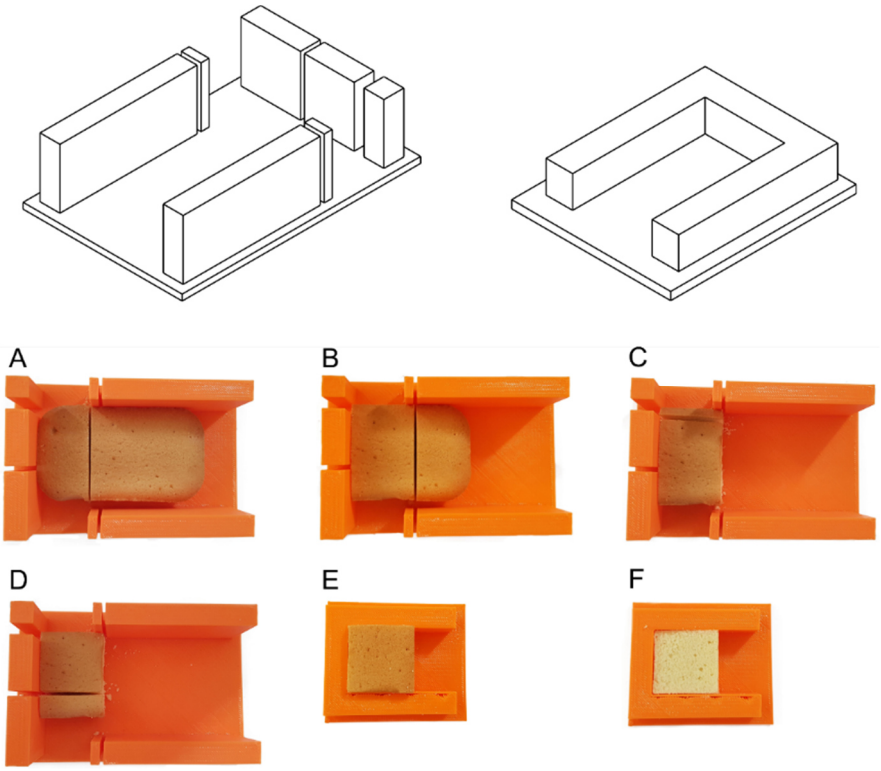

Figure 41. Cutting boards specifically designed to obtain a squared center piece of cake crumb with dimensions of 3 cm × 3 cm × 1 cm. The different steps of cutting are illustrated in the images A-F.

6 EVALUATION OF CHEMICAL REACTIVITY

6.1 CAKE SAMPLE PREPARATION

Prior HS-SPME/GC-MS analysis, the sponge cakes were ground by means of a Grindomix GM200 knife mill equipped with a stainless-steel bowl and titan knifes (Retsch GmbH, Haan, Germany) to minimize the impact of cake particle size on VOC release. The protocol proposed by Cepeda-Vázquez *et al.* (2018) for wheat-based sponge cakes (batches of five, frozen state) comprised three successive grinding steps: (i) 3000 rpm for 10 s, (ii) 6000 rpm for 20 s and (iii) 3000 rpm for 50 s. The different batches were finally mixed together at 2000 rpm for 5 s.

On the basis of this protocol, a preliminary experiment was carried out that aimed at achieving a balance between comparable particle sizes of cakes based on different dry flour ingredients and avoidance of excessive energy input by friction to limit the heat-induced formation or loss of volatile compounds. Thus, the protocol described was broken down into its individual steps and an analysis of the particle size distribution of diverse frozen pulse cakes was carried out after each step using a Mastersizer 2000 equipped with Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK), applying a refractive index of 1.49429 and an absorption index of 0.1.

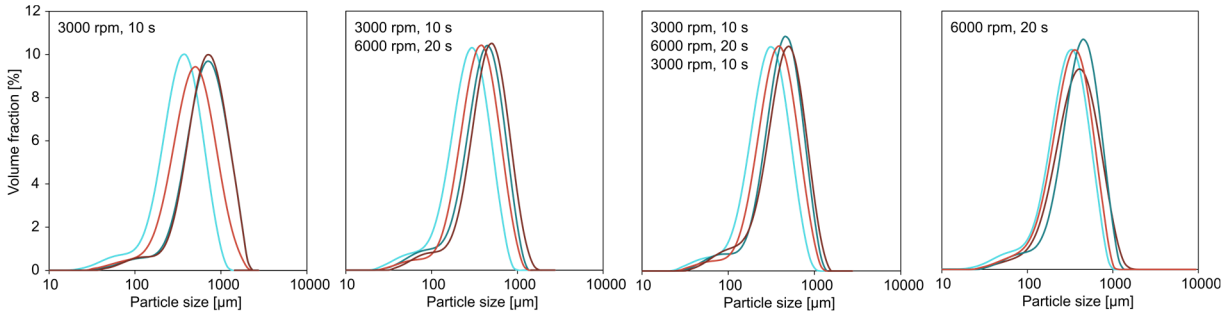

Figure 42. Particle size distributions of sponge cakes ground using different combinations of time and speed. Cakes were made with wheat (■), green pea (■), lentil (■) or chickpea flour (■).

As visible from **Figure 42**, the particle size distribution of the different cakes varied strongly after the first grinding step (3000 rpm, 10 s). After regrinding (6000 rpm, 20 s), the differences between the cakes diminished. Concurrently, however, a distinct cake smell was perceivable, implying high energy input of the grinder that led to cake thawing and volatilization of odorants. This odor was stronger when performing the third grinding step (3000 rpm, 10 s). Thus, a grinding starting with a high speed (6000 rpm, 20 s) was carried out on a new batch of frozen cakes. Interestingly, the resulting particle size distribution was comparable to that after the two/three steps of the original protocol. Moreover, there was no strong smell perceivable. This indicated that the grinding duration and speed were sufficient to grind the cakes to an adequate small particle size, but not strong enough to cause flavor development or release. It was thus decided to grind batches of five frozen cakes for ***20 SECONDS AT 6000 RPM*** for any upcoming experiment. This protocol was likewise applied for the digestibility experiments, where similar particle sizes were likewise desired, as well as chemical analyses. The ground cakes were sealed in glass jars and stored at -20 °C until use.

6.2 HEADSPACE-SOLID PHASE MICROEXTRACTION/GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF VOLATILES

HeadSpace-Solid Phase Microextraction/Gas Chromatography-Mass Spectrometry (HS-SPME/GC-MS) is a simple, fast, solvent-free and widely employed static technique to analyze the volatile compounds in legume-based products (Azarnia *et al.*, 2011b; Ma *et al.*, 2016; Szczygiel *et al.*, 2017; Xu *et al.*, 2019). The method is based on the absorption and desorption of volatiles, which are emitted from a sample enclosed in a vessel, on a fiber that is exposed to the headspace. After equilibria between the sample, headspace and fiber have been reached and the molecules adsorbed on the fiber, the compounds are thermally desorbed from the fiber into the GC injection port for analysis. The fiber is coated with a stationary phase that can affect extraction efficacy due to their different affinity for molecules. Xu *et al.* (2019) compared the performance of different SPME fibers in pea flour and concluded that DVB/CAR/PDMS fibers would be suitable for screening purposes, while CAR/PDMS fibers would be more suitable for quantitative analysis. As our aim was to get more insight into the different reactivity potentials of diverse raw materials, we decided to use a DVB/CAR/PDMS fiber in order to extract a wide variety of different compounds. The method that was used was based on the unpublished work of Hanaei *et al.* (2010). The method described there was optimized to extract high

amounts of alkylpyrazines emerging during the development of wheat-based sponge cakes. The corresponding conditions are listed in **Table 21**. Some adaptations were made and will be described in the following sections.

Table 21. Original HS-SPME/GC-MS conditions reported by Hanaei *et al.* (2010).

Property	Condition
Fiber	DVB/CAR/PDMS (2 cm)
Column	ZB-5ms (29.9 m × 0.32 µm × 0.5 µm)
Incubation	66 °C, 18 min
Extraction	66 °C, 42 min
Desorption	250 °C, 2 min
Oven program	40 °C (5 min) to 240 °C at 4 °C/min
Carrier gas	Helium
MS scan range	*m/z* 33 – 350

6.2.1 SAMPLE AMOUNT

Different sample sizes have been used in the literature to extract volatiles by HS-SPME/GC-MS. For instance, Hanaei *et al.* (2010) used 4 g ground sponge cakes in 20 mL vials, Laleg *et al.* (2016) 2 g pulse flour in 10 mL vials, Szczygiel *et al.* (2017) 5 g bean flour in 50 mL vials and Xu *et al.* (2019) 2 g pea flour in 20 mL vials. Consistently, the commonly used sample-to-headspace ratio varied between 0.1 and 0.2.

In order to select the optimal amount of flour and cake from this range leading to the highest extractability of compounds, a preliminary experiment was carried out by analyzing 2 – 4 g sample in 20 mL vials under the method conditions described in **Table 21**.

The resulting chromatograms are depicted in **Figure 43**. In general, the peak areas were higher in vials containing less flour or cake, which implied saturation of the fiber at higher sample sizes and thus compound desorption into the vial headspace to maintain the partition equilibriums between sample, headspace and fiber (Azarnia *et al.*, 2011b). For this reason, a sample amount of ***2 G*** was selected for the upcoming experiments.

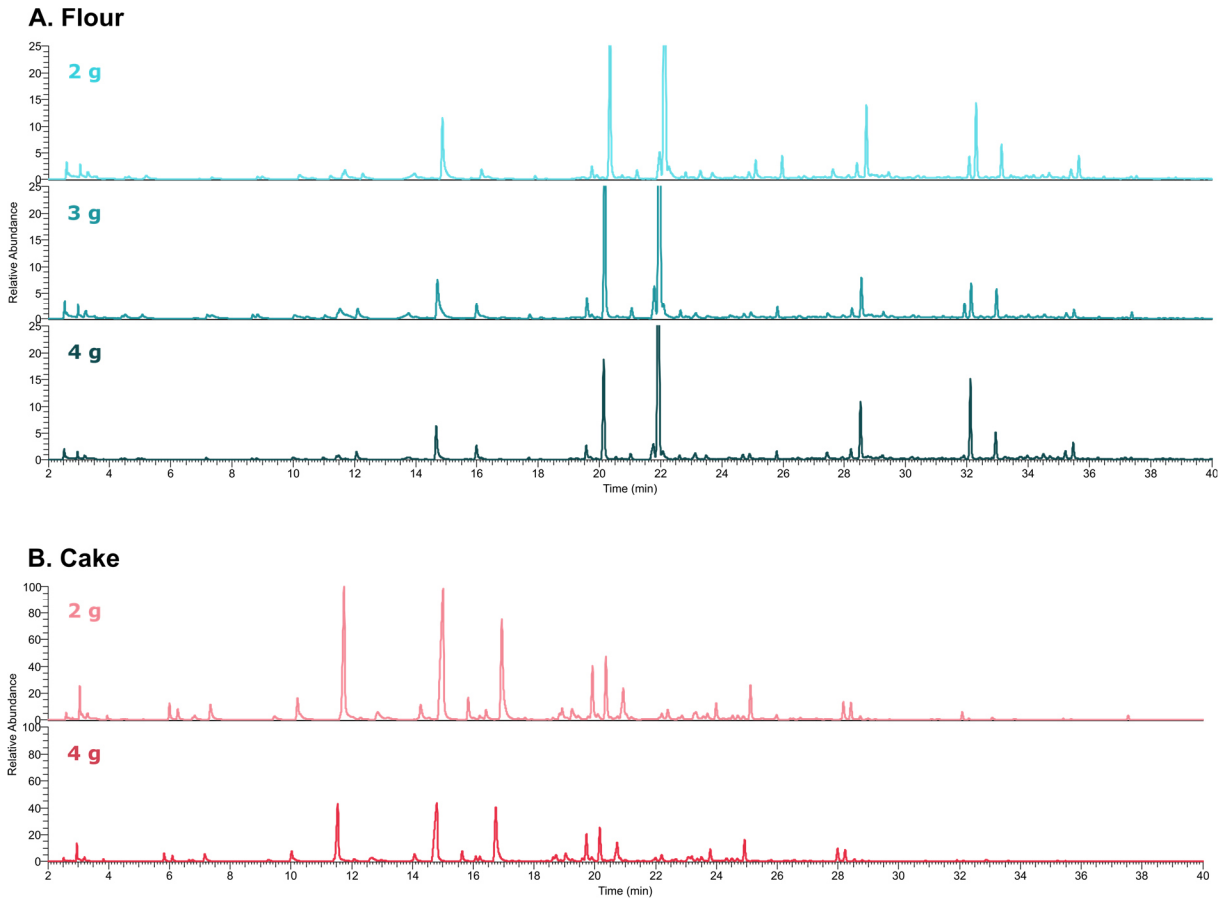

Figure 43. Gas chromatograms of fava bean flour and lentil cake analyzed using different sample amounts (2 – 4 g). The normalization level of the peak intensity is 8×10^6 for the flours and 6×10^6 for the cakes.

6.2.2 TYPE OF CAPILLARY COLUMN

Originally, a non-polar ZB-5 column was used for peak elution with which, however, individual peaks were often only poorly separated from each other. In the search for possibilities for reducing such coelution, the replacement of this column by a polar ZB-wax appeared to be a promising approach, which is recommended to be used for the analysis of alcohols, aldehydes and aromatic compounds that were highly present in the pulse-based products.

Different pulse-based cakes (lentil, green pea, lupin), which complemented each other in their VOC profiles, were therefore analyzed on both columns under the method conditions listed in **Table 21** using a sample size of 2 g. The results of green pea cake are exemplarily shown in **Figure 44**. In general, peak separation could be improved using a ZB-wax column for all products. Moreover, this polar column seemed to be more sensitive as visible from the higher total number of peaks identified. Since the majority of these compounds could be associated with degradation reactions of food ingredients and were not primarily fiber or column components, the ZB-wax column appeared to be a better alternative to the ZB-5 column. Any upcoming experiments were thus performed on the ***ZB-WAX COLUMN***.

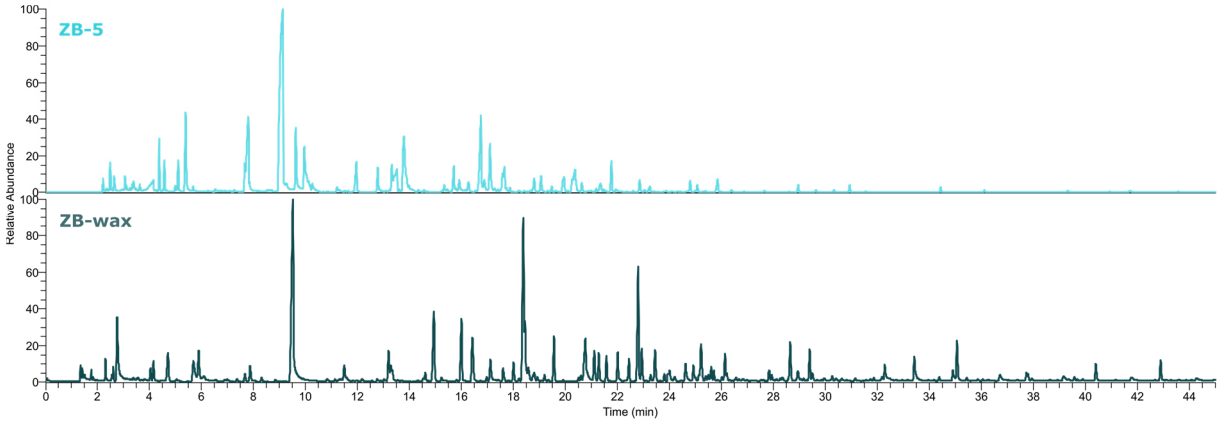

Figure 44. Gas chromatograms of green pea cake analyzed on a ZB-5 or a ZB-wax column.

6.2.3 INTERNAL STANDARDS AND ION SELECTION

Spiking of samples with deuterated internal standards allows the relative quantification of VOCs contained therein by dividing the peak area of the detected component by that of the isotope and multiplying by the known concentration of the isotope. Three isotopes have been chosen for this study, namely ***HEXANAL-D12***, ***FURFURAL-D4*** and ***2-METHYLPYRAZINE-D6***. These quality markers were selected because of their proven occurrence in the products, affiliation to the main classes of compounds generated during product development (2-methylpyrazine and furfural *via* thermal degradation reactions, hexanal *via* lipid oxidation) and different retention times that allow quantification throughout the whole chromatogram (**Figure 45**).

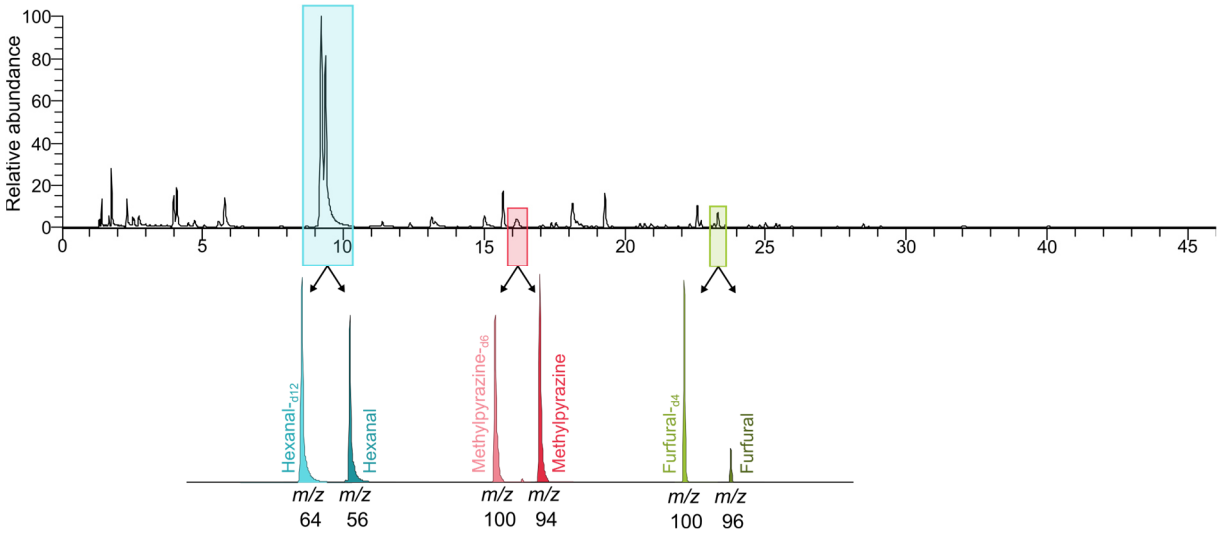

Figure 45. Chromatogram of yellow pea cake recorded by employing the total ion current mode (TIC, top) and selected ion monitoring mode (SIM, bottom) using specific *m/z* characteristic for desired molecules.

During GC-MS analysis, the different inherent compounds and internal standards were detected in both total ion current (TIC) mode and selected ion monitoring (SIM) mode. For the latter, different retention time windows were created throughout the chromatogram running time, in which selected *m/z*

characteristic of desired compounds were recorded to improve sensitivity and specificity. As visible from **Figure 45**, this technique allowed the separation of peak areas of molecules eluting at similar retention times, such as was the case for the internal standards and corresponding inherent compounds. The chosen retention time windows were altered depending on the food matrix (flour, batter or cake) analyzed. An overview of the selected *m/z* is given in **Table 41** in the appendix.

6.2.4 INTERNAL STANDARD EQUILIBRIUM TIME

Before starting the HS-SPME/GC-MS analysis of the spiked samples, it needed to be ensured that the VOCs contained in the sample and the added internal standards reached equilibrium. Therefore, a preliminary test was carried out in which samples were spiked with the respective internal standard solution and stored in the refrigerator for either 7 hours or 17 hours. A period of 7 h corresponds to the time span between the earliest possible preparation on day 1 and the latest possible start of analysis on the same day (morning – afternoon), while a period of 17 h corresponds to the time span between the latest possible preparation on day 1 and the earliest possible start of analysis on day 2 (afternoon – morning).

Since the structure of the food under study could influence the VOC release from the matrix, both the batter and the cake of the sample with the highest number of different volatiles, namely lentil, were analyzed (in triplicate). Due to the significantly lower structural complexity of flour compared to batter and cake, it was assumed that the time-dependent VOC release was lowest in this product, hence it was not integrated into the experimental setup.

Interestingly, the two selected equilibrium times led in both matrices to repeatable ratios of inherent compound to the respective internal standard. For instance, the relative standard deviations for the hexanal-to-hexanal-d_{12} ratios amounted for both storage times to 4% in the cakes and 15% in the batters. The higher relative standard deviations for the batters in comparison to the cakes reflects the retention effects imparted by the more complex batter matrix. Moreover, no new compounds were formed during the two time periods in both batters and cakes, implying that the longer storage did not trigger any reactions and thus adulteration of the VOC profile. Consequently, for strategic reasons, ***OVERNIGHT STORAGE*** for 17 h at 4 °C was selected as the equilibrium time for upcoming experiments.

6.2.5 SEQUENCE LENGTH AND FIBER LIFETIME

Modern GC-MS instruments are equipped with an autosampler, which allows the sequential and fully automated analysis of a great number of samples in a time-optimized manner. Theoretically, around 50 samples could be placed at the same time in the instrument and the corresponding chromatograms retrieved when the sequence was completed. However, it might be possible that the samples changes with the storage time in the GC at ambient temperature. Consequently, the resulting data of the sample analyzed first would not be comparable with that of the sample analyzed at the end of the sequence. In order to avoid such time-induced adulterations, a preliminary test was carried out in which the best set-up and maximal length of a daily sequence were determined. For this purpose, a sequence composed of six lentil cake samples spiked with internal standards was prepared and the samples analyzed after overnight storage with a delay of 3 – 4 h between the samples, resulting in a total sequence length of approximately 20 h. For the batters, a shorter sequence of 10 h was prepared using five lentil batter samples which were run in shorter time intervals (1 – 2 h) owing to the higher risk of changes in the

volatiles profile in the moist and untreated products. In the case of the cakes, the peak areas of inherent hexanal and 2-methylpyrazine were related to the peak areas of their respective internal standards hexanal-d_{12} and 2-methylpyrazine-d_6. In the case of the batters, only the hexanal-to-hexanal-d_{12} ratio was determined due to the absence of thermal reaction markers. The calculated ratios as a function of analysis time for both food matrices are illustrated in **Figure 46**. The experiment was performed once.

It emerges that neither for the cakes nor batters any strong changes in the various ratios occurred over the given period, indicating that theoretically a sequence of 20 hours composed of cake samples and a sequence of 10 hours composed of batters would be possible. The figure also verifies that the chosen overnight storage of spiked samples at 4 °C was an appropriate technique to reach equilibration of the standard and the sample VOCs in the headspace.

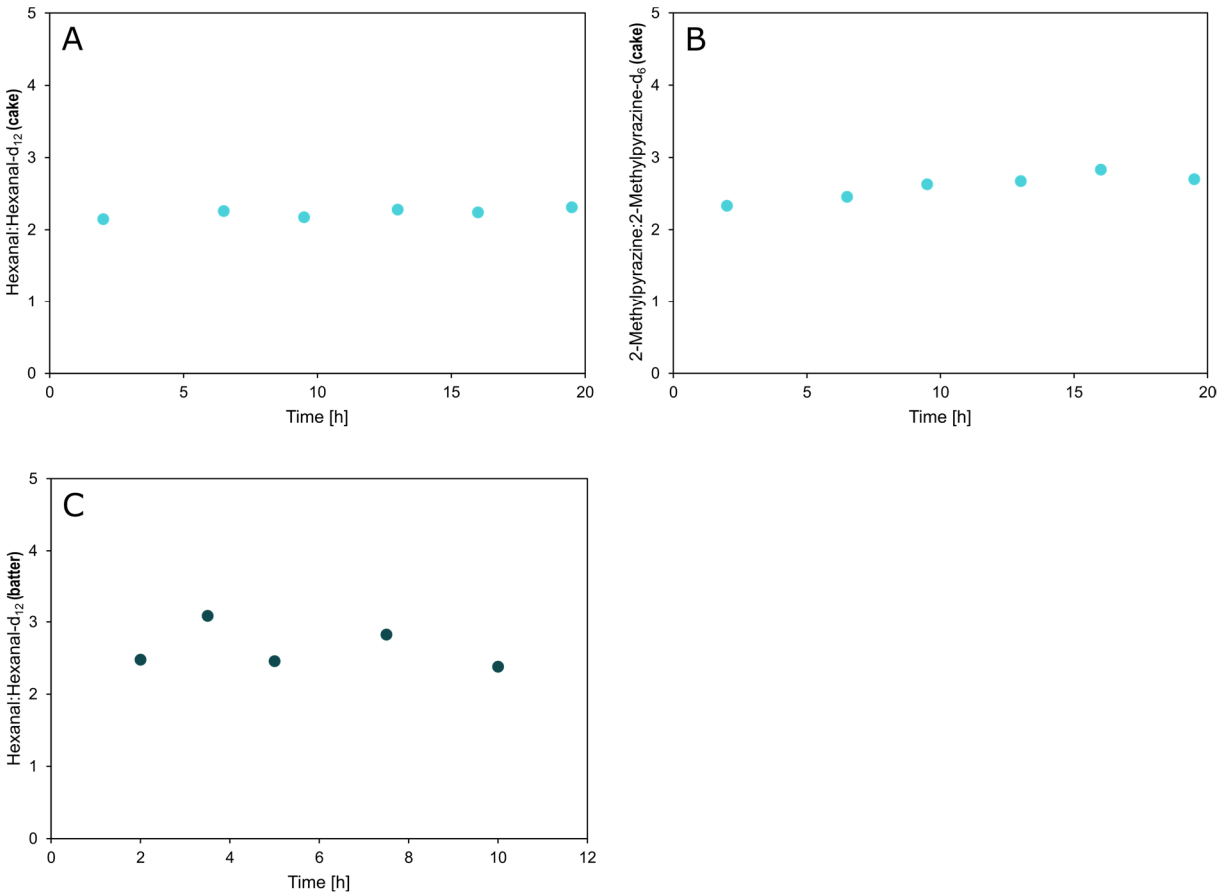

Figure 46. Change of the ratios of inherent compound to internal standard in (A,B) lentil cake and (C) lentil batter as a function of analysis time.

Based on these results, it was decided that a daily sequence would start with the analysis of one batter (in triplicate), followed by the analysis of two cakes or flour in sequence (in triplicate each). Blanks (vials without sample) were additionally integrated into the sequence to monitor the response of fiber and column. Since only a limited number of extractions can be carried out with a single fiber, a control sample was likewise included into the daily sequence and the obtained chromatograms were checked over the entire lifetime of the fiber. For the first major study, this control was yellow pea cake based on IMPROVE flour (article 1); for the second major study, it was yellow pea cake based on Cargill flour (article 2 and 3).

Different fibers were used for the first and second study. As visible from **Table 22**, the concentrations of the internal standards and their corresponding inherent compounds changed only minimally, suggesting that the response of the fiber remained constant over the entire duration of a study.

Table 22. Mean concentrations of inherent compounds and respective internal standards [µg/g] (mean ± SD, *n* = 8 – 9) as well as their ratios determined for control samples that were integrated into the daily sequence over the entire fiber lifetime. Yellow pea cake based on IMPROVE flour was used as control for the pulse flour study, yellow pea cake based on Cargill flour for the pea fraction study.

Compound	Study pulse flours		Study pea fractions	
	Concentration [µg/g cake]	Ratio inherent-to-standard	Concentration [µg/g cake]	Ratio inherent-to-standard
Hexanal	15.86 ± 1.49 *9%*	0.8 ± 0.1 *9%*	17.09 ± 1.67 *10%*	0.8 ± 0.1 *9%*
Hexanal-d_{12}	20.33 ± 0.29 *1%*		20.31 ± 0.27 *1%*	
2-Methylpyrazine	0.20 ± 0.01 *3%*	1.2 ± 0.0 *3%*	0.17 ± 0.01 *6%*	1.0 ± 0.1 *6%*
2-Methylpyrazine-d_6	0.17 ± 0.00 *1%*		0.17 ± 0.00 *1%*	
Furfural	0.12 ± 0.00 *4%*	0.2 ± 0.0 *4%*	0.17 ± 0.01 *6%*	0.2 ± 0.0 *6%*
Furfural-d_4	0.75 ± 0.01 *1%*		0.75 ± 0.01 *1%*	

6.2.6 INCUBATION AND EXTRACTION TEMPERATURE

The incubation and extraction temperature used by Hanaei *et al.* (2010) was 66 °C. To ensure that this temperature did not lead to the formation of artifacts that would have resulted in a falsified VOC analysis, the volatiles contained in lentil flour, batter and cake were additionally extracted in triplicate at 50 °C, which is a commonly used temperature for VOC extraction from pulse ingredients (Azarnia *et al.*, 2011a; Ma *et al.*, 2016; Oomah *et al.*, 2007; Szczygiel *et al.*, 2017).

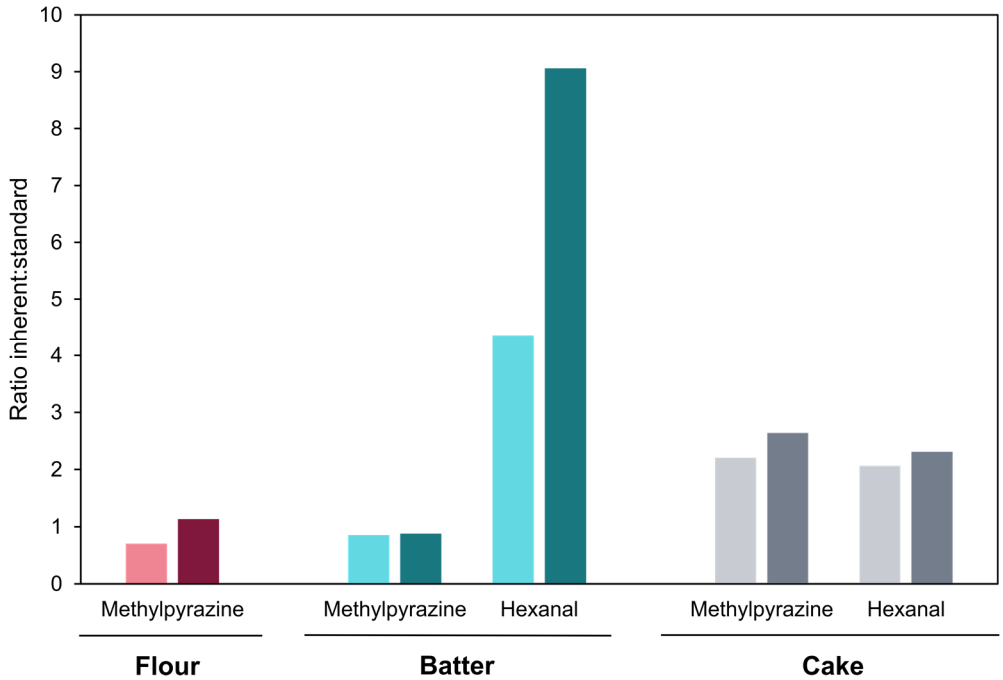

Figure 47. Ratios of inherent compound to internal standard in lentil flours, batters and cakes dependent on the extraction temperature. Light colors correspond to analysis at 50 °C, dark colors to those at 66 °C.

As visible from **Figure 47**, the ratio of inherent compound to corresponding deuterated standard became larger at higher incubation/extraction temperature. Since the deuterated standard possesses high stability, this result implied that the higher temperature initiated reactions that led to the generation of compounds, especially of hexanal in the lentil batter. For this reason, any upcoming experiments were performed using an ***INCUBATION AND EXTRACTION TEMPERATURE OF 50 °C***.

6.2.7 FINAL METHOD CONDITIONS

INTERNAL STANDARD SOLUTIONS

For the relative quantification of VOCs in flours, batters and cakes, aqueous solutions of the isotopic standards furfural-d_4, hexanal-d_{12} and 2-methylpyrazine-d_6 were prepared from which three standard solutions, one for each food matrix, were obtained by mixing in different ratios. The composition of the final flour solution is shown in **Table 23**. The solutions were stored at -20 °C.

Table 23. Composition of final internal standard solutions for flours, batters and cakes.

Standard [mg/L]	Flour	Batter	Cake
Hexanal-d_{12}	139	778	411
2-Methylpyrazine-d_6	0.1	89	3
Furfural-d_4	14	91	15

EXTRACTION OF VOLATILES

Thawed flour, batter or ground cake (2 g) were weighed in triplicate into 20 mL transparent vials, spiked with 100 µL respective internal standard solution and enclosed with aluminum polytetrafluoroethylene-coated silicon septa. In order to ensure that the sample's VOCs and the added standards reached equilibrium in the headspace, the vials were stored overnight at 4 °C. The next day, they were incubated at 50 °C for 18 min in the heating block of a TriPlus Autosampler (Thermo Fisher Scientific). Thereafter, a DVB/CAR/PDMS fiber (50/30 µm, 2 cm, Supelco) was inserted into the vial for 42 min at the same temperature. The compounds were thermally desorbed for 2 min at 250 °C and transferred into the injection port of a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific) at a split flow of 10 mL/min to avoid peak saturation.

DETERMINATION AND ANALYSIS OF VOLATILES

Separation of the volatiles was performed using a ZB-wax capillary column (30 m × 0.25 mm × 0.5 µm) using helium as carrier gas in the constant flow mode (1.2 mL/min). The initial oven temperature was 40 °C which was held for 5 min and then increased to 240 °C at 4 °C/min. Mass spectra were recorded in the electron impact ionization mode at 70 eV by means of an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific). Data was acquired applying a full scan mode (TIC, *m/z* 33-300) as well as a SIM mode using specific *m/z* for selected compounds (see **Table 41** in the appendix).

Identification was performed using Xcalibur 2.2 (Thermo Fisher Scientific) coupled with the MS libraries NIST 08 and Wiley 8. In addition, the compounds' retention indices were calculated as described on the NIST database using a series of *n*-alkanes (C_5-C_{21}) and compared with values reported on the NIST

database. An overview of experimental retention indices and those found in the literature is given in **Table 41** in the appendix.

For VOC quantification, the areas under the TIC or SIM peaks were integrated and related to those of the internal standards (see **Figure 45**). Hexanal, furfural and 2-methylpyrazine were quantified using their deuterated standards. Relative quantification of other compounds was performed using 2-methylpyrazine-d_6 for nitrogenous, furfural-d_4 for furanic and hexanal-d_{12} for the remaining compounds.

6.3 ACID VALUE, CONJUGATED DIENES AND PEROXIDE VALUE

6.3.1 ACID VALUE

Lipid oxidation is an important chemical reaction taking place while processing of pulses. Since only the free fatty acids are deteriorated, it was important to quantify these in the raw materials. This was performed by ***COLORIMETRIC TITRATION*** using the standard method NF EN ISO 660:2020 with slight modifications. 50 mg of oil extracted from the flours were dissolved in 20 mL of a binary mixture of ethanol and petroleum ether (1/1 v/v) which had previously been neutralized. Neutralization was carried out by adding 1 mM potassium hydroxide (KOH) dropwise to the solvent mixture until the color indicator aniline blue (2% w/v in ethanol) turned from blue to pink. The acids present in the oil were immediately titrated with 5 mM ethanolic KOH using an 809 titrando equipped with a LL Solvotrode electrode (Metrohm, Herisau, Switzerland). The concentration of the 5 mM ethanolic KOH was verified using benzoic acid. For this purpose, 0.12 g of benzoic acid was dissolved in 50 mL ethanol and its concentration was checked titrimetrically with a commercially purchased 100 mM KOH solution (→ 20 mM benzoic acid solution). The 100 mM KOH solution was then diluted with ethanol (1/10 v/v) and its concentration was checked with the verified 20 mM benzoic acid solution (→ 10 mM KOH). This 20 mM benzoic acid solution was then diluted with ethanol (1/20 v/v) and its concentration was checked with the verified 10 mM KOH solution (→ 1 mM benzoic acid solution). This 10 mM KOH solution was then diluted with ethanol (1/2 v/v) and its concentration was checked with the verified 1 mM benzoic acid solution (→ 5 mM KOH). The acid value was then calculated from the mass of KOH required to neutralize 1 g fat (**Eq. 22**).

$$Acid\ value\ \left[\frac{mmol}{kg}\right] = \frac{M_{KOH}\ \cdot c_{KOH} \cdot V_{KOH}}{m_{Oil}} \quad (\mathbf{22})$$

where M_{KOH} is the molar mass of KOH [g/mol], c_{KOH} is the concentration of the KOH solution [mol/L], V_{KOH} is the volume of KOH solution required to reach the equilibrium point [mL] and m_{Oil} is the weight of the oil [g].

6.3.2 CONJUGATED DIENES

The oxidative breakdown of fatty acids takes place *via* intermediate products, the so-called hydroperoxides, which are commonly referred to as primary lipid oxidation markers (see **§ 11 of Part II**). The investigation of these intermediate structures can hence serve to gain deeper insight into the oxidation status of the batters and cakes and reveal differences that are not visible from the sole analysis of secondary oxidation products, i.e. volatile compounds (Maire *et al.*, 2013). The analysis of the absorbance of conjugated dienes as well as the peroxide value are appropriate tools to determine primary oxidation markers (Maire *et al.*, 2013).

Conjugated dienes contained in oil (sunflower oil or extracted from eggs, flours, batters and cakes) were determined by measuring their UV absorption using a ***SPECTROPHOTOMETER***. The French standard NF EN ISO 3656 (2011) was modified by weighing 10 – 20 mg of oil into 10 mL volumetric flasks, which was filled up with isooctane and vortexed at maximum speed for 10 s. Dependent on the oxidation status of the sample, a second dilution was necessary to ensure an absorbance in the linear range of the spectrophotometer. Thereby, 500 µL to 5 mL of the primary dilution was pipetted into a second 10 mL volumetric flask and the volume adjusted with isooctane. After another mixing step for 10 s, the absorbance of the dilutions was recorded against isooctane in 1 cm path length quartz cuvettes at 234 nm using a Specord 210 Plus with the software Winaspect Plus Version 4.2.0.0 (Analytik Jena, Jena, Germany). The content of conjugated dienes was expressed as specific absorbance, defined as the absorbance of 1 g oil in 100 mL isooctane at 234 nm.

6.3.3 PEROXIDE VALUE

The peroxide value of oil (sunflower oil or extracted from eggs, batters and cakes) was measured by ***IODOMETRIC TITRATION***. The principle of this method is based on the reaction of hydroperoxides (LOOH) with iodide (I^-) under formation of iodine (I_2) (**Eq. 23**), which is subsequently titrated with sodium thiosulfate ($S_2O_3^{2-}$) to the equivalence point (**Eq. 24**).

$$LOOH + 2I^- + H_2O \rightarrow LOH + I_2 + 2OH^- \quad (\mathbf{23})$$

$$I_2 + 2{S_2O_3}^{2-} \rightarrow {S_4O_6}^{2-} + 2I^- \quad (\mathbf{24})$$

An 814 USB Sample Processor titrator equipped with a Pt Titrode electrode (Metrohm, Herisau, Switzerland) was used to perform the titrimetric method described by Roman *et al.* (2013) with some modifications. The maximal possible amount of oil after analysis of conjugated dienes (0.2 g) was weighed into 75 mL amber flasks and dissolved under stirring for 1 min in 16 mL of glacial acetic acid/isooctane (3/2 v/v) containing 0.002% (w/v) iodine. Thereafter, 1 mL of freshly prepared saturated potassium iodide solution was automatically added and the mixture blended for 90 s. Phase separation was induced by addition of 9 mL distilled water, which had been boiled and cooled to room temperature as suggested by Metrohm. The iodine liberated was then titrated potentiometrically with 5 mM sodium thiosulfate solution, which was kept in an amber glass bottle and used for a maximal duration of 4 h. A blank titration was performed using distilled water instead of oil. The peroxide value was calculated according to **Eq. 25**.

$$Peroxide\ value\ \left[\frac{mmol}{kg}\right] = \frac{[(V_{Oil} - V_{Blank}) \cdot c_{Thio} \cdot 1000]}{m_{Oil}} \cdot 2 \quad (\mathbf{25})$$

where V_{Oil} is the volume of sodium thiosulfate needed to reach the equilibrium point of the oil sample [mL], V_{Blank} is the volume of sodium thiosulfate needed for the blank [mL], c_{Thio} is the molarity of the sodium thiosulfate solution [mM] and m_{Oil} is the weight of the oil [g].

7 EVALUATION OF DIGESTION

Digestion experiments were performed on the cakes developed during the pea fraction studies.

7.1 *IN VITRO* DIGESTION OF PROTEINS AND STARCH

The standardized INFOGEST static in vitro method reported by Brodkorb *et al.* (2019) was performed to digest the cakes in three phases that simulate the oral, gastric and small intestinal digestion processes. Progress of protein and starch digestion was followed during the intestinal phase using nine individual tubes that were sampled after different times points (0, 5, 10, 20, 30, 60, 90, 120 and 180 min) and are considered consecutive and independent repetitions of the same digestive system. A blank was added, which was treated like the sample tubes but did not contain any cake.

7.1.1 PREPARATION OF SIMULATED DIGESTION FLUIDS

Simulated salivary, gastric and intestinal fluids (SSF, SGF and SIF, respectively) are aqueous solutions of different electrolytes, which were prepared as described by Minekus *et al.* (2014) and listed in **Table 24**. SSF was adjusted to pH 7, SGF to pH 3 and SIF to pH 7 with HCl (6 M).

Table 24. Preparation of simulated digestion stock fluids (according to Minekus *et al.* (2014)).

Constituent	Concentration [mmol/L]		
	Simulated salivary fluid (SSF)	Simulated gastric fluid (SGF)	Simulated intestinal fluid (SIF)
KCl	18.9	8.6	8.5
KH_2PO_4	4.6	1.1	1.0
$NaHCO_3$	17.0	31.3	106
NaCl	-	59.0	48.0
$MgCl_2(H_2O)_6$	0.19	0.2	0.41
$(NH_4)_2CO_3$	0.08	0.6	-

7.1.2 ORAL PHASE

The oral phase was initiated by mixing 1.25 g ground cake with 1 mL SSF, 6.25 µL $CaCl_2$ solution (0.3 M) and 119 µL purified water for 2 min to reach the required electrolyte concentrations indicated by Minekus *et al.* (2014). As suggested by Pälchen *et al.* (2021), salivary amylase was excluded from the oral phase due to negligible amylolysis, attributable to short residence times and instant inactivation once the gastric phase commenced. Therefore, it was replaced by 125 µL purified water.

7.1.3 GASTRIC PHASE

The gastric phase was simulated by adding 2 mL SGF (pH 3) and 1.3 µL $CaCl_2$ solution (0.3 M) to the oral bolus. The pH of the mixtures was adjusted to 3 with HCl (2 M) (50 – 150 µL, depending on the cake sample) before 125 µL aqueous porcine pepsin solution were added (2000 U/mL digesta). Afterwards, the volume was completed to 5 mL with purified water to reach the desired electrolyte concentrations and enzyme activity. Then, the samples were incubated for 120 min at 37 °C with constant end-over-end rotation.

7.1.4 SMALL INTESTINAL PHASE

The small intestinal phase was started by mixing the gastric chyme with 2.125 mL SIF (pH 7), 10 µL $CaCl_2$ solution (0.3 M) and 625 µL bile (10 mM in digesta). After adjustment of the pH to 7 using NaOH (2 M) (25 – 80 µL, depending on the cake sample), 1.25 mL of a mixture composed of pancreatic α-amylase (200 U/mL digesta), pure trypsin (100 U/mL digesta) and pure chymotrypsin (25 U/mL digesta) were added and the volume filled up to 10 mL with purified water to reach the desired electrolyte concentrations and enzyme activities. The tubes were incubated at 37 °C with constant end-over-end rotation, sampled after the selected time points and enzymatic activity stopped by placing in a water bath (100 °C) for 5 min. The tubes were centrifuged (2000 *g*, 5 min), the supernatant separated from the pellet, both snap-frozen in liquid nitrogen and stored at -40°C until use.

Unlike the other samples, the samples at 0 min were heated for 5 min at 100 °C before the enzyme solution was added. Addition of the enzyme solution was performed to ensure a comparable composition to the other samples (which might affect absorbance etc.). Thereafter, the samples were heated for another 5 min at 100 °C.

7.2 EVALUATION OF STARCH DIGESTION

7.2.1 3,5-DINITROSALICYLIC ACID (DNS) METHOD

The DNS method is a fast and relatively inexpensive ***SPECTROPHOTOMETRIC*** method, which is based on the property of reducing sugars released during digestion to reduce 3,5-dinitrosalicylic acid (yellow) to 3-amino-5-nitrosalicylic acid (orange-red), which absorbs at 540 nm.

Accordingly, the supernatant of the digested cakes was diluted with purified water (1/40 v/v) and 2 mL dilution mixed with 1 mL dinitrosalicylic color reagent. The latter was prepared by dissolving 2.75 g of 3,5-dinitrosalicylic acid in 125 mL purified water at slightly elevated temperatures (beaker covered by aluminum foil), followed by the addition of 75 g potassium tartrate tetrahydrate and 50 mL NaOH (2 M). After adjustment of the volume to 250 mL with purified water, the solution was stored in an aluminum wrapped bottle to protect from light. After incubation at 100 °C for 15 min, 9 mL purified water were added to the cooled samples, the reactants mixed by repeated inversion and absorbance recorded at 540 nm using a UV-1800 UV/Vis spectrophotometer (Shimadzu, Kyōto, Japan). Reducing sugars were quantified using an external maltose calibration curve (0.05 – 2 mg/mL) and conversion into starch equivalents by multiplication by 0.947. The calibration curves obtained for each newly prepared DNS solution had R^2 of 0.9993 – 0.9995 with similar slope (0.3800 ± 0.0212) and y-intercept (-0.0520 ± 0.0037).

7.2.2 HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY

An alternative to DNS is a method that uses High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD). This technique allows the exact but more time-consuming determination of specific starch degradation products. In order to get an idea about which starch degradation products were mainly present, the different samples were pre-analyzed using an internal method at Cargill. A typical chromatogram is shown in **Figure 48**. It can be seen that the main degradation products of starch were glucose (1), maltose (3) and maltotriose (4), whereas the peaks of larger polymers such as maltotetraose (5) and maltopentaose (6) were almost not detectable.

The latter two peaks also did not increase with ongoing digestion. Therefore, it was decided that only the quantities of glucose, maltose and maltotriose were determined by applying the method described in **§ 4.3.5 of Part IV**.

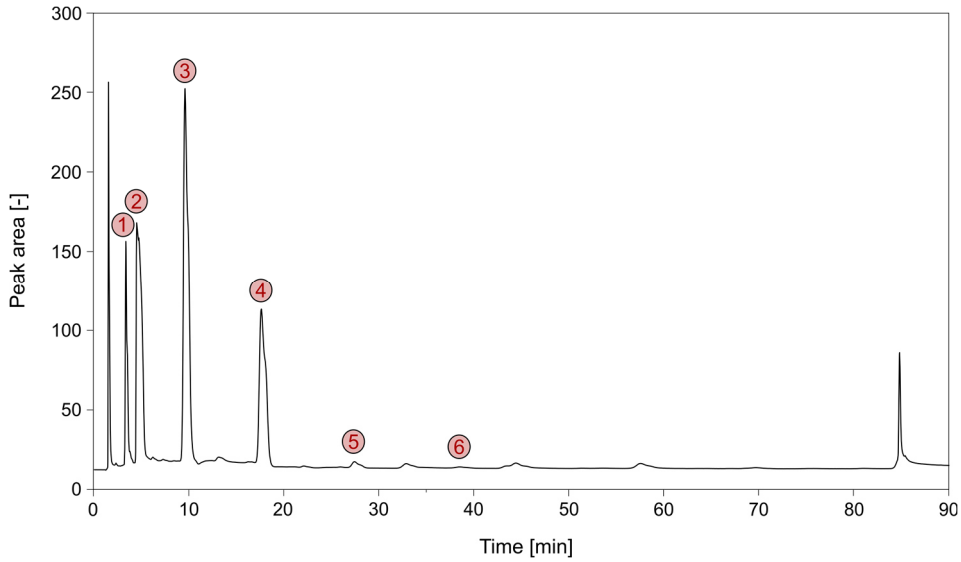

Figure 48. HPAEC-PAD separation of sugars contained in the digestive supernatant of wheat cake after 5 min. 1 = glucose, 2 = sucrose, 3 = maltose, 4 = maltotriose, 5 = maltotetraose and 6 = maltopentaose.

7.3 EVALUATION OF PROTEIN DIGESTION

Protein digestion was evaluated using the ***SPECTROPHOTOMETRIC OPA METHOD*** reported by Nielsen *et al.* (2001) and modified by Zahir *et al.* (2018). This method is based on the reaction of amino groups with *o*-phthaldialdehyde (OPA) in the presence of dithiothreitol (DTT), which forms a colored compound that absorbs at 340 nm.

Duplicates were prepared by adding trichloroacetic acid (TCA) to the digestive supernatant (final concentration 3.1% w/v) to separate larger peptides and proteins by precipitation from soluble amino acids and oligopeptides (Gwala *et al.*, 2020). This TCA-fraction was considered the readily bioaccessible protein fraction, which can be directly absorbed at the brush-border *via* passive diffusion, tight junctions, endocytosis and/or transcellular carrier-mediated transport (Ozorio *et al.*, 2020). After centrifugation (10000 *g*, 30 min), the supernatant was filtered through 0.25 µm Minisart NML filters, diluted with purified water (1/40 v/v) and 0.4 mL mixed with 3 mL light-protected OPA reagent. The latter was prepared by solubilization of 7.62 g disodium tetraborate decahydrate in 150 mL purified water, to which 160 mg OPA in 4 mL ethanol as well as 200 mg sodium dodecyl sulfate and 176 mg DTT were added. The solution was filled up to 200 mL with purified water and protected from light. It was used within 4 hours. After addition of OPA reagent, the samples were incubated for exactly 2 min in the dark before the absorbance was recorded at 340 nm using a Ultrospec 2100 pro UV/Vis spectrophotometer (GE Healthcare, Buckinghamshire, UK). Free α-amino groups were quantified using an external serine calibration curve (12.5 – 100 mg/L). Serine was selected as reference amino acid due to its response that reflects the average response of amino acids (Nielsen *et al.*, 2001). The calibration curves obtained

for each newly prepared OPA reagent had R^2 of 0.9995 – 1 with similar slope (0.0560 ± 0.0037) and y-intercept (0.0003 ± 0.0002).

For comparison with the amino groups contained in the undigested cakes, the proteins contained in the cakes needed to be fully hydrolyzed. Accordingly, 5 mg cakes were mixed with 1 mL HCl (6 M) and subjected to acid hydrolysis during 48 h at 110 °C. This time was sufficient to ensure full hydrolysis of proteins as it revealed identical results to that performed for 72 h. Thereafter, the solvent was evaporated at 60 °C under vacuum until dryness. After re-dilution in 5 mL purified water, the samples were filtered through 0.45 µm Minisart filters and analyzed with OPA reagent following the aforementioned procedure.

Since the TCA fraction contained not only α-amino groups but also a soluble protein fraction, interpretation of the degree of proteolysis might be improved by conversion of this soluble protein fraction into α-amino groups due to the same unit as the undigested cakes. Consistently, the TCA fraction was subjected to acid hydrolysis as described for the undigested cakes and the α-amino groups were quantified using OPA reagent.

7.4 REPEATABILITY OF DIGESTION EXPERIMENT

Consecutive data from a particular digestive system were integrated using kinetic modelling approaches (**§ 7.4.1 of Part IV**). In addition, repeatability was further ensured by repetition of the digestion experiment. The results on both protein and starch digestion of pea starch cake digested at two different days are shown in **Figure 49**. ANOVA analysis revealed non-significant differences at each point of digestion for both analysis methods.

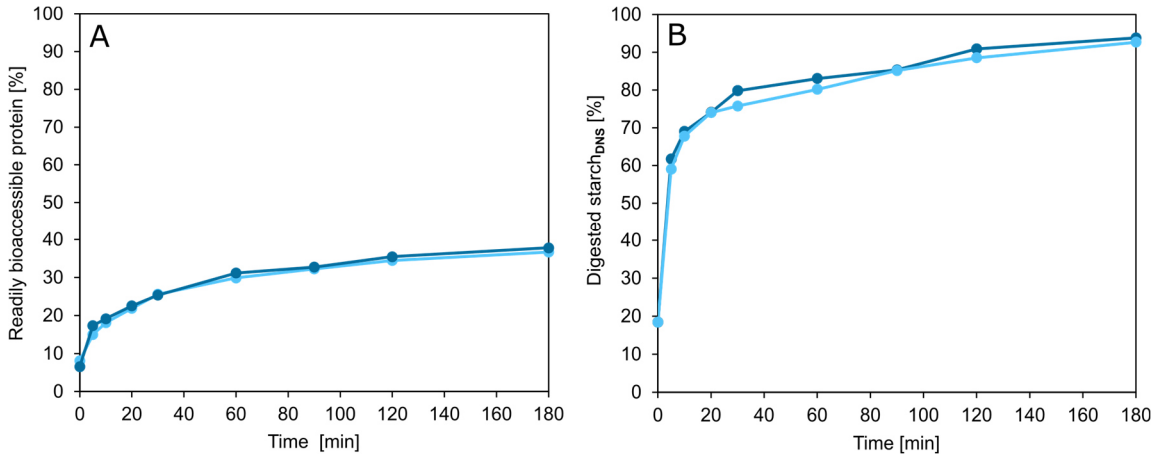

Figure 49. Repeatability of *in vitro* protein (A) and starch digestion (B) of sponge cakes based on pea starch performed at two independent days. Kinetics during small intestinal digestion are presented.

7.4.1 KINETIC MODELLING OF *IN VITRO* DIGESTION

Experimental data of protein digestion were modeled using a first order equation as described by Gwala *et al.* (2020) (**Eq. 26**).

$$Protein_t = Protein_f + (Protein_i - Protein_f) \times e^{-kt} \quad (26)$$

where $Protein_t$ is the readily bioaccessible protein at any time t [%]/[mg/g cake], $Protein_f$ is the estimated plateau of readily bioaccessible protein at extended digestion times [%]/[mg/g cake], $Protein_i$ is the readily bioaccessible protein at the beginning of the small intestinal phase [%]/[mg/g cake] and k is the estimated reaction rate constant of digestion [1/min].

Experimental data of starch digestion were modeled using a biphasic model composed of two first order equations as discussed by Edwards *et al.* (2014) (**Eq. 27**).

$$Starch_t = Starch_2 - Starch_1 \times e^{-k_1 t} + (Starch_1 - Starch_2) \times e^{-k_2 t} \quad (27)$$

where $Starch_t$ is the digested starch at time t [%]/[mg/g cake], $Starch_2$ is the estimated plateau of digested starch at the end of the second digestion phase at extended digestion times [%]/[mg/g cake], $Starch_1$ is the digested starch at the end of the first digestion phase [%]/[mg/g], k_1 is the estimated reaction rate constant of the first digestion phase [1/min] and k_2 is the estimated reaction rate constant of the second digestion phase [1/min].

The initial reaction rate of proteolysis or amylolysis was calculated from the slope of the tangent to the respective models at time $t = 0\ min$.

7.5 MICROSCOPIC ANALYSIS OF DIGESTION PELLETS

The progress of protein and starch digestion was qualitatively assessed by microscopic analysis of the remaining starch and protein in the pellets obtained after selected digestion times (0, 30 and 180 min).

The ability of proteins to auto-fluoresce due to the presence of aromatic amino acids(Arntfield *et al.*, 1987) was used to visualize proteins contained in 10 µL diluted pellet (50 mg in 100 µL purified water) under the epi-fluorescent microscope (excitation at 460-490 nm). For the analysis of starch, 100 µL diluted pellet were mixed with 1 µL Lugol's iodine reagent (5% w/v iodine and 10% w/v potassium iodide) and 10 µL stained sample observed under the normal light microscope. Representative images were captured at 10× magnification by means of an BX51 microscope couplet to an epifluorescence illumination X-Cite 120Q and equipped with a XC50 digital camera (Olympus, Tokyo, Japan).

PART V

RESULTS & DISCUSSION

CHAPTER 1 *REACTIVITY POTENTIAL OF PULSE FLOURS (ARTICLE 1)*

CHAPTER 2 *ODOR ACTIVITY OF VOLATILES IN PULSE CAKES (PROCEEDINGS)*

CHAPTER 3 *EFFECT OF BATTER BEATING ON REACTIVITY AND STRUCTURE (ARTICLE 2)*

CHAPTER 4 *IMPACT OF PEA INGREDIENTS ON STRUCTURE AND REACTIVITY OF CAKES (ARTICLE 3)*

CHAPTER 5 *DIGESTION OF PEA-BASED CAKES (ARTICLE 4)*

CHAPTER 1

REACTIVITY POTENTIAL OF PULSE FLOURS

The first step in better understanding the ability of pulse ingredients to participate in reactions that lead to the release of potentially odorous volatile compounds during sponge cake development was to fully replace traditionally used wheat flour with whole pulse flours. Diverse pulse flours were selected, which derived from peas (green and yellow), lentils, chickpeas and lupins in order to gain a global idea of the different reactivity potentials of pulses and their effects on the overall cake quality. In addition, a yellow pea flour, which was produced and stored under nitrogen was used to elucidate the influence of the flour pre-processing on their susceptibility to reactions. In order to track the individual reactions that take place during the different steps of cake development, the change in the profile of volatile compounds from the raw materials to the batter and to the final cake was measured. Due to the frequently discussed high susceptibility of pulse lipids to oxidative degradation, the oxidative status of the individual products was also measured at each production step by analyzing the intermediates of lipid oxidation, i.e. conjugated dienes and hydroperoxides. In order to discriminate the diverse cakes based on their profile of volatile compounds, a PCA analysis was carried out to associate certain molecules with specific products and thus to draw conclusions about their potential effect on the cake odor. Apart from this sensory aspect, other essential cake quality criteria were also evaluated, which included color and volume, to obtain a holistic understanding of the application potential of pulse flours in gluten-free cakes.

FROM FLOURS TO CAKES: REACTIVITY POTENTIAL OF PULSE INGREDIENTS TO GENERATE VOLATILE COMPOUNDS IMPACTING THE QUALITY OF PROCESSED FOODS

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HIGHLIGHTS

- Pulses generate a larger amount and variety of process-induced volatiles than wheat
- Lipoxygenase plays a major role in lipid oxidation during batter beating
- Pea flour made under N_2 is highly prone to lipid oxidation during product making
- Pea cakes are the richest in oxidation markers typically linked to green odors
- Lentil and chickpea flours induce most odor-active Maillard compounds

ABSTRACT

This study investigated the impact of wheat substitution by pulse flours (lentil, chickpea, lupin, green and yellow pea) on chemical reactivity during different steps of sponge cake development. Pulse ingredients exhibited greater abilities to generate volatiles with probable odor activity. Batter beating initiated lipid oxidation which depended on lipoxygenase activity and fatty acid profile of the flours. Among the pulses, pea batters were richest in oxidation markers, whereas lupin was least reactive probably due to thermal pre-treatment. Baking triggered caramelization and Maillard reactions, particularly in the pulse products, which were especially enriched in pyrazines and furanic compounds. Principle component analysis revealed that pea cakes were associated with lipid oxidation markers typically possessing green-beany flavors, while Maillard markers known to impart nutty, roasted notes were assigned to lentil and chickpea cakes. These findings highlight the importance of ingredient type and its pre-processing in the development of quality-related markers of new gluten-free products.

KEYWORDS

Lipid oxidation, Maillard reaction, gluten-free, hexanal, flavor, baking

1 INTRODUCTION

In recent years, the high prevalence of protein malnutrition in the rapidly growing global population is promoting the development of healthy and nutritious eating practices based on plant foods. Owing to their sustainability, low cost and nutritional value, pulses represent a popular source of plant-based protein (Hall *et al.*, 2017; Sozer *et al.*, 2017). Apart from their protein contents, these commodities not only provide dietary fiber and minerals but also possess a low glycemic index (Hall *et al.*, 2017). thus making them promising ingredients for diverse formulations, including baked goods.

Wheat-based bakery products have been part of the human diet for centuries. However, the associated ingestion of gluten can cause diverse clinical disorders, such as celiac disease, wheat allergy and non-celiac gluten sensitivity (Laleg *et al.*, 2016). Consequently, the market for gluten-free foods is expanding and the creation of value-added pulse products is being increasingly spotlighted.

Despite the appealing composition and health-promoting benefits of pulses, exploitation of their advantages is impeded by their unpleasant green-beany off-flavor (Murat *et al.*, 2013). The lipoxygenase-catalyzed oxidation of unsaturated fatty acids is considered to be a major contributor to the release of odor-active compounds during harvest and storage and has been reviewed for pea, chickpea, lentil and lupin (Azarnia *et al.*, 2011a; Jakobsen *et al.*, 1998; Kaczmarska *et al.*, 2018; Murat *et al.*, 2013; Paucean *et al.*, 2018; Xu *et al.*, 2019). By contrast, the literature lacks information on the ability of pulses to generate aroma markers and contribute to overall flavor during the development of pulse-based foods subjected to thermomechanical treatments.

Sponge cakes are baked systems of particular interest when studying the link between quality and reactivity because of their complex composition (Cepeda-Vázquez *et al.*, 2019; Maire *et al.*, 2013). They are prepared in a two-step process of batter beating and baking, which can favor the oxidative and thermal degradation of lipids, proteins and sugars (Maire *et al.*, 2013; Xu *et al.*, 2019). Replacing wheat with pulse ingredients might therefore affect the presence of precursors likely to undergo changes *via* lipid oxidation, Maillard reaction and caramelization. It was therefore considered to be interesting, to evaluate the reactivity potential of pulse flours to generate volatiles that could impact food quality during the different steps of cake development compared to a wheat reference. Moreover, the occurrence of newly-formed compounds was studied alongside other cake quality criteria.

2 MATERIALS AND METHODS

2.1 INGREDIENTS

Sucrose was purchased from Tereos (Lille, France), sunflower oil from Lesieur (Asnières-sur-Seine, France), whole pasteurized eggs from Ovoteam (Locmine, France), wheat flour from Grands Moulins de Paris (Ivry-sur-Seine, France), green lentil flour and chickpea flour from Celnat (Saint-Germain Laprade, France) and lupin flour and green pea flour from Moulin Meckert-Diemer (Krautwiller, France). Two yellow pea flours (Improve, Dury, France) were used, which had either been produced during exposure to air or under nitrogen.

2.2 CHEMICALS

Furfural-d_4 (≥96%) and 2-methylpyrazine-d_6 (98.4%) were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada), hexanal-d_{12} (≥96%), Celite® 545, iodine, potassium hydroxide (0.1 M), Alkali Blue 6B and glyceryl triheptadecanoate (≥96%) from Sigma Aldrich (Saint-Quentin- Fallavier, France), petroleum ether, isopropanol, heptane, sodium hydroxide, methanol, glacial acetic acid, sodium thiosulfate pentahydrate, isooctane, potassium iodide and iodine (0.02M) from Carlo Erba (Val de Reuil, France) and hydrochloric acid (37%) from Prolabo (Fontenay-sous-Bois, France).

2.3 BATTER AND CAKE FORMULATIONS

Eggs (45% w/w) and sucrose (25% w/w) were whipped together for 10 min (maximum speed) using a mixer equipped with a vertical whisk (KitchenAid Artisan 5KSM150, St. Joseph, Michigan, USA). Non-sifted wheat or pulse flour (25% w/w) was gradually incorporated within 90 s (minimum speed) and the mixture was beaten for a further 30 s. Thereafter, sunflower oil (5% w/w) was added within 15 s (minimum speed) and the whole batter was whipped for a further 60 s. Each formulation was prepared in triplicate. A portion of each batter was immediately stored at -20 °C until reaction marker analysis in order to slow down oxidation reactions, whereas the remainder was used for immediate pH and density measurements.

Sponge cakes were generated from a new batch of batter (total 630 g), where 21 aluminum molds (9.8×6.2×3.3 cm^3) were each filled with 25 g of batter and baked at 170 °C for 25 min in an insulated convection oven (Bongard, Wolfisheim, France) guaranteeing thermal homogeneity (Fehaili *et al.*, 2010). While 15 cakes were frozen immediately at -20 °C in hermetically sealed glass jars until reaction marker analysis, the remaining six were cooled in order to characterize density, moisture content and pH. Each batch of 21 cakes was only made once because a preliminary test had confirmed insignificant variability between three batches using the same flour ($p<0.05$) in terms of selected analyses described in the sections below.

2.4 CAKE GRINDING

Prior analysis of moisture content and pH, the six cakes cooled to ambient temperature were ground after density measurement for 1 min at turbo speed using a Moulinex AT710131 (SEB, Ecully, France). By contrast, a different protocol was applied to the cakes used to analyze reaction markers in order to ensure similar particle size distribution and limit the formation or loss of volatiles induced by friction heat. Accordingly, 15 frozen cakes from one baking were milled in batches of five for 20 s at 6000 rpm using a Grindomix GM200 knife mill equipped with a stainless-steel bowl and titanium blades (Retsch GmbH, Haan, Germany) and stored at -20 °C.

2.5 CHEMICAL COMPOSITION

2.5.1 MOISTURE, ASH, PROTEIN, FREE AMINO ACID, STARCH AND FIBER CONTENTS

The moisture contents of the ingredients and cakes were determined in triplicate by differential weighing before and after drying for 24 hours at 105 °C. The ash content of eggs and flours were analyzed by dry combustion for twelve hours at 550 °C, while their protein contents were determined using the Kjeldahl method (NF EN ISO 5983-2) with a nitrogen-to-protein conversion factor of 6.25 for pulses and 5.7 for wheat. The flours were further characterized in terms of total starch (EC 152/2009), dietary fiber (AOAC 985.29) and free amino acids (ISO 13903:2005 and ISO 13904:2016). The ash, protein, total starch, dietary fiber and free amino acid contents were determined in duplicate by Improve (Dury, France).

2.5.2 PH VALUE

The pH of 2 g batter or cake was analyzed in triplicate in 20 mL distilled water using a SensION^{TM}+ PH3 meter (Hach Lange, Lognes, France).

2.6 PHYSICAL PROPERTIES

2.6.1 DENSITY

Batter density was determined in triplicate by slowly drawing the batter up into 10 mL open-mouth syringes to avoid gas cell destruction. After weighing, density was calculated from the ratio of batter mass to syringe volume, previously defined using water.

Similarly, the density of six pre-weighed cakes was obtained by calculating the mass to volume ratio. The latter was determined using a laser-based scanner (VolScan Profiler, Stable Micro Systems, Surrey, UK).

2.6.2 COLOR

The CIEL*a*b* color parameters of three thawed cakes were recorded at illuminant D65 and a 10° observer angle using a CD-6834 spectro-guide sphere gloss colorimeter (BYK-Gardner, Geretsried, Germany). Color was measured at three points on the cake crust, then the cake was halved in a horizontal plane and three further points were analyzed on the crumb of the lower half.

2.7 ANALYSIS OF LIPID OXIDATION

2.7.1 LIPOXYGENASE (LOX) ACTIVITY

LOX activity was determined externally by Eurogerm (Saint-Apollinaire, France) according to the method described by Delcros *et al.* (1998). Briefly, crude enzyme extracts were prepared in triplicate by blending 2 g flour with 20 mL phosphate buffer (pH 7.5). After centrifugation, 20-200 µL enzymatic extract was mixed with linoleic acid in phosphate buffer (pH 6.5) and saturated with air. LOX activity was expressed as nmol oxygen consumed per second.

2.7.2 OIL EXTRACTION

To analyze lipid oxidation precursors and markers, oil was extracted from the products using accelerated solvent extraction adapted from Maire *et al.* (2013). Samples (in triplicate) were mixed with Celite® 545 at ratios (w/w) of 1/1, 3/2, 10/9 and 14/5 for eggs, flours, batters and cakes, respectively, and extracted at 40 °C with heptane/isopropanol (5/1 v/v) for 25 min using a Dionex ASE 350 (Thermo Fisher Scientific, Waltham, MA, USA). The solvents were evaporated at 40 °C under a vacuum; yield was then determined by differential weighing before extraction and after evaporation. The oil was stored under nitrogen at -20 °C until analysis.

2.7.3 FATTY ACID (FA) PROFILE

The FA profile of extracted oils was determined by gas chromatography coupled with mass spectrometry (GC-MS) after trans-methylation as described by Roman *et al.* (2013) with slight modifications. Approximately 30 mg extracted oil (in triplicate) was dissolved in 1 mL petroleum ether containing 3 mg internal standard heptadecanoic acid. After gentle mixing for 10 s, 200 µL NaOH in methanol (2 M) was added and the mixture was shaken for 10 s. The tubes were then incubated in a water bath at 52 °C under constant agitation for 40 s. The reaction was stopped by the addition of 400 µL HCl in methanol (1 M). Samples were allowed to stand for 2 min for phase separation before the upper phase was transferred into GC vials.

Fatty acid methyl esters (FAME) were eluted on a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific) equipped with an SP-2560 GC column (100 m×250 µm×0.2 µm, Supelco, Bellefonte, PA, USA) using helium as the carrier gas (1.20 mL/min). A 1 µL sample was injected at 250 °C with a split flow of 448 mL/min. The FAMEs were separated by increasing the temperature by 5 °C/min increments from 100 °C (held for 4 min) to 200 °C (held for 8 min) and then to 250 °C (held for 12 min). Mass spectra (*m/z* 40-400) were detected at 250 °C using an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific). Peaks were identified by comparing their mass spectra and retention times with those of a FAME standard mixture containing 37 compounds (Supelco) subjected to the same procedure. Relative FAME quantities were calculated by relating the integrated areas under the peaks to those of the internal standard.

2.7.4 CONJUGATED DIENES (CD)

The UV-spectrophotometric measurement of CD in sunflower and extracted oils was performed in triplicate according to Roman *et al.* (2013) with some modifications. 10-20 mg oil was weighed into 10 mL volumetric flasks, filled quantitatively with isooctane and vortexed at maximum speed for 10 s. Depending on the oxidation status of the sample, a second dilution (0.5-5 mL in 10 mL isooctane) was necessary to ensure absorbance within the linear range of the spectrophotometer. The absorbance of the dilutions was recorded against isooctane in 1 cm quartz cuvettes at 234 nm using a Specord 210

Plus (Analytik Jena, Jena, Germany). The CD content was expressed as a specific absorbance, defined as the absorbance of 1 g oil in 100 mL isooctane at 234 nm.

2.7.5 PEROXIDE VALUE (PV)

The PV of sunflower and extracted oils was analyzed in triplicate by adapting the method described by Roman *et al.* (2013). 0.2 g oil was weighed into 75 mL amber beakers and dissolved in 16 mL glacial acetic acid/isooctane (3/2 v/v) containing 0.002% w/v iodine. 1 mL saturated potassium iodide solution was added and the mixture was blended for 90 s. 9 mL distilled water was added and the released iodine was titrated potentiometrically with sodium thiosulfate solution (5 mM). Water was used for the blank titration. The PV was calculated according to the equation Eq. (**1**).

$$PV\ \left[\frac{mmol}{kg}\right] = \frac{(V_{Oil} - V_{Blank}) \cdot c_{Thio} \cdot 2000}{m_{Oil}} \qquad (\mathbf{1})$$

where V_{Oil} and V_{Blank} are the volumes of sodium thiosulfate required to reach the equilibrium point of the oil or blank sample [mL], c_{Thio} is the molarity of the sodium thiosulfate solution [mM] and m_{Oil} is the weight of the oil [g].

2.7.6 ACID VALUE

The acid value of extracted oils from the flours was analyzed in triplicate according to the standard method NF EN ISO 660:2020 with slight modifications. Briefly, 50 mg oil was dissolved in 20 mL neutralized ethanol/petroleum ether (1/1 v/v) and the acids present titrated with ethanolic potassium hydroxide (KOH) solution (5 mM) using Alkali Blue 6B as color indicator. The acid value was expressed as the mass of KOH required to neutralize 1 g fat using the equation Eq. (**2**).

$$Acid\ value = \frac{M_{KOH} \cdot c_{KOH} \cdot V_{KOH}}{m_{Oil}} \qquad (\mathbf{2})$$

where M_{KOH} is the molar mass of KOH [g/mol], c_{KOH} is the concentration of the KOH solution [mol/L], V_{KOH} is the volume of KOH solution required to reach the equilibrium point [mL] and m_{Oil} is the weight of the oil [g].

2.8 ANALYSIS OF VOLATILE ORGANIC COMPOUNDS (VOCS)

2.8.1 INTERNAL DEUTERATED STANDARD SOLUTION

For each food matrix (flours/batters/ cakes), an aqueous solution containing furfural-d_4, 2-methylpyrazine-d_6 and hexanal-d_{12} at concentrations of 14/91/15 mg/L, 0.1/89/3 mg/L and 139/778/411 mg/L, respectively, were prepared and stored at -20 °C until use.

2.8.2 HEADSPACE-SOLID PHASE MICRO EXTRACTION (HS-SPME) OF VOLATILE COMPOUNDS

For the GC-MS analysis of VOCs, preliminary experiments were performed to optimize the sample, equilibrium, extraction and injection conditions. Accordingly, 2 g thawed flour, batter or ground cake were weighed in triplicate into 20 mL transparent vials, spiked with 100 µL of their respective standard solution and enclosed with aluminum polytetrafluoroethylene-coated silicon septa. To ensure that the VOCs of the sample and added standards reached equilibrium in the headspace, the vials were stored overnight at 4 °C. The next day, they were incubated at 50 °C for 18 min in a TriPlus autosampler (Thermo Fisher Scientific). A DVB/CAR/PDMS fiber (50/30 µm, 2 cm, Supelco) was then inserted for 42 min at 50 °C. The compounds were thermally desorbed for 2 min (250 °C) and transferred into the injection port of a TRACE GC Ultra gas chromatograph at a split flow of 10 mL/min.

2.8.3 DETERMINATION AND ANALYSIS OF VOCS

Separation of the volatiles was performed on a ZB-wax capillary column (30 m×0.25 mm× 0.5 µm, Phenomenex, Aschaffenburg, Germany) using helium as the carrier gas (1.2 mL/min). The initial oven temperature of 40 °C (held for 5 min) was increased to 240 °C at 4 °C/min. Mass spectra were recorded in the electron impact ionization mode at 70 eV by means of an ISQ single quadrupole mass spectrometer (Thermo Fischer Scientific). Data were acquired by applying both full scan (TIC, *m/z* 33-300) and SIM mode using specific *m/z* values for selected compounds.

Identification was performed using Xcalibur 2.2 (Thermo Fisher Scientific) coupled with the MS libraries NIST 08 and Wiley 8, as well as by calculating the retention indices of the compounds using a series of *n*-alkanes (C5-C21), which were compared with the values reported on the NIST database (detailed information in **Table 41**). For VOC quantification, the areas under the TIC or SIM peaks were integrated and related to those of the internal standards. Hexanal, furfural and 2-methylpyrazine were quantified using their deuterated standards. The relative quantification of other compounds was performed using 2-methylpyrazine-d_6 for nitrogenous, furfural-d_4 for furanic and hexanal-d_{12} for the remaining compounds.

2.9 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was performed and significant differences were evaluated by Tukey's test at $p<0.05$ using SAS studio version 3.8 (SAS Institute Inc., Cary, NC, USA). The data acquired were reported with two significant figures. Principal component analysis (PCA) was conducted using XLSTAT 2020.1 (Addinsoft, Paris, France).

3 RESULTS AND DISCUSSION

Wheat, lupin, lentil, chickpea, green pea, yellow pea and yellow pea processed under nitrogen are abbreviated to W, LU, LE, CP, GP, YP and YPN, respectively. The letters "F", "B" and "C" indicate whether the flour, batter or cake was being examined.

3.1 VOLATILE COMPOUNDS IDENTIFIED IN THE FLOURS

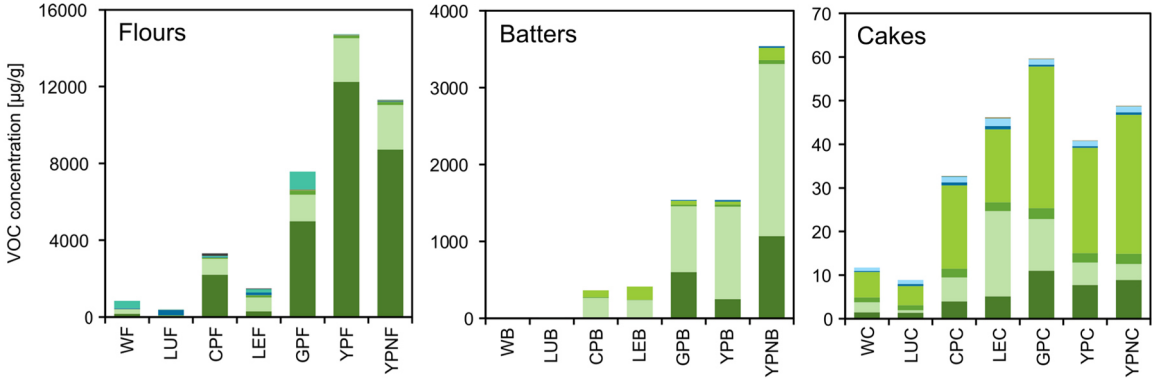
Figure 50 shows the profiles of VOCs extracted from the flours, batters and cakes made using wheat and pulses. An overview of all compounds and their quantities can be found in **Table 42** – **Table 46**.

The identification of the VOCs present in the flours is an important prerequisite to understand their potential to form molecules during cake development. As shown in **Figure 50**, the pulse flours, and particularly YPF/YPNF, contained significantly higher relative VOC amounts compared to WF, with the exception of LUF. Moreover, distinct differences in the VOC composition between the raw materials could be determined.

In WF, monoterpenes were observed as the dominant group of volatiles (**Figure 50**), which was not the case for the pulse flours, even though high levels were also detected. The molecules found included limonene, *β*-myrcene, *β*-pinene, 3-carene and linalool, which might have been products of endogenous isoprenoid biosynthesis or carotenoid degradation (Azarnia *et al.*, 2011a; Jakobsen *et al.*, 1998; Paucean *et al.*, 2018).

By contrast, the most abundant constituents of CPF, GPF and YPF/YPNF included a complex mixture of alkanes (**Figure 50**). These compounds might have been produced by the oxidation of lipids (Paucean *et al.*, 2018; Stevenson *et al.*, 1996) or they could have migrated from the packaging (Chang *et al.*, 2016; Rivas-Cañedo *et al.*, 2009).

In LEF, however, the main group of volatiles was alcohols. Relative to WF and LUF, the concentrations determined were significantly higher, but not when compared to CPF and the pea flours (**Figure 50**). Ethanol generally accounted for a large share of alcohols in all the flours, and particularly in pea cultivars (**Table 42**). This might suggest microbial spoilage, as the compound can be produced from the amino acid alanine *via* the Ehrlich pathway (Pico *et al.*, 2015). The presence of 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol and 2-phenylethanol in the different flours could have confirmed fungal infection as they can form *via* the same mechanism from valine, leucine, isoleucine and phenylalanine, respectively (Pico *et al.*, 2015). The comparatively low proportions of these molecules in LUF, however, could indicate that this flour or its seeds had been subjected to thermal treatment, thereby suppressing microbial growth.



Sample		VOC concentration [µg/g sample]										
		Alkanes	Alcohols	Ketones	Aldehydes	Furanic	Nitrogenous	Acids/Esters	Pyrones	Sulfurous	Terpenes	Total
Flour	W	160.2 ± 5.5^{c}	235 ± 40^{c}	16.2 ± 2.5^{d}	6.19 ± 0.32^{b}	45 ± 12^{c}	0.013 ± 0.011^{b}	n.d.	n.d.	n.d.	381 ± 14^{b}	843 ± 54^{d}
	LU	23.1 ± 2.1^{c}	30.9 ± 1.3^{c}	29.6 ± 1.4^{d}	2.054 ± 0.014^{c}	291 ± 11^{a}	0.0575 ± 0.0038^{a}	11.45 ± 0.66^{c}	n.d.	n.d.	0.54 ± 0.14^{d}	389 ± 11^{d}
	CP	2200 ± 550^{c}	835 ± 100^{b,c}	69.8 ± 9.7^{c,d}	n.d.	27.4 ± 1.3^{c,d}	0.0178 ± 0.0067^{b}	n.d.	n.d.	126 ± 17^{a}	57.8 ± 9.2^{d}	3310 ± 680^{c,d}
	LE	293 ± 29^{c}	729 ± 99^{b,c}	105.4 ± 1.8^{b,c}	12.9 ± 1.8^{a}	144.6 ± 5.0^{b}	0.022 ± 0.012^{b}	n.d.	n.d.	29.0 ± 1.1^{b}	174 ± 66^{c}	1490 ± 180^{d}
	GP	4990 ± 180^{b,c}	1390 ± 320^{b}	218 ± 39^{a}	6.871 ± 0.048^{b}	5.02 ± 0.44^{e}	0.0090 ± 0.0037^{b}	42.95 ± 0.86^{a}	n.d.	n.d.	922 ± 21^{a}	7570 ± 480^{b,c}
	YP	12200 ± 2000^{a}	2280 ± 480^{a}	124 ± 20^{b,c}	14.54 ± 0.36^{a}	14.66 ± 0.43^{d,e}	0.0077 ± 0.0046^{b}	30.6 ± 8.6^{b}	n.d.	n.d.	21.7 ± 4.9^{d}	14700 ± 2400^{a}
	YPN	8700 ± 4700^{a,b}	2320 ± 480^{a}	152 ± 35^{b}	13.4 ± 1.3^{a}	32.6 ± 3.4^{c,d}	0.0124 ± 0.0055^{b}	41.2 ± 3.5^{a,b}	n.d.	15.0 ± 7.3^{b}	22.9 ± 2.8^{d}	11300 ± 4500^{a,b}
Batter	W	0.62 ± 0.43^{c}	4.83 ± 0.37^{c}	n.d.	0.531 ± 0.019^{d}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.98 ± 0.62^{c}
	LU	3.85 ± 0.29^{c}	1.580 ± 0.027^{c}	0.598 ± 0.034^{d}	0.1292 ± 0.0074^{d}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.20 ± 0.24^{c}
	CP	9.5 ± 4.8^{c}	255 ± 19^{c}	9.28 ± 0.64^{c,d}	89.2 ± 4.9^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	363 ± 29^{c}
	LE	0.84 ± 0.40^{c}	232 ± 26^{c}	7.4 ± 1.1^{d}	172 ± 10^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	413 ± 31^{c}
	GP	600 ± 100^{a,b}	857 ± 74^{b}	20.81 ± 0.76^{b,c}	48.5 ± 2.9^{c}	12.9 ± 1.9^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	1540 ± 140^{b}
	YP	247 ± 69^{b,c}	1200 ± 170^{b}	28.8 ± 3.6^{b}	37.7 ± 2.9^{c}	21.0 ± 6.8^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	1540 ± 100^{b}
	YPN	1070 ± 480^{a}	2240 ± 520^{a}	50.4 ± 8.2^{a}	159.1 ± 9.4^{a}	21.4 ± 3.7^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	3540 ± 930^{a}
Cake	W	1.456 ± 0.014^{d}	2.299 ± 0.071^{e}	1.143 ± 0.032^{c}	5.84 ± 0.34^{e}	0.197 ± 0.011^{f}	0.790 ± 0.025^{d}	n.d.	n.d.	n.d.	n.d.	11.72 ± 0.46^{e}
	LU	1.35 ± 0.10^{d}	0.593 ± 0.026^{f}	1.168 ± 0.034^{c}	4.37 ± 0.14^{f}	0.472 ± 0.013^{d}	0.8409 ± 0.0069^{d}	n.d.	0.00542 ± 0.00078^{a}	n.d.	n.d.	8.81 ± 0.27^{e}
	CP	3.993 ± 0.053^{c}	5.48 ± 0.15^{c}	2.013 ± 0.058^{b}	19.06 ± 0.43^{c}	0.713 ± 0.028^{b}	1.211 ± 0.010^{b,c}	0.1579 ± 0.0070^{b}	0.112 ± 0.085^{a}	n.d.	n.d.	32.74 ± 0.84^{d}
	LE	5.13 ± 0.71^{c}	19.56 ± 0.81^{a}	2.00 ± 0.13^{b}	16.74 ± 0.35^{d}	0.770 ± 0.021^{a}	1.69 ± 0.12^{a}	0.241 ± 0.021^{a}	n.d.	n.d.	n.d.	46.1 ± 1.9^{b}
	GP	10.98 ± 0.61^{a}	11.68 ± 0.11^{b}	2.527 ± 0.042^{a}	32.47 ± 0.33^{a}	0.4312 ± 0.0029^{d,e}	1.261 ± 0.039^{b,c}	0.1453 ± 0.0091^{b}	n.d.	n.d.	n.d.	59.49 ± 0.48^{a}
	YP	7.7 ± 1.1^{b}	5.15 ± 0.11^{c}	2.180 ± 0.030^{b}	24.11 ± 0.48^{b}	0.415 ± 0.018^{e}	1.171 ± 0.051^{c}	0.1136 ± 0.0071^{c}	n.d.	n.d.	n.d.	40.9 ± 1.6^{c}
	YPN	8.9 ± 1.2^{b}	3.666 ± 0.055^{d}	2.390 ± 0.071^{a}	31.80 ± 0.65^{a}	0.592 ± 0.017^{c}	1.356 ± 0.012^{b}	0.1419 ± 0.0059^{b,c}	n.d.	n.d.	n.d.	48.8 ± 1.6^{b}

Figure 50. Profile of VOCs [µg/g sample] extracted from flours, batters and cakes. Compounds are categorized into alkanes (■) alcohols (■), ketones (■), aldehydes (■), furanic compounds (■), nitrogenous compounds (■), pyrans (■), acids/esters (■), terpenes (■) and sulfurous compounds (■). N.d.: not detected. Relative amounts were estimated based on the internal deuterated standards hexanal-d_{12}, furfural-d_4 and 2-methylpyrazine-d_6. Different letters within a column of one food matrix indicate significantly different means ($p<0.05$).

According to the literature, the remaining alcohols can be assumed as being more likely to derive from lipid oxidation, although some have also been described as fungal metabolites (Azarnia *et al.*, 2011a; Maire *et al.*, 2013). Among these, 1-hexanol was one of the most abundant, particularly in LEF, GPF and YPF. Other alcohols typical of lipid oxidation, such as 1-penten-3-ol, 2-hexen-1-ol and 3-hexen-1-ol (Kochhar, 1996), were exclusively detected in LEF. Moreover, this flour contained two aldehyde oxidation markers, namely hexanal and 2-hexenal (Belitz *et al.*, 2009), the latter of which was not detected in any other flour, thereby emphasizing the higher degree of oxidation of LEF. The opposite could be concluded for CPF based on the absence of aldehydes.

Interestingly, no difference in alcohols and aldehydes profiles could be measured between the two yellow pea flours. Both flours were produced from the same raw material, but were processed and stored under exposure to air (YPF) or under nitrogen (YPNF). From this it could be deduced that lipid oxidation in this ingredient tended to be initiated during harvest and transport rather than during seed grinding or storage.

With regard to aldehydes, LUF occupied a special position. Unlike the other flours, LUF contained 2- and 3-methylbutanal (**Table 43**), which are both indicators of heat treatment, generated by Strecker degradation of either isoleucine or leucine (Cepeda-Vázquez *et al.*, 2018). The assumption of the heat exposure of LUF was affirmed by the identification of furanic compounds, which made up 75% of the VOCs. These included furfural, a known marker of the Maillard reaction (Belitz *et al.*, 2009). In addition, LUF contained traces of pyrazines (0.01-0.03 µg/g) and ketones linked to the Maillard reaction such as 2,3-butanedione, 2,3-pentanedione and 3-hydroxy-2-butanone, which validated the thermal processing of lupin seeds/flour.

3.2 CHANGE OF VOC PROFILE DURING BATTER BEATING

The distribution of dry ingredients in the moist matrix, along with air entrapment during batter whipping, seemed to initiate reactions. This could be seen from the marked changes in VOC profiles compared to the flours (**Figure 50**). The highest levels of compounds were extracted from batters made with green and yellow pea, while the lowest quantities were recorded in WB and LUB. In terms of the number of VOCs, the batters could be ranked in a similar order (**Table 42** – **Table 46**).

Alcohols represented the dominant group of VOCs in all batters with the exception of LUB, where the profile was primarily characterized by alkanes. The highest relative amounts were determined in pea batters, the alcohol levels in which exceeded those in WB by up to 460× (**Figure 50**). Taking account of the fact that the batters only contained 25% flour, and that the contributions of inherent egg and oil volatiles to the VOC profile were negligible (Maire *et al.*, 2013), it emerged that the amounts of alcohols increased sharply during batter beating. Most of these alcohols were associated with FA breakdown, attributable to the incorporation of air. 1-Hexanol was one of the most abundant compounds in the batters (**Table 42**), which was found at concentrations up to 35× higher than those detected in the flours when considering their 25% content. The widely accepted pathway for 1-hexanol production involves the oxidation of linoleic acid under the formation of hexanal, which is reduced to 1-hexanol by the action of an alcohol dehydrogenase (Jakobsen *et al.*, 1998). In addition, several other alcohols were generated, some of which are also believed to originate from linoleic acid degradation. These included 1-octen-3-ol and 1-pentanol, which appeared at high levels, particularly in GPB, YPB and YPNB (**Table 42**). These batters were moreover highly enriched in 1-penten-3-ol, which was more likely to have been produced from linolenic acid oxidation catalyzed by an alcohol dehydrogenase (Murray *et al.*, 1976). Among the yellow pea batters, YPNB stood out for its significantly higher concentrations

of lipid oxidation markers compared to YPB (**Figure 50**). This might indicate that producing pea flour under nitrogen protected unsaturated FAs from oxidation. Consistent with this, it could be hypothesized that any contact with atmospheric oxygen would cause an instant reaction, leading to VOC formation.

In addition, large quantities of ethanol were measured in the batters, which probably derived from the raw materials or arose in the course of fermentation processes during batter preparation. Indeed, it was the principal alcohol in both WB and LUB (**Table 42**), suggesting their low susceptibility to lipid oxidation.

LEB and CPB could be discriminated from the other batters as having higher aldehyde than alcohol contents (**Figure 50**). Hexanal, in particular, was of considerable importance as its level increased by up to 80× compared to the respective flours, when considering that only 25% flour was used during batter making. High values were also extracted from YPB, GPB and CPB, which thus greatly exceeded those in WB and LUB, where only traces of aldehydes were found. Apart from hexanal, the oxidative degradation of linoleic acid also seemed to have caused the release of 2,4-nonadienal, 2-nonenal, 2-heptenal, 2-octenal, pentanal and heptanal (Belitz *et al.*, 2009; Murray *et al.*, 1976), most of which were solely identified in LEB. The same was true for the breakdown products of linolenic acid, which included 2-hexenal, 2,4-heptadienal and 2-pentenal (Murray *et al.*, 1976). Based on these findings, it could be assumed that CPF, and notably LEF, contained alcohol dehydrogenases that were either at lower levels or with weaker activities, thus transforming fewer aldehydes into alcohols, unlike peas.

The greater vulnerability of unsaturated FAs to oxidative degradation in LEB, CPB, GPB and YPB/YPNB was further substantiated by the presence of ketones such as 3-octanone, that were lacking in LUB and WB. After alcohols and aldehydes, these molecules made up the third largest group of VOCs. Finally, furanic components were also found, but only accounted for a maximum of 1% of volatiles (**Figure 50**).

3.3 FACTORS INFLUENCING THE REACTIVITY POTENTIAL DURING BATTER BEATING

3.3.1 AMOUNT OF INCORPORATED AIR

The generation of VOCs during batter preparation was largely attributed to the oxidation of unsaturated FAs. In order to understand whether the amount of air incorporated was correlated with the potential of the flours to form VOCs, the density of the batters was analyzed and associated with the VOC quantities. As shown in **Table 25**, YPNB and GPB appeared to be the most voluminous, while LUB was the densest. Since the former products were distinctively enriched in VOCs compared to the latter (**Figure 50**), it was supposed that enhanced air enclosure promoted oxidative reactions. However, this hypothesis was refuted by the behavior of YPB, which contained similar amounts of volatiles as GPB but displayed significantly higher density. Furthermore, its density did not differ significantly from that of WB, LEB, CPB and LUB, but far exceeded their VOC concentrations. This led to the conclusion that the amount of incorporated air was not the principal cause of lipid oxidation, so it appeared essential to address the enzymatic activity of the different flours.

3.3.2 LOX ACTIVITY AND FA PROFILE

Unlike the other batters, LUB and WB contained fewer lipid oxidation markers than their respective flours used at a proportion of 25%. As discussed in **§ 3.2**, these molecules are well-known breakdown products of the polyunsaturated FAs linoleic and linolenic acid. Owing to their 1-*cis*,4-*cis*-pentadiene system, they are suitable substrates for LOX, catalyzing their conversion into unstable hydroperoxides, which are subsequently transformed into volatiles (Belitz *et al.*, 2009). Because of the low level of oleic acid degradation products, it was assumed that enzyme-mediated lipid oxidation was the main pathway through which VOCs were released. This was confirmed by analyzing the LOX activities of the flours at a pH similar to that of the batters

(~ pH 7). While the enzyme was practically inactive in WF and LUF, its activity in the other flours ranged from 2510 nkat/g (CPF) to 4350 nkat/g (YPF). This finding provided a substantiated explanation for the low concentrations of lipid oxidation markers in the batters made with LUF or WF. In the former case, the suppression of LOX could indicate heat-induced denaturation during seed/flour roasting, which could be supported by the presence of thermal reaction markers (e.g. pyrazines). However, this assumption was not applicable to WF owing to its lack of such molecules. But for the other flours, a logical link between LOX activity and volatiles could be established: the more the enzyme was active, the more VOCs were identified.

The high levels of linoleic and linolenic acid in the pea flours, combined with their striking LOX activity, could further underline their pronounced susceptibility to oxidation (**Table 25**). Although these flours were characterized by a significantly lower acid value than wheat, which reflects the quantity of free FA that can be easily oxidized, they had a higher ability to undergo oxidation and form volatile compounds. This implies, that the fewer FA freely available in the pea flours were probably polyunsaturated fatty acids, which were rapidly oxidized by the highly active LOX. Interestingly, however, CPF and LEF had a weaker ability to generate oxidation markers despite high concentrations of polyunsaturated fatty acids (**Table 25**). In addition, a large number of these seemed to be freely available, in particular in LEF, however were much less degraded compared to the pea flours. This could not only be related to reduced LOX activity but potentially also to a high antioxidative capacity, as described in the literature (Hall *et al.*, 2017). In light of their reported elevated polyphenol contents, a deceleration of FA breakdown could be imagined (Hall *et al.*, 2017).

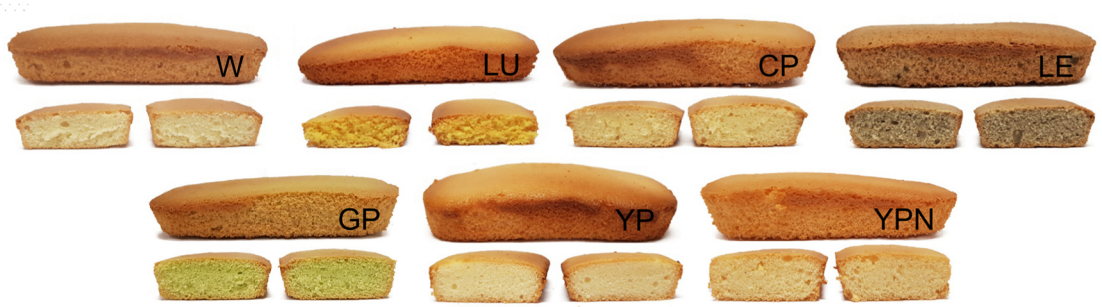
3.4 DEVELOPMENT OF DENSITY AND COLOR IN CAKES DURING BAKING

Baking induces starch gelatinization and protein denaturation that lead to the setting of a solid cake structure. This affects the density of the cakes, which is considered a good indicator of cake quality. As depicted in **Figure 51**, the complete substitution of WF with the different pulse flours resulted in the successful production of sponge cakes with a slightly higher volume. These findings coincide with the results published by de la Hera *et al.* (2012), who reported an increase in sponge cake volume after the incorporation of lentil flour. The authors assigned this behavior to the elevated level of protein, which has important emulsifying properties. However, other studies have demonstrated the opposite effect with chickpea (Gómez *et al.*, 2008) or green pea flour (Monnet *et al.*, 2020). These differences could be related to the different recipes chosen. Monnet *et al.* (2020) observed cracks on the surface of their cakes, indicating crust rupture. Unlike the present study, the authors used baking

Table 25. Batter densities, LOX activities and profile of unsaturated fatty acids of flours.

Flour	Batter density	LOX activity	Unsaturated fatty acids [g/100 g fat]			Acid value
	[g/cm³]	[nkat/g flour]	C18:1	C18:2	C18:3	[-]
W	0.6046 ± 0.0077^{b}	<20^{d}	5.61 ± 0.25^{d}	24.1 ± 1.2^{d}	1.88 ± 0.18^{d}	23.22 ± 0.74^{b}
LU	0.645 ± 0.027^{a}	<1^{d}	37.0 ± 1.3^{a}	12.07 ± 0.31^{e}	5.29 ± 0.15^{b,c}	4.512 ± 0.089^{d}
CP	0.618 ± 0.013^{a,b}	2510 ± 140^{c}	18.25 ± 0.80^{b}	38.14 ± 0.35^{a}	1.35 ± 0.24^{d}	5.01 ± 0.27^{d}
LE	0.603 ± 0.013^{b}	3200 ± 250^{b}	15.26 ± 0.51^{c}	22.77 ± 0.86^{d}	4.50 ± 0.58^{c}	42.522 ± 0.038^{a}
GP	0.560 ± 0.023^{c}	3870 ± 110^{a}	15.550 ± 0.070^{c}	28.22 ± 0.18^{c}	5.63 ± 0.31^{a,b}	10.59 ± 0.49^{c}
YP	0.621 ± 0.017^{a,b}	4350 ± 300^{a}	19.80 ± 0.39^{b}	35.51 ± 0.82^{b}	6.050 ± 0.060^{a,b}	10.94 ± 0.52^{c}
YPN	0.533 ± 0.028^{c}	4340 ± 160^{a}	19.70 ± 0.69^{b}	34.54 ± 0.95^{b}	6.42 ± 0.36^{a}	10.31 ± 0.46^{c}

Different letters within a column indicate significantly different means (p<0.05). W = wheat, LU = lupin, CP = chickpea, LE = lentil, GP = green pea, YP = yellow pea and YPN = yellow pea processed and stored under nitrogen. C18:1 = oleic acid, C18:2 = linoleic acid and C18:3 = linolenic acid.



Cake	Density [g/cm^3]	Dry matter [g/100 g]	pH value	Crumb color			Crust color		
				L*	a*	b*	L*	a*	b*
W	0.3423 ± 0.0094^{b}	95.36 ± 0.10^{d}	7.55 ± 0.10^{a}	87.32 ± 0.83^{a}	1.40 ± 0.16^{d}	22.78 ± 0.71^{f}	64.9 ± 2.1^{d}	14.15 ± 0.61^{b}	31.27 ± 0.57^{d}
LU	0.409 ± 0.034^{a}	97.10 ± 0.14^{a}	6.180 ± 0.046^{e}	74.7 ± 2.2^{e}	7.24 ± 0.87^{a}	50.15 ± 0.67^{a}	69.7 ± 3.9^{a}	14.1 ± 1.7^{b}	40.7 ± 2.0^{a}
CP	0.309 ± 0.011^{c}	96.19 ± 0.46^{c}	6.91 ± 0.10^{d}	83.82 ± 0.63^{c}	2.82 ± 0.19^{b}	29.46 ± 0.72^{b}	67.7 ± 2.4^{a,b}	14.23 ± 0.73^{b}	33.12 ± 0.47^{b}
LE	0.305 ± 0.010^{c}	95.46 ± 0.26^{c,d}	6.847 ± 0.026^{d}	64.9 ± 1.1^{f}	1.92 ± 0.32^{c}	15.66 ± 0.81^{g}	61.5 ± 1.6^{e}	9.1 ± 1.1^{c}	26.13 ± 0.99^{e}
GP	0.2971 ± 0.0048^{c}	96.333 ± 0.090^{b}	7.157 ± 0.065^{b}	78.9 ± 1.1^{d}	-4.58 ± 0.27^{e}	28.01 ± 0.76^{c}	65.3 ± 1.7^{c,d}	9.1 ± 1.3^{c}	31.56 ± 0.52^{c,d}
YP	0.2944 ± 0.0043^{c}	95.521 ± 0.073^{c,d}	6.950 ± 0.035^{c,d}	85.9 ± 1.6^{b}	2.65 ± 0.43^{b}	25.63 ± 0.65^{e}	67.3 ± 3.6^{b,c}	14.6 ± 1.2^{a,b}	32.11 ± 0.36^{c}
YPN	0.2810 ± 0.0054^{c}	93.71 ± 0.28^{e}	7.10 ± 0.10^{b,c}	83.4 ± 1.6^{c}	2.52 ± 0.30^{b}	26.25 ± 0.82^{d}	65.9 ± 3.2^{b,c,d}	15.3 ± 1.2^{a}	33.23 ± 0.44^{b}

Figure 51. Appearance and characteristics of sponge cakes prepared with different flours. Different letters within a column indicate significantly different means (p<0.05). W = wheat, LU = lupin, CP = chickpea, LE = lentil, GP = green pea, YP = yellow pea and YPN = yellow pea processed and stored under nitrogen.

powder in their formula, which presumably led to an additional formation of gas during cake production. The resulting increase in internal pressure caused the crust to burst and allowed the vapor to escape, triggering shrinkage.

According to Gómez *et al.* (2012), cake shrinkage can also be correlated to the proportion of the starch fraction. In addition to proteins, these macromolecules play a decisive role in the formation of a structural framework during baking based on their heat-induced gelatinization, which in turn affects viscosity and thus the ability to retain air incorporated in the batter (Monnet *et al.*, 2020; Ronda *et al.*, 2011). With a reduced starch-to-protein ratio, the gas cells cannot be sufficiently stabilized during batter solidification, which therefore causes the development of a dense cake. This phenomenon might be used to describe the extraordinary behavior of LUC, which was characterized by the highest density (**Figure 51, Table 26**). In addition, the elevated fiber content in this flour (**Table 26**) might have contributed to the reduced volume owing to its effect on the gelatinization and pasting properties of the starch and disorganization of the starch-protein network, being accompanied by a lower capacity for gas retention (Aydogdu *et al.*, 2018; BeMiller, 2011; Sasaki *et al.*, 2000).

In addition to density, the use of pulse flours was associated with modifications to the color properties of the cakes, which is another decisive criterion for the cake quality (**Figure 51**). Pulse flours induced a significant reduction in lightness *L** of the crumb compared to WF, which was most pronounced with LEF. In contrast, redness *a** was significantly increased except with GPC, for which negative *a** values were measured. This implied a greenish hue of the crumb, probably due to the presence of chlorophyll in the raw material. Additionally, color generally shifted toward more yellow tints, as perceptible from the higher *b** values, which could be assigned to the high proportion of carotenoids. It has been reported that temperatures ≥120°C and water activities ≤0.6 are required to trigger the color formation of baked goods. Working on similar sponge cakes, Srivastava *et al.* (2018) measured temperatures lower than 120°C in the crumb, while those at the surface were sufficiently high to initiate browning reactions, thereby causing darkening of the crust. As visible in **Figure 51**, the *L**, *a** and *b** values were very similar in the different products, especially for the crust. This could be explained by the Maillard reaction which leads to the formation of brown pigments capable of masking the differences due to the flours in the crust.

In addition to their attractive appearance, the developed cakes were also characterized by an improved nutritional quality compared to the wheat-based reference. This could be concluded from the particular chemical composition of the flours used, which led to pulse-based cakes with

Table 26. Chemical composition [g/100 g] of different flours.

Flour	Dry matter	Ash	Starch	Protein	Fiber	Fat
W	87.463 ± 0.028^{c}	0.76 ± 0.40^{d}	70.2 ± 7.4^{a}	8.65 ± 0.36^{e}	2.9 ± 1.2^{c}	1.112 ± 0.031^{e}
LU	92.90 ± 0.11^{a}	4.79 ± 0.40^{a}	$<0.4^{c}$	42.4 ± 1.3^{a}	26.7 ± 3.3^{a}	11.93 ± 0.12^{a}
CP	$91.787 \pm 0.047^{a,b}$	$3.60 \pm 0.40^{b,c}$	39.3 ± 4.3^{b}	22.19 ± 0.67^{c}	13.6 ± 2.4^{b}	6.354 ± 0.018^{b}
LE	$91.134 \pm 0.013^{a,b}$	3.79 ± 0.40^{b}	40.7 ± 4.4^{b}	27.64 ± 0.83^{b}	$8.9 \pm 2.0^{b,c}$	1.746 ± 0.043^{d}
GP	$90.091 \pm 0.039^{b,c}$	2.67 ± 0.40^{c}	51.9 ± 5.5^{b}	18.68 ± 0.56^{d}	$8.2 \pm 1.9^{b,c}$	1.652 ± 0.056^{d}
YP	$90.11 \pm 0.17^{b,c}$	2.741 ± 0.033^{c}	48.8 ± 5.2^{b}	22.98 ± 0.10^{c}	$8.8 \pm 2.0^{b,c}$	2.064 ± 0.024^{c}
YPN	$90.13 \pm 0.12^{b,c}$	2.741 ± 0.033^{c}	48.6 ± 5.2^{b}	22.98 ± 0.10^{c}	$8.1 \pm 1.9^{b,c}$	2.024 ± 0.022^{c}

Different letters within a column indicate significantly different means ($p<0.05$). W = wheat, LU = lupin, CP = chickpea, LE = lentil, GP = green pea, YP = yellow pea and YPN = yellow pea processed and stored under nitrogen.

elevated levels of protein and fiber as well as lower levels of starch.

3.5 HEAT-DRIVEN GENERATION OF VOLATILE COMPOUNDS DURING BAKING

Apart from structure and color changes, the energy-driven degradation of molecules can be triggered during baking and involve lipid oxidation, the Maillard reaction and caramelization. As shown in **Figure 50**, the cakes were characterized by substantially lower quantities of VOCs compared to the batters, most probably due to volatilization, consecutive reactions or matrix effects. A total of 59 VOCs was detected, most of them in GPC and LEC, while LUC and WC contained the fewest (**Table 42** – **Table 46**). Moreover, the latter two stood out for their remarkably low total VOC amounts among all products (**Figure 50**).

Except for LEC, aldehydes formed the main group of components in all the cakes, reaching particularly high concentrations in GPC and YPNC. Although the amounts in LEC, CPC and YPC were significantly lower, they clearly surpassed those found in WC and LUC. Among these aldehydes, hexanal predominated in most cakes (**Table 43**). This marker of linoleic acid oxidation is typically characterized by a low odor threshold, and thus is considered to be relevant regarding the perception of a grassy, beany odor (Buttery *et al.*, 1998; Pozo-Bayón *et al.*, 2007). Further oxidation products resulting from linoleic acid degradation were present, such as pentanal, 2-octenal and 2,4-decadienal as well as heptanal and octanal, although the latter two might also have derived from the breakdown of oleic acid (Kochhar, 1996). The first occurrence of some of these compounds might indicate heat-triggered autoxidation of the aforementioned precursors, while others might have been retained from the batter, rendering the aldehyde profiles more complex than those of the batters. Together they may not only enhance the green scent, but also impart a fatty, nutty and toasted fragrance (Burdock, 2009; Murat *et al.*, 2013; Szczygiel *et al.*, 2017) to the cakes, especially those based on pea.

The VOC profiles of WC and LUC, on the other hand, contained fewer lipid oxidation markers but more of the aldehydes assigned to thermal amino acid degradation through the Strecker reaction (**Table 43**). This led to the assumption that the wheat and lupin products were generally less susceptible to lipid oxidation, regardless of whether they were enzymatically catalyzed or heat-induced. The globally accepted mechanism for the formation of Strecker aldehydes involves the reaction between amino acids and *α*-dicarbonyl compounds such as the deoxyosones generated through the Maillard reaction (Belitz *et al.*, 2009). The Strecker aldehydes identified comprised 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, acetaldehyde and phenylacetaldehyde, which are associated with the oxidative decarboxylation of valine, isoleucine, leucine, alanine and phenylalanine, respectively (Belitz *et al.*, 2009). While 2- and 3-methylbutanal totaled about 60-70% of the aldehydes in both WC and LUC, the percentage contributions in the other cakes reached only 10-19%, although in quantitative terms, the concentrations in all cakes were similar (**Table 43**). Strecker aldehydes typically have a marked malty and sweet aroma (Garvey *et al.*, 2021; Pico *et al.*, 2015).

LEC differed from the other cakes in terms of its higher alcohol content (**Figure 50**). The majority of this class was composed of 1-hexanol, followed by ethanol and 1-penten-3-ol (**Table 42**). These three components were also most strongly represented in the other products, with 1-penten-3-ol dominating in YPC/YPNC and ethanol in GPC, LUC and WC. According to the literature, these compounds exhibit more than 1000× higher odor threshold values in water than aldehydes (Belitz *et al.*, 2009; Buttery *et al.*, 1998). For this reason, they may have contributed only marginally to the overall cake aroma despite their relatively elevated concentrations (**Table 42**).

The same conclusion could be drawn for ketones, which were present at much lower concentrations than alcohols (**Figure 50**). Unlike

the batters, the VOC profiles of the cakes were enriched in markers characteristic of Maillard and caramelization reactions, including 1-hydroxy-2-propanone (sweet, burnt), 3-hydroxy-2-butanone (buttery), 2,3-butanedione (buttery) and 2,3-pentanedione (buttery) (**Table 44**). Yaylayan *et al.* (2000) proposed two pathways for the generation of 2,3-butanedione, including either glucose alone or in combination with glycine *via* pyruvaldehyde. Subsequently, 2,3-butanedione can be reduced to form 3-hydroxy-2-butanone (Yaylayan *et al.*, 2000). Moreover, these authors suggested that 2,3-pentanedione is produced from pyruvaldehyde, followed by chain elongation with two carbon atoms from amino acids like alanine (Yaylayan *et al.*, 2000). For 1-hydroxy-2-propanone, it was assumed that it could derive from isomerized 1-deoxyosone or dehydrated Amadori product through retro-aldol cleavage (Yaylayan *et al.*, 2000).

Apart from ketones, a large number of other browning reaction products were detected, including furanic compounds such as furfural, 5-methylfurfural and 2-furanmethanol, which were found at higher concentrations in the pulse cakes than in WC (**Figure 50**, **Table 44**). According to the literature, furfural can be generated at elevated temperatures *via* 1,2-enolization from pentoses or glucose through either caramelization or the Maillard reaction (Belitz *et al.*, 2009; Yaylayan *et al.*, 2000). The latter pathway involves condensation of the sugars with free amino acids, followed by further enolization and deamination reactions, giving rise to 3-deoxyosones from which furfural can be released (Srivastava *et al.*, 2018). The generation of 2-furanmethanol from 3-deoxyosone has also been reported (Hollnagel *et al.*, 2002). Furanic molecules are generally associated with a burnt, caramel-like scent (Cepeda-Vázquez *et al.*, 2018; Garvey *et al.*, 2021). However, other molecules from this class and not originating from thermal degradation reactions but lipid oxidation (such as 2-pentylfuran) were present, which could have contributed to the green, nutty notes of the cakes (except LUC).

Nitrogenous compounds, i.e. pyrazines and pyrroles, represented another class of VOCs typically arising from Maillard reactions. Pyrazine formation is widely explained by a condensation reaction between two *α*-aminocarbonyl compounds, which themselves are generated next to Strecker aldehydes through Strecker degradation from the reaction between *α*-dicarbonyl compounds and amino acids (Belitz *et al.*, 2009). Among the different cakes, the highest levels were found in LEC, and the lowest in LUC and WC (**Figure 50**) Despite their low levels, their percentage contributions to all the VOCs in WC and LUC were the highest. The structures identified ranged from simple pyrazine to its single or multiple alkyl-substituted or amidated form. In addition, pyrrole and its acetylated derivative were detected. In all cases, 2,5-dimethylpyrazine accounted for more than half of the nitrogenous VOCs, followed by 2-methylpyrazine (**Table 45**). These heterocyclic compounds are known to have roasted, nutty and earthy smells (Garvey *et al.*, 2021; Pico *et al.*, 2015; Pozo-Bayón *et al.*, 2007).

3.6 MONITORING LIPID OXIDATION THROUGHOUT CAKE DEVELOPMENT

To verify that lipid oxidation was initiated during batter preparation and continued during baking, the concentrations of primary markers, namely CD and hydroperoxides, were monitored throughout the different steps of production (**Figure 52**). The starting values for CD were determined from the specific absorbance obtained for oil, egg and flour, taking the proportions used into account. This calculation was not possible for the hydroperoxides because of the limited fat contents in the flours.

As expected, mixing the ingredients into a foam initiated lipid oxidation (**Figure 52A**). The significant increment in specific absorbance of CD was more pronounced with pulses than with wheat, thus corroborating their higher susceptibility to FA breakdown. The extraordinary behavior of YPNB, which was already characterized by extremely high oxidation-derived VOC concentrations (**§ 3.2**), was underlined by its specific absorbance values. This implied that the

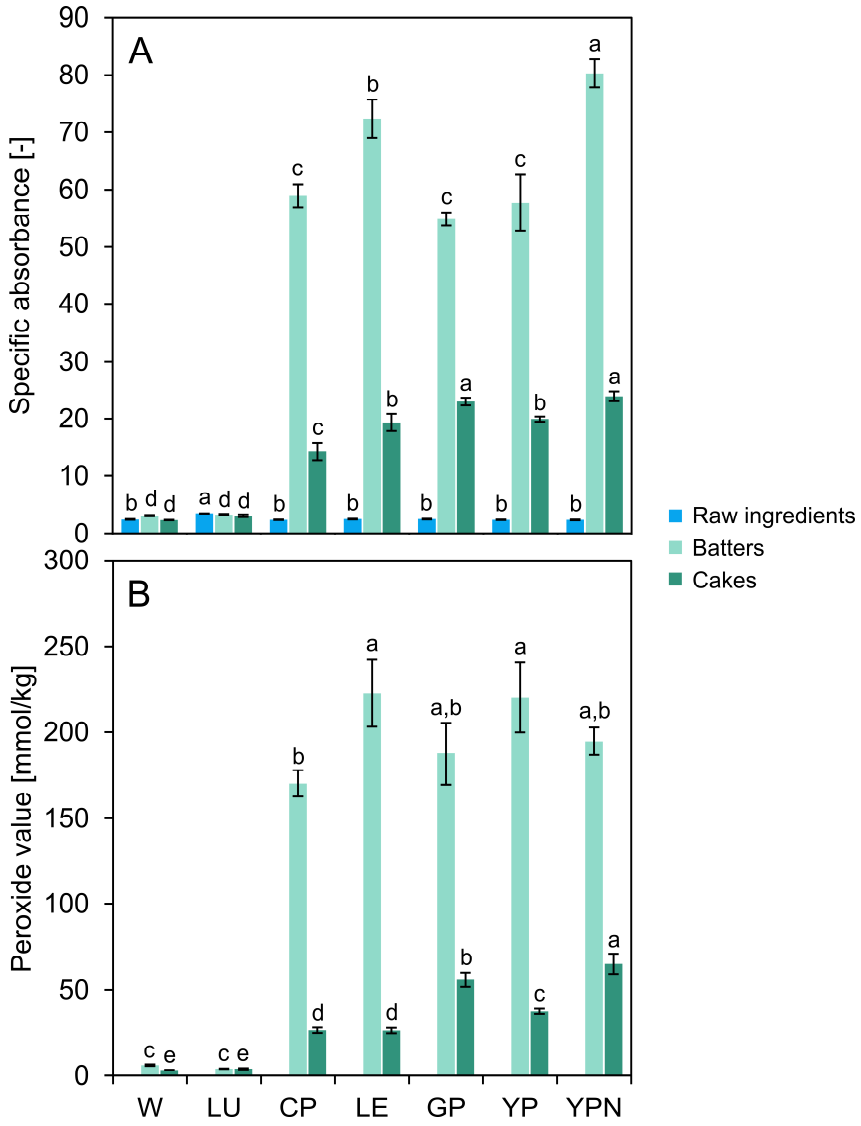


Figure 52. Evolution of (A) specific absorbance of conjugated dienes and (B) peroxide value from raw ingredients (■) to batters (■) to cakes (■). Different letters within one step of making indicate significantly different means (p<0.05). W = wheat, LU = lupin, CP = chickpea, LE = lentil, YP = yellow pea and YPN = yellow pea processed and stored under nitrogen

air-free processing of the associated flour protected important precursors from oxidation and made them prone to immediate degradation when they came into contact with oxygen. Moreover, lupin displayed striking oxidative stability as could be noted from its similar specific absorbance of CD when passing from the raw materials to the batter (**Figure 52A**). This affirmed that lipid oxidation was mainly enzyme-mediated.

Coinciding with the literature, baking led to a decline of both CD and hydroperoxide levels (**Figure 52A**, **Figure 52B**), thereby emphasizing the instability of the markers and their subsequent transformation into volatiles (Maire *et al.*, 2013). This trend was more pronounced with pulses (except lupin) than wheat, thus indicating the pursuit of oxidation during baking and explaining their higher VOC levels in the cakes.

3.7 IMPACT OF FLOUR TYPE ON REACTIVITY AND SENSORY-RELATED VOLATILE MARKERS OF CAKES

As described in **§ 3.5**, complex profiles of VOCs deriving mainly from lipid oxidation, the Maillard reaction and caramelization during

different cake making steps were identified in the various cakes. The majority of these molecules are known to exhibit intense odor activities, so are therefore likely to contribute to the flavor quality of the final food. A multivariate approach made it possible to visualize which VOCs were responsible for the main differences between pulse-based products and how reaction markers were correlated to each other. Closer inspection revealed that WC and LUC differed considerably from the other cakes by their distinctively lower potential to form volatiles through the aforementioned pathways. Based on this finding, it was of interest to gain deeper insight into the discrimination of LEC, CPC, GPC, YPC and YPNC in order to associate specific reaction markers with individual products, and hence deduce hypotheses regarding possible impacts on flavor perceptions and cake qualities. PCA was therefore performed on all the VOCs identified in the five selected cakes.

As depicted in **Figure 53**, PC1, PC2 and PC3 were responsible for 47.75%, 24.59% and 13.65% of variability, respectively. Two clusters of cakes were opposed on PC1: (1) LEC and CPC, and (2) YPC, YPNC and GPC. This differentiation was primarily driven by lipid oxidation markers (alcohols, aldehydes, ketones) which were associated with positive loadings and thus with the pea products. This indicated that fatty acid breakdown played a major role during cake development using pea flours, probably attributable to their increased LOX activity compared to LEF and CPF (**Table 25**). Additionally, the literature has reported high levels of polyphenols (e.g. chlorogenic acid, sinapic acid, catechins, epicatechins) in chickpeas and lentils, suggesting that their antioxidant effects may have decelerated oxidative reactions in both products (Hall *et al.*, 2017). Due to the significantly lower concentrations of lipid oxidation markers in these cakes compared to their respective batters, it was assumed that the majority of molecules tended to be residues of batter beating rather than formed during baking. It could therefore be imagined that the particular assignment of 1-hexanol and hexanoic acid to LEC rather than the pea cakes may have been due to matrix effects markedly affecting the reactivity of the products, for instance by retarding volatilization of the named components during baking. According to the literature, the lipid oxidation markers detected are typically linked to green, beany and grassy odors (Xu *et al.*, 2019), implying that the pea products were probably marked by such off-flavors.

Further differentiation was achieved when considering PC2 and PC3. On PC1 *vs* PC2 and PC1 *vs* PC3 it emerged that pyrazines were mainly assigned to LEC and CPC (**Figure 53**). Chemical characterization revealed that both LEF and CPF were distinctively enriched in free amino acids (7 µg/g each) compared to the pea flours (3-4 µg/g) (data not shown). According to Adams *et al.* (2008), alanine, serine and arginine are important precursors for the generation of 2-ethyl-3,5-dimethylpyrazine, 2-ethyl-6-methylpyrazine, 2-methylpyrazine and 2,5-dimethylpyrazine. The former two amino acids were identified at elevated concentrations in LEF, thus possibly explaining the greater presence of the aforementioned pyrazines in LEC. Amino acid analysis further revealed high arginine contents in all the flours (1-4 µg/g), which might have been responsible for the high levels of 2,5-dimethylpyrazine in all cakes (**§ 3.5**). In addition, other studies have discussed the ability of polyphenols to accelerate hexose degradation into *α*-dicarbonyls. This hypothesis is based on the potential for polyphenols to reduce metals, and particularly iron, which may participate in the Fenton reaction (García-Lomillo *et al.*, 2016). The hydroxy radicals thus generated may promote sugar transformation into *α*-dicarbonyls, which can undergo Strecker degradation, subsequently leading to pyrazines (García-Lomillo *et al.*, 2016). As indicated above, LEF and CPF are known to contain high levels of

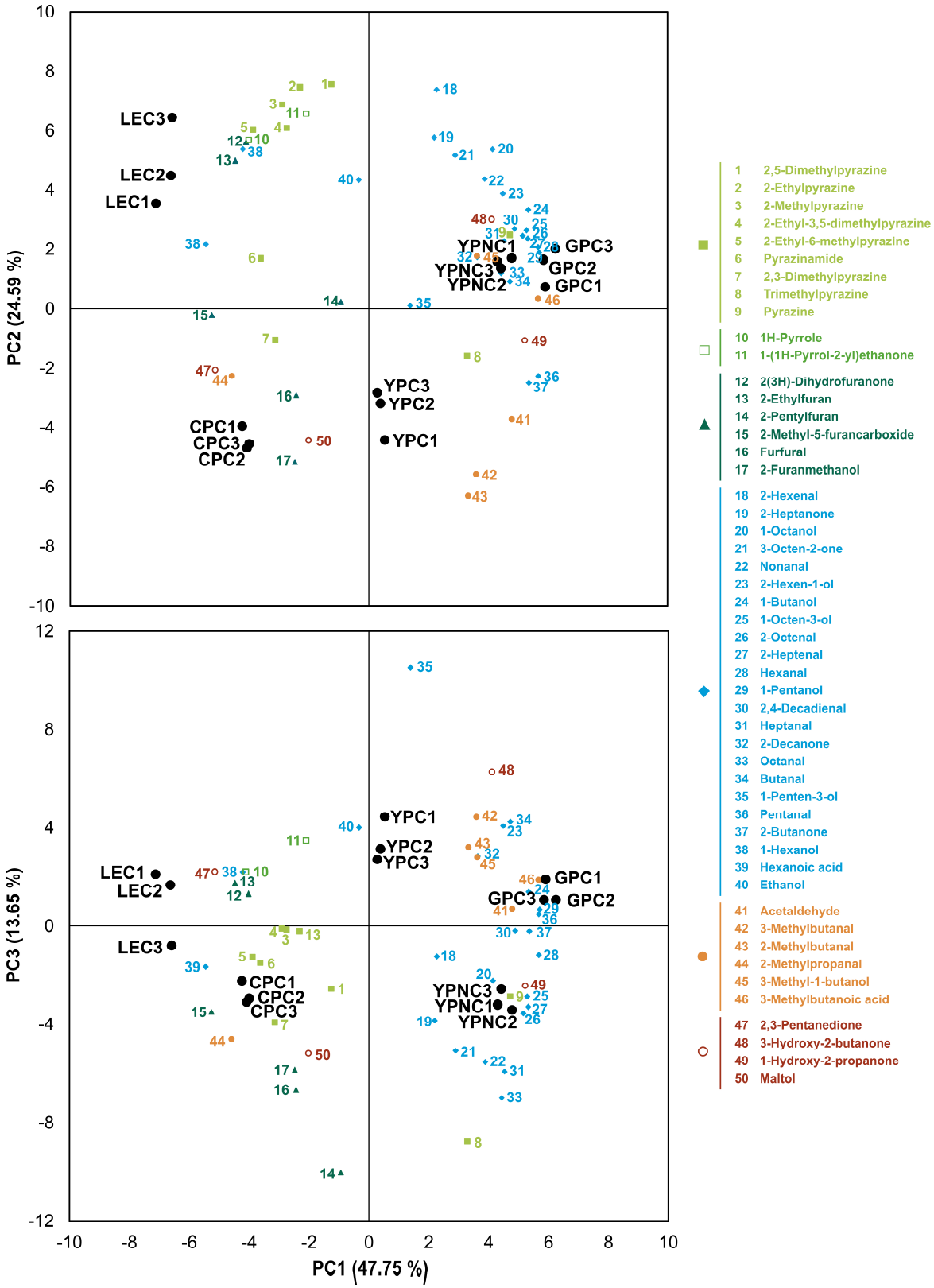


Figure 53. PCA biplots on volatile markers, categorized into pyrazines (■), pyrroles (□), furanic compounds (▲) lipid oxidation markers (♦) Strecker aldehydes and derivatives (●) and further Maillard and/or caramelization markers (○). LEC = lentil cake, CPC = chickpea cake, GPC = green pea cake, YPC = yellow pea cake and YPNC = cake made with yellow pea flour stored and processed under nitrogen.

phenolic acids, which in combination with their high ash contents compared to the pea flours (**Table 26**) might have stimulated pyrazine formation. In light of these results, it appeared that the VOC profiles of CPC, and particularly LEC, were influenced to a major extent by pyrazines, thus possibly contributing to a roasted odor in the cakes typically associated with pyrazines (Pico *et al.*, 2015).

Another result worth mentioning is the central location of 2-pentylfuran on PC1 *vs* PC2, from which it was concluded that this molecule might not only be produced through lipid oxidation but concurrently through alternative pathways involving amino group interactions, thus coinciding with findings in the literature (Cepeda-Vázquez *et al.*, 2018).

4 CONCLUSION

The complete replacement of wheat with pulse flours yielded sponge cakes with attractive nutritional and structural properties. At the same time, these products were characterized by a more complex profile of volatile compounds with probable effects on sensory quality. The majority of molecules derived from oxidative processes, which were initiated during batter beating. Lipoxygenase was identified as the main catalyst of lipid oxidation due to its significantly higher activity in the pulse flours, particularly in yellow and green pea flours, compared to wheat. One exception was lupin, in which the enzyme was practically inactive (probably due to heat treatment), thus explaining its elevated stability versus lipid degradation. By contrast, yellow pea flour produced and stored under nitrogen stood out for its oxidation lability, which might be linked to the greater susceptibility of protected precursors to oxidation.

Baking not only perpetuated lipid oxidation but also triggered the decomposition of sugars and amino acids *via* caramelization and the Maillard reaction, being accompanied by the release of pyrazines, Strecker aldehydes and furanic compounds. Larger quantities of both lipid oxidation and thermal reaction markers were detected in the pulse-containing products than in those made using wheat, most likely due to compositional and matrix effects. Principle component analysis revealed that lipid oxidation markers were mainly associated with green and yellow pea cakes, while pyrazines were assigned to lentil and chickpea cakes. Based on odor descriptors reported in the literature, it was assumed that the global flavor of the pea products might be impacted by a green-beany odor imparted by the lipid oxidation markers, whereas chickpea and especially lentil cakes tended to be characterized by pleasant aroma compounds arising from the Maillard reaction. Nonetheless, further studies need to be carried out in order to gain more insight into the most odor-active compounds in pulse-based applications such as cakes.

This outcome indicates that flour type and pre-processing exert a considerable impact on the reactivity potential throughout cake making, thus enabling products to be tailored to the desired odor profile.

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5 ADDITIONAL RESULTS

5.1 PARTICLE SIZE DISTRIBUTION OF CAKES

Whether similar particle sizes between the cakes was achieved with the grinding protocol applied could be confirmed by analysis of their volume distribution by laser diffraction (**§ 6.1 of Part IV**). As can be seen in **Figure 54**, the particle sizes of all products were in a very similar size range.

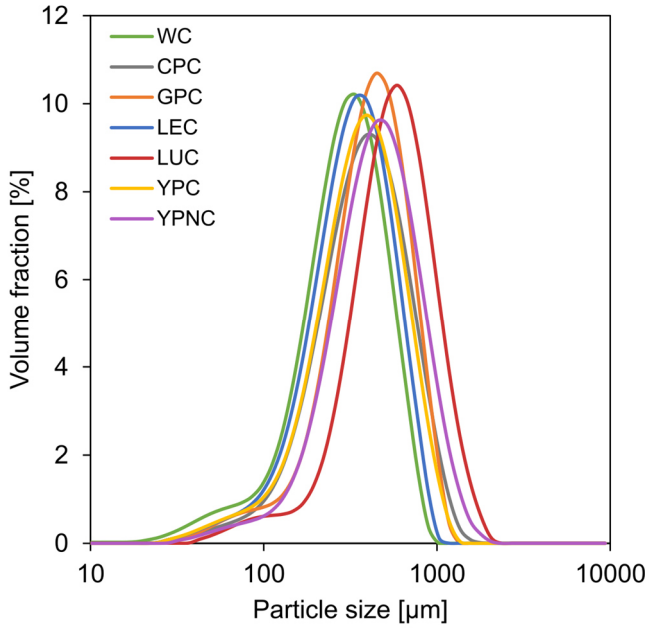

Figure 54. Particle size distribution of ground sponge cakes. WC = wheat cake, LUC = lupin cake, LEC = lentil cake, CPC = chickpea cake, GPC = green pea cake, YPC = yellow pea cake, YPNC = cake based on yellow pea flour processed and stored under nitrogen.

5.2 PRECURSORS FOR THERMAL REACTIONS

As discussed in **§ 12 of Part II**, amino acids and sugars are very important precursors to generate a wide variety of different volatiles, including pyrazines, Strecker aldehydes and furanic compounds. Therefore, we kindly commissioned Improve (Dury, France) to perform the analysis of the free amino acid and sugar composition of the flours. The results are presented in **Table 27**.

As discussed in the article, increased concentrations of certain amino acids could possibly help explain the higher susceptibility of LEF and CPF to pyrazine formation. By contrast, the detected concentrations could not be used to interpret the different abilities of the flours to form Strecker aldehydes, since the levels of relevant precursors (see **Table 17 of Part II**) were below the detection limit of 0.02%. Unfortunately, this was also the case with regard to sugars, which implies that more sensitive analysis methods would be needed.

Table 27. Concentration of free amino acids as well as mono- and disaccharides in the different flours.

Compound	Concentration [g/100g]						
	WF	LUF	CPF	LEF	GPF	YPF	YPNF
Free amino acids							
Cysteine	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Methionine	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Glycine	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Threonine	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Tyrosine	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Lysine	<0.02	0.05 ± 0.03	0.04 ± 0.03	<0.02	<0.02	<0.02	<0.02
Histidine	<0.02	0.05 ± 0.03	<0.02	<0.02	<0.02	<0.02	<0.02
Serine	<0.02	0.02 ± 0.03	<0.02	0.09 ± 0.03	<0.02	<0.02	<0.02
Alanine	<0.02	0.03 ± 0.03	<0.02	0.02 ± 0.03	<0.02	0.01 ± 0.00	0.01 ± 0.00
Aspartic acid	0.02 ± 0.03	0.09 ± 0.03	0.03 ± 0.03	0.06 ± 0.03	0.04 ± 0.03	0.03 ± 0.00	0.03 ± 0.00
Glutamic acid	<0.02	0.15 ± 0.03	0.11 ± 0.03	0.13 ± 0.03	0.15 ± 0.03	0.17 ± 0.01	0.17 ± 0.01
Arginine	<0.02	0.72 ± 0.06	0.4 ± 0.03	0.32 ± 0.03	0.09 ± 0.03	0.19 ± 0.02	0.19 ± 0.02
Isoleucine	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Proline	<0.02	0.04 ± 0.03	<0.02	<0.02	<0.02	0.03 ± 0.00	0.03 ± 0.00
Phenylalanine	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Valine	<0.02	0.04 ± 0.03	0.05 ± 0.03	0.03 ± 0.03	0.03 ± 0.03	<0.02	<0.02
Leucine	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Tryptophane	<0.02	<0.02	0.06 ± 0.01	<0.02	<0.02	<0.02	<0.02
Mono- & disaccharides							
Glucose	<0.2	<0.2	<0.2	<0.2	<0.2	0.9 ± 0.7	<0.2
Fructose	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Sucrose	0.2 ± 0.3	3.4 ± 0.9	2.7 ± 0.8	0.8 ± 0.5	2.9 ± 0.9	2.1 ± 0.7	2.3 ± 0.8
Lactose	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Maltose	1.5 ± 0.6	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2

5.3 COLOR PROPERTIES OF THE BATTERS

The important role of the raw materials in the coloring of the cake crumbs could be verified by analyzing the colors of the corresponding batters. These are illustrated in **Figure 55** as color charts, which were obtained by conversion of the CIEL*a*b* color values into RGB values. A similar trend in the colors is visible compared to the cake crumb. In particular the batter made with lupin flour appears yellowish, whereas the batter based on green pea flour has a greenish tint.

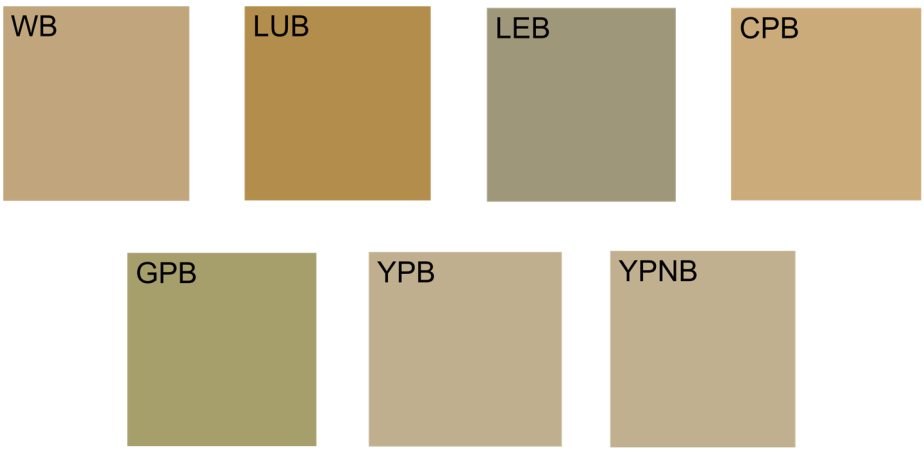

Figure 55. Color charts of sponge cake batters made with different flours. WB = wheat batter, LUB = lupin batter, LEB = lentil batter, CPB = chickpea batter, GPB = green pea batter, YPB = yellow pea batter, YPNB = batter based on yellow pea flour processed and stored under nitrogen.

6 FINAL CONCLUSION

This study demonstrated that the complete replacement of wheat flour with pulse flours can result in products with an interesting appearance and texture. Moreover, the combination of different analytical methods used in this study made it possible to gain deeper insights into the complexity of the reactions that take place during the development of sponge cakes based on wheat or pulse flours. The higher susceptibility of products made using pulses could be followed throughout the cake development by analysis of intermediate and final reaction markers. The latter are particularly interesting in cakes, as they can influence their sensory quality. By associating the identified volatile compounds with their aroma descriptors reported in the literature, a good understanding of the potential odor differences between the products could be obtained. From this it could be deduced that the choice of flour and its preprocessing can have an essential influence on the aroma quality of the developed foods.

However, it may be possible that not all of the VOCs that were detected impart odor to the final cakes. It is therefore particularly interesting to identify the potential key odorants. Due to the sudden outbreak of COVID 19, our plan to carry out a GC-O analysis was unfortunately thwarted. A rapid and easier alternative is the determination of odor activities, which is described in the next chapter.

CHAPTER 2

ODOR ACTIVITY OF VOLATILES IN PULSE CAKES

Study 1 demonstrated the distinct ability of pulse flours to undergo a number of different reactions to a greater extent than wheat during the production of sponge cakes, thereby forming various volatile components. In order to assess the potential influence of these molecules on the overall aroma of the final products, the calculation of their odor activity values seems to be a promising approach. For this purpose, the determined concentrations of all identified VOCs need to be divided by their corresponding odor threshold value reported in the literature. The greater the odor activity value, the stronger the aroma power of the respective compounds. In the best case, the odor threshold values were measured in a matrix that corresponds to the examined object. However, since no threshold studies have been performed in cakes to date, we aimed to retrieve these values from studies that had been carried out in similar matrices, such as starch. Unfortunately, however, the available information using such systems is also very limited, which is why we had to base our calculations on odor threshold values that were determined in water. The results obtained are explained in the following study, which was published in the proceedings book of the 16th Weurman Flavour Research Symposium.

ODOUR COMPOUNDS IN THERMO-MECHANICALLY PROCESSED FOOD BASED ON PULSE INGREDIENTS

Proceedings of the 16th Weurman Flavour Research Symposium
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ABSTRACT

This study investigates the effect of pulse flours (chickpea, lentil, lupin, yellow and green pea) on the formation of key volatiles with likely impact on the odour of sponge cakes compared to a wheat reference. Twenty molecules with an odour activity value of five or higher were determined from a wide variety of volatile compounds identified using HS-SPME/GC-MS and quantified by internal isotope standards. While the majority of these originated from lipid oxidation, the remaining compounds were generated through Maillard reaction. Principle component analysis revealed that the lipoxidation-derived volatiles typically possessing green, fatty odours were mainly related to cakes made with lentil, chickpea, yellow and especially green pea flour. Among these, hexanal was assumed to be of substantial sensory importance based on its outstanding odour activity values. 3-Methylbutanal (malty, chocolate notes) was identified as most odour-active compound in all formulations and might have contributed to the overall flavour, in particular in wheat, green pea and yellow pea cakes. Moreover, lentil formulas were associated with 2-ethyl-3,5-dimethylpyrazine, which is known to be responsible for an earthy aroma. From these results it can be concluded that the raw material determines the potential to generate odour-active compounds during product development and mastering the reactions likely to occur might offer an opportunity to tailor the global flavour quality of food.

KEYWORDS

Lipid oxidation, Maillard reaction, odour activity, reactivity, cake

1 INTRODUCTION

The increasing interest for sustainable and plant-based eating practices has prompted the incorporation of pulses into the human diet. Owing to their high protein content and attractive nutritional value, pulses are emerging ingredients in baked goods, particularly in gluten-free or healthy food design applications. The substitution of traditional ingredients, such as wheat flour, by novel constituents might, however, alter the chemical reactivity during food processing due to a modified profile of nutrients with diverging susceptibilities to oxidative and thermal degradation. This can further be accompanied by the formation of volatile compounds, which in turn might affect the aroma quality of the final bakery products.

Therefore, this study aimed at identifying key volatile compounds with high probability to contribute to the aroma of sponge cakes made with different pulse flours (lentil, chickpea, lupin, green and yellow pea) in comparison to a wheat reference. Accordingly, the molecules identified using HS-SPME/GC-MS and quantified by internal isotope standards were associated with their odour activity value and submitted to multivariate data analysis to estimate the potent odours in the different pulse-based applications.

2 EXPERIMENTAL

2.1 SPONGE CAKE ELABORATION AND CHARACTERIZATION

Wheat and pulse cakes were elaborated as reported by Krause *et al.* (2021a) from 45% (w/w) pasteurized liquid whole eggs, 25% (w/w) sucrose, 25% (w/w) wheat or pulse flour and 5% (w/w) sunflower oil. Twenty-one cakes were generated per cakes recipe, applying the three-step development process described by Cepeda-Vázquez *et al.* (2018), comprising (1) whipping of sugar and eggs, (2) addition of flour and oil and (3) batter baking at 170 °C for 25 min. In the pulse-based formulations, wheat flour (T55, Grands Moulins de Paris, France) was completely replaced by certified organic flours from lentils (Celnat, France), chickpeas (Celnat, France), lupins (Moulin Meckert-Diemer, France), green peas (Moulin Meckert-Diemer, France) and yellow peas (Improve, France). Immediately after baking, samples were deep-frozen at -20 °C in hermetically closed glass vessels until chemical analysis.

For each recipe, composite sampling was performed on ten cakes randomly chosen and ground in frozen state to a similar particle size distribution (d50 ~250 µm). Reproducibility and repeatability of processing conditions were assessed at each step of cake development by means of classic physical and chemical measurements, including dry matter content, pH value, density and colour, as well as HS-SPME/GC-MS analysis (Krause *et al.*, 2021a).

2.2 IDENTIFICATION AND QUANTIFICATION OF VOLATILE ORGANIC COMPOUNDS

Gas chromatographic analysis of volatile compounds was carried out as described by Krause *et al.* (2021a). Samples (2 g) were weighed in triplicates, spiked with 100 µL aqueous solution of deuterated internal standards (411 mg/L hexanal-d_{12}, 15 mg/L furfural-d_4 and 3 mg/L 2-methylpyrazine-d_6), hermetically sealed in 20 mL headspace vials with PTFE-coated silicon septa and stored overnight at 4 °C.

Head-Space Solid Phase Microextraction (HS-SPME) was performed by applying the following parameters: DVB/CAR/PDMS fibre (50/30 µm, 2 cm, Supelco); incubation (18 min) and extraction (42 min) at 50 °C. Analyses were carried out using a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific) equipped with a ZB-wax capillary column (30 m × 0.25 mm × 0.5 µm). Compounds were thermally desorbed at 250 °C during 2 min and separated using the oven temperature program from 40 °C with an isotherm of 5 min to 240 °C at a rate of 4 °C/min. Helium carrier gas was used at a flow rate of 1.2 mL/min.

For detection, an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific) was operated in the EI mode at 70 eV, scanning

masses from *m/z* 33 to 300 and in SIM mode for selected molecules. Compounds were identified through mass spectra libraries search (NIST 08, Wiley 8) and by comparing their experimental retention indices obtained using a series of *n*-alkanes (C5-C21) with those from the NIST database. Hexanal, furfural and 2-methylpyrazine were quantified using their deuterated internal standards by isotopic dilution method. In addition, relative quantification of other specific classes of compounds was performed using 2-methylpyrazine-d_6 for nitrogenous-containing heterocycles, furfural-d_4 for furanic compounds and hexanal-d_{12} for the remaining compounds (aldehydes, ketones, alcohols).

2.3 Determination of odour activity values

Odour activity values of the extracted compounds were determined as the ratio of compound concentration to its odour threshold in water described in literature. Nonetheless, sponge cakes are complex food systems, for which reason the utilization of odour threshold values in water can only give an idea of the most powerful odorants in cakes. Odour thresholds in matrices similar to cakes, such as starch, would give better insights into the odour activity values of individual compounds. However, this information is only available for a limited number of molecules in literature, for instance in simple starch models, which, however, are still far from the realistic cake matrix. Consequently, in order to take this uncertainty into account, only odour activity values ≥5 were considered for multivariate data analysis. Moreover, this value helped to select and hierarchise those compounds with probably highest contribution to the odour of the final products.

2.4 Statistical analysis

One-way analyses of variance (ANOVA) were performed on determined concentrations of volatile compounds and significant differences evaluated by Tukey's test at $p<0.05$, using SAS studio version 3.8 (SAS Institute Inc., Cary, NC, USA). Principal component analysis (PCA) was conducted on both concentration and odour activity values of extracted volatiles using XLSTAT version 2020.1 (Addinsoft, Paris, France).

3 Results and Discussion

Complex profiles of volatile organic compounds were detected in the wheat and different pulse-based cakes, which mainly belonged to the chemical classes of alcohols, aldehydes, ketones, pyrazines and furans. In order to identify the compounds that are likely to affect the odour of the individual cakes, the odour activity values of all 59 identified volatiles were calculated and those with an odour activity value of 5 or higher selected.

As shown in **Table 28**, a total of 20 odour-active molecules was found with an odour activity value of 5 or higher. Among these, the majority of components were identified as typical markers of lipid oxidation, denoting the degradation of unsaturated fatty acids in the presence of oxygen, which can be either catalysed by endogenous lipoxygenase or triggered by heat, light and metals (Murat *et al.*, 2013; Rackis *et al.*, 1979). In the case of sponge cakes, lipid oxidation is assumed to be initiated during batter beating, attributable to the entrapment of atmospheric oxygen while mixing the ingredients into a foam, and persists during baking due to heat-induced autoxidation (Krause *et al.*, 2021a; Maire *et al.*, 2013). In the course of this reaction, hydrogen atoms are abstracted from the unsaturated fatty acids, thereby yielding hydroperoxides, which in turn can be converted into volatile compounds *via* subsequent reactions such as β-scission (Belitz *et al.*, 2009). The odour-active molecules generated through such pathway and extracted from the diverse cakes were majorly deriving from linoleic acid (C18:2). Typical representatives included pentanal, hexanal, heptanal, octanal, 2-heptenal, 2-octenal and 2,4-decadienal as well as 1-octen-3-ol and 2-pentylfuran (**Table 28**) (Adams *et al.*, 2008; Belitz *et al.*, 2009; Murray *et al.*, 1976). However, hexanal and 2-heptenal are likewise known as possible oxidation products of linolenic

Table 28. Odour activity values, odour thresholds in water (OT) and odour descriptors of volatiles identified in sponge cakes made with wheat (WC), lupin (LUC), chickpea (CPC), green pea (GPC), lentil (LEC) or yellow pea flour (YPC). Only key compounds with odour activity values ≥5 are shown.

Compound	Odour activity value						OT	Odour descriptor
	WC	LUC	CPC	GPC	LEC	YPC	[µg/L]	
3-Methylbutanal	10264	8163	8963	10570	5802	11600	0.2[d]	Malty, roasty, chocolate[a,b,g]
Hexanal	327	183	2641	5266	2675	3502	4.5[d]	Green, grassy, tallow[a,b]
2,4-Decadienal			1174	1991	1209	1256	0.07[d]	Deep fat fried[b]
2-Ethyl-3,5-dimethylpyrazine	352	535	786	781	1207	869	0.04[d]	Earthy[b]
2-Methylbutanal	557	468	606	652	353	742	3[d]	Almond, malty[b]
2-Methylpropanal	108		523		271		1[f]	Malty[b]
Pentanal	19	14	162	248	100	215	12[e]	Pungent, strong acrid[b]
Nonanal	34	49	161	204	168	147	1[d]	Citrus, soapy[a,b]
1-Octen-3-ol	15		132	289	142	179	1[d]	Mushroom, vegetable[b,c]
Octanal	23	28	133	151	112	119	0.7[d]	Citrus, flowery[a,b]
Heptanal	25	17	122	140	117	120	3[d]	Green, fatty, rancid[a,c]
2-Octenal			33	72	33	43	3[d]	Fatty, nutty, roasted[b]
2-Pentylfuran	10	5	57	36	48	38	6[d]	Green, floral, fruity[a]
Phenylacetaldehyde	16	28					4[d]	Honey-like, sweet[a,b]
2,3-Butanedione	27						3[d]	Buttery, caramel[a,b]
2,3-Pentanedione	11	13	12	7	13	13	20[d]	Buttery[b]
Acetaldehyde	8	8	10	17		14	15[e]	Fruity[b]
Butanal			8	13	10	13	9[d]	Malty[b]
2-Heptenal			5	13	5	6	13[d]	Green, fatty[b]
2-Hexenal				5	5		17[d]	Green, fatty[b]

[a] from Birch *et al.* (2013)
[b] from Pico *et al.* (2015)
[c] from Murat *et al.* (2013)
[d] from Buttery *et al.* (1998)
[e] from Buttery *et al.* (1988)
[f] from Belitz *et al.* (2009)
[g] from Rega *et al.* (2009)

acid (C18:3), whereas heptanal and octanal could have alternatively originated from oleic acid (C18:1) (Belitz *et al.*, 2009; Murray *et al.*, 1976). Both oleic and linolenic acid might have also been the precursors of the aldehydes nonanal and 2-hexenal, respectively (Belitz *et al.*, 2009; Murray *et al.*, 1976).

Apart from these lipoxidation markers, the remaining odour-active components were associated with the thermal-induced breakdown of sugars and amino acids mainly *via* Maillard reaction occurring during batter baking. These included Strecker aldehydes (2-methylpropanal, 2-methylbutanal, 3-methylbutanal, acetaldehyde, phenylacetaldehyde), 2,3-butanedione, 2,3-pentanedione and 2-ethyl-3,5-dimethylpyrazine (**Table 28**). Maillard reaction involves the condensation of amino acids and reducing sugars at elevated temperatures to produce aminoketoses (Amadori compounds) or aminoaldoses (Heyns compounds) (Belitz *et al.*, 2009). In subsequent steps, these constituents are degraded through enolization, dehydration and hydrolysis into 1-deoxyosones or 3-deoxyosones (Belitz *et al.*, 2009). Owing to their high reactivity, these α-dicarbonyl compounds are likely to undergo further reactions, resulting in the release of a wide variety of molecules. Among these, Strecker aldehydes are commonly found, deriving from the reaction of α-dicarbonyl

compounds with amino acids (Belitz *et al.*, 2009). In the present study, multiple Strecker aldehydes were identified as key odour-active compounds in the diverse cake formulations, comprising 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, acetaldehyde and phenylacetaldehyde, which originated from valine, isoleucine, leucine, alanine and phenylalanine, respectively (Belitz *et al.*, 2009). In addition to Strecker aldehydes, the nitrogen-containing compound 2-ethyl-3,5-dimethylpyrazine was present, being characterized by a very low odour threshold (**Table 28**). This heterocyclic molecule may have been formed from α-aminocarbonyls obtained next to the Strecker aldehydes in the condensation reaction of α-dicarbonyl compounds and amino acids. It is generally supposed that two α-aminocarbonyls react under formation of dihydropyrazine, which in turn can be substituted to create alkylpyrazines [9]. In this context, the generation pathway of 2-ethyl-3,5-dimethylpyrazine is postulated to involve the incorporation of acetaldehyde into the corresponding dihydropyrazine carbanion (Shibamoto *et al.*, 1979). Acetaldehyde could not only emerge from Strecker degradation as previously discussed, but likewise from oxidation of ethanol (Shibamoto *et al.*, 1979), an alcohol that was largely present in the diverse batters and cakes (data not shown). Moreover, two further compounds with pronounced odour activity value were determined, namely 2,3-butanedione and 2,3-pentanedione (**Table 28**), which have been reported to likewise originate from Maillard reaction (Yaylayan *et al.*, 2000). According to Yaylayan *et al.* (1999) and Yaylayan *et al.* (2000), the former structure might be either directly formed from glucose upon dehydration and retro-aldol cleavage or from the α-dicarbonyl compound 3-deoxyosone *via* pyruvaldehyde, followed by chain elongation by one carbon atom coming from amino acids like glycine. The latter pathway can be similarly applied to describe the production of 2,3-pentanedione, however requiring the incorporation of two carbon units into pyruvaldehyde, which, for instance, could be donated by alanine (Yaylayan *et al.*, 2000). The authors further underlined that both structures could be considered precursors for Strecker degradation (Keyhani *et al.*, 1996).

The twenty odour-active compounds listed in **Table 28** were subsequently subjected to principle component analysis in order to visualize how they drive the discrimination of the wheat and pulse cakes.

As depicted in **Figure 56**, PC1, PC2 and PC3 accounted for 63.61%, 19.54% and 8.72% of total variability. Along PC1, two groups of highly correlated cakes were distinguished. While wheat cakes (WC) and lupin cakes (LUC) clustered in the negative part of PC1, positive loadings were associated with cakes made from lentil (LEC), chickpea (CPC) green pea (GPC) and yellow pea (YPC). This differentiation was majorly driven by lipoxidation markers, which were characterized by positive signs, and thus, were directly linked to the second group of cakes. From these findings, it could be concluded that the unsaturated fatty acids contained in the associated flours possessed higher susceptibility towards oxidative degradation, hence promoting the generation of lipoxidation-derived volatiles. According to Krause *et al.* (2021a), chickpea, lentil, yellow pea and green pea flours were not only significantly enriched in oleic, linoleic and linolenic acid compared to wheat and lupin, but concurrently comprised lipoxygenase with considerably higher activity, thus accelerating fatty acid oxidation. Following the general consensus of literature, the thereby released volatiles are typically responsible for a green, citrus and fatty odour (**Table 28**) (Birch *et al.*, 2013; Murat *et al.*, 2013; Pico *et al.*, 2015). Owing to its outstanding odour activity values among all lipoxidation markers identified, ranging from 183 to 5266, hexanal was assumed to be of substantial sensory importance. Interestingly, green pea cakes were characterized by the highest odour activity values of almost all lipoxidation markers. From this it was inferred that the associated green notes will probably be strongly perceived in this product. Lupin and wheat cakes, on the other hand, were correlated in the negative part of PC1 with phenylacetaldehyde, 2,3-butaedione and 2,3-

pentanedione, probably emerging from Maillard reaction. These molecules are generally described to exert sweet, buttery and caramel-like odours (Birch *et al.*, 2013; Pico *et al.*, 2015), however, had only low odour activity values (7-28) (**Table 28**).

Further distinction of the individual cakes was obtained by consideration of PC2. As visible from both PC1 vs PC2 and PC3 vs PC2 biplots, the yellow and green pea products as well as the wheat cakes were spotted in the positive part of PC2, hence being associated with 3-methylbutanal, 2-methylbutanal and acetaldehyde formed during Strecker degradation (**Figure 56**). Of all odour-active compounds observed, 3-methylbutanal appeared to have the highest odour activity values in all products, varying from 5802 to 11600 (**Table 28**). This molecule is known to cause a malty, roasty and chocolate odour (Birch *et al.*, 2013; Pico *et al.*, 2015; Rega *et al.*, 2009), which is likely to be easily perceived in the various cakes and could have been a key odorant especially in cakes made with wheat, yellow and green pea flours. In these products, the malty odour might have been intensified by 2-methylbutanal, although the correlated odour activity values were relatively lower. From the PC3 vs PC2 biplot, it further emerged that chickpea and lentil cakes were linked to 2-methylpropanal likewise reported to possess a malty but also almond-like aroma (Pico *et al.*, 2015), however having similar odour activity values as 2-methylbutanal (**Table 28**). Consequently, it could be concluded that Strecker degradation was an important chemical reaction occurring in all products, yielding substances with similar flavour despite different amino acid precursors.

Lentil cakes further differed from the other cakes in the clear correlation with 2-ethyl-3,5-dimethylpyrazine (earthy notes) linked with negative loadings on PC2. **Table 28** indicates a very low odour threshold for this heterocycle (Pico *et al.*, 2015), for which reason it was supposed to be a strong contributor to the aroma especially of lentil cakes. Krause *et al.* (2021a) assigned the higher ability of lentil flour to form pyrazines during sponge cake development to the exclusive presence of specific amino acids known to favour pyrazine production.

Interestingly, the discussed discrimination of the cakes based on the odour activities of relevant volatiles was comparable to that based on the concentrations of all extracted volatiles as reported by Krause *et al.* (2021a). This indicated that the selection of key odour-active compounds did not distort the global positioning of cakes on the PCA biplots and thus seemed to be an adequate approach to describe principle differences between the products.

In general, it needs to be considered that the overall odour of food is affected by a complex mixture of many odour-active compounds occurring at specific proportions. Consistently, the results presented in this study are primarily helping to understand and predict major differences between thermo-mechanically treated products prepared from diverse raw materials. Further techniques should thus be applied to gain deeper insight into the sensory discrimination of bakery products, such as cakes.

4 CONCLUSION

Total replacement of wheat by pulse flours seemed to considerably change the profile of volatile compounds with potent odour in sponge cakes. The volatile compounds were extracted from the individual bakery products by means of HS-SPME/GC-MS and quantified using isotopic dilution of internal standards. Odour activity values of all analysed components were determined on the basis of their calculated concentrations and known odour threshold values. Those molecules with an odour activity value greater than or equal to five were selected as main odour contributors and submitted to principle component analysis in order to evaluate their role in the discrimination of the diverse cakes. The majority of the twenty aroma-active compounds appeared to originate from the oxidative degradation of unsaturated fatty acids, while the remaining components were assigned to the thermally induced breakdown of sugars and amino acids mainly via Maillard reaction. This implied that different chemical

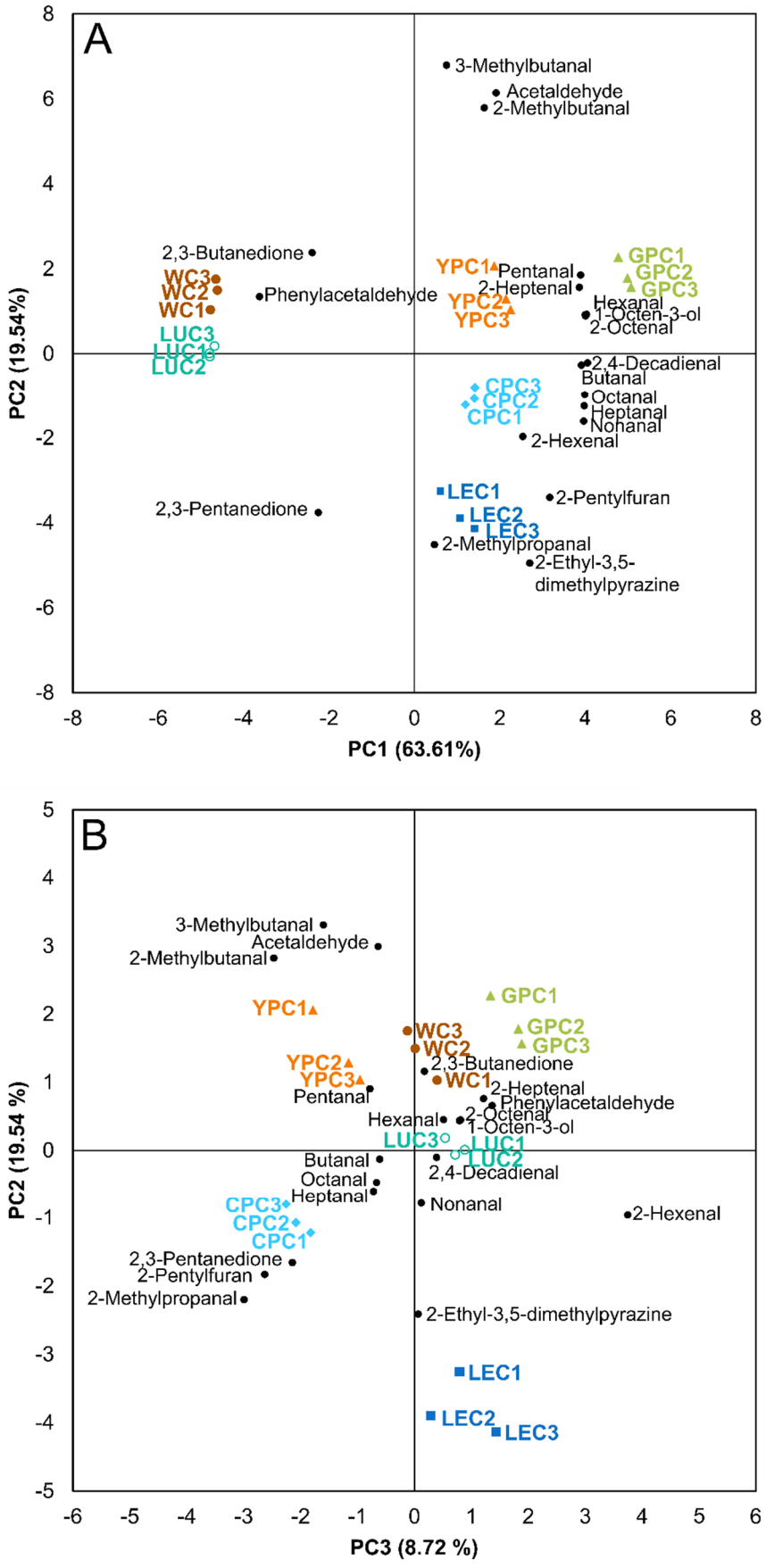

Figure 56. Biplots of (A) PC1 and PC2 and (B) PC2 and PC3 on odour-active compounds in sponge cakes made with wheat (WC), lupin (LUC), chickpea (CPC), green pea (GPC), lentil (LEC) or yellow pea flour (YPC).

reactions took place during sponge cake making, through which diverse compounds were released with interesting odour characteristics.

Multivariate data analysis disclosed that the pulse-based cakes mainly differed from the wheat cakes in being strongly correlated with lipoxidation markers usually possessing green, fatty odours. Among these pulse products, lupin represented an exception as it was rather related to Maillard-derived compounds with known buttery, sweet notes, which were concurrently associated with the wheat cakes. Nonetheless, Maillard reaction also seemed to play a not negligible role in the pulse-containing products. In the course of this reaction, Strecker aldehydes seemed to be released with probable sensory importance (typically malty odours) in all samples examined, particularly in wheat, yellow pea and green pea cakes. In addition, lentil cake could be further discriminated by its association with 2-ethyl-3,5-dimethylpyrazine, which was suggested to be responsible for an earthy aroma.

These results highlight that diverse odour-active volatile compounds can be produced during development of baked goods dependent on the raw material used. Therefore, the right choice of ingredient type is crucial in processing food with designed sensory attributes.

ACKNOWLEDGEMENTS

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5 FINAL CONCLUSION

The link between flour type and potential cake aroma, which was initially established in study 1 only on the basis of the complex multitude of VOCs, could be underlined by the determination of the odor activity values of selected volatiles, even though the odor threshold values used had not been determined in cakes or similar matrices but in water. The combination of these methods is therefore a promising approach to rapidly obtain information about the possible sensory quality of food.

CHAPTER 3

EFFECT OF BATTER BEATING ON REACTIVITY AND STRUCTURE

In Study 1, yellow pea flour in particular emerged as a highly reactive raw material that promoted oxidative processes during batter preparation. As discussed there, this was mainly due to an exceptionally high activity of the enzyme lipoxygenase, which catalyzed the breakdown of abundantly present linoleic and linolenic acids into volatile organic compounds. Since these molecules are often described in the literature as undesirably green and beany, we wanted to understand how the application potential of peas in food products can be exploited despite their reactivity. This was particularly important because peas are not only highly attractive because of their nutritive value but also because they are one of the most commonly grown pulses and are therefore globally available at a low price. Consequently, a first approach was to gain a better understanding of which step in cake preparation is the relevant trigger for oxidative processes. For this purpose, the beating process of the batters was modified in order to change not only the amount of air incorporated but also the exposure time of lipoxygenase to its substrates. Since the enzyme and the majority of fatty acids relevant for lipid oxidation are known to be contained in the flour and oil, these ingredients were selected and the mixing time after their addition extended. To guarantee that the prolonged mixing did not lead to the collapse of the batter, a preliminary experiment was performed to carefully select the maximum beating times. The details are described in **§ 3.2 of Part IV**. The sunflower oil was purified from endogenous antioxidations in order to maximize the oxidative response. Since the change in the process may not only affect the extent of chemical reactions, but also the structural properties of the batter, an innovative strategy of coupling the quantification of reaction markers (conjugated dienes, peroxide value, volatile compounds) with the analysis of the batter microstructure (by confocal laser scanning microscopy) was implemented. This approach was important to clarify the influence of the structure on the reactivity potential in complex formulated products. The results were combined in a publication that was submitted to the journal LWT and will be presented in the following pages.

LIPID OXIDATION DURING THE BEATING OF CAKE BATTER CONTAINING YELLOW PEA (*PISUM SATIVUM* L.) FLOUR

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HIGHLIGHTS

- Extended beating of flour and oil favors the formation of volatile compounds
- 13- and 10-lipoxygenases are the main catalysts that oxidize fatty acids
- The degree of lipid oxidation depends on the batter microstructure and mixing time
- Reactivity increases in line with the surface of lipid droplets and air bubbles

ABSTRACT

The aim of this study was to understand the impact of ingredients and processes on reactivity during sponge cake batter making using yellow pea flour. Numerous volatiles were identified, mainly originating from the degradation of linoleic and linolenic acid catalyzed by 10- and 13-lipoxygenases. The extent of lipid oxidation depended on the beating process, owing to the effect on local ingredient distribution and the exposure time of lipoxygenase to its substrates. Although prolonged beating after the addition of either flour or oil did not favor volatile formation, the latter led to elevated quantities of hydroperoxides. This intensified oxidation was assigned to an improved homogeneity and reduced size of lipid droplets and air cells. A sequential increase in both mixing times significantly increased the amount of volatiles, which could be attributed to a larger reaction interface and longer contact time between reactive ingredients. These results highlight the importance of parameter controls to limiting the generation of volatiles with odor activity.

KEYWORDS

Volatile organic compounds, confocal microscopy, structure, hydroperoxide, legume

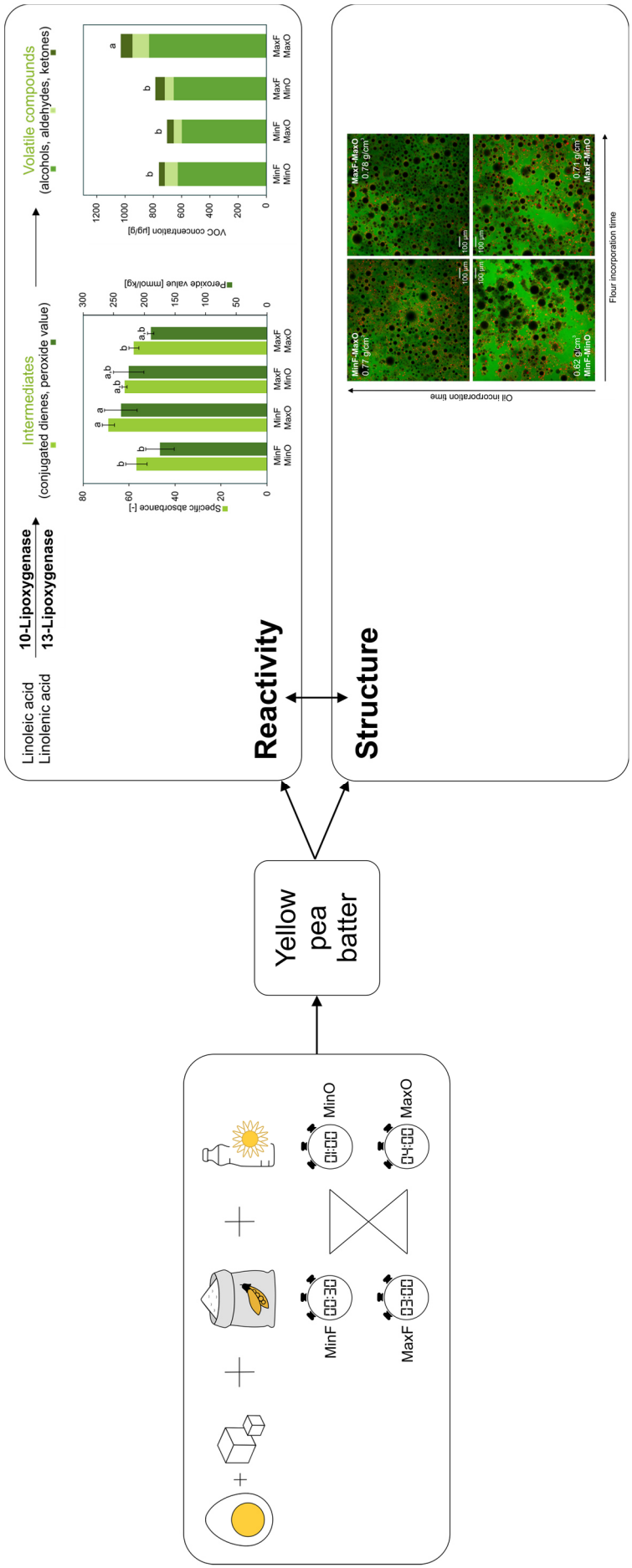

Figure 57. Graphical abstract of article 2.

1 INTRODUCTION

Growing interest in plant-based products to improve health, environmental sustainability and food security is continuously driving the incorporation of legumes in the human diet (Day, 2013; Hall *et al.*, 2017). Among these, pea (*Pisum sativum* L.) represents a popular legume crop due to its abundant availability, low cost, attractive source of high-quality protein and low glycemic index (Dahl *et al.*, 2012; Lam *et al.*, 2018; Marinangeli *et al.*, 2011). Whole pea flour, in particular, can be considered an emerging ingredient for various food formulations because of its low degree of refining as opposed to isolated pea proteins.

Nonetheless, the applicability of such novel flour ingredients to replace traditional raw materials remains a challenge for the food industry because of their differing chemical composition which might alter reactivity during food processing, and hence product quality and safety. In the case of pea, exploitation of the benefits is especially limited because of the occurrence of inherent odor-active compounds and precursors to green-beany off-flavors (Krause *et al.*, 2021a). These volatile organic compounds typically originate from the lipoxygenase-catalyzed degradation of unsaturated fatty acids in the presence of oxygen (Hall *et al.*, 2017; Sessa *et al.*, 1977). Lipoxygenase (EC 1.13.11.12) is an iron-containing enzyme occurring in the raw material which introduces dioxygen into the carbon chain of fatty acids with a *cis,cis*-1,4-pentadiene system as found in linoleic and linolenic acid (Belitz *et al.*, 2009). In the course of this reaction, hydroperoxides are formed and are subsequently cleaved into secondary structures, such as odorous alcohols, aldehydes and ketones, all with a probable impact on the sensory properties of the final food (Belitz *et al.*, 2009; Krause *et al.*, 2021b).

Lipid oxidation plays a particular role in food systems that contain a complex mixture of different ingredients that undergo beating. This process is characteristic of the production of baked goods such as sponge cakes, which can be prepared using pea flour (Krause *et al.*, 2021a). The basis for these products is a foam obtained by whisking egg and sugar, into which flour particles are gently dispersed and oil droplets emulsified. During this mixing process, air and thus oxygen is entrapped in the batter matrix, which can trigger the oxidative degradation of unsaturated fatty acids (Krause *et al.*, 2021a; Maire *et al.*, 2013). The occurrence of this phenomenon when using pulse flours, and particularly pea flour, compared to traditional wheat flour was demonstrated in our previous publication (Krause *et al.*, 2021a). It is therefore of considerable importance to gain a clearer understanding of the options available to control lipid oxidation during batter making so as to improve the sensory quality of cakes based on pulse flours. The aforementioned study also showed that the existing processing strategy of producing pea flour under nitrogen did not mitigate oxidative reactions but, on the contrary, it intensified them (Krause *et al.*, 2021a). A new approach was pursued subsequently; this consisted in varying the process conditions during batter preparation and thus identifying the importance of individual process steps in the development of lipid oxidation markers during batter making using yellow pea flour. To achieve this, the mixing times after the incorporation of flour and oil were separately or jointly extended and their impact on the release of intermediate and final lipid oxidation products was analyzed. At the same time, process-induced modifications to the batter microstructure were recorded so that their relevance to the degree of lipid oxidation could be determined.

2 MATERIALS AND METHODS

2.1 INGREDIENTS

Yellow pea flour (23.8% protein, 47.6% starch, 8.8% moisture, 8.2% dietary fiber, 3.5% ash) was supplied by Cargill (Vilvoorde, Belgium). Sucrose was purchased from Tereos (Lille, France), sunflower oil from Lesieur (Asnières-sur-Seine, France) and whole pasteurized liquid eggs from Ovoteam (Locmine, France).

2.2 CHEMICALS

Furfural-d_4 (≥96%) and 2-methylpyrazine-d_6 (98.4%) were obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada), hexanal-d_{12} (≥96%), Celite® 545, iodine (≥99.8%) and aluminum oxide from Sigma Aldrich (Saint-Quentin-Fallavier, France), isopropanol, *n*-heptane, sodium thiosulfate pentahydrate, isooctane, glacial acetic acid, potassium iodide and iodine (0.02 M) from Carlo Erba (Val de Reuil, France) and DyLight 488 and Bodipy 655/676 from Thermo Fisher Scientific (Illkirch, France).

2.3 PURIFICATION OF SUNFLOWER OIL

In order to evidence the progress of lipid oxidation during batter preparation, the sunflower oil was purified by stripping its tocopherols as described by Pernin *et al.* (2019). In brief, 1 L sunflower oil was filtered three times at reduced pressure (960 mbar) through 200 g aluminum oxide activated overnight at 200 °C. The success of stripping was verified by recording the absorbance at 290-299 nm in isooctane using a fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies, Les Ulis, France). The absence of absorbance bands within this wavelength range indicated the effective removal of tocopherols. The stripped oil was characterized by significantly lower peroxide values (2.31 ± 0.26 mmol O_2/kg) and specific absorbances values for conjugated dienes at 234 nm (1.52 ± 0.04) compared to the untreated oil (3.11 ± 0.08 mmol O_2/kg and 2.62±0.14, respectively) because stripping purifies oils from polar compounds in general. Immediately after preparation, the stripped oil was stored in glass bottles under nitrogen and in the dark at -20 °C until use within two weeks.

2.4 EXPERIMENTAL DESIGN FOR BATTER PREPARATION

Sponge cake batters were prepared according to the procedure published by Krause *et al.* (2021a). Egg (45% w/w) and sucrose (25% w/w) were thus whipped for 10 min into a stable foam, to which non-sifted pea flour (25% w/w) was added gently within 90 s. After mixing for 30 s, sunflower oil was slowly incorporated within 15 s and the batter then beaten for 60 s (**Figure 58**). This mixing protocol is denoted below as *MinF–MinO*.

Because of the known presence of relevant precursors or catalysts for lipid oxidation in flour and oil, it was deemed interesting to extend the mixing times after the incorporation of each ingredient. The aim was to alter not only the exposure time of the lipoxygenase contained in the flour to unsaturated fatty acids but also the amount of air, and thus oxygen, embedded in the batter, in order to identify the relevant process parameters that favor lipid oxidation and their effects on batter microstructure. A preliminary test was thus performed, during which beating after the addition of flour was prolonged from 30 s to 3 min (*MaxF*), followed by the extension of mixing after the addition of oil from 1 min to 4 min (*MaxO*). Over both periods, batter density was monitored in order to select maximal beating times after adding flour and oil that would be sufficiently long to modify air entrapment and exposure time of the enzyme to its substrates while concurrently

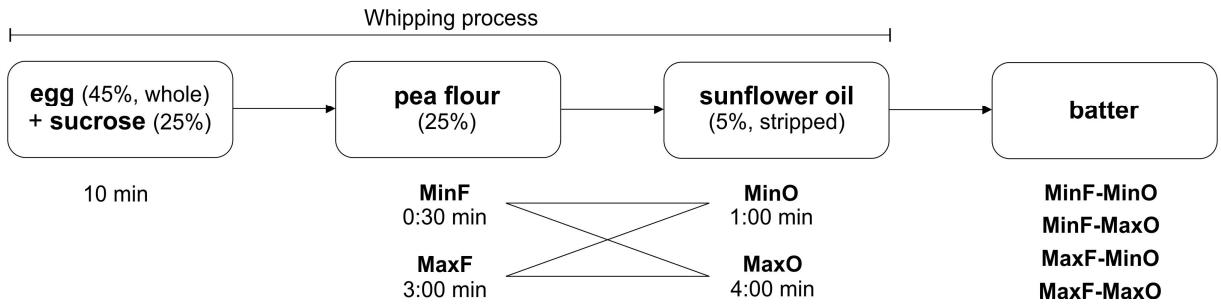

Figure 58. Schematic illustration of batter preparation using different incorporation times for flour and oil.

avoiding collapse of the batter structure. Interestingly, batter density changed only slightly over both time periods when compared to *MinF–MinO*. For this reason, an experimental plan was set up in which four batters were prepared by crosslinking the maximal limits thus selected with the minimum conditions described in *MinF–MinO* (**Figure 58**). Each batter was only made once because a preliminary test had confirmed insignificant variability ($p<0.05$) between three independently prepared *MinF–MinO* batters in terms of the analyses described in the sections below. In order to prevent further oxidative reactions after making, the batters were frozen immediately at -20 °C under nitrogen in glass vessels sealed with septum-containing lids until analysis.

2.5 Physical and chemical characterization of batters

2.5.1 Batter density

Batter density was determined in triplicate directly after preparation at room temperature by slowly drawing it up into 10 mL open-mouth syringes to prevent the destruction of gas cells. After weighing, density was calculated from the ratio of the mass of the batter to the volume of the syringes which was previously been defined using water.

2.6 Microstructure analysis by Confocal Laser Scanning Microscopy (CLSM)

The microstructure of the batters was analyzed using a Leica TC2 SP8 confocal laser scanning microscope (Wetzlar, Germany). 1 g freshly prepared product was stained with 3 µL of a mixture of DyLight 488 and Bodipy 665/676 (1/1 v/v) in order to the label proteins and lipids in green and red, respectively. After excitation at 488 nm (green) and 638 nm (red) using two lasers, the emitted fluorescence was detected in two channels within the wavelength ranges of 497-601 nm and 643-765 nm. Images were acquired at 10× magnification using a scan speed of 400 Hz, 70.7 µm pinhole and xyz scan mode.

14.3.1 pH value

To determine the pH, 2 g batter were dispersed in triplicate in 20 mL distilled water and the pH value recorded using a Sension+ PH3 meter (Hach, Loveland, CO, USA).

2.7 Analysis of lipid oxidation

2.7.1 Lipoxygenase (LOX) activity

LOX activity of the pea flour was determined in triplicate by adapting the method described by Delcros *et al.* (1998). Briefly, 20 mL phosphate buffer (pH 7.5) was homogenized with 2 g flour and centrifuged, and 20-200 µL of the supernatant was mixed with linoleic acid in phosphate buffer (pH 6.5). LOX activity was expressed as nmol oxygen consumed per second.

2.7.2 Fatty acid profile, conjugated dienes and peroxide value

The oxidation status of the batters was monitored by measuring conjugated dienes, peroxide values and the profile of fatty acids after the extraction of fatty matter according to the accelerated solvent extraction procedure described by Krause *et al.* (2021a). In brief, the batters were pounded in a mortar with Celite® 545 (10/9 w/w), transferred into 34 mL stainless-steel extraction cells and extracted for 25 min at 40 °C with a binary mixture of heptane and isopropanol (5/1 v/v) using a Dionex ASE 350 (Thermo Fisher Scientific, Waltham, MA, USA). After solvent evaporation at reduced pressure and 40 °C, the oil was deep-frozen at -20 °C under nitrogen until analysis of the reaction markers.

Conjugated dienes were determined in triplicate by adapting the method described by Roman *et al.* (2013). Firstly, dilutions of 10 mg extracted oil in isooctane (1:100-1:200) were prepared and the mixtures vortexed for 10 s. Absorbance was then recorded against isooctane in 1 cm quartz cuvettes at 234 nm using a Specord 210 (Analytik Jena, Savigny-le-Temple, France). The content in conjugated dienes was expressed as specific absorbance, which was defined as the absorbance of 1 g oil in 100 mL isooctane at 234 nm.

The **peroxide value** was analyzed in triplicate by iodometric titration according to Krause *et al.* (2021a). In brief, 200 mg extracted oil were dissolved in glacial acetic acid/isooctane (3/2 v/v, 16 mL), followed by the addition of saturated potassium iodide solution (1 mL) and distilled water (9 mL). Titration of the released iodine was performed using sodium thiosulfate (5 mM). The peroxide value was expressed as millimole of hydroperoxide per kg of oil.

The **profile of polyunsaturated fatty acids** was analyzed in triplicate as reported by Krause *et al.* (2021a). In short, 30 mg extracted oil were dissolved in petroleum ether (1 mL) containing heptadecanoic acid as standard, and the fatty acids were esterified at 52 °C for 40 s using NaOH in methanol (2 M, 200 µL). The reaction was stopped by HCl in methanol (1 M, 400 µL) and the fatty acid methyl esters (FAMEs) present in 1 µL of the upper phase were separated on a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a SP-2560 GC column (100 m × 250 µm × 0.2 µm, Supelco, Bellefonte, PA, USA). The oven program was set as follows: 100 °C isotherm (4 min); 100 °C to 200 °C at 5 °C/min; 200 °C isotherm (8 min); 200 °C to 250 °C at 5 °C/min, 250 °C isotherm (12 min). Helium carrier gas was used at a split flow of 448 mL/min. For detection, an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific) was operated at 250 °C, scanning masses from *m/z* 40 to 400. Compounds were identified by comparing the of mass spectra and retention times with those of a standard mixture containing 37 compounds (Supelco) subjected to the same procedure. Relative FAME quantities were calculated using the internal standard.

14.3.2 PROFILE OF VOLATILE ORGANIC COMPOUNDS (VOCS)

VOCs were extracted by HeadSpace-Solid Phase Micro Extraction (HS-SPME) using the optimized procedure reported by Krause *et al.* (2021a). Two grams of batter were weighed in triplicate in 20 mL glass vials, spiked with 100 µL of an aqueous solution containing 778 mg/L of the deuterated standard hexanal-d_{12} and sealed using aluminum caps with PTFE/silicone septa. After overnight storage at 4 °C to equilibrate the standard within the sample, the vials were incubated at 50 °C for 18 min in a Triplus autosampler (Thermo Fisher Scientific). Volatiles were absorbed on a DVB/CAR/PDMS fiber (50/30 µm, 2 cm, Supelco) for 42 min at 50 °C and finally desorbed for 2 min at 250 °C in a TRACE GC Ultra (10 mL/min) injector port.

The separation of VOCs was achieved on a ZB-wax capillary column (30 m × 0.25 mm × 0.5 µm) by increasing the temperature from 40 °C (maintained for 5 min) to 240 °C at 4 °C/min. Helium was used as the carrier gas (1.2 mL min). Mass spectrometry was performed using an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific) operated in the EI mode at 70 eV, scanning in full scan (TIC, *m/z* 33-300) and SIM mode for selected molecules. VOCs were identified by matching (i) experimental mass spectra with MS libraries (NIST 08, Wiley 8) and (ii) by comparing experimental retention indices obtained using a series of *n*-alkanes with the reference values of the NIST database. Hexanal was quantified by comparing the peak areas with those of the internal standard hexanal-d_{12}. Similarly, relative quantities were determined for the remaining compounds (alcohols, aldehydes and ketones).

2.8 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was performed and significant differences were evaluated by Tukey's test at $p<0.05$ using XLSTAT 2020.1 (Addinsoft, Paris, France).

3 RESULTS AND DISCUSSION

3.1 VOCS AS INDICATORS OF LIPID OXIDATION DURING BATTER BEATING

Table 29 shows the volatile organic compounds extracted from the yellow pea batters prepared using different mixing times after flour and oil incorporation.

A total of 18 different VOCs were identified in the batter produced according to the standard protocol (*MinF–MinO*), the majority of which are alcohols (10), followed by ketones (5) and aldehydes (4). From a quantitative point of view, alcohols were observed as the dominant group of volatiles (626 µg/g), and thus accounted for 82% of the total VOC concentration (760 µg/g). The remaining fifth was mostly composed of aldehydes (91 µg/g), while ketones contributed only marginally (43 µg/g).

According to the general consensus in the literature, most of the alcohols, aldehydes and ketones detected were typical markers of the oxidative degradation of unsaturated fatty acids. Nevertheless, molecules such as ethanol also appeared at high concentrations (226 µg/g), which were assumed to have emerged from the flour or during fermentation reactions. Among the lipid oxidation-derived compounds, 1-penten-3-ol and 1-hexanol were the most abundant in *MinF–MinO*, each reaching values of around 160 µg/g (**Table 29**). The formation pathway for 1-penten-3-ol likely involves the oxidation of linolenic acid to 13-hydroperoxide, which in turn can be cleaved to form 1-penten-3-ol (Fisher *et al.*, 2003; Gardner *et al.*, 1996; Salch *et al.*, 1995; Shen *et al.*, 2014). Alternatively, de Lumen *et al.* (1978) proposed its generation from 16-hydroperoxide deriving from the same fatty acid, which could be isomerized enzymatically into 1-penten-3-one, followed by a reduction to 1-penten-3-ol catalyzed by an alcohol dehydrogenase (Ma *et al.*, 2016). By contrast, 1-hexanol has been reported to arise from linoleic acid after peroxidation to the corresponding 13-hydroperoxide, from which hexanal can be released upon cleavage (Belitz *et al.*, 2009; Xu *et al.*, 2019). In a subsequent step, this aldehyde can be further transformed into 1-hexanol in the presence of an alcohol dehydrogenase (Belitz *et al.*, 2009; Jakobsen *et al.*, 1998). The occurrence of this consecutive reduction reaction in *MinF–MinO* was reflected by the lower relative concentration of hexanal compared to 1-hexanol (**Table 29**). Apart from the compounds mentioned above, other volatiles, similarly associated with the degradation of the 13-hydroperoxides described were also found. These included 3-hexen-1-ol (linolenic acid), 2-pentenal (linolenic acid) and 1-pentanol (linoleic acid), which however accumulated in considerably smaller amounts (1-51 µg/g) (**Table 29**) (Frankel, 2012b; Luning *et al.*, 1995; Przybylski *et al.*, 1995a; Schaich, 2013). Furthermore, Sakouhi *et al.* (2016) and Sánchez-Ortiz *et al.* (2013) had suggested that the ketone 3-pentanone, which totaled 32 µg/g, might have been produced through a similar reaction scheme.

Moreover, trace quantities of 1-octen-3-ol and 2-octenal were detected (1-6 µg/g), the synthesis of which is typically based on the oxidation of linoleic acid (Frankel, 2012b; Kakumyan *et al.*, 2009) Unlike the previous volatiles, this oxidative breakdown takes place under the formation of an intermediate non-conjugated 10-hydroperoxide (Belitz *et al.*, 2009; Luning *et al.*, 1995).

Interestingly, oxidation products of monounsaturated oleic acid were also present in low concentrations (1-4 µg/g) (**Table 29**), comprising 2-heptanone, 1-octanol and 2-octanone (Cao *et al.*, 2014; Kochhar, 1996; Przybylski *et al.*, 1995a).

In our previous publication on the ability of diverse pulse flours to generate VOCs during sponge cake making (Krause *et al.*, 2021a), we reported a significantly higher susceptibility of pulse flours to oxidative deterioration compared to wheat flour, which is traditionally used in bakery products. We postulated that LOX is the main catalyst of lipid oxidation during batter beating when using pulse flours, which could be attributed to a positive correlation between enzyme activity and VOC formation: while the sum of oxidation-derived alcohols, aldehydes and alcohols was negligible (<1 µg/g) in batters made with wheat flour, in which LOX was practically inactive (<20 nkat/g), those in batters based on flours from chickpeas, lentils or peas were several hundred higher because of LOX activities of up to 4350 nkat/g. In the present study, an average LOX activity of 5379 nkat/g yellow pea flour as well as a total relative VOC amount of 760 µg/g were determined, of which around 70% were assumed

Table 29. Occurrence of volatile organic compounds [µg/g batter] identified in yellow pea batters prepared using different mixing times after the addition of flour and oil (mean ± SD, *n* = 3). Relative amounts are estimated based on the internal deuterated standard hexanal-d_{12}.

Compound	Selected ion (*m/z*)	Experimental retention index	Reference retention index[1]	Relative concentration of volatile organic compounds [µg/g batter]			
				MinF–MinO	*MaxF–MinO*	*MinF–MaxO*	*MaxF–MaxO*
Alcohols							
Ethanol	45	944	942	226 ± 18^{a}	214 ± 48^{a}	237 ± 56^{a}	249 ± 16^{a}
1-Propanol	59	1047	1049	5.59 ± 0.39^{b}	5.75 ± 0.83^{b}	5.11 ± 0.48^{b}	7.56 ± 0.10^{a}
1-Butanol	56	1153	1147	4.47 ± 0.49^{a}	2.76 ± 0.65^{b}	2.73 ± 0.48^{b}	4.265 ± 0.076^{a}
1-Pentanol	70	1257	1255	51.2 ± 3.7^{b}	44.3 ± 6.7^{b}	41.4 ± 4.9^{b}	64.1 ± 1.2^{a}
1-Hexanol	69	1360	1360	159.4 ± 8.2^{a,b}	131 ± 23^{b}	123 ± 16^{b}	190.3 ± 2.6^{a}
1-Octanol	56	1567	1569	0.829 ± 0.044^{b}	0.78 ± 0.13^{b}	0.70 ± 0.10^{b}	1.226 ± 0.026^{a}
1-Penten-3-ol	57	1169	1165	161 ± 15^{c}	235 ± 20^{b}	171 ± 18^{c}	287.7 ± 9.9^{a}
1-Octen-3-ol	99	1457	1458	5.65 ± 0.35^{a}	4.42 ± 0.45^{b}	4.34 ± 0.65^{b}	6.64 ± 0.19^{a}
3-Pentanol	59	1047	1049	10.64 ± 0.96^{b}	15.6 ± 2.8^{a,b}	11.2 ± 2.8^{b}	17.56 ± 0.83^{a}
3-Hexen-1-ol	82	1392	1394	1.47 ± 0.17^{a}	1.48 ± 0.34^{a}	1.64 ± 0.27^{a}	1.79 ± 0.11^{a}
Total				*626 ± 48^{b}*	*655 ± 103^{a,b}*	*598 ± 99^{b}*	*830 ± 31^{a}*
Aldehydes							
Hexanal	56	1091	1091	83.4 ± 3.8^{b}	55.4 ± 2.0^{c}	50.2 ± 3.9^{c}	104 ± 12^{a}
2-Pentenal	84	1137	1132	2.76 ± 0.31^{c}	4.239 ± 0.038^{b}	2.83 ± 0.51^{c}	5.83 ± 0.77^{a}
2-Heptenal	83	1332	1332	3.79 ± 0.24^{b}	2.69 ± 0.35^{c}	2.72 ± 0.20^{c}	5.47 ± 0.26^{a}
2-Octenal	70	1437	1437	1.150 ± 0.043^{b}	0.846 ± 0.077^{c}	0.793 ± 0.073^{c}	1.54 ± 0.16^{a}
Total				*91.1 ± 4.4^{b}*	*63.1 ± 2.5^{c}*	*56.6 ± 4.7^{c}*	*117 ± 14^{a}*
Ketones							
2-Butanone	72	911	900	1.77 ± 0.13^{b}	1.97 ± 0.13^{b}	1.65 ± 0.21^{b}	2.51 ± 0.14^{a}
2-Heptanone	58	1189	1185	3.68 ± 0.23^{b,c}	4.69 ± 0.43^{b}	3.52 ± 0.53^{c}	6.50 ± 0.28^{a}
2-Octanone	58	1292	1297	1.997 ± 0.093^{b}	2.40 ± 0.24^{b}	1.99 ± 0.36^{b}	3.301 ± 0.061^{a}
3-Pentanone	86	985	986	32.1 ± 2.5^{c}	54.5 ± 4.5^{b}	37.5 ± 3.8^{c}	64.3 ± 1.7^{a}
3-Octanone	99	1261	1261	3.22 ± 0.22^{b}	3.07 ± 0.44^{b}	2.68 ± 0.42^{b}	4.260 ± 0.044^{a}
Total				*42.8 ± 3.2^{c}*	*66.6 ± 5.7^{b}*	*47.4 ± 5.3^{c}*	*80.9 ± 2.2^{a}*
Total				760 ± 56^{b}	785 ± 111^{b}	702 ± 109^{b}	1028 ± 46^{a}

to most likely originate from lipid oxidation. This corresponded to a concentration of typical oxidation volatiles that was more than 500 times higher compared to the wheat flour batter of our previous study (Krause *et al.*, 2021a). It was thus hypothesized that LOX might likewise have played a crucial role in VOC development through fatty acid breakdown in the present yellow pea batter. This enzyme targets the *cis,cis*-1,4-pentadiene system of polyunsaturated fatty acids, such as linoleic and linolenic acid, thereby introducing dioxygen into the carbon chain to form the corresponding hydroperoxides. In accordance with Krause *et al.* (2021a), the oxygen solubilized in the batter during beating seemed to be sufficient to trigger an instant reaction as soon as the dry ingredients were dispersed in the moist batter matrix.

Pea may contain three, four or five isoenzymes of LOX, which were grouped by Fischer *et al.* (2020) in one heat-stable LOX with a pH optimum above 8 and 13-regiospecificity (LOX-1) as well as three heat-labile LOX with optimal activity at pH 6-7 but different regiospecificities of 9, 10 and 13 (LOX-3, LOX-5 and LOX-2, respectively). The remaining LOX-4 has not yet been identified. Owing to the quantitative dominance of 1-hexanol and 1-penten-3-ol in pea batter, which has been shown to originate from the 13-hydroperoxides of linoleic and linolenic acids, it is reasoned that the activity of a 13-LOX was of major importance. Because the pH values of the batters were around 7.2 (**Table 30**), it could be imagined that LOX-1 and/or LOX-2 were active. This was further corroborated by the presence of traces of other molecules at the same time, which also arose from these 13-hydroperoxides. On the other hand, however, the occurrence of volatiles formed *via* 10-hydroperoxides might also indicate the catalysis of fatty acid oxidation by a 10-LOX (LOX-5).

Although LOX activity is generally assumed to be the main driver for lipid oxidation during batter beating, small amounts of VOCs arising from oleic acid were monitored. Based on the lack of a *cis,cis*-1,4-pentadiene system in this fatty acid, which is a necessary requirement for LOX catalysis, it was concluded that non-enzymatic lipid oxidation had taken place, but only to a limited extent. Ongoing autoxidation was supported by the presence of 2-heptenal (**Table 29**), which has been reported to arise from linoleic acid 12-hydroperoxide. To date, no 12-LOX has been detected in pea, so the enzymatically-controlled formation of 12-hydroperoxide, and hence 2-heptenal could be ruled out.

3.2 Effects of mixing times on VOC levels

As shown in **Table 29**, extending mixing times after the addition of either flour or oil from 30 s to 3 min (*MaxF–MinO*) and from 1 to 4 min (*MinF–MaxO*), respectively, as well as their combined extension (*MaxF–MaxO*) led to the release of identical volatile compounds but in different quantities.

In general, the individual extensions of either flour or oil beating times by 2.5 or 3 min, respectively, resulted in batters characterized by total VOC concentrations that did not differ significantly from that determined in *MinF–MinO* (785 µg/g and 702 µg/g vs. 760 µg/g). Apart from

Table 30. Density and pH value of yellow pea batters prepared using different mixing times after the addition of flour and oil (mean ± SD, n = 3).

Batter	Density [g/cm^3]	pH value [-]
MinF–MinO	0.62 ± 0.01^{c}	7.22 ± 0.03^{a}
MinF–MaxO	0.77 ± 0.02^{a}	7.23 ± 0.00^{a}
MaxF–MinO	0.71 ± 0.01^{b}	7.24 ± 0.02^{a}
MaxF–MaxO	0.78 ± 0.02^{a}	7.21 ± 0.01^{a}

Different letters within a column indicate significantly different means (p<0.05).

this, the percentage distribution of volatile levels between the chemical classes of alcohols, aldehydes and ketones remained almost constant. It was thus deduced that both mixing times were insufficient to drive the formation of volatile compounds. However, closer inspection of *MaxF–MinO* revealed that molecules known to originate from the oxidative degradation of linolenic acid (i.e. 1-penten-3-ol and 2-pentenal) appeared to be formed in increasing quantities (**Table 29**). Considering the proportions of the individual ingredients, as well as their fat content and fatty acid profile, it emerged that linolenic acid mainly derived from the egg and flour. This indicated that extending the mixing time after adding the flour increased the probability of contact between the enzyme and linolenic acid, thus leading to a higher proportion of linolenic acid-derived volatiles. This assumption was corroborated by non-significant differences in the levels of linolenic acid oxidation markers in *MinF–MaxO* compared to *MinF–MinO* (**Table 31**). During the short mixing after flour addition (30 s), competition for the active site of the LOX only occurred between the linoleic and linolenic acids arising from the egg and flour. The subsequent incorporation of oil rich in linoleic acid thus diluted the amount of linolenic acid in the batter. The extended exposure of LOX to this complex mixture of fatty acids therefore led to a similar VOC profile as that seen with the standard protocol.

A different picture emerged when both periods were extended at the same time, as the *MaxF–MaxO* batter thus produced was considerably enriched in all the lipid oxidation markers identified. In the case of alcohols, this increase was mainly ascribed to 1-penten-3-ol, reaching values around 288 µg/g, which corresponded to an approximately 80% increment compared to *MinF–MinO* (**Table 29**). Unlike the standard batter, this compound clearly dominated the profile of volatile compounds in *MaxF–MaxO*. Nevertheless, 1-hexanol and its precursor hexanal concurrently accumulated at high relative concentrations (190 and 104 µg/g, respectively), thus markedly contributing to the total VOC content. Interestingly, substantial increases in the amounts of minor compounds by up to 111% (2-pentenal) were likewise recorded. These changes were not only assigned to molecules known to originate from the LOX-mediated oxidation of linoleic and linolenic acid, but also to those arising from the autoxidation of oleic acid. It was therefore concluded that the combined prolongation of mixing times after addition of both flour and oil, reaching a total of 5.5 min, was sufficient to prompt not only enzymatic but also non-enzymatic lipid oxidation.

3.3 EVOLUTION OF LIPID OXIDATION AS A FUNCTION OF MIXING TIME

The GC-MS analysis of VOCs contained in the diverse batters revealed that extended mixing times for both flour and oil (*MaxF–MaxO*) favored molecule generation when compared to *MinF–MinO* (**§ 3.2**). By contrast, non-significant modifications were observed following the separate extension of beating times after the incorporation of either flour or oil (*MaxF–MinO* and *MinF–MaxO*, respectively). Volatile compounds, however, are the end products of fatty acid degradation, for which reason they are often

Table 31. Peroxide value, specific absorbance of conjugated dienes and profile of polyunsaturated fatty acids of yellow pea batters prepared using different mixing times after the addition of flour and oil (mean ± SD, n = 3).

Batter	Peroxide value [mmol O_2/kg fat]	Conjugated dienes [-]	Linoleic acid [g/100 g fat]	Linolenic acid [g/100 g fat]
MinF–MinO	175 ± 23^{b}	56.8 ± 4.6^{b}	21.7 ± 2.3^{a}	0.35 ± 0.05^{a,b}
MinF–MaxO	238 ± 27^{a}	69.1 ± 2.7^{a}	18.5 ± 1.2^{a}	0.27 ± 0.04^{a,b}
MaxF–MinO	225 ± 25^{a,b}	61.9 ± 1.1^{a,b}	22.4 ± 5.0^{a}	0.38 ± 0.05^{a}
MaxF–MaxO	190 ± 5^{a,b}	57.9 ± 2.1^{b}	20.4 ± 1.2^{a}	0.25 ± 0.05^{b}

Different letters within a column indicate significantly different means (p<0.05).

described as secondary markers of oxidation. In fact, the oxidative breakdown of fatty acids takes place *via* intermediate products, or hydroperoxides, which are commonly referred to as primary lipid oxidation markers. Investigation of these intermediate structures can therefore serve to gain greater insight into the oxidation status of the batters and reveal differences that are not visible from the analysis of secondary oxidation products alone (Maire *et al.*, 2013).

Analysis of the peroxide value and absorbance of conjugated dienes are appropriate tools to determine primary oxidation markers. **Table 31** depicts the outcomes with the four yellow pea batters using both techniques. At closer inspection, it became apparent that the two approaches described a similar trend: while *MaxF–MinO* and *MaxF–MaxO* could not be discriminated from the standard *MinF–MinO*, a significant accumulation of primary markers was measurable in the case of *MinF–MaxO*. This finding indicated intensified lipid oxidation in the batter produced using a longer beating time after oil addition, which contrasted with the results of the VOC analysis. It was therefore assumed that lipid oxidation occurred during the initial stage and the beating time was insufficiently long to convert the hydroperoxides thus generated into volatiles. Conversely, this appeared to be the case for the batter with maximum mixing duration (*MaxF–MaxO*) as it was characterized by a significantly higher quantity of VOCs (**Table 29**) and a reduced concentration of hydroperoxides (**Table 31**), thus implying the successful transformation of primary into secondary markers. The greater susceptibility of polyunsaturated fatty acids to oxidative degradation during prolonged flour and oil mixing was likewise noticeable from the significantly lower content of linolenic acid in *MaxF–MaxO* compared to the other products (**Table 31**).

3.4 Correlation between mixing time and the local distribution of reactive ingredients

Cake batter is considered as a liquid foam that consists of a continuous aqueous phase containing proteins in which lipids are emulsified and both gas cells and starch granules are dispersed. In order to prevent foam collapse, the stabilization of these air bubbles is achieved by surface-active proteins and lipids, which are naturally present in the ingredients; i.e. egg, flour and oil (Godefroidt *et al.*, 2019). The microstructure of batters is determined by the beating protocol applied. Modifications to mixing might therefore be accompanied by changes to the distribution of air cells, water-soluble proteins (e.g. enzymes) and lipid droplets, which could affect their availability to participate in lipid oxidation. In addition, the duration of beating is typically correlated with the amount of air, and thus oxygen, entrapped in the continuous phase of the batter (Bousquières *et al.*, 2017b; Monnet *et al.*, 2020), thereby potentially impacting the degree of oxidative fatty acid degradation. For this reason, it was of particular importance to investigate changes in the structural properties of the batters as a function of mixing times after the incorporation of flour and oil.

Figure 59 shows CLSM images of the four batters using red and green dyes to stain lipids and proteins, respectively. In the case of *MinF–MinO*, a non-homogenous distribution of proteins and lipids could be seen. Moreover, dark disks of various diameters were observable, representing the incorporated air bubbles that caused a batter density of 0.62 g/cm^3 (**Table 30**). This large and uneven size distribution of gas cells might indicate only partial stabilization by surface-active compounds such as lipids, which appeared to be incompletely emulsified as visible from the occurrence of large lipid droplets.

A similar image was captured when increasing the mixing time after flour addition from 30 s to 3 min (*MaxF–MinO*). Compared to the standard batter, however, *MaxF–MinO* was characterized by a significantly higher batter density of 0.71 g/cm^3 (**Table 30**), indicating a loss of previously entrapped air. According to Bousquières *et al.* (2017b), this might be explained by the mechanical disruption of air cells by the solid flour particles.

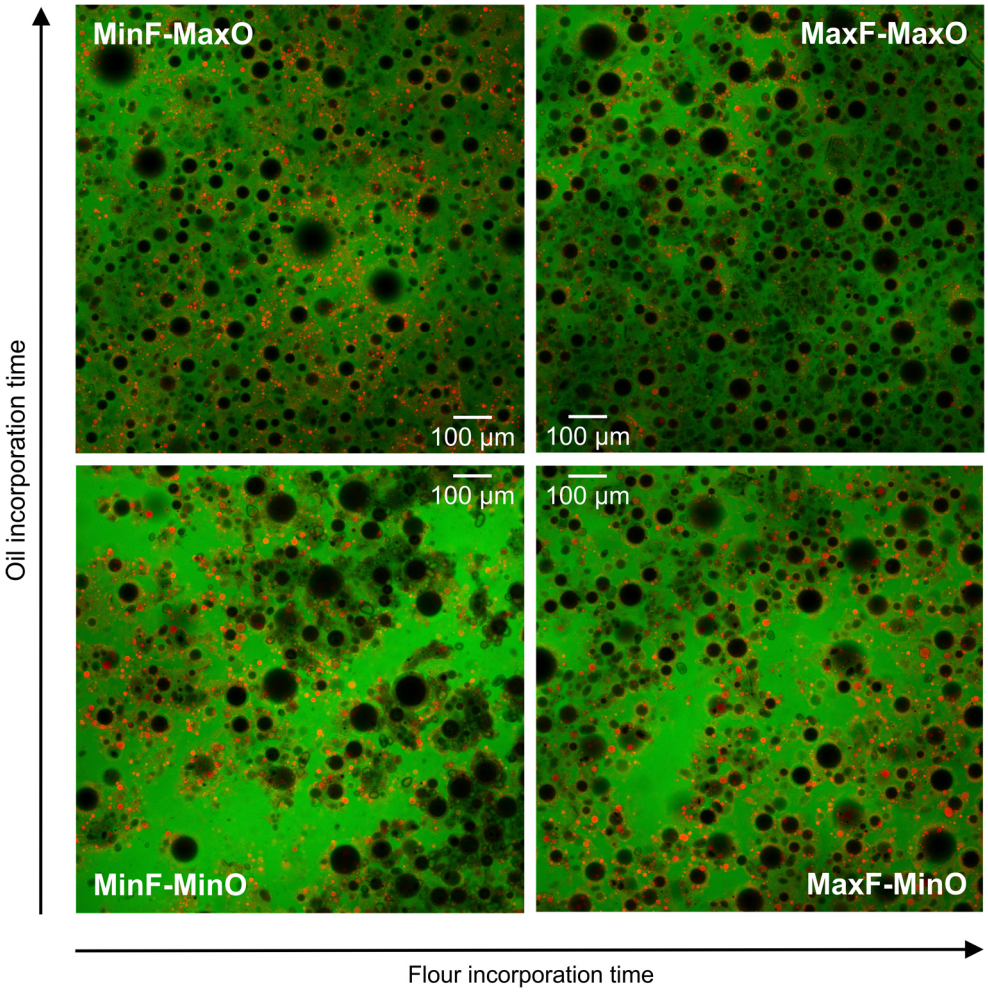

Figure 59. Microstructure of yellow pea batters prepared using different mixing times after the addition of flour and oil. Proteins are stained in green, lipids in red. Dark circles represent air bubbles. Magnification: 10×, scale bar: 100 µm.

On the other hand, the sole prolongation of mixing after oil incorporation by 3 min perceptibly modified the microstructure of the corresponding *MinF–MaxO* batter (**Figure 59**). Despite a total batter preparation time similar to that of *MaxF–MinO*, a significantly higher density in *MinF-MaxO* was determined (0.77 g/cm^3) (**Table 30**). This indicated a greater loss of air, which was presumably due to the degassing effect of the sunflower oil. This phenomenon had likewise been reported by Monnet *et al.* (2020) who studied the effects of the order of ingredient incorporation on the properties of cake batters. At the same time, this beating protocol appears to have led to a reduction in the size not only of air cells but also of fat droplets, which was accompanied by an enlargement of the reaction interface, promoting lipid oxidation. As a second consequence, an improved emulsification of lipids as well as a more homogenous distribution of lipids, proteins and air bubbles was achieved, which increased the likelihood of contact between LOX and its substrates. These structural properties of *MinF–MaxO* therefore provided a substantiated explanation for the elevated levels of primary lipid oxidation markers (i.e. conjugated dienes and hydroperoxides) compared to *MinF–MinO* and *MaxF–MinO* (**§ 3.3**).

As soon as the batter beating times after flour and oil addition were both extended, synergistic effects became apparent. From a structural perspective, no clear distinction

between *MaxF–MaxO* and *MinF–MaxO* could be made as the distribution of macromolecules and air cells, as well as batter density, were very similar (**Table 30**, **Figure 59**). However, significantly higher concentrations of secondary oxidation markers (i.e. volatile compounds) were identified in this product (**Table 29**). In addition to the surface enlargement effects, this could also be attributed to a significantly longer mixing time, which favored the enzymatically catalyzed breakdown of fatty acids. Interestingly, the increase in batter density did not affect fatty acid degradation, although it reflected a reduced amount of air, and thus of incorporated oxygen, which was required for lipid oxidation. This led to the conclusion that solubilized oxygen was sufficient to trigger oxidation, which was in line with the results reported by Krause *et al.* (2021a).

4 CONCLUSION

Lipid oxidation played a crucial role in the development of sponge cake batters containing yellow pea flour, which was mainly attributable to the activity of lipoxygenase present in the flour. The batter we produced was characterized by a complex mixture of volatile organic compounds, essentially composed of alcohols, followed by ketones and aldehydes. Most of these molecules are known to originate from 13- and 10-hydroperoxides of linoleic and linolenic acid, thus suggesting the predominant activity of 13- and 10-lipoxygenases, preferably introducing dioxygen at positions 13 or 10 of the fatty acid carbon chain.

Interestingly, the intensity of fatty acid degradation appeared to be influenced by the ingredient mixing process applied, owing to a combined impact on the local distribution of reactive ingredients and the exposure time of lipoxygenase to its substrates. When the batter beating time was increased after the addition of flour from 30 sec initially to 3 min, neither the quantities of hydroperoxides nor those of volatile compounds varied significantly; this indicated that the longer contact time between lipoxygenase and fatty acids from egg and flour was insufficient to intensify lipid oxidation. This could be assigned to the uneven partitioning and high proportion of large air bubbles and lipid droplets in the batter, which limited their probability of contact. In contrast, prolonging batter beating after the addition of oil by a similar duration of 3 min led to a significant accumulation of hydroperoxides, which implied an elevated degree of lipid oxidation. This supposedly resulted not only from improved batter homogeneity but also from a reduced size, thus enlarging the reaction surface of both lipid droplets and air bubbles, promoting chemical reactivity. Notwithstanding this, the higher potential for fatty acid breakdown after a modified oil mixing time was not reflected in a higher level of volatile compounds. This signified that during the selected extension to beating, the intermediate lipid oxidation markers had not yet been subjected to subsequent cleavage. However, this phenomenon was visible as soon as both mixing times after oil and flour incorporation were extended sequentially by a total of 5.5 min. While the corresponding batter was significantly enriched in volatile compounds, a lower level of hydroperoxides was detected, thus implying the successful transformation of primary into secondary lipid oxidation markers. Because the microstructure of this batter was similar to that obtained after prolonged oil mixing time, the greater potential to generate volatiles was also assigned to a longer contact between the enzyme and its substrates.

Based on these findings, it was concluded that by varying the mixing process parameters, a significant effect can be achieved on the degree of lipid oxidation during the development of legume-based products. This valuable knowledge of the opportunities to control the generation of volatiles with potential effects on the sensory attributes of the final food will help to exploit the potential of less refined legume ingredients, such as whole pea flour, and thus to contribute to the design of more sustainable foods.

ACKNOWLEDGEMENTS

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5 ADDITIONAL RESULTS

5.1 BATTER MICROSTRUCTURE AT HIGHER MAGNIFICATION

In addition to the CLSM images of the different yellow pea batters shown in the article, other CLSM images were also taken, but at a higher magnification (40×) and are shown in **Figure 60**. These images make it possible to see a certain section of the batter enlarged and thus the distribution of lipid droplets, starch granules and air bubbles.

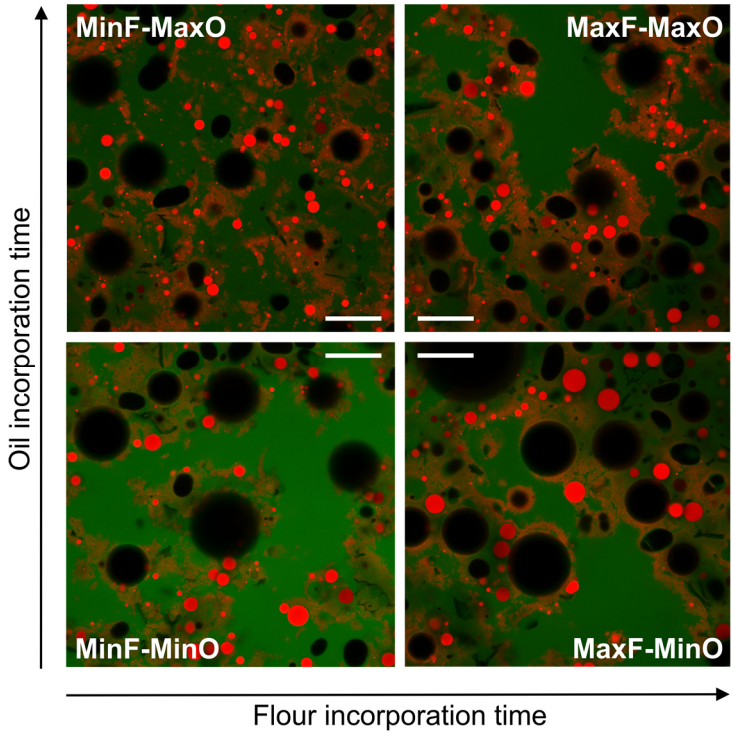

Figure 60. Microstructure of yellow pea batters prepared using different mixing times after the addition of flour and oil. Proteins are stained in green, lipids in red. Dark circles represent air bubbles. Magnification: 40×, scale bar: 50 µm.

As discussed in the text, the *MinF–MinO* formulation appeared to be less homogenous, which was noticeable in the presence of large areas in which only proteins were present, the broad size distribution of the air bubbles and the occurrence of large lipid droplets. This was also the case in the batter that

was mixed for a longer time after the flour had been incorporated (*MaxF–MinO*). Here, however, a part of the fat seemed to be better emulsified, while at the same time some lipids coalesced together locally to form larger droplets. A much more homogeneous distribution and improved emulsification of lipids could be observed in the batter in which the oil had been incorporated for a long time instead of the flour (*MinF–MaxO*). Moreover, these lipids as well as the air bubbles were considerably smaller, which implied larger surface areas that might have favored lipid oxidation. A similar image was captured when the mixing times were extended after the incorporation of both flour and oil (*MaxF–MaxO*). In addition, the lipids appeared to be clearly situated around the air bubbles to reduce interfacial tension and improve batter stability. These structural properties as well as the extended mixing times may have been reasons for the higher degree of lipid oxidation.

6 FINAL CONCLUSION

The innovative combination of methods for analyzing both reactivity and product structure used in this study was a successful approach to gain better insights into their interdependence. The knowledge gained in this way lays an important basis for the future formulation of pulse-based foods, as it provides information on how the overall aroma quality of the final product can be controlled by regulating critical process parameters.

CHAPTER 4

IMPACT OF PEA INGREDIENTS ON STRUCTURE AND REACTIVITY DURING CAKE MAKING

In addition to whole pea flour, other pea ingredients are also used in the food industry such as isolated pea proteins because of their good emulsifying, foaming and gelling properties. However, the extraction of these proteins is associated with an increased consumption of resources, which is thus reflected in an elevated environmental footprint compared to the unrefined flour (Lie-Piang *et al.*, 2021). On the other hand, it has recently been found that isolated pea proteins contain a lower quantity of volatile compounds with beany-related odor compared to the flour from which they were extracted (Xu *et al.*, 2020). However, whether this difference has a relevant influence on the sensory quality of the final product, and whether these isolated pea proteins as well as pea starch have a reduced ability to form volatile compounds during food development than the whole pea flour (see study 1) has not yet been examined. It was therefore of great interest to investigate the reactivity potentials of purified pea fractions (pea protein, pea starch) and to compare them not only to the whole pea flour but also to wheat flour and maize starch which are common cereals used in bakery products.

Since the functional properties of these purified pea fractions may differ from those of the whole pea, it could be imagined that the products formulated with these fractions had different structural properties, which might affect the susceptibility of the ingredients to reactions. In order to elaborate the potential correlation between the ability to form volatile compounds and the structural properties of the products, the analysis of reaction markers (conjugated dienes, hydroperoxides, volatile compounds, some precursors) was therefore linked with the analysis of structural properties (density, consistency, microstructure). Moreover, PCA was used to discriminate the developed cakes based on the profile of volatile compounds, which allows to conclude about their potential sensory characteristics. The overall cake quality was further assessed by investigating their color and volume to obtain a global view of the applicability of diverse pea ingredients in complex food formulations. The results have been combined in an article, which has been submitted to Food Chemistry, and will be described in the following pages.

APPLICABILITY OF PEA INGREDIENTS IN BAKED PRODUCTS: LINKS BETWEEN FORMULATION, REACTIVITY POTENTIAL AND PHYSICOCHEMICAL PROPERTIES

Article 3 – accepted in Food Chemistry (with modifications)

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HIGHLIGHTS

- Replacing wheat with pea ingredients produces cakes with an attractive appearance
- Lipoxygenase activity affects batter oxidation more than microstructure
- Replacing pea flour with purified fractions leads to fewer volatiles from oxidation
- Maillard compounds are the main volatiles in cakes made with pea protein and starch

ABSTRACT

This study aimed to evaluate the applicability of purified pea ingredients (starch and protein isolate) by assessing their potential to form volatile compounds during the different steps of sponge cake development compared to pea flour and wheat flour. While pea flour was highly susceptible to lipid oxidation during batter beating, the combination of purified pea starch and pea protein yielded significantly fewer oxidation markers with known green-beany off-odors. This was due more to the inactivation of lipoxygenase during flour fractionation than to differences in batter structure. However, fractionated ingredients were highly prone to participating in the Maillard reaction and caramelization during baking, leading to a more complex mixture of pyrazines, Strecker aldehydes and furanic compounds with potential malty and roasted notes compared to cakes based on pea flour or wheat flour. These findings confirm that using purified pea fractions can create high-quality products with an attractive nutritional composition.

KEYWORDS

Lipid oxidation, Maillard reaction, structure, pea protein, pea starch

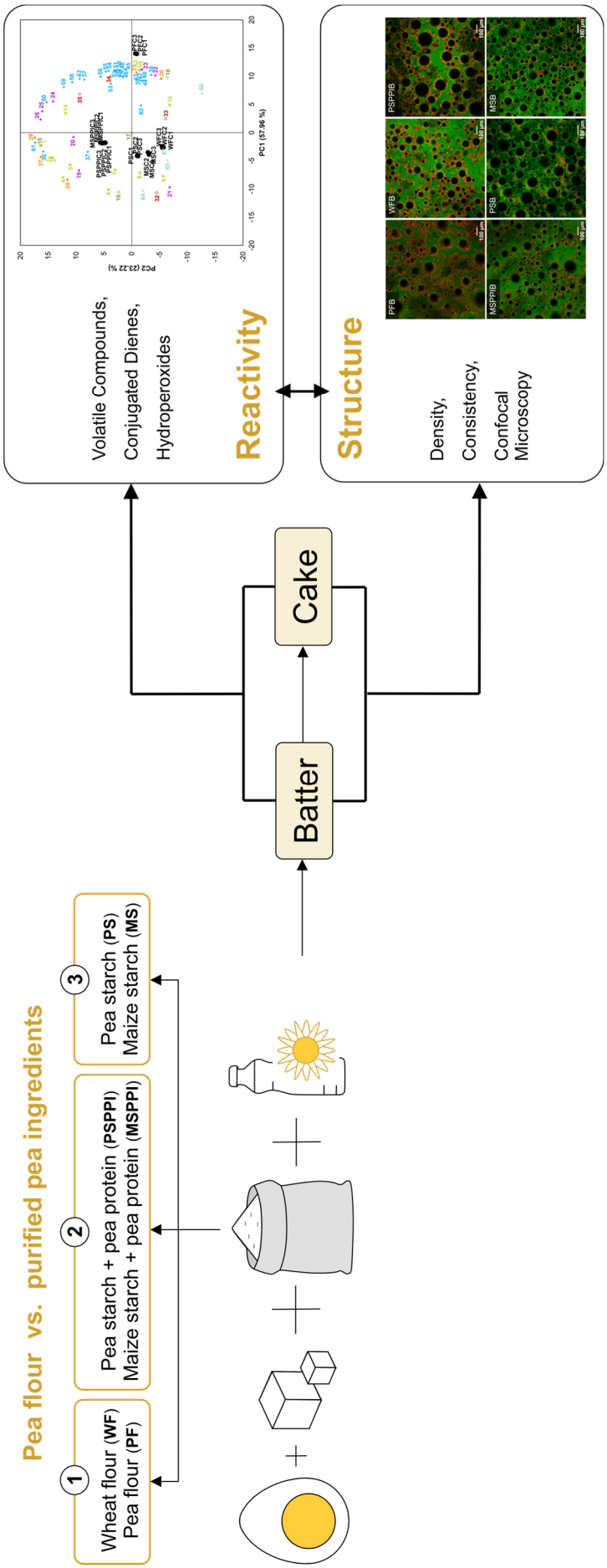


Figure 61. Graphical abstract of article 3.

1 INTRODUCTION

In response to the shift in consumer preferences toward sustainable and healthy products, the food industry is trying to find novel plant-based proteins that can replace those derived from wheat and soy (Lam *et al.*, 2018). Among these potential sources, pea (*Pisum sativum* L.) has gained considerable popularity owing to its high protein content, nutritional value, availability and low cost (Lam *et al.*, 2018). Pea proteins are commonly available in the form of flour (up to 30% protein), concentrates (50-75% protein) and isolates (>80% protein). Of these, pea protein concentrates and isolates, in particular, are used as nutritional and/or functional ingredients (emulsifiers, foaming agents or gelling agents) to create protein-enriched foods with attractive textural properties. On the other hand, the search for more sustainable protein alternatives demands a simplification of processes to meet the desire for product naturalness and reductions in costs and environmental footprints. From this point of view, the use of flours would appear to be more interesting than that of refined concentrates and isolates that necessitate other resources such as water, chemicals and energy (Lie-Piang *et al.*, 2021). Furthermore, it is interesting to note that the fractionation process does not only produce pea protein but also pea starch, which accumulates as a by-product that is still rarely incorporated in foods (except e.g. in Asian noodles) (Wang *et al.*, 2014) and needs to be valorized.

Nevertheless, pea flour exhibits great potential to generate volatiles with an unpleasant green-beany aroma, which poses a difficult challenge in terms of its applicability (Krause *et al.*, 2021a). This off-flavor formation can be ascribed to the activity of endogenous lipoxygenase that catalyzes the oxidation of polyunsaturated fatty acids to hydroperoxides, which can be cleaved into odor-active molecules (Belitz *et al.*, 2009; Krause *et al.*, 2021b). Lipid oxidation can occur during flour storage and processing (Roland *et al.*, 2017), but is also particularly stimulated when the flour is incorporated in complex products such as sponge cakes (Krause *et al.*, 2021a). Cake development involves the mixing of different ingredients to produce a moist batter, which results in the immediate activation of lipoxygenase due to air entrapment and homogenous substrate distribution, and hence the release of multiple volatiles.

In order to exploit the benefits of pea without compromising sustainability, it is therefore essential to better understand the behavior of pea ingredients in complex applications and to find new strategies to improve the overall quality of pea-based foods. These include controlling the oxidative processes that might affect sensory characteristics.

Unfortunately, an initial attempt to limit undesirable oxidation by processing pea flour under nitrogen generated an unexpected intensification of off-flavor (Krause *et al.*, 2021a). By contrast, we were able to show that the degree of lipid oxidation could be altered significantly by modifying individual process parameters during the making of pea flour batters (Krause *et al.*, 2021c).

Alternatively, isolated pea proteins could be used, as they have been reported to contain lower levels of aroma compounds than the corresponding flour (Xu *et al.*, 2020). However, the sensory importance of these volatiles in final foods, and especially the effect of the extraction process on the ability of the isolate to produce volatiles during product development, have not yet been clarified.

The objective of this study was therefore to evaluate the ability of purified pea fractions (starch and protein isolate) to generate volatiles during sponge cake making when compared to a whole pea flour from the same batch and a wheat-based reference. Process-induced reactions and sensory and safety markers were therefore monitored and their presence linked to the physicochemical characteristics of the raw materials and the structural properties of the batters.

2 MATERIALS AND METHODS

2.1 INGREDIENTS

Flour (PF), starch (PS) and protein isolate (PPI) from the same batch of yellow peas were supplied by Cargill (Vilvoorde, Belgium). The fractionation of PF was achieved by (i) suspension of the flour in water, (ii) protein extraction at pH 8-11 and slightly elevated temperatures, (iii) pea starch recovery by centrifugation, (iv) isoelectric protein precipitation at pH 4-5, (v) protein recovery by centrifugation and (vi) spray drying. Wheat flour (WF) was purchased from Axiane Meunerie (Olivet, France), maize starch (MS) from Cargill (Vilvoorde, Belgium), sucrose from Tereos (Lille, France), sunflower oil from Lesieur (Asnières-sur-Seine, France) and whole pasteurized liquid eggs from Ovoteam (Locmine, France). All compositions are described in **Table 47**.

2.2 CHEMICALS

Furfural-d_4 (≥96%) and 2-methylpyrazine-d_6 (98.4%) were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada), hexanal-d_{12} (≥96%), Celite® 545, iodine, urea, sodium acetate anhydrous, glyceryl triheptadecanoate (≥96%), D(+)-glucose anhydrous and amyloglucosidase from *Aspergillus niger* from Merck (Darmstadt, Germany), petroleum ether, isopropanol, heptane, sodium hydroxide, methanol, glacial acetic acid, sodium thiosulfate pentahydrate, isooctane, potassium iodide and iodine (0.02M) from Carlo Erba (Val de Reuil, France), hydrochloric acid (37%) from Prolabo (Fontenay-sous-Bois, France) and dimethyl sulfoxide from Fisher Scientific (Merelbeke, Belgium).

2.3 BATTER AND CAKE FORMULATIONS

Sponge cake batters and cakes were produced according to the method described by Krause *et al.* (2021a). Eggs (45% w/w) and sucrose (25% w/w) were beaten for 10 min after which the non-sifted dry ingredients (25% w/w) were gently folded in within 1.5 min. After mixing for 30 s, the sunflower oil (5% w/w) was incorporated within 15 s and the batter beaten for a further 1 min.

WF was selected as the dry ingredient for the reference because of its traditional use in baked goods. The PF product was prepared by totally replacing WF with PF. To examine the effect of PF fractionation on the reactivity potential of the purified fractions, PF was fully replaced with a mixture (1/2 w/w) of PPI and either PS or MS to obtain similar proportions as in PF. MS was chosen because of its frequent use in the food industry and limited chemical reactivity (Bousquières *et al.*, 2017a), and thus served as a control versus PS. These recipes were called “composite formulations”. Alongside, two products based on PS or MS alone and without the further addition of protein, were also generated. These starch recipes were designed to gain a deeper understanding of the role of each individual fraction in the formation of volatiles compared to PF only. Each formulation was prepared in triplicate, and a portion was deep-frozen immediately at -20 °C until reaction marker analysis, in order to decelerate oxidation. The remaining was used for instant pH, density and consistency measurements.

A new batch of each batter was prepared to make the sponge cakes. From a total of 630 g batter, 21 identical cakes were obtained by filling 25 g into aluminum molds (9.8×6.2×3.3 cm^3) and baking for 25 min at 170 °C. Five cakes were stored immediately at -20 °C in hermetically sealed glass jars until reaction marker analysis. The remaining cakes were cooled to ambient temperature for color, moisture, pH and density measurements.

2.4 CAKE GRINDING

The cakes were ground as described by Krause *et al.* (2021a) after baking (analysis of chemical composition, pH, moisture content) or in a frozen state (reaction marker analysis) for 20 s at 6000 rpm using a Grindomix GM200 knife mill equipped with a stainless-steel bowl and titanium blades (Retsch GmbH, Haan, Germany).

2.5 CHEMICAL CHARACTERISTICS

The ingredients and cakes were characterized in terms of their ***moisture*** (moisture analyzer MA30, Sartorius, Göttingen, Germany; in triplicate) and ***protein content*** (Kjeldahl method NF EN ISO 5983-2 with a nitrogen-to-protein conversion factor of 5.7 for wheat and 6.25 for pea and maize; in duplicate). ***Starch content*** was determined in triplicate by adapting the method described by Morrison *et al.* (1983). Flours/fractions or cakes were dispersed in dimethyl sulfoxide/urea (6 M) (90/10 v/v), centrifuged (2000*g*, 10 min) and enzymatically hydrolyzed at 55 °C for 72 h by mixing 1 mL supernatant with 4 mL sodium acetate buffer (0.2 M, pH 4.5) containing amyloglucosidase. The glucose released was determined by High Performed Liquid Chromatography (HPLC) using a 717plus autosampler, 515 HPLC pump, column heater and 2410 refractive index detector (Waters, Antwerp, Belgium). The digested samples were filtered through 0.45 µm Minisart NML filters and diluted (1/50 v/v) with purified water. 20 µL of the dilution were injected on two Aminex HPX-87C columns connected in series (300×7.8 mm) and protected by a micro-guard de-ashing cartridge (30×4.6 mm) (BioRad, Temse, Belgium). The isocratic elution of glucose was performed at 75 °C using degassed purified water (0.5 mL/min). Glucose was quantified using an external glucose calibration curve (1-100 g/L).

Ash (dry combustion for 12 h at 550 °C) and ***dietary fiber*** (AOAC 985.29) in the ingredients were analyzed in triplicate. ***Lipoxygenase (LOX) activity*** was determined by Eurogerm (Saint-Apollinaire, France) in triplicate according to Delcros *et al.* (1998) by dispersing 2 g flour or PPI in 20 mL phosphate buffer (pH 7.5), centrifugation and subsequent mixing with linoleic acid in phosphate buffer (pH 6.5) under an oxygen-saturated atmosphere. LOX activity was expressed as nmol oxygen consumed per second.

The ***pH value*** of 2 g batter or cake (in triplicate) was recorded in 20 mL purified water using a SensION^{TM}+ PH3 meter (Hach Lange, Lognes, France).

2.6 PHYSICAL AND STRUCTURAL PROPERTIES

Density of the batters and cakes was determined in triplicate from their mass-to-volume ratio as described by Krause *et al.* (2021a) using open-mouth syringes of known volume and a laser-based scanner for batters and cakes, respectively.

Consistency of the batters was measured in triplicate according to Monnet *et al.* (2020) with some modifications. A 20 mm aluminum plate was attached to a TA-XT2i texture analyzer equipped with a 5 kg load cell (Stable Micro Systems, Surrey, UK) and inserted into 40 mL batter at a speed of 2 mm/s until a distance of 20 mm was reached. The resistive force to compression was plotted against the compression distance, and batter consistency was determined from the average force over the constant part at the end of the curve.

CIEL*a*b* color of the three cakes was analyzed at three points on both cake crust and crumb using a spectro-guide sphere gloss colorimeter CD-6834 (BYK-Gardner, Geretsried, Germany) at illuminant D65 and a 10° observer angle.

Batter ***microstructure*** was investigated in triplicate using confocal laser scanning microscopy (CLSM) as reported by Krause *et al.* (2021c). In brief, 3 µL of Dylight 488 and Bodipy 665/676 (1/1 v/v) were used to stain the proteins (green) and lipids (red) contained in 1 g fresh batter. The stains were excited at 488 nm and 638 nm and the emitted fluorescence recorded at 497-601 nm and 643-765 nm by means of a Leica TC2 SP8 (Wetzlar, Germany). Images were captured at ×10 magnification.

The ***pasting properties*** of the ingredients were analyzed in duplicate using a Rapid Visco Analyzer RVA 4500 (Perten Instruments, New South Wales, Australia). Flours/starches were suspended in purified water (9% w/w, 30 g total) and the viscosity profiles recorded using the following temperature program: 40 °C isothermal for 2 min, heating to 90 °C at 5 °C/min, 90°C isothermal for 5 min,

cooling to 20 °C at 2.5 °C/min and 20 °C isothermal for 5 min.

Particle size distribution of the flours was detected in triplicate by laser diffraction using a Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK), applying a refractive index of 1.49429 and an absorption index of 0.1.

2.7 ANALYSIS OF THE OXIDATION STATUS

The oxidation status of the ingredients, batters and cakes was investigated by determining conjugated dienes, peroxide values and acid values according to Krause *et al.* (2021a). The oil was isolated by mixing flours/fractions, batters and cakes with Celite® 545 at ratios (w/w) of 3/2, 10/9 and 14/5, respectively, followed by accelerated solvent extraction (25 min, 40 °C, 5/1 v/v heptane/isopropanol) using a Dionex ASE 350 (Thermo Fisher Scientific, Waltham, MA, USA). After solvent evaporation, the oils were deep-frozen at -20 °C under nitrogen until analysis.

Conjugated dienes were analyzed in triplicate by diluting 10 mg oil in 10-200 mL isooctane and measuring the absorbance at 234 nm with a Specord 210 (Analytik Jena, Savigny-le-Temple, France). The content in conjugated dienes was expressed as specific absorbance, which was defined as the absorbance of 1 g oil in 100 mL isooctane.

The ***peroxide value*** was determined in triplicate by dissolving 200 mg oil in 16 mL glacial acetic acid/isooctane (3/2 v/v), to which 1 mL saturated potassium iodide solution and 9 mL purified water were added. Iodometric titration was carried out with 5 mM sodium thiosulfate and the peroxide value was expressed as millimole of hydroperoxide per kg oil.

The ***acid value*** was determined in triplicate according to NF EN ISO 660:2020 with slight modifications. Briefly, 50 mg oil was dissolved in 20 mL neutralized ethanol/petroleum ether (1/1 v/v) and the acids were titrated with ethanolic potassium hydroxide solution (5 mM). The acid value was expressed as the mass of potassium hydroxide required to neutralize 1 g fat.

2.8 PROFILE OF VOLATILE ORGANIC COMPOUNDS (VOCS)

To evaluate the potential of the flours/fractions to generate VOCs during cake production, HeadSpace-Solid Phase Micro Extraction (HS-SPME) was performed according to the optimized method described by Krause *et al.* (2021a). Briefly, 2 g of flour/fraction, batter or cake were transferred in triplicate into 20 mL glass vials to which were added 100 µL of an aqueous solution containing the deuterated standards hexanal-d_{12}, furfural-d_4 and 2-methylpyrazine-d_6 at varying concentrations (ingredients/batters/cakes): 14/91/15 mg/L furfural-d_4; 0.1/89/3 mg/L 2-methylpyrazine-d_6; 139/778/411 mg/L hexanal-d_{12}. The vials were sealed with aluminum polytetrafluoroethylene-coated silicon septa, kept overnight at 4°C to equilibrate the standard within the sample and then incubated at 50 °C for 18 min in a Triplus autosampler (Thermo Fisher Scientific). Molecules were extracted over 42 min at 50 °C and then desorbed from a DVB/CAR/PDMS fiber (50/30 µm, 2 cm) at 250 °C for 2 min (10 mL/min) into a TRACE GC Ultra injector port (Thermo Fisher Scientific).

VOCs were separated on a ZB-wax capillary column (30 m×0.25 mm×0.5 µm) by increasing the initial oven temperature from 40 °C (maintained for 5 min) to 240 °C at 4 °C/min using helium as the carrier gas (1.2 mL/min). An ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific) recorded the mass spectra in the EI mode at 70 eV by scanning in full (TIC, *m/z* 33-300) and SIM mode for selected molecules. VOCs were identified by matching the experimental mass spectra with MS libraries (NIST 08, Wiley 8) and comparing the experimental retention indices obtained using a series of *n*-alkanes (C5-C21) with a NIST database (see **Table 41**). Hexanal, furfural and 2-methylpyrazine were quantified using their deuterated standards. The relative quantification of other compounds was performed using 2-methylpyrazine-d_6 for nitrogenous,

furfural-d_4 for furanic and hexanal-d_{12} for the remaining compounds

2.9 STATISTICAL ANALYSIS

XLSTAT 2020.5.1 (Addinsoft, Paris, France) was used to perform principle component analysis and one-way analysis of variance (ANOVA) as well as to evaluate significant differences using Tukey's test at $p<0.05$. The data acquired were reported with two significant numbers on the total uncertainty.

3 RESULTS AND DISCUSSION

3.1 VOCS IN FLOURS AND FRACTIONS

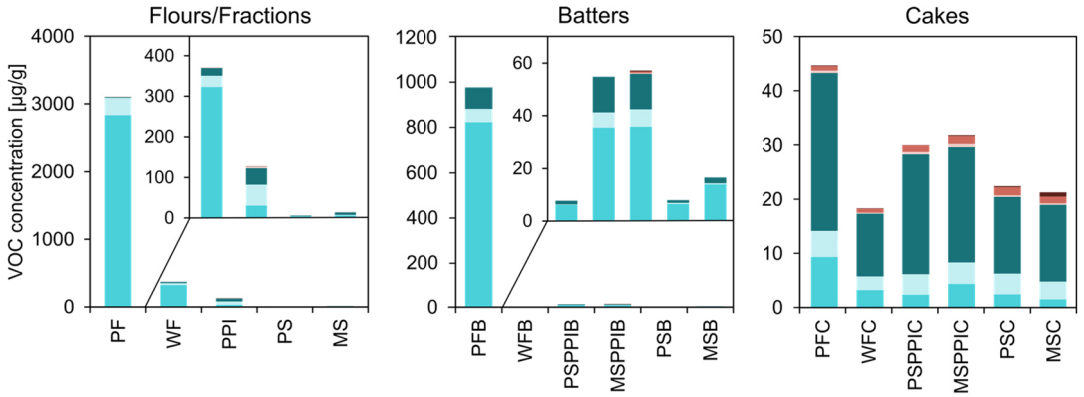
Figure 62 depicts the profiles of VOCs extracted from the flours/fractions, batters and cakes. An overview of all compounds and their relative amounts can be found in **Table 48** – **Table 51**.

Analysis of the VOCs in raw materials is important to understand their starting conditions and potential to undergo changes during product development. As can be seen in **Figure 62**, the highest VOC quantities were determined in PF and WF, whereas the purified ingredients PPI and, in particular, PS and MS, contained significantly fewer volatiles. Within the flours, PF stood out for its substantially higher VOC concentrations.

In both flours, alcohols represented the dominant group of volatiles, followed by ketones, aldehydes and furanic compounds, the latter two only occurring as traces. The profile of alcohols appeared to be largely determined by metabolites typically deriving from microbial activity (**Table 48**). These comprised ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-phenylethanol as well as their corresponding aldehydes, which can be synthesized *via* the Ehrlich pathway from alanine, valine, leucine and phenylalanine, respectively (Pico *et al.*, 2015). Apart from these, several alcohols were found that most likely originated from the breakdown of fatty acids, including 1-hexanol and 1-pentanol. Both components are markers of oxidative linoleic acid degradation which occurs *via* their aldehydes in the presence of alcohol dehydrogenases (Belitz *et al.*, 2009; Jakobsen *et al.*, 1998; Ma *et al.*, 2016). Other oxidation-derived alcohols, aldehydes and ketones arising from linolenic acid (1-penten-3-ol, 3-hexen-1-ol) and oleic acid (2-heptanone, nonanal) were also detected, but only in WF. This implied a greater degree of oxidation that might have taken place during harvesting, grinding or storage.

The fractionation of PF into PPI and PS resulted not only in an enormous loss of molecules (**Figure 62**) but also in visible changes to the VOC profiles (**Table 48** – **Table 51**). In order to compare VOC levels in PF with those of PPI and PS, the VOC quantities reported needed to be corrected by the protein and starch contents of the flour (23.8% and 47.6%, respectively). When this was done, it emerged that PPI contained approximately 100× fewer volatiles than PF, which was even more pronounced with regard to PS (more than 1000×). The almost negligible quantities of VOCs in the latter mainly comprised ethanol, hexanal and acetone, which were probably residues from the flour (**Table 48** – **Table 51**). The same was seen for MS, which was being investigated as a control to PS (**Figure 62**).

Despite the quantitative inferiority of PPI to PF, however, PPI contained considerably larger numbers of individual molecules (25 vs. 16). Around half of these potentially originated from fatty acid degradation, which clearly outnumbered those detected in PF (**Table 48 – Table 50**). The most abundant oxidation marker was hexanal, which might have formed during linoleic acid breakdown (Belitz *et al.*, 2009; Jakobsen *et al.*, 1998). In addition, VOCs also originating from linoleic acid (1-pentanol, heptanal, octanal) as well as oleic acid (1-octanol, nonanal, 2-heptanone, 2-nonanone, 2-decanone, heptanal, octanal) and linolenic acid (3,5-octadien-2-one, 1-penten-3-ol, 2-penten-1-ol) were present at low levels (Belitz *et al.*, 2009; Cao *et al.*, 2014; Kochhar, 1996; Ma *et al.*, 2016; Murray *et al.*, 1976). Based on these findings, it was concluded that the isolation of PPI from the PF took place under conditions favorable to oxidation (see **§ 2.1**). This reaction might have been initiated when suspending the flour in water



Sample		VOC concentration [µg/g sample]							
		Alcohols	Ketones	Aldehydes	Esters	Furanic compounds	Nitrogenous compounds	Sulfurous compounds	Total
Flours/ Fractions	PF	2816 ± 11^{a}	260.0 ± 8.4^{a}	10.30 ± 0.40^{c}	n.d.	8.43 ± 0.85^{a}	n.d.	n.d.	3109.3 ± 2.0^{a}
	WF	323 ± 46^{b}	27.7 ± 4.6^{c}	19.4 ± 2.0^{b}	n.d.	1.48 ± 0.14^{c}	n.d.	n.d.	372 ± 51^{b}
	PPI	30.5 ± 1.9^{c}	51.8 ± 2.9^{c}	40.7 ± 2.1^{a}	n.d.	4.14 ± 0.22^{b}	0.3167 ± 0.0033	0.35 ± 0.10	127.8 ± 7.0^{c}
	PS	4.26 ± 0.11^{c}	0.648 ± 0.060^{d}	1.423 ± 0.013^{e}	n.d.	n.d.	n.d.	n.d.	6.33 ± 0.10^{d}
	MS	6.323 ± 0.069^{c}	1.468 ± 0.085^{d}	6.19 ± 0.27^{d}	n.d.	0.696 ± 0.024^{c}	n.d.	n.d.	14.67 ± 0.29^{d}
Batters	PFB	784 ± 85^{a}	55.0 ± 3.0^{a}	115.1 ± 3.7a	n.d.	2.47 ± 0.71^{a}	n.d.	n.d.	957 ± 89^{a}
	WFB	6.18 ± 0.66^{b}	0.169 ± 0.015^{c}	1.38 ± 0.10^{c}	n.d.	n.d.	n.d.	n.d.	7.73 ± 0.78^{b}
	PSPPIB	35.39 ± 0.79^{b}	5.86 ± 0.23^{b}	13.73 ± 0.84^{b}	n.d.	n.d.	n.d.	n.d.	55.0 ± 1.6^{b}
	MSPPIB	35.8 ± 1.6^{b}	6.75 ± 0.40^{b}	13.90 ± 0.93^{b}	n.d.	0.260 ± 0.015^{b}	0.62 ± 0.19	0.36 ± 0.19	56.7 ± 2.6^{b}
	PSB	6.49 ± 0.48^{b}	0.497 ± 0.018^{c}	0.982 ± 0.051^{c}	n.d.	n.d.	n.d.	n.d.	7.97 ± 0.50^{b}
	MSB	14.0 ± 1.7^{b}	0.399 ± 0.023^{c}	2.26 ± 0.17^{c}	n.d.	n.d.	n.d.	n.d.	16.6 ± 1.9^{b}
Cakes	PFC	9.36 ± 0.14^{a}	4.868 ± 0.091^{a}	29.24 ± 0.43^{a}	0.0342 ± 0.0017^{c}	0.425 ± 0.012^{b}	0.860 ± 0.021^{d}	0.1329 ± 0.0046^{b,c}	44.93 ± 0.67^{a}
	WFC	3.23 ± 0.17^{c}	2.53 ± 0.11^{d}	11.65 ± 0.52^{d}	n.d.	0.1804 ± 0.0053^{d}	0.733 ± 0.025^{e}	0.0775 ± 0.0025^{c,d}	18.41 ± 0.79^{d}
	PSPPIC	2.354 ± 0.054^{d}	3.816 ± 0.054^{b}	22.26 ± 0.43^{b}	0.0925 ± 0.0018^{a}	0.424 ± 0.051^{b}	1.236 ± 0.016^{c}	0.0506 ± 0.0020^{d}	30.23 ± 0.59^{b}
	MSPPIC	4.36 ± 0.10^{b}	4.01 ± 0.11^{b}	21.40 ± 0.74^{b}	n.d.	0.599 ± 0.017^{a}	1.424 ± 0.026^{b}	0.144 ± 0.015^{c,d}	31.93 ± 0.97^{b}
	PSC	2.448 ± 0.035^{d}	3.850 ± 0.022^{b}	14.23 ± 0.24^{c}	0.0641 ± 0.0023^{b}	0.298 ± 0.010^{c}	1.519 ± 0.044^{a}	0.1845 ± 0.0063^{b}	22.60 ± 0.28^{c}
	MSC	1.503 ± 0.081^{e}	3.31 ± 0.23^{c}	14.22 ± 0.63^{c}	n.d.	0.3196 ± 0.0048^{c}	1.240 ± 0.030^{c}	0.789 ± 0.063^{a}	21.38 ± 0.92^{c}

Figure 62. Profile of VOCs [µg/g sample] extracted from flours/fractions, batters and cakes. Molecules are categorized into alcohols (■), ketones (■), aldehydes (■), esters (■), furanic (■), nitrogenous (■) and sulfurous compounds (■). n.d. = not detected. Relative amounts are estimated based on the internal deuterated standards furfural-d_4 (furanic compounds), 2-methylpyrazine-d_6 (nitrogenous compounds) and hexanal-d_{12} (remaining compounds). Different letters within a column of one food matrix indicate significantly different means (p<0.05). PF = pea flour, WF = wheat flour, PS = pea starch, MS = maize starch, PPI = pea protein isolate.

owing to pH conditions that were optimal for the enzyme LOX (Fischer *et al.*, 2020). In addition, oxidation might have been triggered during centrifugation because of the potential introduction of oxygen. Finally, it could be imagined that fatty acids were degraded by heat-induced autoxidation during spray drying. This latter hypothesis might be corroborated by the occurrence of volatiles originating from oleic acid that lacked the *cis,cis*-1,4-pentadiene system, so were not oxidized by LOX. Nonetheless, spray drying might have partially volatilized VOCs, explaining their low total concentrations in PPI.

3.2 Change of VOC profile during batter beating

The batters were made by beating eggs and sugar into a foam to which the dry ingredients (flours/fractions) and oil were added. This preparation seemed to have initiated certain reactions, as visible from the marked changes to the VOC profiles compared to the dry ingredients alone (**Figure 62**). Similar to the flours/fractions, alcohols contributed the most to total VOC amounts, but notably larger proportions of aldehydes and ketones were observed. The highest total VOC levels were detected in pea flour batter (PFB), which far exceeded those in batters made with wheat flour (WFB), a combination of isolated proteins and pea or maize starch (PSPPIB, MSPPIB) or pure pea or maize starch without additional protein (PSB, MSB).

Apart from its quantitative superiority, PFB stood out for its 2-4× higher number of different molecules than in the other batters (**Table 48** – **Table 51**). Moreover, on closer inspection of the type of compounds, it became apparent that most of the volatiles in WFB, the composite and starch batters were correlated with fermentation (e.g. ethanol, acetone), while those in PFB mainly derived from the oxidative breakdown of fatty acids. Nevertheless, high levels of fermentation markers were also present in PFB, which might either have been residues from the flour or have developed during batter preparation. This indicated a generally higher reactivity and, in particular, pronounced susceptibility to oxidation of PF compared to the other ingredients.

The most abundant oxidation markers in PFB included 1-penten-3-ol, 1-hexanol, hexanal and 3-pentanone, which are known products of linoleic or linolenic acid degradation (**Table 48 – Table 50**) (Belitz *et al.*, 2009; Jakobsen *et al.*, 1998; Salch *et al.*, 1995; Sánchez-Ortiz *et al.*, 2013). A direct comparison of their amounts with those in PF revealed increases of 17× in the case of 1-hexanol and almost 30× in the case of hexanal when considering that the proportion of flour used in the batter was only 25%. 1-Penten-3-ol and 3-pentanone were not present in the flour. In addition, other process-induced volatiles were detected in smaller quantities, which might have originated from the decomposition of linoleic (1-pentanol, 1-octen-3-ol, 2-heptenal, 2-octenal) linolenic (3-hexen-1-ol, 2-penten-1-ol, 2-pentenal) and oleic acid (1-heptanol, 1-octanol and 2-octanone) (Belitz *et al.*, 2009; Kochhar, 1996; Luning *et al.*, 1995; Maire *et al.*, 2013; Murray *et al.*, 1976; Przybylski *et al.*, 1995a).

Although oxidation played a significantly less important role in the other batters, their vulnerability to oxidative deterioration varied distinctively (**Figure 62**). Contrary to WFB, the composite batters (PSPPIB, MSPPIB) contained significantly larger numbers and quantities of volatiles arising during the course of oxidation (**Table 48** – **Table 50**). For instance, the concentration of the main oxidation marker hexanal was around 10× higher than in WFB, but only a seventh of that detected in PFB. Notwithstanding, this amount corresponded to an increment of 3-4× compared to the sum determined in the dry ingredients used at a combined proportion of 25%, which was nevertheless well below the 30× calculated for PFB. From this, it was concluded that the fractionation of PF into its main constituents led to a reduced susceptibility to oxidation during batter beating, which remained higher than that of WF.

Interestingly, PSPPIB and MSPPIB were characterized by nearly identical VOC profiles and concentrations, which suggested that the starch component had no apparent effect on the

formation of volatiles and could have been attributed to the absence of fat (<0.1% in both starches) and hence precursors for oxidation. This was corroborated by analyzing the molecules present in PSB and MSB, which were produced using starch alone without supplementary proteins. These formulations contained VOC levels similar to those in WFB. Of the volatiles identified, only two were associated with oxidation, namely hexanal and nonanal, the latter only being present in MSB. The concentration of hexanal in this product was also significantly higher than in PSB, but generally low compared to the other formulations. Owing to the absence of lipids in both starches, it could be assumed that the oxidation markers detected originated from fatty acids contained in the eggs and oil. Minimal differences between PSB and MSB were possibly due to structural differences (see **§ 3.3**).

3.3 ROLE OF LOX ACTIVITY AND FATTY ACID COMPOSITION IN VOC GENERATION

As previously described, lipid oxidation was initiated during batter beating, and its degree depended on the dry fraction used. According to our previous publications, LOX is the main catalyst of oxidation during the batter preparation using wheat or pulse flours (Krause *et al.*, 2021b; Krause *et al.*, 2021a). This was attributed to a high prevalence of VOCs deriving from linoleic and linolenic acid, as well as a positive correlation between LOX activity and VOC formation (Krause *et al.*, 2021b; Krause *et al.*, 2021a). This latter dependence was also found during the present study since markedly higher LOX activity was determined for PF (5379 nkat/g) than WF (43 nkat/g) and PPI (<1 nkat/g). Consequently, the extraction of proteins from PF resulted in the inactivation or removal of LOX. The key role of LOX was substantiated when analyzing the VOC profiles of PSB and MSB as they did not contain any additional protein apart from egg and therefore no source of LOX, which presumably explains the observed absence of oxidation markers. Moreover, the pH conditions for PFB were optimal for LOX activity (Fischer *et al.*, 2020), which probably favored oxidative processes (**Table 32**).

Despite the lack of LOX in PPI, however, the corresponding batters were enriched in oxidation markers compared to WFB, implying the occurrence of non-enzymatic autoxidation. But why were PSPPIB and MSPPIB more susceptible to oxidation than WFB? As discussed in **§ 3.2**, fatty acids from egg and oil were less degraded *via* autoxidation during batter preparation due to the low levels of oxidation markers in PSB and MSB, which were made using lipid-free starches. It was therefore assumed that the volatiles in PSPPIB and MSPPIB mainly originated from the fatty acids contained in PPI. Although PPI and WF had similar fat contents (1.2% and 1.6%, respectively), it should be borne in mind that WF made up 25% of the batter ingredients but PPI only 8%. Despite the resulting smaller quantities of precursors, fatty acid degradation took place to a greater extent in PPI-based batters. This could be attributed to the higher content in linoleic and linolenic acids in pea compared to wheat (Krause *et al.*, 2021a). The importance of the fatty acid profile was corroborated by analyzing the unit-free acid value, which provides information on the quantity of free fatty acids that can easily be oxidized. While the acid value in WF was 24.0, that in PPI was only 8.3 (data not shown). Although this implied that fewer fatty acids were freely available in PPI, they were more degraded, presumably because a large proportion of them were linoleic and linolenic acids, unlike WF. Alternatively, it is also conceivable that WF contained antioxidants which protected fatty acid oxidation and were probably absent from the purified PPI and PS.

3.4 CORRELATION BETWEEN STRUCTURAL BATTER PROPERTIES AND REACTIVITY

Apart from chemical and enzymatic factors, the structural batter properties were investigated in order to determine their potential impact on the degree of VOC generation and release.

As can be seen in **Table 32**, PFB displayed both the highest batter density and consistency, which suggested the smallest amount of air incorporated and the highest resistance to flow. The opposite was concluded for the starch formulations, based on their lowest batter densities and consistencies. This ranking appeared to be correlated with the protein levels in the products: higher protein contents led to higher batter densities and consistencies. These results coincided with those obtained by de la Hera *et al.* (2012), Gómez *et al.* (2008) and Monnet *et al.* (2020), who observed increments in cake batter densities and viscosities when replacing wheat with protein-rich lentil, chickpea or pea flours. Despite similar protein contents, however, the densities and consistencies of the composite formulations were lower than those of PFB, which could be related to the high purity of the fractions and hence the absence of components, e.g. fiber. Moreover, it could be imagined that the proteins were partially unfolded during the isolation process, which might enhance their ability to stabilize the air-liquid interface and therefore produce batters with densities as low as those of PSB and MSB. Nevertheless, the higher protein contents in PSPPIB and MSPPIB than in the starch formulations might have improved lipid emulsification, thus yielding batters with thicker consistencies.

When these structural properties were related to VOC levels (**§ 3.2**), it emerged that a greater air entrapment was not associated with an increased formation of oxidation markers. This conclusion was in line with our previous findings on sponge cake batters made with various pulse flours (Krause *et al.*, 2021a), indicating that the oxygen dissolved was sufficient to trigger oxidation. Moreover, it seemed that the potential impact of structure on VOC release was negligible since formulations with similar densities and consistencies displayed different VOC concentrations.

In addition, the local distribution of reactive ingredients in the batters could be relevant to oxidation so it was therefore captured using CLSM. As illustrated in **Figure 63**, MSB and PSB were characterized by the highest number of incorporated air bubbles compared to the flour formulations, and particularly PFB, which contained a visibly smaller air fraction. MSPPIB and PSPPIB were positioned in between, thus complying with the density and consistency results (**Table 32**). Interestingly, the air bubbles in maize batters were considerably smaller than in PSB. de la Hera *et al.* (2012) suggested that smaller air bubbles might result from a reduction in particle size, which could have been a suitable explanation in the present case due to a smaller size distribution of MS (d_{50} = 14 µm) compared to PS (d_{50} = 24 µm).

Air bubbles in the batter were stabilized by amphiphilic proteins and lipids, which absorb at air-liquid interfaces to reduce interfacial tension (Godefroidt *et al.*, 2019). In each product, proteins were well dispersed, whereas differences in the partitioning of fat droplets were noted (**Figure 63**). In the case of starch formulations, lower

Table 32. pH value, consistency and density of sponge cake batters.

Batter	pH value [-]	Consistency [N]	Density [g/cm^3]
PFB	7.260 ± 0.017^{e}	2.27 ± 0.14^{a}	0.615 ± 0.063^{a}
WFB	7.783 ± 0.050^{d}	1.972 ± 0.048^{b}	0.5525 ± 0.0086^{b}
PSPPIB	7.947 ± 0.038^{b}	1.961 ± 0.028^{b}	0.4220 ± 0.0042^{c}
MSPPIB	7.9233 ± 0.0058^{b,c}	1.871 ± 0.022^{b}	0.4365 ± 0.0044^{c}
PSB	8.4567 ± 0.0058^{a}	0.843 ± 0.035^{c}	0.432 ± 0.014^{c}
MSB	7.863 ± 0.012^{b}	0.8010 ± 0.0035^{c}	0.440 ± 0.010^{c}

Different letters within a column indicate significantly different means (p<0.05). PFB = pea flour batter, WFB = wheat flour batter, PSPPIB = pea protein + pea starch batter, MSPPIB = pea protein + maize starch batter, PSB = pea starch batter, MSB = maize starch batter

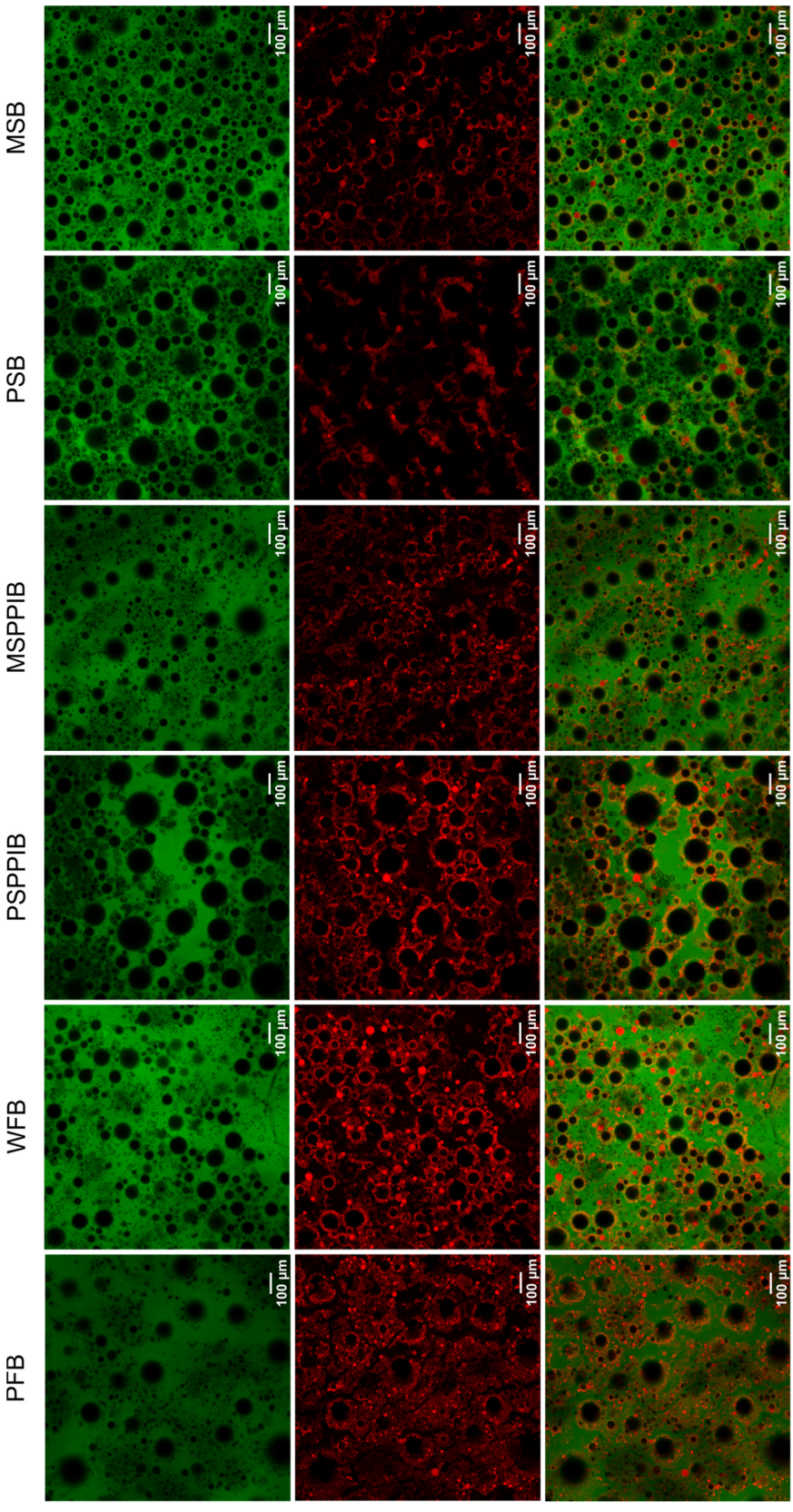


Figure 63. Confocal laser scanning microscopy images of sponge cake batters. Proteins are stained in green, lipids in red. Dark circles represent air bubbles. Magnification: 10×, scale bar: 100 µm. PFB = pea flour batter, WFB = wheat flour batter, PSPPIB = pea protein + pea starch batter, MSPPIB = pea protein + maize starch batter, PSB = pea starch batter, MSB = maize starch batter.

fluorescence indicated reduced lipid contents, attributable to the absence of lipids from the high-purity starches, meaning that those present derived exclusively from egg and oil. In PSB, these seemed to cover a smaller air bubble surface than in MSB, which might be due to steric hindrances imparted by the comparatively larger starch granules. Presumably, this structural characteristic was favoring oxidation in MSB, as reflected by the slightly higher VOC quantity (**Figure 62**). As soon as PPI was added, these steric limitations were less apparent. In PSPPIB and MSPPIB, the lipid and protein content of PPI contributed markedly to stabilizing the air bubbles, thus promoting fatty acid degradation when compared to the starch formulations. A similar picture emerged in WFB. Nonetheless, some lipids participated in the formation of larger droplets, which might therefore have been less available to be oxidized *via* autoxidation. In contrast, lipids in PFB were not only dispersed homogenously throughout the system, but the droplets were also smaller, implying larger reaction surfaces that could favor oxidation.

3.5 Development of cake color and density during baking

During baking, aerated batter is transformed into a solid sponge due to starch gelatinization and protein denaturation. As visible in **Figure 64**, each formulation gave rise to an attractive product with specific densities that followed the order WFC > PFC > PSPPIC/MSPPIC > PSC/MSC. The higher volumes of the PFC and composite cakes could be assigned to their elevated protein contents that might exert their functional properties (**Figure 64**). These findings coincided with those reported by de la Hera *et al.* (2012) and Krause *et al.* (2021a), who observed more voluminous sponge cakes when wheat was replaced with lentil, pea or chickpea flours. Additionally, RVA analysis revealed that starch pasting in WF commences at 79-80°C, which was visibly higher compared to PF (69-70 °C) and the purified starches (71-72 °C each) (data not shown). According to Singh *et al.* (2015), this might be associated with delayed setting of the cake structure, which in turn could have led to a loss of incorporated air and hence a lower cake volume. On the other hand, the remarkably low densities in PSC and MSC might have been attributable to the high starch-to-protein ratio, favoring an increase in viscosity during heating and thus air retention. In addition, it is possible that the egg proteins might develop their full emulsifying and foaming potentials better in the absence of vegetable proteins.

Contrary to density, only minimal differences in CIEL*a*b* color were observed between the six cakes (**Figure 64**). In general, the crust was darker than the crumb, as reflected by lower *L** values. This indicated that whichever ingredient was used, the inherent precursors were available to initiate browning reactions, such as the Maillard reaction and caramelization, because of higher cake surface temperatures than those inside the product (Srivastava *et al.*, 2018).

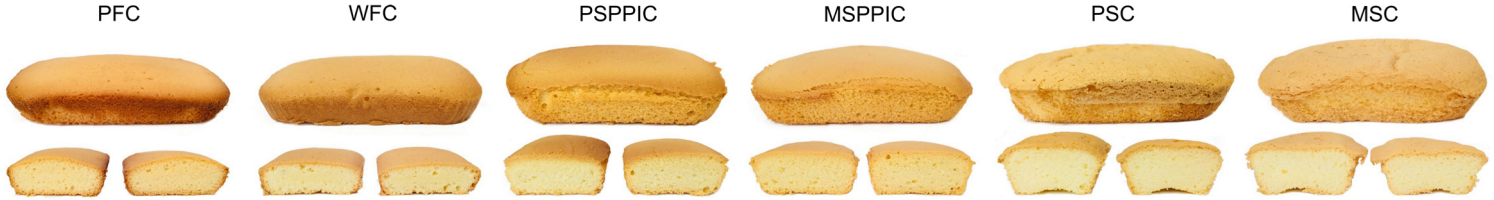
3.6 Heat-driven change of reactivity during baking

3.6.1 Degradation of lipid oxidation intermediates

In addition to structural changes, it was interesting to analyze changes to reactivity during the transition from batter to cake. The quantities of primary lipid oxidation markers, namely conjugated dienes and hydroperoxides, were therefore monitored in both matrices.

Coinciding with the VOC results, PFC was linked with a pronounced susceptibility to fatty acid breakdown, as could be seen from immense increments in peroxide levels and the specific absorbance of conjugated dienes (**Figure 65**). Among the other products, WFC in particular displayed oxidative stability, thus harmonizing with the low volatile contents detected. Similarly, the composite batters only contained traces of intermediates, which confirmed that the fractionation of PF into PPI and PS had led to the inactivation or removal of LOX.

In accordance with data in the literature, baking generally resulted in a reduction in



Cake	Dry matter [%]	Protein [%]	Starch [%]	pH value	Density [g/cm^3]	Crumb color			Crust color		
						L*	a*	b*	L*	a*	b*
PFC	83.935 ± 0.021^{a,b}	15.42 ± 0.46^{b}	18.3 ± 1.7^{c}	6.977 ± 0.012^{c}	0.3194 ± 0.0015^{b}	81.52 ± 0.52^{d}	3.34 ± 0.24^{a}	26.09 ± 0.62^{a}	66.7 ± 1.4^{c}	14.15 ± 0.58^{a}	30.92 ± 0.37^{b}
WFC	84.16 ± 0.86^{a,b}	10.10 ± 0.40^{c}	25.32 ± 0.79^{b}	7.6333 ± 0.0058^{d}	0.3638 ± 0.0018^{a}	84.41 ± 0.84^{b}	1.71 ± 0.21^{b}	23.43 ± 0.63^{c}	66.8 ± 1.1^{c}	12.68 ± 0.22^{c}	29.52 ± 0.36^{c}
PSPPIC	84.52 ± 0.59^{a}	16.50 ± 0.50^{a,b}	23.2 ± 1.6^{b}	8.007 ± 0.040^{b}	0.2735 ± 0.0052^{c}	81.83 ± 0.83^{d}	1.77 ± 0.16^{b}	23.74 ± 0.60^{c}	66.9 ± 1.4c	13.38 ± 0.50^{b}	31.29 ± 0.33^{a}
MSPPIC	84.32 ± 0.33^{a,b}	16.66 ± 0.50^{a}	22.15 ± 0.64^{b}	7.840 ± 0.017^{c}	0.2615 ± 0.0032^{d}	82.47 ± 0.52^{c}	1.85 ± 0.13^{b}	24.44 ± 0.78^{b}	69.5 ± 1.1^{b}	12.44 ± 0.63^{c}	30.78 ± 0.42^{b}
PSC	82.88 ± 0.10^{a,b}	7.61 ± 0.40^{d}	32.3 ± 1.1^{a}	8.520 ± 0.046^{a}	0.2298 ± 0.0037^{e}	87.28 ± 0.74^{a}	0.84 ± 0.15^{c}	17.40 ± 0.36^{e}	70.9 ± 1.3^{a}	10.91 ± 0.24^{d}	27.89 ± 0.37^{d}
MSC	82.48 ± 0.54^{b}	7.65 ± 0.40^{d}	31.67 ± 0.93^{a}	7.997 ± 0.032^{b}	0.2529 ± 0.0047^{d}	87.74 ± 0.77^{a}	0.89 ± 0.17^{c}	18.63 ± 0.55^{d}	70.4 ± 1.2^{a,b}	10.58 ± 0.28^{d}	27.68 ± 0.32^{d}

Figure 64. Appearance and characteristics of sponge cakes. Different letters within a column indicate significantly different means (p<0.05). PFC = pea flour cake, WFC = wheat flour cake, PSPPIC = pea protein + pea starch cake, MSPPIC = pea protein + maize starch cake, PSC = pea starch cake, MSC = maize starch cake.

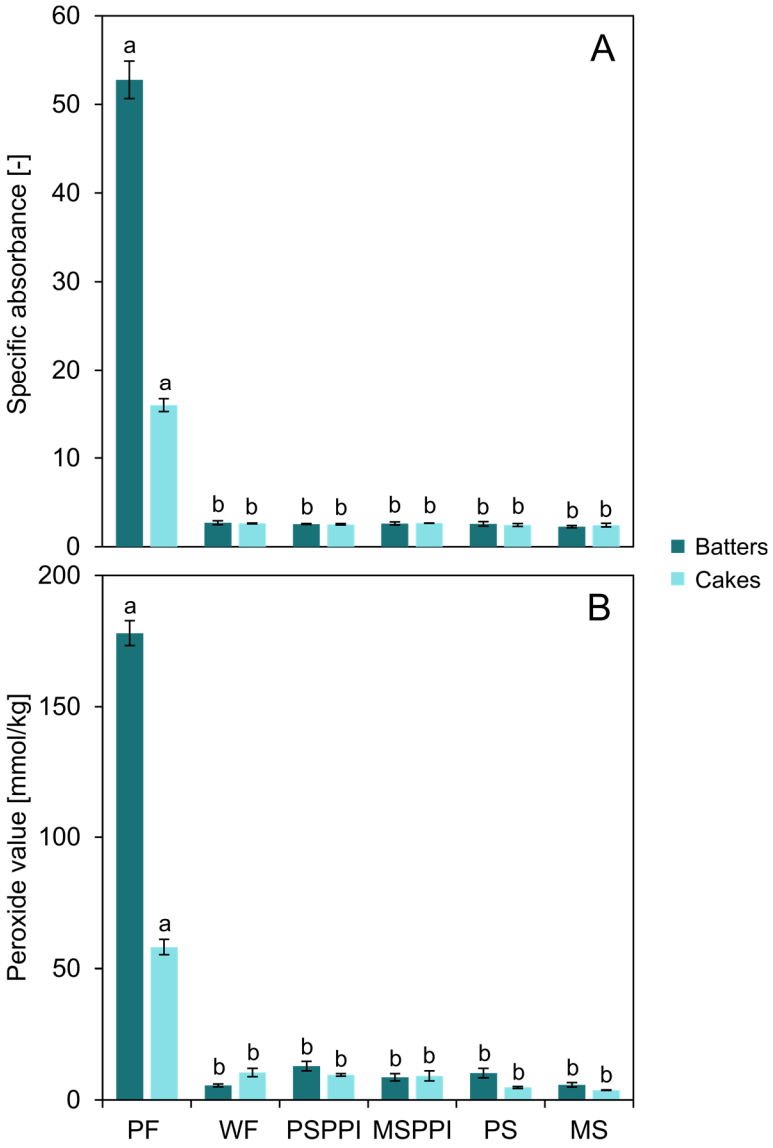


Figure 65. Evolution of (A) conjugated dienes expressed as specific absorbance and (B) hydroperoxides from batters (■) to cakes (■). Different letters within one step of making indicate significantly different means ($p<0.05$). PF = pea flour, WF = wheat flour, PS = pea starch, MS = maize starch, PPI = pea protein isolate.

hydroperoxides because of their transformation into VOCs (Krause *et al.*, 2021a; Maire *et al.*, 2013). This loss was most pronounced in PFC (by 67%) (**Figure 65**). A similar decline was seen regarding conjugated dienes, although they were absent from the other products. Nevertheless, PFC remained significantly enriched in both intermediates, suggesting ongoing lipid oxidation during baking. However, a significant (89%) increase in hydroperoxides was observed in WFC, which indicated elevated reactivity during baking than at the batter beating stage and thus a high degree of lability of the fatty acids in WF towards heat-induced autoxidation. To verify these assumptions, an analysis of the VOC profiles of the cakes was of paramount importance.

3.6.2 Generation of final VOCs

Baking triggered different chemical reactions which gave rise to numerous VOCs that were not present in the flours or batters (**Figure 62**). From a quantitative perspective, however, a direct comparison with the batters revealed smaller quantities of molecules in the case of PFC and the composite cakes (PSPPIC, MSPPIC),

which could be attributed to volatilization, consecutive reactions or matrix effects. By contrast, WFC and the starch cakes (PSC, MSC) were visibly enriched in VOCs, indicating higher reactivity during baking than batter making. Overall, however, the lowest total VOC levels were detected in WFC, while the highest values were found in PFC (**Figure 62**).

Aldehydes formed the main group of volatiles in all cakes, and originated from different pathways depending on the dry ingredients used. While the aldehyde profiles of WFC and the composite and starch cakes were determined by thermal degradation markers, the profile of PFC was clearly dominated by molecules deriving from lipid oxidation (**Table 48** – **Table 51**). In the latter case, it is assumed that a large proportion of the molecules identified remained as residues from batter preparation, but the first appearance of certain compounds might also indicate the heat-induced autoxidation of unsaturated fatty acids. The extracted volatiles comprised 2-hexenal, 2-octenal, 2,4-decadienal, pentanal, heptanal, octanal, nonanal and hexanal, the latter of which accounted for more than half of all aldehydes (**Table 49**). Some of these lipid oxidation markers were also present in the other cakes but at significantly lower levels, particularly in WFC and the starch formulations, which corroborated their limited susceptibility to oxidation as already reported for their batters. This was further underlined with regard to alcohols, which were associated in all cakes with oxidation (e.g. 1-penten-3-ol and 1-hexanol) but only occurred at high levels in PFC.

By contrast, most of the aldehydes extracted from WFC, the starch and composite cakes were markers of amino acid and sugar degradation. These comprised phenylacetaldehyde, 2-methylpropanal, 2-methylbutanal and, in particular, 3-methylbutanal; they are all formed by Strecker degradation at elevated temperatures *via* the oxidative decarboxylation of phenylalanine, valine, leucine and isoleucine, respectively (Belitz *et al.*, 2009; Lee *et al.*, 2020). Among the cakes, PSPPIC and MSPPIC contained the highest relative quantities, followed by the starch formulations, all of which were significantly higher than those in PFC (**Table 49**). This indicated that the fractionation of PF produced purified constituents which were more available to participate in thermal reactions.

This assumption was corroborated when looking at nitrogenous compounds such as pyrazines as they accumulated in significantly larger quantities in the composite and starch cakes than in PFC (**Figure 62**). Formation of these molecules is correlated with the Strecker degradation, which gives rise to not only Strecker aldehydes but also *α*-aminocarbonyl compounds that can condensate to produce dihydropyrazines and then substituted to create alkylpyrazines (Adams *et al.*, 2008; Belitz *et al.*, 2009). In all cakes, 2,5-dimethylpyrazine and 2-methylpyrazine were the most abundant. Despite the generally low levels of pyrazine, PFC differed from the other products in containing several ethylated pyrazines, presumably deriving from the incorporation of acetaldehyde into dihydropyrazine (Shibamoto *et al.*, 1979). This aldehyde might result from the oxidation of ethanol, an alcohol that was abundantly present in the corresponding batter (**Table 48**). By contrast, the presence of acetylated pyrazines in the composite and starch cakes indicated the substitution of dihydropyrazine by formaldehyde, which might have been produced *via* the Strecker degradation of glycine (Adams *et al.*, 2008) but was not detected due to an *m/z* outside the range of detection.

The greater susceptibility of the composite and starch formulations to thermal reactions when compared to PFC was further underlined by the high levels of furanic compounds typically originating from Maillard and caramelization reactions (Belitz *et al.*, 2009; Lee *et al.*, 2020). Among others, these included furfural, 2-furanmethanol and furan, the latter only being present in PSPPIC, MSPPIC, PSC and MSC. In general, formation of these structures is based on the reaction of sugars such as glucose with amino acids, which can undergo enolization and deamination reactions to release 1- or 3-deoxyosones (Perez Locas *et al.*, 2004;

Srivastava *et al.*, 2018). While 3-deoxyosones can give rise to furfural and 2-furanmethanol, 1-deoxyosones can lead to furan (Belitz *et al.*, 2009; Hollnagel *et al.*, 2002; Srivastava *et al.*, 2018). Apart from these, further furanic compounds that tend to derive from lipid oxidation rather than thermal degradation were detected. One example is 2-pentylfuran, which occurred at significantly higher concentrations in PFC, PSPPIC and MSPPIC compared to WFC and the starch cakes, thus substantiating their generally greater predisposition to lipid oxidation.

A similar conclusion could be drawn with regards to ketones, the profile of which was dominated by thermal markers generated *via* the Maillard reaction in all cakes. These included 2,3-butanedione, 2,3-pentanedione, 3-hydroxy-2-butanone and 1-hydroxy-2-propanone, which are formed either directly from glucose or through its reaction with amino acids (Maire *et al.*, 2013; Pico *et al.*, 2015; Yaylayan *et al.*, 2000). In WFC, PSC and MSC, these volatiles accounted for 86-89% of the ketones, whereas slightly smaller percentages were found for PFC, PSPPIC and MSPPIC (60-64%). However, the concentrations were similar in all cakes. This implied that other ketones were present in PFC, PSPPIC and MSPPIC, which originated from fermentation (acetone) or lipid oxidation (2-heptanone, 2-octanone, 2-heptanone, 3,5-octadien-2-one, 3-octen-3-one), thus further emphasizing their higher reactivity potential.

3.6.3 Potential sensory and safety impact of reaction markers in cakes

To gain a deeper understanding of the principal volatiles responsible for the distinctive features of the different cakes, principle component analysis was performed to associate specific reaction markers with individual products and to reach a conclusion regarding their potential impact on the overall odor of the final food. The corresponding PCA biplots are shown in **Figure 66**. As depicted, PC1, PC2 and PC3 accounted for 57.96%, 23.22% and 11.53% of variability. Along PC1, a clear separation of PFC from the other five cakes could be observed, which was mainly driven by lipid oxidation markers that strongly correlated with PFC; this enabled the assumption that the breakdown of unsaturated fatty acids played a crucial role during the development of cakes based on PF. This could be attributed to the high LOX activity which promoted the formation of lipid oxidation-derived volatiles, which are typically associated with green, beany and grassy odors (Xu *et al.*, 2020). Among these, particularly hexanal, with its known green-grassy odor, might be of significant sensory importance owing to its high concentrations and low odor threshold (Buttery *et al.*, 1998; Krause *et al.*, 2021b; Pico *et al.*, 2015).

By contrast, the wheat, composite and starch cakes were correlated on PC1 with VOCs originating from the Maillard reaction and caramelization, which emphasized their pronounced stability against lipid oxidation compared to PFC. Further differentiation was achieved when considering PC2 and PC3. While PSPPIC and MSPPIC clustered in the positive part of PC2, negative loadings were associated with PSC, MSC and WFC as well as PFC. This distribution could be attributed to Strecker aldehydes, furanic compounds and pyrazines, which were characterized by positive signs and thus directly linked to the composite cakes. On PC2 *vs* PC3, however, it emerged that certain pyrazines and furanic compounds also correlated with the starch cakes. Among the thermal reaction markers, Strecker aldehydes with known malty odors were characterized by high odor activities (Buttery *et al.*, 1998; Pico *et al.*, 2015; Rega *et al.*, 2009), and were thus forecast to be key odorants in the composite cakes. In addition, pyrazines have been reported to possess nutty and roasted aromas and might have been important contributors to overall flavor, particularly of the composite and starch cakes (Buttery *et al.*, 1998; Pico *et al.*, 2015; Rega *et al.*, 2009).

Overall, these findings suggested that formulations based on purified starch, and in particular combined with isolated proteins, were more likely to undergo heat-induced reactions than the whole flour. This might indicate the occurrence of molecular changes during the

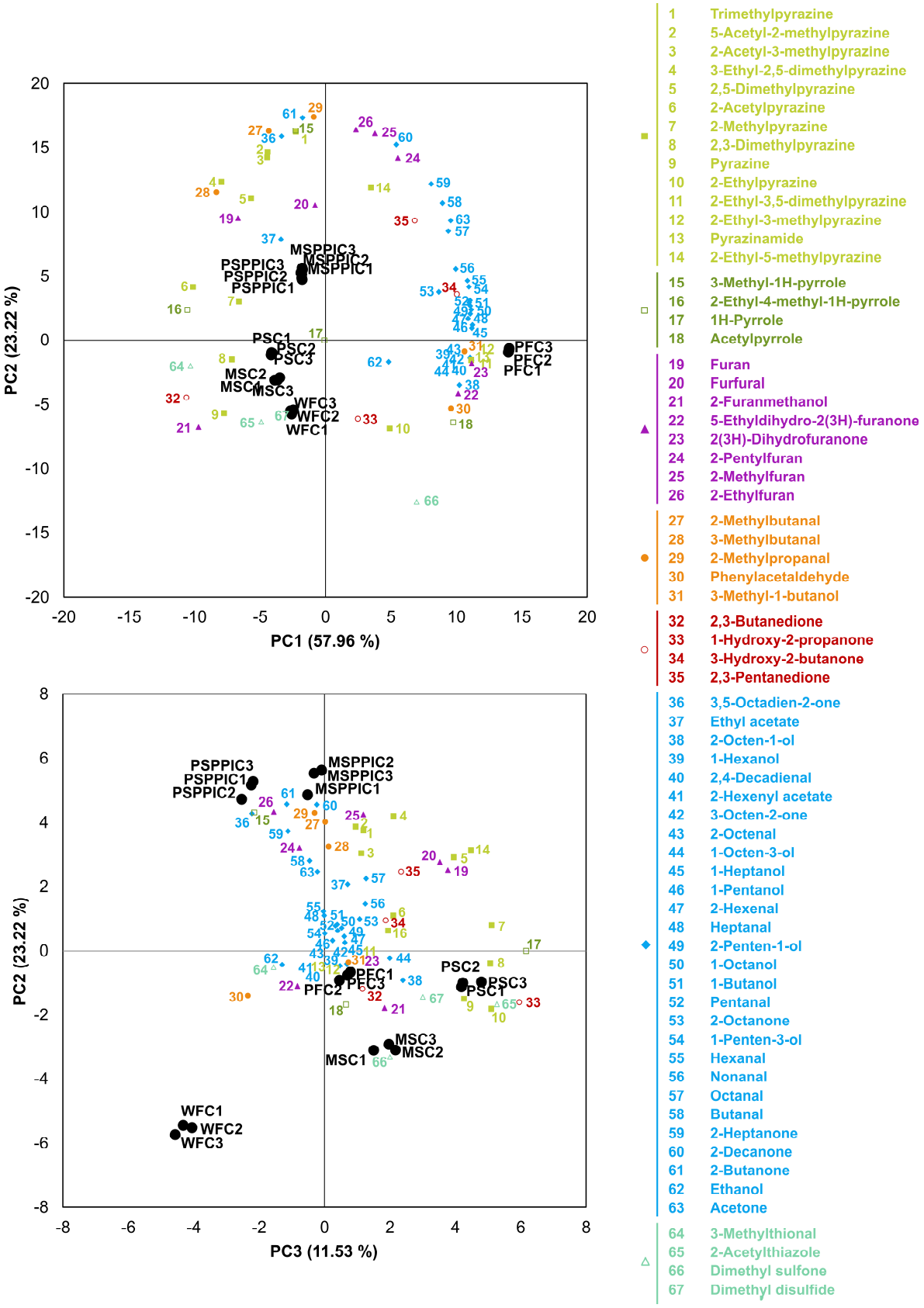


Figure 66. PCA biplots on volatile reaction markers, categorized into pyrazines (■), pyrroles (□), furanic compounds (▲), Strecker aldehydes and derivatives (●), further Maillard and/or caramelization markers (○), lipid oxidation and fermentation markers (♦) and sulfurous compounds (△). PFC = pea flour cake, WFC = wheat flour cake, PSPPIC = pea protein + pea starch cake, MSPPIC = pea protein + maize starch cake, PSC = pea starch cake, MSC = maize starch cake.

fractionation process, possibly resulting in a greater availability of precursors (i.e. glucose and amino acids) for thermal reactions. Moreover, protein changes were reflected by the inactivity of LOX, thus leading to considerably less fatty acid degradation. Notwithstanding, the composite products appeared to be associated with selected lipid oxidation markers on PC2 as opposed to PSC, MSC and WFC, which implied that fractionation produced a protein isolate rich in unsaturated fatty acids that were rapidly degraded *via* non-enzymatic oxidation compared to those derived from wheat flour, egg or oil. Consistent with this, it could be presumed that some lipid oxidation markers might be perceived in PSPPIC and MSPPIC if they had not been masked by the Maillard aroma compounds mentioned above.

Moreover, PC2 *vs* PC3 revealed a clear separation of WFC from the other products (**Figure 66**), indicating the lowest potential to form volatiles through any reaction pathway, potentially due to a limited availability of precursors to undergo changes. Moreover, the corresponding flour was characterized by a low ash content (0.9%) compared to PPI (6.0%) and PF (3.5%) (**Table 47**), which thus might have stimulated the Maillard reaction in products containing PPI and PF (Hayase *et al.*, 1996).

Another result worth mentioning was the similar behavior of pea and maize starch in both the composite and starch formulations (**Figure 66**), which confirmed that chemical composition rather than product microstructure influenced reactivity during cake development.

Finally, particular attention should be paid to furan because of its classification as “possibly carcinogenic to humans” (Group 2B) by the International Agency for Research on Cancer (IARC, 1995). This component was detected in the composite and starch cakes, but not in the products based on pea or wheat flour. From this, it was deduced that flour fractionation led to an increased probability of furan formation and thus counteracted the positive influence on the formation of lipid oxidation-associated off-flavors. However, a more sensitive quantification method needs to be applied in order to accurately quantify furan in the cakes and determine whether its occurrence raises any safety concerns.

4 CONCLUSION

Pea ingredients were used successfully to produce sponge cakes with similar or improved nutritional, structural and optical properties compared to wheat. Moreover, their use was accompanied by the development of a more complex profile of VOCs with potential impacts on the sensory and safety quality of the cakes.

Lipid oxidation emerged as a critical reaction occurring during the beating of pea flour batter, leading to a remarkable increase in intermediate and volatile reaction markers. Pea flour batter contained small and homogenously distributed lipid droplets with large reaction surfaces which might have favored the catalysis of highly active endogenous lipoxygenase. This phenomenon was inhibited when pea flour was replaced with pea protein isolate extracted from it, due to the inactivation of enzymes involved in oxidative reactions during isolation. Consistent with this, products based on isolated pea protein and pea starch mixed at the same ratio as in pea flour were characterized by significantly lower susceptibility to generate compounds *via* oxidation.

On the other hand, baking appeared to trigger the Maillard reaction and caramelization to a greater extent in products made from pea starch and protein isolate, as reflected by the significant development of Strecker aldehydes and pyrazines. This suggested greater availability of amino acids and sugars for thermal reactions in the purified ingredients. We therefore believe that these heat-induced markers with typical malty-nutty odors were important contributors to the overall flavor of the composite cakes, whereas oxidation-derived volatiles linked to green-beany off-notes were of sensory importance in the pea flour cake. Nevertheless, further sensory studies need to be carried out in order to confirm the most odor-active compounds in these applications and their individual contributions to the overall perception.

This study further revealed that pea starch has great potential for use in bakery products, as it behaved similarly to maize starch in terms of both textural and sensory aspects. Overall, the results highlighted the possibility of tailoring food products to desired functionalities and odor profiles by crosslinking processes and formulations.

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5 ADDITIONAL RESULTS

5.1 PARTICLE SIZE DISTRIBUTION OF RAW MATERIALS AND CAKES

In the article it was discussed that the particle size distribution of maize starch was smaller compared to pea starch, with their d_{50} around 14 µm and 24 µm, respectively. In order to substantiate this statement, the associated volume distributions are presented in **Figure 67A**.

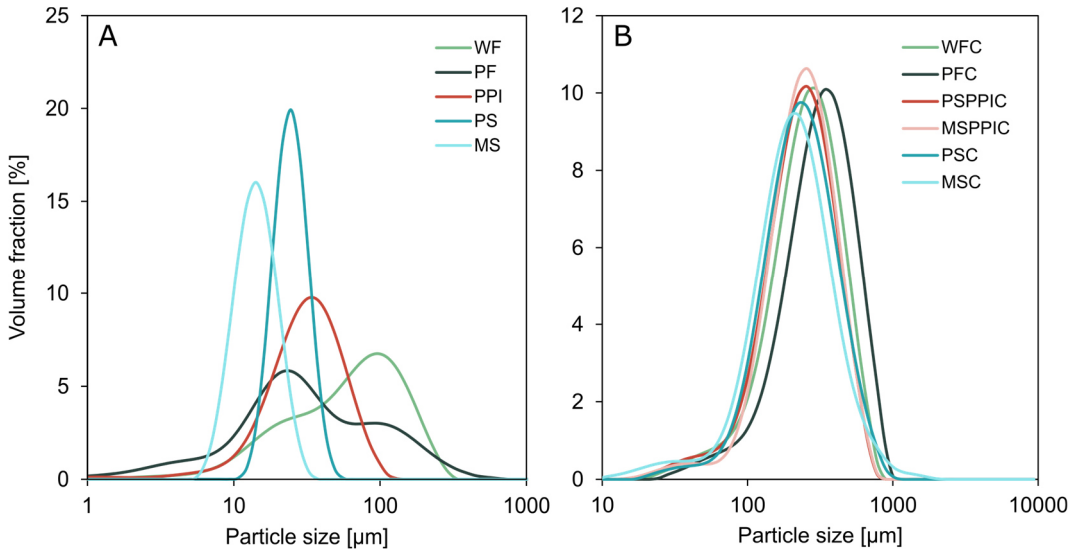

Figure 67. Particle size distribution of raw materials (A) and ground cakes (B). WF = wheat flour, PF = pea flour, PPI = pea protein isolate, PS = pea starch and MS = maize starch.

This graph also shows the particle size distributions of wheat flour and pea flour, which are characterized by two modes: the first mode might correspond to starch granules (peak at around 25 µm), while the second mode might correspond to cotyledon cells (peak at around 98 µm) (Monnet *et al.*, 2019). In

agreement with the literature, the particle size distribution of pea flour was broader (1 – 678 µm) than that of wheat flour (1 – 340 µm) (Monnet *et al.*, 2019).

Moreover, it had to be verified whether the particle sizes of the cakes after grinding were in a similar range in order to minimize adverse effects on the release of VOCs. As can be seen in **Figure 67B**, the volume distributions of all ground cakes were overlapping, indicating that the applied grinding protocol was adequate to reach comparable sizes.

5.2 STARCH GRANULE MORPHOLOGY

As described in **§ 5.1.1 of Part IV**, the morphological characteristics of the starch granules contained in the ingredients were observed under the light microscope in order to get a better understanding of the general properties of the raw materials. In good agreement with the literature, the starch granules contained in the wheat flour were spherical, those of pea starch oval and those in maize starch polyhedral (**Figure 68**) (BeMiller *et al.*, 2009; Bertoft *et al.*, 1993; Hoover *et al.*, 2010; Jane, 2006; Mishra *et al.*, 2006; Ratnayake *et al.*, 2002; Tester *et al.*, 2004). In addition, the wheat and maize starch granules appeared to be smaller than those of the pea, despite the suspension in water and associated swelling.

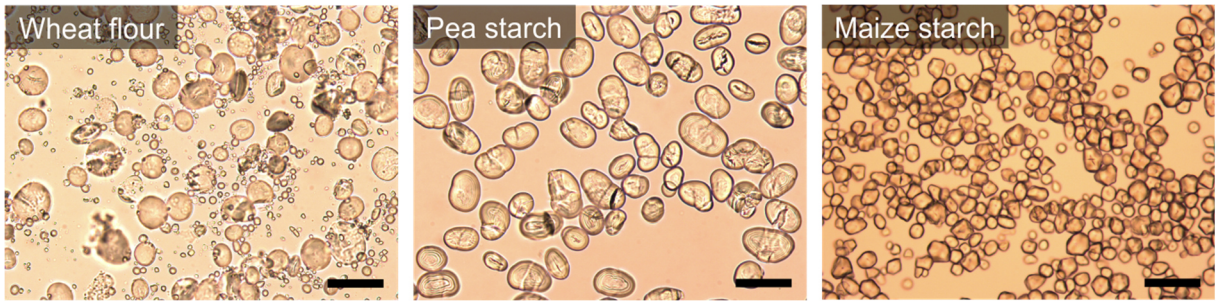

Figure 68. Light micrographs of in water suspended starch granules contained in wheat flour, pea starch and maize starch. Magnification: 50×. Scale bar: 50 µm.

5.3 PASTING PROPERTIES OF THE FLOURS AND FRACTIONS

The different pasting temperatures discussed in the article were determined by analyzing the viscosity curves recorded using the Rapid Visco Analyzer (**§ 5.2.1 of Part IV**). These curves are illustrated in **Figure 69** and the corresponding properties listed in **Table 33**. Apart from the higher pasting temperature in the case of wheat flour compared to pea flour, it can be seen that pea flour had a considerably lower peak viscosity than wheat flour, which might be a consequence of its elevated amylose content that limits granular swelling owing to the development of strong internal hydrogen bonds (Hoover *et al.*, 2010; Hoover *et al.*, 1991; Tester *et al.*, 1990). This seemed to be underlined by the fact that the peak viscosity of pea starch was well below that of maize starch, which is known to contain a higher proportion of amylopectin (see **Table 1**). In addition, the high amylose content of the pea ingredients appeared to affect the trough viscosity, which deviated only slightly from the peak viscosity as opposed to those in wheat flour and maize starch, which were characterized by a more pronounced breakdown. This implied high resistance of pea starch to disintegration under shear. Moreover, the purified pea starch had a high tendency to retrograde during cooling as visible from the high final viscosity, which was remarkably higher compared to purified maize starch.

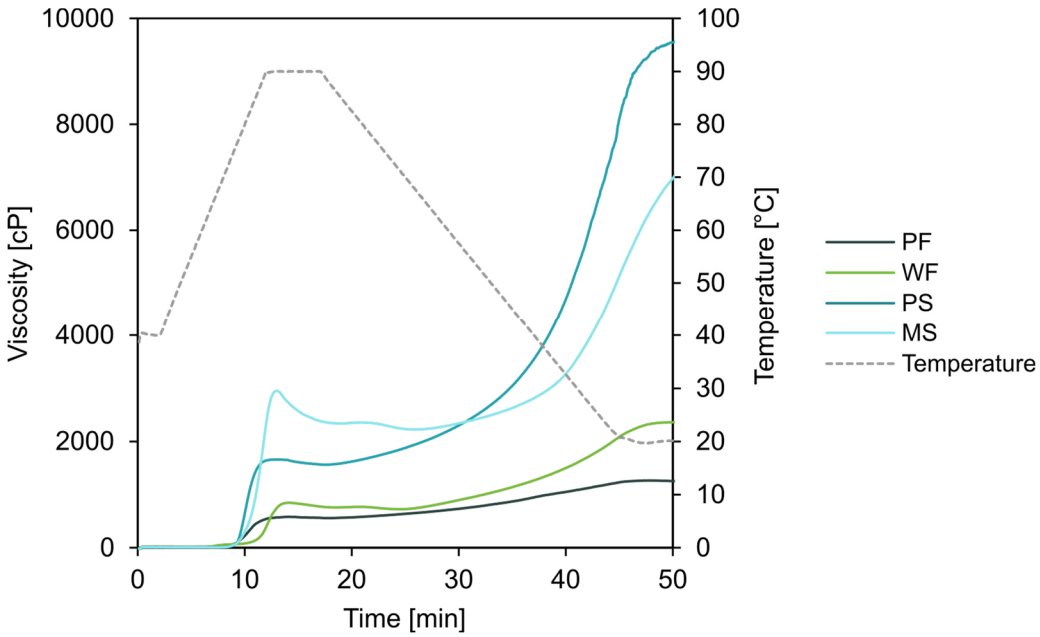

Figure 69. Pasting curves of flours and purified starches. PF = pea flour, WF = wheat flour, PS = pea starch, MS = maize starch.

Table 33. Pasting properties of flours and fractions.

Ingredient	Pasting temperature [°C]	Peak viscosity [cP]	Trough viscosity [cP]	Final viscosity [cP]
Pea flour	69 – 70	607 ± 31	552 ± 13	1219 ± 51
Pea starch	71 – 72	1660 ± 8	1600 ± 49	9547 ± 49
Wheat flour	79 – 80	829 ± 27	746 ± 21	2305 ± 76
Maize starch	71 – 72	3010 ± 82	2375 ± 54	7027 ± 91

5.1 PRECURSORS FOR THERMAL REACTIONS

As discussed in the article, amino acids and sugars are precursors for pyrazines, Strecker aldehydes and furanic compounds. In order to verify that the fractionation of the pea flour into pea starch and proteins led to presence of more precursors that could undergo the Maillard reaction and caramelization, we kindly commissioned Improve (Dury, France) to perform the analysis of the free amino acid and sugar composition of the flours and fractions. The results are presented in **Table 34**.

Unfortunately, however, the concentrations measured by Improve could not be used to interpret the different abilities of the ingredients to form the mentioned reaction markers. This is due to the limited sensitivity of the method applied, which provided no reveal relevant information for either amino acids or sugars. Therefore, it would be recommended to implement a more precise but robust method in order to detect minimal differences between the ingredients.

Table 34. Concentration of free amino acids as well as mono- and disaccharides in the different flours/fractions.

Compound	Concentration [g/100g]				
	PF	WF	PPI	PS	MS
Free amino acids					
Cysteine	<0.02	<0.02	<0.02	<0.02	<0.02
Methionine	<0.02	<0.02	<0.02	<0.02	<0.02
Glycine	<0.02	<0.02	<0.02	<0.02	<0.02
Threonine	<0.02	<0.02	<0.02	<0.02	<0.02
Tyrosine	<0.02	<0.02	<0.02	<0.02	<0.02
Lysine	<0.02	<0.02	<0.02	<0.02	<0.02
Histidine	<0.02	<0.02	<0.02	<0.02	<0.02
Serine	<0.02	<0.02	<0.02	<0.02	<0.02
Alanine	<0.02	<0.02	<0.02	<0.02	<0.02
Aspartic acid	0.07 ± 0.03	<0.02	<0.02	<0.02	<0.02
Glutamic acid	0.16 ± 0.03	<0.02	0.02 ± 0.03	<0.02	<0.02
Arginine	0.20 ± 0.03	<0.02	0.04 ± 0.03	<0.02	<0.02
Isoleucine	<0.02	<0.02	<0.02	<0.02	<0.02
Proline	<0.02	<0.02	<0.02	<0.02	<0.02
Phenylalanine	<0.02	<0.02	<0.02	<0.02	<0.02
Valine	<0.02	<0.02	<0.02	<0.02	<0.02
Leucine	<0.02	<0.02	<0.02	<0.02	<0.02
Tryptophane	<0.02	<0.02	<0.02	<0.02	<0.02
Mono- & disaccharides					
Glucose	<0.2	<0.2	<0.2	<0.2	<0.2
Fructose	<0.2	<0.2	<0.2	<0.2	<0.2
Sucrose	2.3 ± 0.8	0.4 ± 0.4	<0.2	0.3 ± 0.3	<0.2
Lactose	<0.2	<0.2	<0.2	<0.2	<0.2
Maltose	<0.2	1.7 ± 0.7	<0.2	<0.2	<0.2

5.2 COLOR PROPERTIES OF THE BATTERS

The influence of the original color of the ingredients on the color of the developed products can be determined not only from the analysis of the color of the final cakes but also of the corresponding batters. Consequently, the CIEL*a*b* color values of the six batters were measured and the data obtained were converted into RGB values, which are illustrated as color charts in **Figure 70**. As it can be seen, the batters had very similar colors, implying that the influence of the slightly differently colored raw materials was minimal.

Figure 70. Color charts of sponge cake batters made with different raw materials. WFB = wheat batter, PFB = pea flour batter, PSPPIB = pea starch + pea protein batter, MSPPIB = maize starch + pea protein batter, PSB = pea starch batter and MSB = maize starch batter.

5.3 LOCAL DISTRIBUTION OF INGREDIENTS IN THE CAKE CRUMB

Apart from the batters, also the cakes were observed under the confocal laser scanning microscope. As shown in **Figure 71**, however, the captured images were difficult to interpret due to the dominance of dark areas, which complicated the analysis of the structural properties, i.e. organization of lipids, proteins and starch granules, of the cakes. Nevertheless, for the sake of completeness, these figures are included in this work.

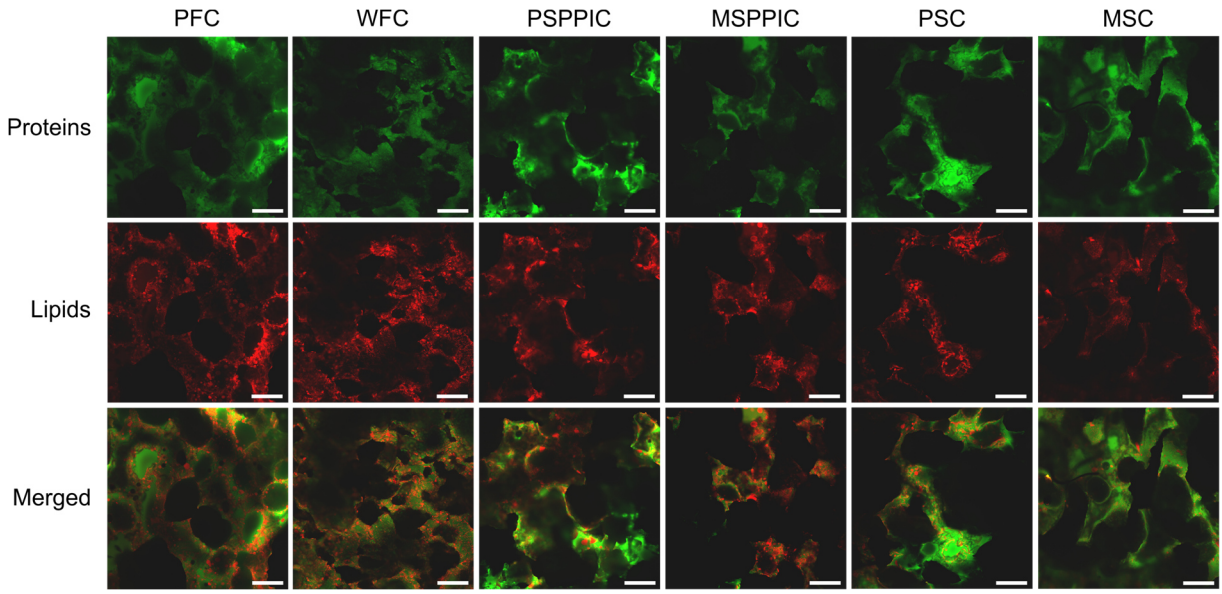

Figure 71. Confocal laser scanning microscopy images of sponge cakes. Proteins are stained in green, lipids in red. Dark circles represent air bubbles. Magnification: 10×, scale bar: 200 µm. PFC = pea flour cake, WFC = wheat flour cake, PSPPIC = pea protein + pea starch cake, MSPPIC = pea protein + maize starch cake, PSC = pea starch cake, MSC = maize starch cake.

5.4 MECHANICAL PROPERTIES OF THE CAKES

As discussed in the article, the structural properties of the cakes could be distinguished at macroscopic level in terms of their densities. Further discrimination of the textural differences between the cakes can be achieved by the mesoscopic analysis of their response to mechanical stress. Consistently, an uniaxial compression test was performed in quintuplicate (see details in **§ 5.2.5 of Part IV**), which provides information about the stiffness of the crumb at small deformations as well as the stiffness of cell walls at large deformations. Both information were expressed as Young moduli, which corresponded to the

slopes of the stress-versus-strain curves during compression from 1% to 6% and 87% to 90% of the initial height, respectively. The results are presented in **Table 35**.

As visible from **Table 35**, the method applied yielded less repeatable Young moduli due to large standard errors, which complicates the detection of significant differences between the samples. In the case of the Young moduli of the crumb, this observation could be attributed to the use of a high force load (750 kg), which was not sensitive enough to measure trigger forces of less than 1 N that occurred at small deformations. By contrast, the large standard errors of the Young moduli of the cell walls may be explained by moisture loss of the cake samples, modifying the responses during compression. For these reasons, it was decided that the calculated values were not representative and the applied method was not appropriate for measuring differences between the products. This was corroborated by the results of Pedro Martinez Noguera, who analyzed a new batch of the same cake formulations and found large variations in the measured values (data not shown), which did not allow to conclude about the responses of the different cakes to mechanical stress. For future experiments it is therefore recommended to analyze products with a height greater than 1 cm (as was the case in the present study) and to use more suitable load cells to measure small deformations.

Table 35. Mechanical properties of sponge cakes.

Cake	Young modulus of the crumb [kPa]	Young modulus of the cell walls [kPa]
WFC	24.3 ± 9.3^{b}	5467 ± 4190^{a}
PFC	45 ± 15^{a}	1527 ± 425^{b}
PSPPIC	23.8 ± 5.2^{b}	1723 ± 773^{b}
MSPPIC	25.3 ± 7.4^{b}	1251 ± 235^{b}
PSC	14.9 ± 3.8^{b}	742 ± 234^{b}
MSC	12.2 ± 3.0^{b}	835 ± 347^{b}

5.5 CELLULAR STRUCTURE OF THE CAKE CRUMBS

The textural characteristics of the different cakes could be further evaluated by assessing the crumb cellular structure using image analysis based on mathematical morphology (see **§ 5.2.4 of Part IV**). This method allows to conclude about cell size and homogeneity as well as cell wall thickness and degree of openness. Only elements with sizes ranging from 0.13 mm to 3.4 mm are taken into consideration.

The granulometric curves obtained by mathematical morphology for the different cake crumb images were subjected to principle component analysis. The corresponding PCA plot is shown in **Figure 72**. Interestingly, the distinction of the different crumb structures was almost exclusively driven by PC1, which accounted for 80% of the total variability. In order to better interpret the structural properties being responsible for this distribution, the PC loadings were observed in detail.

As indicated in **Figure 73**, the left side of the loadings corresponded to the dilatation steps, which provide information about the resistance of dark objects, i.e. cells, to brightening, while the right side corresponded to the erosion steps, which provide information about the resistance of bright objects, i.e. cell walls, to darkening. The unit of the abscissa is millimeters, which has been calculated on the basis of the known image pixel size. The two horizontal lines at 0.4 and -0.4 indicate the significance level.

Above or below these boundaries, the particle size is considered relevant to drive the distribution of the different cake crumb images on the PCA plot.

PC1 (blue color) showed four significant bands, two of which were in the positive and two in the negative part (**Figure 73**). The positive bands corresponded to the dilatation and erosion steps, which involved larger structuring elements (0.8 – 3.5 mm and 1.0 – 3.5 mm, respectively) and the negative bands corresponded to the dilatation and erosion steps, which involved smaller structuring elements (0.13 – 0.7 mm and 0.13 – 0.9 mm, respectively). Consistently, the cake crumb images that were positioned in the positive part of PC1 in **Figure 72** contained larger structuring elements (WFC, PSC, MSC), while those situated in the negative part of PC1 contained smaller structuring elements (PFC, PSPPIC, MSPPIC). When considering that the dilatation steps are correlated with the darker elements of the cake crumb, it could be concluded that WFC, PSC and MSC contained cells of larger sizes (0.8 mm – 3.4 mm) compared to PFC, PSPPIC and MSPPIC (0.13 – 0.7 mm). Based on the knowledge about erosions, it was also deduced that WFC, PSC and MSC contained thicker cell walls (1.0 – 3.5 mm) than PFC, PSPPIC and MSPPIC (0.13 – 0.9 mm). Accordingly, the crumb structures of WFC, PSC and MSC were characterized by large open cells with thick walls.

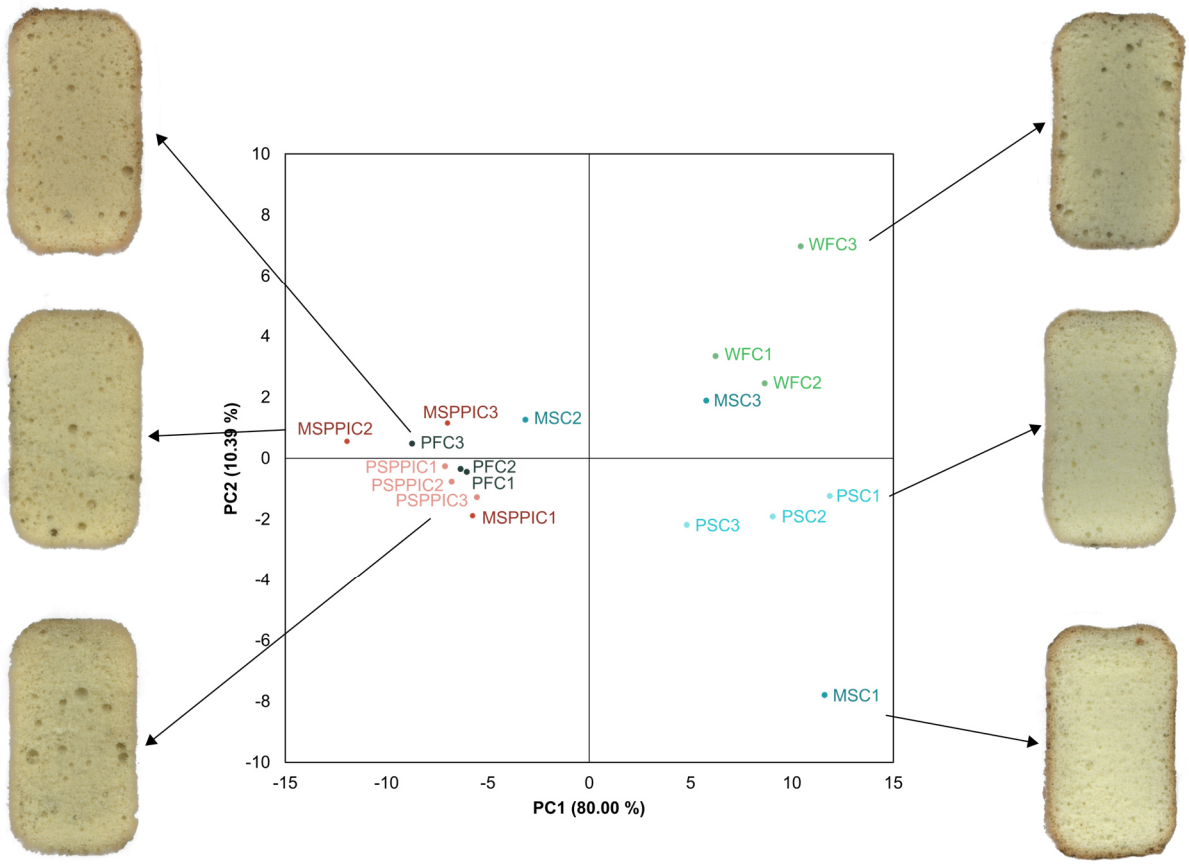

Figure 72. PCA plot representing the cake crumb images (*n* = 3) of the different sponge cakes. Only elements of sizes ranging from 0.13 mm to 3.4 mm were considered.

By contrast, PC2 (orange color) showed only one significant band in the positive part (**Figure 73**), which was related to the dilatation steps and sizes of 0.4 – 0.9 mm. This implied that cakes, that were associated with positive scores on PC2 contained small cells. At the same time, however, it also

emphasized that PC2 was less important (only 10% of the total variability) in driving the distinction of the different cakes.

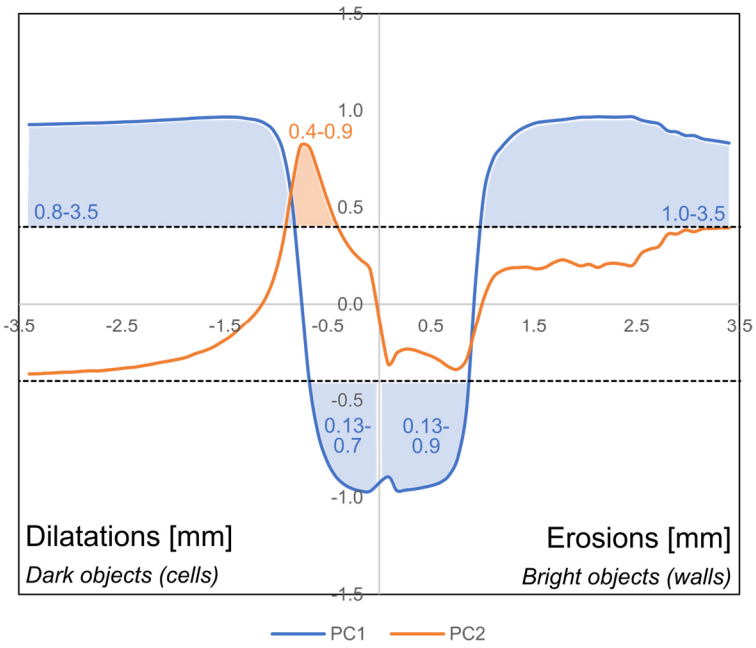

Figure 73. First and second loading corresponding to the principle components PC1 and PC2 of the general PCA. The level of significance was set to 0.4.

5.6 CORRELATIONS BETWEEN STRUCTURAL AND CHEMICAL CHARACTERISTICS

In order to elaborate correlations between the structural properties of the cakes, i.e. degree of cell openness and wall thickness, crumb stiffness, cell wall stiffness and cake densities, and their chemical characteristics (protein, starch, dry matter and fiber content), a PCA was performed. It has to be reminded that the mechanical properties assessed by uniaxial compression were not fully representative (see **§ 5.4 of Chapter 5 of Part V**), for which reason the results described in the following section will only help to explain some tendencies. Moreover, the fiber contents were based on those determined in the flours instead of the cakes. The correlation matrix between the parameters is given in **Table 36**.

A significant negative correlation (-0.497) between the crumb stiffness and the degree of openness/wall thickness was found, indicating that smaller cells with thinner cell walls led to a firmer cake crumb. This appeared to be in particular the case with cakes based on whole pea flour. Due to the negative correlation between the degree of openness/wall thickness and protein content (-0.874), it was concluded that the highly abundant proteins in the pea-based cakes were able to stabilize the incorporated air in the form of small bubbles, attributable to their favorable foaming ability (see **§ 3.2.4 of Part II**). Moreover, the positive correlation between crumb stiffness and both protein (0.601) and fiber content (0.833) implied that the high prevalence of these components were responsible for the increased crumb stiffness, possible because of they are binding water and form of a rigid network.

In addition, a significant positive correlation between density and cell wall stiffness was observed (0.645), which suggested that a higher cell wall stiffness resulted in a higher cake density. These values were highest in the cakes based on wheat flour, which is known to contain a high proportion of proteins

with sulfur-containing amino acids, which can form inflexible disulfide bonds (see **§ 3.1 of Part II**). Therefore, it could be imagined that this wheat gluten could have formed a firm film around the air bubbles, resulting in an elevated cell wall stiffness. The fact that the air bubbles in this cake were assumed to be larger in size compared to those in cakes containing pea protein (either in the form of flour or protein isolate), suggesting that the pea proteins possessed better surface activities and thus foaming abilities than the wheat gluten, thereby leading to smaller cells with thinner cell walls.

Table 36. Correlation matrix between mechanical properties, structural parameters, density and chemical characteristics of sponge cakes.

Variables	Openness	Density	$Stiffness_{Crumb}$	$Stiffness_{Walls}$	Protein	Starch	Dry matter	Fiber
Openness	**1**	0.032	**-0.497**	0.199	**-0.874**	**0.753**	-0.345	-0.154
Density	0.032	**1**	0.461	**0.645**	0.176	**-0.533**	**0.711**	**0.614**
$Stiffness_{Crumb}$	**-0.497**	0.461	**1**	0.318	**0.601**	**-0.712**	**0.514**	**0.833**
$Stiffness_{Walls}$	0.199	**0.645**	0.318	**1**	-0.009	-0.124	**0.479**	0.299
Protein	**-0.874**	0.176	**0.601**	-0.009	**1**	**-0.871**	**0.644**	0.234
Starch	**0.753**	**-0.533**	**-0.712**	-0.124	**-0.871**	**1**	**-0.739**	**-0.556**
Dry matter	-0.345	**0.711**	**0.514**	**0.479**	**0.644**	**-0.739**	**1**	0.388
Fiber	-0.154	**0.614**	**0.833**	0.299	0.234	**-0.556**	0.388	**1**

Values in bold are different from zero with a significance level $\alpha = 0.05$.

6 FINAL CONCLUSION

This study showed that the use of different pea ingredients led to products with an attractive appearance, which was similar to a wheat-based reference. Furthermore, the methodological approach chosen could provide valuable insights into the varying susceptibility of the various ingredients to undergo changes *via* lipid oxidation, Maillard reaction and caramelization during the different steps of sponge cake production. Noteworthy was the fact that the enormous differences in the VOC quantities, that were observed between the batters based on either whole pea flour (PFB) or a combination of isolated pea proteins and pea starch (PSPPIB), were of much less importance in the final cakes. Although the whole pea flour cake was associated more strongly with VOCs with typical green odors, numerous thermal reaction markers were also present that may mask or compensate for the undesirable aroma. It is therefore of great importance to evaluate the actual relevance of the individual compounds for the sensory quality of the cakes by means of further sensory experiments.

In addition, this study succeeded in identifying the most important factors that were responsible for the different abilities of the raw materials to form volatile compounds by analyzing not only the chemical composition and enzymatic activities in the raw materials but also the local distribution of reactive ingredients in the batters. This knowledge can be helpful for the formulation of appealing pulse-based products with high quality.

The additional results on the structural properties of the cake crumbs could give some insight into the differences between the products on macroscopic and microscopic level, which are relevant parameters that can affect the overall quality of the cakes. The outcome of the uniaxial compression test should be verified by repeating the experiment with adequate cake dimensions and loading cells. Complementary experiments could be performed, including the non-destructive analysis of the three-dimensional internal structure of the cakes using X-ray microtomography in order to gain a better understanding of the

complex food matrix. These instrumental results should then be coupled with sensory studies to relate the experimental data to quality-determining attributes, such as mouthfeel and texture. This allows to obtain a global view on the applicability of new pulse ingredients in foods.

CHAPTER 5

DIGESTION OF PEA-BASED CAKES

In addition to the influence of unrefined pea flour and purified pea fractions on the aroma and texture of sponge cakes, it is also important to clarify possible differences in their digestion behaviors in order to obtain a holistic picture of their suitability in food and ultimately their attractiveness for the consumer.

Based on the particular chemical composition of pea ingredients, their use to replace wheat flour in bakery products is correlated with an increased protein content as well as lower starch content. However, whether one can benefit from these nutritional advantages depends on how well these macronutrients are digested. With regard to protein, a high degree of hydrolysis would be beneficial, whereas to opposite would be desired for starch due to a slower release of glucose into the blood, leading to an increased stability in the insulin response, which reduces the risk of obesity, high blood cholesterol and type 2 diabetes mellitus (Jenkins *et al.*, 2012; Rizkalla *et al.*, 2002).

Researchers categorized pulses such as peas as foods with low glycemic index (GI < 55), which is a measure of the effect of products on the postprandial blood glucose response (Jenkins *et al.*, 1980; Lu *et al.*, 2018; Rizkalla *et al.*, 2002). So far, research has often focused on the digestibility of pulses as such, while knowledge about their behavior in complex formulated food is still very limited. One of the few studies available has been performed by Marinangeli *et al.* (2011) and deals with the ability of pea flour to reduce risk factors associated with cardiovascular diseases and diabetes in overweight hypercholesterolaemic individuals. The authors were able to demonstrate that the intake of muffins made with pea flour could effectively reduce fasting insulin concentrations and insulin resistance as well as the android adiposity of women when compared to a wheat-based control (Marinangeli *et al.*, 2011). It was therefore concluded that pea flour is a promising ingredient for the preparation of complex foods with high nutritional quality.

We wanted to build on this idea and evaluate the digestion of sponge cakes made with pea flour compared to a wheat flour reference. In addition, it was considered to be interesting to evaluate the different digestibility of unrefined and purified pea ingredients (pea flour versus pea protein isolate and pea starch) which has not yet been clarified. This aimed to understand whether ingredient purification altered their susceptibility to enzymatic hydrolysis. In the literature, it has been discussed that fractionation of pulse flours into their major constituents can eliminate some anti-nutritional factors, such as tannins and phytates, which are known to be present in the flour and can interfere with protein

digestion (Fernández-Quintela *et al.*, 1997; Sarwar Gilani *et al.*, 2012). At the same time, however, other components such as dietary fiber with positive impact on digestive health are also removed. In addition, we were able to show in our previous study that the purified pea fractions were more prone to undergo changes *via* thermal reactions compared to the whole pea flour, which implied changes in their chemical composition associated with the isolation process. Whether these structural alterations also play a role in their digestibility is yet to be investigated.

For this reason, study 5 aimed to compare the *in vitro* digestion of protein and starch in sponge cakes made using either whole pea flour or purified pea fractions. The wheat- and maize-based products that were developed in study 4 were also included in this digestion study in order to better categorize the observed digestion kinetics and to draw conclusions about the global nutritional quality of the developed cakes. Apart from the different chemical characteristics of the raw materials that could influence the enzymatic breakdown of the macronutrients, an influence of the structural properties of the final cakes was also conceivable. Consistently, the structural properties of the cake crumbs were assessed to elaborate correlations with the measured digestion kinetics.

The experiments were performed at the research facilities of KU Leuven and Cargill in Belgium during a 6-month secondment. The results will be discussed in the following pages and are planned to be published in the journal Food & Function.

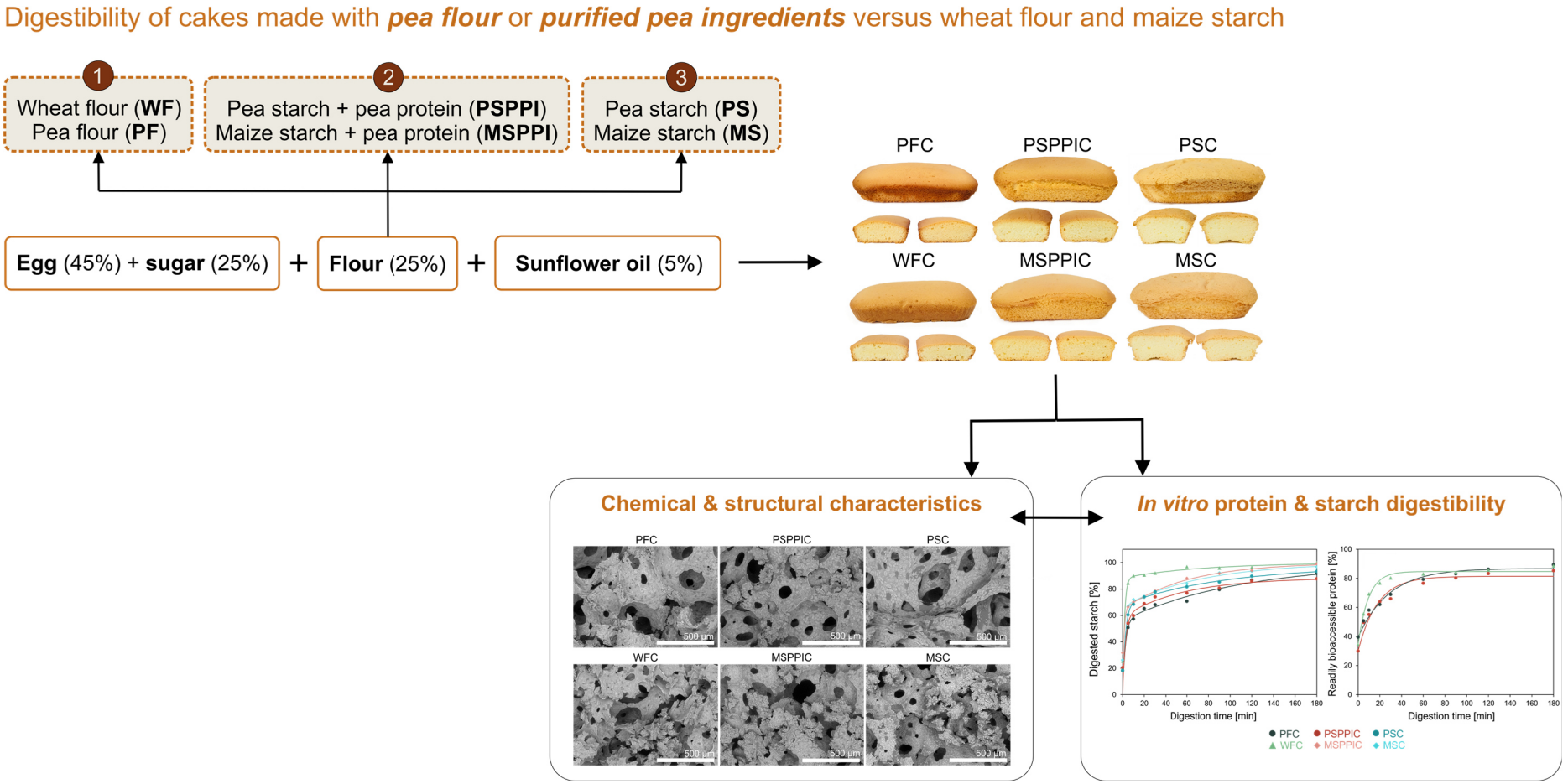

Figure 74. Graphical abstract of article 4.

IN VITRO DIGESTION OF PROTEIN AND STARCH IN SPONGE CAKES FORMULATED WITH PEA (*PISUM SATIVUM* L.) INGREDIENTS

Article 4 – accepted in Food & Function (with modifications)

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ABSTRACT

This study investigated the *in vitro* digestion of purified pea fractions (protein isolate and starch) in sponge cakes when compared to unrefined pea flour and to the whole wheat flour and purified maize starch commonly used in the food industry. Proteins in the wheat cake were hydrolysed more rapidly than those in cakes made with either pea flour or a combination of pea proteins and purified starch. In absolute terms, however, more readily bioaccessible protein was released from these pea cakes (by around 40%). By contrast, cakes containing wheat flour or maize starch were more susceptible to amylolysis compared to those based on pea starch in the form of the purified ingredient or whole flour. This could be attributed to a higher proportion of amylose and resistant starch in the pea cakes as well as structural characteristics that might have decelerated enzyme-substrate interactions. Interestingly, similar digestion patterns were observed regarding the purified pea ingredients and unrefined whole pea flour. It was therefore concluded that pea ingredients, and particularly the less purified and thus more sustainable whole pea flour, are promising plant-based alternatives for use in gluten-free baked products.

1 INTRODUCTION

In recent years, pea (*Pisum sativum* L.) has gained considerable importance as a plant-based food ingredient due to its high content in proteins with a balanced amino acid ratio, low glycaemic index, and abundant availability and affordability (Boukid *et al.*, 2021; Boye *et al.*, 2010a; Lam *et al.*, 2018). Furthermore, pea is considered an appropriate alternative to soy and wheat because of its non-GMO status and low allergenicity (Stone *et al.*, 2015). Wheat flour, in particular, is the main ingredient in a wide variety of commercial products, including baked goods such as sponge cakes. However, with increasingly common diagnoses of coeliac disease and wheat allergy, the demand for gluten-free products is rising steadily (Woomer *et al.*, 2021). The global gluten-free product market is therefore projected to reach an average annual growth rate (CAGR) of 9.2% during a forecast period from 2020 to 2027 (Grand View Research, 2020).

Various pea protein ingredients are used in the food industry; they include flour (up to 30% protein), concentrate (50-75% protein) and isolate (>80% protein). Of these, pea protein isolates in particular have gained in popularity because of their promising functional properties (emulsifying, foaming and gelling), which can be exploited to generate products with appealing textural characteristics. On the other hand, however, the protein purification process necessitates the use of resources such as energy, water and chemicals, which increases the footprint of protein isolates compared to the unrefined flour (Lie-Piang *et al.*, 2021). Moreover, large quantities of pea starch are produced as a by-product of protein extraction, but it is still rarely incorporated in food products (Ratnayake *et al.*, 2002). Apart from this sustainability perspective, the fractionation of flour into proteins and starch is linked to the removal of dietary fibre, which is known to retard starch digestion and thus slow down glucose release (Liu *et al.*, 2020; Schuchardt *et al.*, 2016). In addition, it may alter the structure and composition of the proteins and starch, which could affect their susceptibility to enzymatic hydrolysis during digestion (Chao *et al.*, 2018; Shevkani *et al.*, 2019).

To date, and when compared to traditional wheat flour, information on the digestibility of pulse proteins and starch in complex formulated foods such as cakes is still very limited (Assad-Bustillos, *et al.*, 2020a; Assad-Bustillos *et al.*, 2020b; Gularte *et al.*, 2012). In particular, the potential differences in the digestion behaviour of unrefined and purified ingredients have not yet been elucidated. In our previous study, we were able to demonstrate that both purified pea fractions and whole pea flour could be used with success to produce visually attractive sponge cakes similar to a wheat reference, as well as interesting sensory-related qualities (Krause *et al.*, 2021d). Knowledge of their different digestion kinetics would therefore help to obtain a holistic picture of the applicability of diverse pea ingredients in nutritious gluten-free products.

The aim of this study was therefore to investigate the *in vitro* digestion of purified pea fractions (protein isolate and starch combined) in sponge cakes compared to whole pea flour from the same batch as well as a wheat-based reference. Moreover, sponge cakes based purely on pea starch were developed in order to explore the roles of proteins from different sources (egg, pea, wheat) by omission. The digestion behaviour of this cake was compared versus a formulation prepared with maize starch only (which is a purified starch often used in the food industry) in order to gain a greater understanding of the digestion of pea starch that is still rarely used in foods.

The progress of protein digestion was assessed by determining the readily bioaccessible fraction, while starch digestion was investigated by quantifying the sugars released. In the latter case, the rapid and relatively inexpensive spectrophotometric analysis of reducing sugars was compared with the exact but time-consuming chromatographic determination of individual starch degradation products. In addition, further chemical and structural characterisations of the raw materials and cakes were performed in order

to determine potential correlations with the digestion kinetics thus measured.

2 MATERIALS AND METHODS

2.1 INGREDIENTS

Flour, starch and protein isolate from the same batch of yellow pea, as well as maize starch, were provided by Cargill (Vilvoorde, Belgium). Wheat was purchased from Axiane Meunerie (Olivet, France), sucrose from Tereos (Lille, France), sunflower oil from Lesieur (Asnières-sur-Seine, France) and whole pasteurised liquid egg from Ovoteam (Locmine, France).

2.2 CHEMICALS

Anhydrous citric acid, dimethyl sulfoxide, disodium tetraborate decahydrate (DT), ethanol, hydrochloric acid (HCl), magnesium chloride hexahydrate, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride and trichloro acetic acid (TCA) were obtained from Fisher Scientific (Merelbeke, Belgium), glacial acetic acid, ammonium carbonate, amyloglucosidase (EC 3.2.1.3, from *Aspergillus niger*), porcine bile extract, calcium chloride, chymotrypsin (EC 3.4.21.1, from bovine pancreas), D(-)-fructose, D(+)-glucose anhydrous, D(+)-maltose monohydrate, 3,5-dinitrosalicylic acid (DNS), 1,4-dithiothreitol (DTT), iodine, maltotriose, *o*-phthaldialdehyde (OPA), pancreatin (EC 232.468.9, from porcine pancreas), pepsin (EC 3.4.23.1, from porcine gastric mucosa), potassium iodide, potassium sodium tartrate tetrahydrate (PST), L-serine, anhydrous sodium acetate, sodium dodecyl sulphate (SDS), sodium thiosulfate, sucrose, trypsin (EC 3.4.21.4, from porcine pancreas) and urea from Merck (Darmstadt, Germany) and sodium hydroxide (NaOH) from VWR (Leuven, Belgium).

2.3 SPONGE CAKE FORMULATION

Sponge cakes were prepared according to the method described by Krause *et al.* (2021d). Eggs (45% w/w) and sucrose (25% w/w) were beaten together for 10 min using a stand mixer equipped with a vertical whisk (KitchenAid Artisan 5KSM150, St. Joseph, Michigan, USA). For the *flour formulations*, non-sifted pea flour or non-sifted wheat flour (25% w/w) was gradually added within 1.5 min. Wheat flour was used as the reference for pea flour as it is the traditional flour component of baked goods. For the *composite formulations*, flour was replaced with a mixture (1/2 w/w) of pea protein isolate and either pea starch or maize starch, to obtain similar proportions as in the corresponding pea flour. Maize starch was used because it is one of the commonly used purified starches in the food industry and thus served as a reference for pea starch. For the *starch formulations*, flour was replaced by either pea starch or maize starch, without the addition of supplementary protein. After beating the different mixtures for 30 s, the sunflower oil (5% w/w) was slowly incorporated within 15 s and the batter mixed for a further 60 s. The compositions of all formulations and their respective abbreviations are indicated in **Table 37**. A total of 630 g batter was produced, which was filled in 21 aluminium moulds (25 g each, 9.8×6.2×3.3 cm^3) and baked for 25 min at 170 °C. The cakes were then deep-frozen at -20°C in hermetically sealed glass jars until the digestion experiments.

2.4 CAKE GRINDING

To analyse the chemical composition of the cakes and perform digestion experiments, 15 frozen cakes were ground in batches of five for 20 s at 6000 rpm using a Grindomix GM200 knife mill equipped with a stainless-steel bowl and titanium blades to a similar particle size distribution (**Figure 67**) (Retsch GmbH, Haan, Germany) and stored at -20 °C.

2.5 CHEMICAL CHARACTERIZATION OF INGREDIENTS AND CAKES

The **moisture content** of the ingredients and cakes was determined thermogravimetrically using a moisture analyser MA30 (Sartorius, Göttingen, Germany) (in triplicate). The **ash content** of the ingredients was determined by dry

Table 37. Sponge cake formulations.

Ingredient [g]	Flour cakes		Composite cakes		Starch cakes	
	WFC	PFC	PSPPIC	MSPPIC	PSC	MSC
Egg	283.5	283.5	283.5	283.5	283.5	283.5
Sucrose	157.5	157.5	157.5	157.5	157.5	157.5
Wheat flour	157.5	-	-	-	-	-
Pea flour	-	157.5	-	-	-	-
Pea protein isolate	-	-	52.5	52.5	-	-
Pea starch	-	-	105.0	-	157.5	-
Maize starch	-	-	-	105.0	-	157.5
Sunflower oil	31.5	31.5	31.5	31.5	31.5	31.5
Total	*630*	*630*	*630*	*630*	*630*	*630*

WFC = wheat flour cake; PFC = pea flour cake; PSPPIC = pea starch + pea protein isolate cake; MSPPIC = maize starch + pea protein isolate cake; PSC = pea starch cake; MSC = maize starch cake

combustion for twelve hours at 550 °C (in triplicate). The **dietary fibre content** of the ingredients was measured according to AOAC 985.29 (in triplicate). The **protein content** of the ingredients and cakes was analysed using the Kjeldahl method (NF EN ISO 5983-2) with a nitrogen-to-protein conversion factor of 5.7 for wheat and 6.25 for pea and maize (in duplicate). The **lipid content** of the ingredients and cakes was determined using accelerated solvent extraction as described by Krause *et al.* (2021d). The **resistant starch content** of the cakes was measured according to AOAC 2002.02 (in triplicate). The **damaged starch content** of the ingredients was determined in triplicate using the amperometric method reported by Monnet *et al.* (2019). The **total starch, apparent amylose and total amylose contents** of the cakes were analysed in duplicate by adapting the method described by Morrison *et al.* (1983). The ingredients and cakes were weighed to an anhydrous starch content of 0.4-0.5 g, dissolved in 50 mL UDMSO (90% v/v dimethyl sulfoxide, 10% v/v 6 M urea) and centrifuged (2000 g, 10 min). The *content in total starch* was determined by High Performance Liquid Chromatography (HPLC) according to Krause *et al.* (2021d). In brief, 1 mL supernatant was enzymatically hydrolysed for 72 h at 55 °C using amyloglucosidase in sodium acetate buffer (0.2 M, pH 4.5). The supernatant was then filtered (0.45 µm), and diluted with purified water (1/50 v/v), and 20 µL were injected on two serially-connected Aminex HPX-87C (300 × 7.8 mm) and protected by a micro-guard deashing cartridge (30 × 4.6 mm) (BioRad, Temse, Belgium). The glucose was eluted at a column temperature of 75 °C using degassed purified water (0.5 mL/min). An external calibration curve of glucose standard (1-100 g/L) was used for quantification. The *contents in apparent and total amylose* were analysed according to the iodine reagent method reported by Morrison *et al.* (1983), where 1 mL supernatant was either transferred directly into a 100 mL volumetric flask (apparent amylose) or firstly diluted with 9 mL ethanol, centrifuged (2000 g, 10 min) and the pellet resuspended in 1 mL UDMSO before transfer (total amylose). Subsequently, approximately 95 mL purified water was added, followed by 2 mL of an aqueous solution containing 0.1% iodine and 1% potassium iodide. The flask was filled up to 100 mL with purified water and the content mixed by repeated inversion. Absorbance was recorded after 15 min in 1 cm quartz cuvettes at 635 nm against diluted iodine reagent (1/50 v/v) using a Lambda 650 UV/Vis Spectrophotometer (PerkinElmer, Zaventem, Belgium). The amylose contents were calculated from the absorbance of 10 mg anhydrous starch in 100 mL iodine reagent at 635 nm and 20 °C. An overview of the chemical composition of the ingredients is given in **Table 47**. The particle size distribution of the cakes was measured in triplicate by laser diffraction using a Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments,

Worcestershire, UK). A refractive index of 1.49429 and an absorption index of 0.1 were used.

2.6 Analysis of cake crumb morphology by Scanning Electron Microscopy (SEM)

SEM imaging was performed on a piece (0.8 cm × 0.8 cm × 0.3 cm) taken from the centre of the cakes to visualise the morphology of the cake crumb. The samples were mounted on metal stubs and placed in a TM4000 Plus microscope (Hitachi, Maidenhead, UK). The surface of the cake crumb was scanned under low vacuum in the charge-up reduction mode by an electron beam accelerated with 15 kV. Back-scattered electron images were collected at 100× magnification.

2.7 *In vitro* digestion of cakes

Digestion of the cakes was performed according to the standardized INFOGEST static *in vitro* method developed by Brodkorb *et al.* (2019). This procedure involves three successive phases simulating oral, gastric and intestinal digestion. The progress of protein and starch digestion was followed during the intestinal phase using nine individual tubes that were sampled after different times points (0, 5, 10, 20, 30, 60, 90, 120 and 180 min). These tubes could therefore be considered as consecutive and independent repetitions of the same digestive system. Consecutive data from a particular digestive system were integrated using kinetic modelling approaches (see **§ 2.10**). Repeatability was further ensured by repeating particular digestion experiments (data not shown). A blank was processed like the sample tubes but did not contain any cake. The electrolyte solutions of simulated salivary, gastric and intestinal fluids (SSF, SGF and SIF, respectively) were prepared according to Minekus *et al.* (2014).

2.7.1 Oral phase

The oral phase was initiated by mixing 1.25 g ground cake with 1 mL SSF (pH 7), 6.25 µL $CaCl_2$ solution (0.3 M) and 244 µL purified water for 2 min. As suggested by Pälchen *et al.* (2021), no salivary amylase was added during the oral phase because of negligible amylolysis, which could be attributed to short residence times and instant inactivation once the gastric phase commenced.

2.7.2 Gastric phase

The gastric phase was simulated by adding 2 mL SGF (pH 3) and 1.3 µL $CaCl_2$ solution (0.3 M) to the oral bolus. The pH of the mixture was adjusted to 3 with HCl (2 M) before 125 µL of an aqueous porcine pepsin solution was added (2000 U/mL digesta). The volume was then completed to 5 mL with purified water and the samples incubated for 120 min at 37 °C with constant end-over-end rotation.

2.7.3 Small intestinal phase

The small intestinal phase was started by mixing the gastric chyme with 2.125 mL SIF (pH 7), 10 µL $CaCl_2$ solution (0.3 M) and 625 µL bile (160 mM in SIF). After adjusting the pH to 7 using NaOH (2 M), 1.25 mL of a mixture composed of pancreatin (with an *α*-amylase activity of 200 U/mL digesta) as well as pure trypsin and pure chymotrypsin to reach the desired proteolytic activities (100 U/mL digesta and 25 U/mL digesta, respectively) was added and the volume filled up to 10 mL with purified water. The tubes were incubated at 37 °C with constant end-over-end rotation and sampled after the selected time points. Enzymatic activity was stopped by placing them in a water bath (100 °C) for 5 min. The tubes were centrifuged (2000 *g*, 5 min) and the supernatant separated from the pellet, after which both were snap-frozen in liquid nitrogen and stored at -40°C until use.

2.8 Qualitative and quantitative evaluation of starch digestion

2.8.1 Determination of reducing sugars using the DNS method

The reducing sugars solubilized in the digestive supernatants were analysed spectrophotometrically (in duplicate) using the DNS method (Miller, 1959). The supernatant was diluted with purified water (1/40 v/v) and 2 mL of

the dilution were mixed with 1 mL dinitrosalicylic colour reagent (composed of 1.1% w/v DNS, 30% w/v PST and 20% v/v 2 M NaOH in purified water). After incubation at 100 °C for 15 min, 9 mL purified water were added to the cooled samples. The reactants were mixed by repeated inversion and absorbance was recorded at 540 nm using a UV-1800 UV/Vis spectrophotometer (Shimadzu, Kyōto, Japan). The reducing sugars were quantified using an external maltose calibration curve (0.05-2 mg/mL) and conversion into starch equivalents by multiplying by 0.947. *Relative digested starch [%]* was determined by dividing the maltose concentration by the initial starch content of the cakes. *Absolute digested starch [mg/g cake]* was determined by dividing the maltose concentration by the amount of cake used.

2.8.2 DETERMINATION OF INDIVIDUAL STARCH HYDROLYSIS PRODUCTS USING THE HPAEC-PAD METHOD

High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) was carried out to quantify the malto-oligosaccharides released during starch digestion. Preliminary experiments revealed that starch was essentially degraded into glucose, maltose and maltotriose, as visible from the lack of glucose polymers with a degree of polymerization of four and higher (data not shown). To analyse the three aforementioned saccharides, the ion chromatographic method reported by Vennard *et al.* (2020) was implemented with slight modifications. Prior to analysis, the supernatants of the digested samples were purified in duplicate by centrifugation (15000 *g*, 10 min) using a molecular weight filter (<3 kDa). After diluting with purified water, 25 µL of filtrate were injected on a Dionex ICS-5000 ion chromatograph system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an AS-AP autosampler, a dual pump, a CarboPac PA20 analytical column (3 × 150 mm) protected by a CarboPac PA20 guard column (3 × 30 mm), and an electrochemical detector. Separation of the sugars was performed at 30 °C and a flow rate of 0.5 mL/min using 600 mM NaOH, 300 mM NaOH and purified water as eluents A, B and C, respectively. The gradient programme was set as follows: 0% A, 3.3% B and 96.7% C isocratic (13 min); 0% A, 3.3% to 66.6% B and 96.7% to 33.3% C (12 min); 0% A, 66.6% B and 33.3% C isocratic (2 min); 0% to 100% A, 66.6% to 0% B, 33.3% to 0% C (0.1 min); 100% A, 0% B and 0% C isocratic (2.9 min); 100% to 0% A, 0% to 3.3% B and 0% to 96.7% C (0.1 min). The sugars were detected using a gold working electrode and an AgCl reference electrode applying the quadrupole potential waveform of E1 = +0.1 V (400 ms), E2 = -2.0 V (20 ms), E3 = +0.6 V (10 ms) and E4 = -0.1 V (70 ms). The quantities of saccharide were determined from external calibration curves of the glucose, maltose and maltotriose standards (0.5-100 mg/L). *Relative digested starch [%]* was calculated as the sum of glucose, maltose and maltotriose divided by the initial starch content of the cakes. Each sugar was thus converted into a starch equivalent by multiplying by 0.900, 0.947 and 0.964 for glucose, maltose and maltotriose, respectively. Alternatively, the sum of sugars was related to the amount of cake in order to determine the *absolute digested starch [mg/g cake*].

2.9 QUALITATIVE AND QUANTITATIVE EVALUATION OF PROTEIN DIGESTION

Protein digestion was evaluated using the spectrophotometric OPA method reported by Nielsen *et al.* (2001) and modified by Zahir *et al.* (2018).

2.9.1 READILY BIOACCESSIBLE PROTEIN (RBP)

In order to assess the readily bioaccessible protein, duplicates were prepared by adding TCA to the digestive supernatant (final concentration: 3.1% w/v). This led to the precipitation of larger peptides and proteins from the soluble amino acids and oligopeptides (Gwala *et al.*, 2020). The latter are considered as the readily bioaccessible protein fraction (TCA fraction), which can be directly absorbed at the brush-border *via* passive diffusion, tight junctions, endocytosis and/or transcellular carrier-mediated

transport (Ozorio *et al.*, 2020). After centrifugation (10000 *g*, 30 min), the supernatant was filtered through 0.25 µm Minisart NML filters, diluted with purified water (1/40 v/v) and then 0.4 mL were mixed with 3 mL light-protected OPA reagent (composed of 3.81% w/v DT, 2% v/v ethanol, 0.1% w/v SDS, 0.09% w/v DTT and 0.08% w/v OPA in purified water). The samples were incubated for exactly 2 min in the dark before absorbance was recorded at 340 nm using an Ultrospec 2100 pro UV/Vis spectrophotometer (GE Healthcare, Buckinghamshire, UK). The free *α*-amino groups of *α*-amino acid monomers and small peptides were quantified using an external serine calibration curve (12.5-100 mg/L). The concentration thus obtained was expressed as *relative readily bioaccessible protein* [%] after dividing by the total concentration of the *α*-amino groups (*α*-amino acid monomers) contained in the undigested cake. The latter was determined in cake samples (5 mg) that had been subjected to acid hydrolysis for 48 h at 110 °C using 1 mL HCl (6 M). After solvent evaporation and re-dilution in 5 mL purified water, the samples were filtered through 0.25 µm Minisart NML filters and mixed with OPA reagent as described previously. Alternatively, the RBP content was divided by the cake amount to express it as *absolute readily bioaccessible protein* [mg/g cake].

2.9.2 READILY BIOACCESSIBLE PROTEIN$_{HYDROLYSED}$ (RBP$_{HYDROLYSED}$)

As described in **§ 2.9.1**, the TCA-fraction contained not only *α*-amino acids but also small peptides. Since the protein content of the undigested cakes was, however, expressed as *α*-amino acid monomers released after acid hydrolysis, we decided to additionally hydrolyse the TCA-fraction in order to convert the small peptides into *α*-amino acid monomers (Gwala *et al.*, 2020; Pälchen *et al.*, 2021). This would allow to express the readily biaccessible protein in terms of *α*-amino acid monomers and thus to better compare it with the *α*-amino acid monomers of the undigested cakes and thus to better interpret protein digestion. The acid hydrolysis was thus performed for 48 h at 110 °C using 1 mL HCL (6 M) on the TCA-fractions of selected cakes (WFC, PFC and PSPPIC; 0.5 mL each). The solvents were evaporated, 5 mL purified water were added and the samples were filtered through 0.25 µm Minisart NML filters. Subsequently, the *α*-amino acid monomers were quantified using the OPA reagent. The resulting content in serine equivalents was expressed as either *relative* [%] or *absolute* [mg/g cake] *readily bioaccessible protein*$_{hydrolysed}$.

2.10 KINETIC MODELING OF *IN VITRO* DIGESTION

Experimental data on the *in vitro* digestion kinetics were modelled using XLSTAT 2020.5.1 (Addinsoft, Paris, France). The fit of the models was evaluated by calculating the $R^2_{adjusted}$ and the root mean square errors (RMSE), and by visual analysis of the residual and parity plots. The initial reaction rate of proteolysis or amylolysis was calculated from the slope of the tangent to the respective models at time t_{0min}.

2.10.1 PROTEIN DIGESTION KINETICS

The fractional conversion model described by Gwala *et al.* (2020) was used (**Eq. 1**).

$$Protein_t = Protein_f + (Protein_i - Protein_f) \times e^{-kt} \quad (1)$$

where $Protein_t$ is the RBP at any time t [%]/[mg/g], $Protein_f$ is the estimated plateau of RBP at extended digestion times [%]/[mg/g], $Protein_i$ is the RBP at the start of the small intestinal phase [%]/[mg/g] and k is the estimated reaction rate constant of digestion [1/min].

2.10.2 STARCH DIGESTION KINETICS

Edwards *et al.* (2014) discussed the potential occurrence of different phases of starch digestion in certain foods, characterised by specific kinetic parameters. Such biphasic digestion appeared to take place during the present study, so the biphasic model comprising two first order equations as described in **Eq. 2** was implemented:

$$Starch_t = Starch_2 - Starch_1 \times e^{-k_1 t} + (Starch_1 - Starch_2) \times e^{-k_2 t} \quad (2)$$

where $Starch_t$ is the digested starch at time t [%]/[mg/g], $Starch_2$ is the estimated plateau of digested starch at the end of the second digestion phase at extended digestion times [%]/[mg/g], $Starch_1$ is the digested starch at the end of the first digestion phase [%]/[mg/g], k_1 is the estimated reaction rate constant of the first digestion phase [1/min] and k_2 is the estimated reaction rate constant of the second digestion phase [1/min].

For the different cakes, the area under the curve (AUC) from t_{0min} to t_{90min} was integrated and related to that of the most digestible cake (WFC) in order to calculate the Hydrolysis Index (HI) as shown in **Eq. 3** (Edwards *et al.*, 2019; Goñi *et al.*, 1997; Jenkins *et al.*, 1981).

$$HI = \frac{AUC_{sample}}{AUC_{reference}} \times 100 \quad (3)$$

2.11 MICROSCOPIC ANALYSIS OF DIGESTION PELLETS

The progress of protein and starch digestion was assessed qualitatively by microscopic analysis of the starch and protein remaining in the pellets after selected digestion times (0, 30 and 180 min). The ability of proteins to fluoresce due to the presence of aromatic amino acids (Arntfield *et al.*, 1987) was utilised to visualise the proteins contained in 10 µL diluted pellet (50 mg in 100 µL purified water) under the epifluorescent microscope (excitation at 460-490 nm). To analyse the starch, 100 µL diluted pellet were mixed with 1 µL Lugol's iodine reagent (5% w/v iodine and 10% w/v potassium iodide) and 10 µL stained sample were observed under the normal light microscope. Representative images were captured at 10× magnification using a BX51 microscope coupled to an epifluorescence illumination X-Cite 120Q and equipped with a XC50 digital camera (Olympus, Tokyo, Japan).

2.12 FURTHER STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was performed and significant differences were evaluated using Tukey's test at $p<0.05$ using XLSTAT 2020.5.1 (Addinsoft, Paris, France).

3 RESULTS AND DISCUSSION

3.1 CHARACTERIZATION OF CAKES

As discussed in our previous publication, cakes made with either pea flour or purified ingredients all had attractive visual and textural properties, as evidenced by their appealing volume and colour, which were comparable to the wheat reference (Krause *et al.*, 2021d).

From a nutritional point of view, the cakes were distinctive for their different chemical compositions (**Table 38**). As targeted, the composite cakes formulated with pea protein isolate and either pea starch or maize starch (PSPPIC, MSPPIC) had similar protein and starch contents as PFC. Compared to WFC, these three pea-based cakes were significantly enriched in protein and contained much lower quantities of starch. The highest starch contents, however, were determined for the starch recipes (PSC, MSC), which at the same time contained the least

Table 38. Chemical characteristics of sponge cakes. Values are expressed on either cake or starch dry basis (db).

Cake	Dry matter [% cake]	Lipid [% db cake]	Protein [% db cake]	Starch [% db cake]	Resistant starch [% db starch]	Amylose [% db starch]		
						Total	Apparent	Lipid complexes
WFC	84.2 ± 0.9^{a,b}	17.2 ± 0.7^{a}	11.8 ± 0.5^{c}	29.6 ± 0.6^{b}	5.7 ± 0.3^{c}	20.9 ± 0.2^{b}	15.4 ± 0.0^{c}	5.6 ± 0.2^{a}
PFC	83.9 ± 0.0^{a,b}	16.1 ± 1.5^{a}	18.1 ± 0.5^{b}	21.5 ± 1.4^{d}	22.3 ± 1.3^{a}	26.8 ± 1.5^{a}	26.2 ± 0.6^{a}	0.6 ± 0.9^{c}
PSPPIC	84.5 ± 0.6^{a}	16.4 ± 0.7^{a}	19.3 ± 0.6^{a,b}	27.1 ± 1.4^{b,c}	18.7 ± 1.0^{b}	28.2 ± 0.1^{a}	26.1 ± 0.2^{a}	2.1 ± 0.1^{b,c}
MSPPIC	84.3 ± 0.3^{a,b}	17.2 ± 0.4^{a}	19.7 ± 0.6^{a}	26.2 ± 0.5^{c}	6.9 ± 0.6^{c}	21.0 ± 0.2^{b}	16.1 ± 0.2^{c}	4.9 ± 0.1^{a}
PSC	82.9 ± 0.1^{a,b}	16.9 ± 1.0^{a}	9.1 ± 0.5^{d}	38.6 ± 0.9^{a}	18.7 ± 0.6^{b}	29.0 ± 0.0^{a}	27.6 ± 0.4^{a}	1.4 ± 0.4^{b,c}
MSC	82.5 ± 0.5^{b}	16.5 ± 1.1^{a}	9.2 ± 0.5^{d}	38.2 ± 0.8^{a}	7.7 ± 0.9^{c}	21.3 ± 0.2^{b}	17.9 ± 0.7^{b}	3.4 ± 0.9^{a,b}

Different letters within a column indicate significantly different means ($p<0.05$).

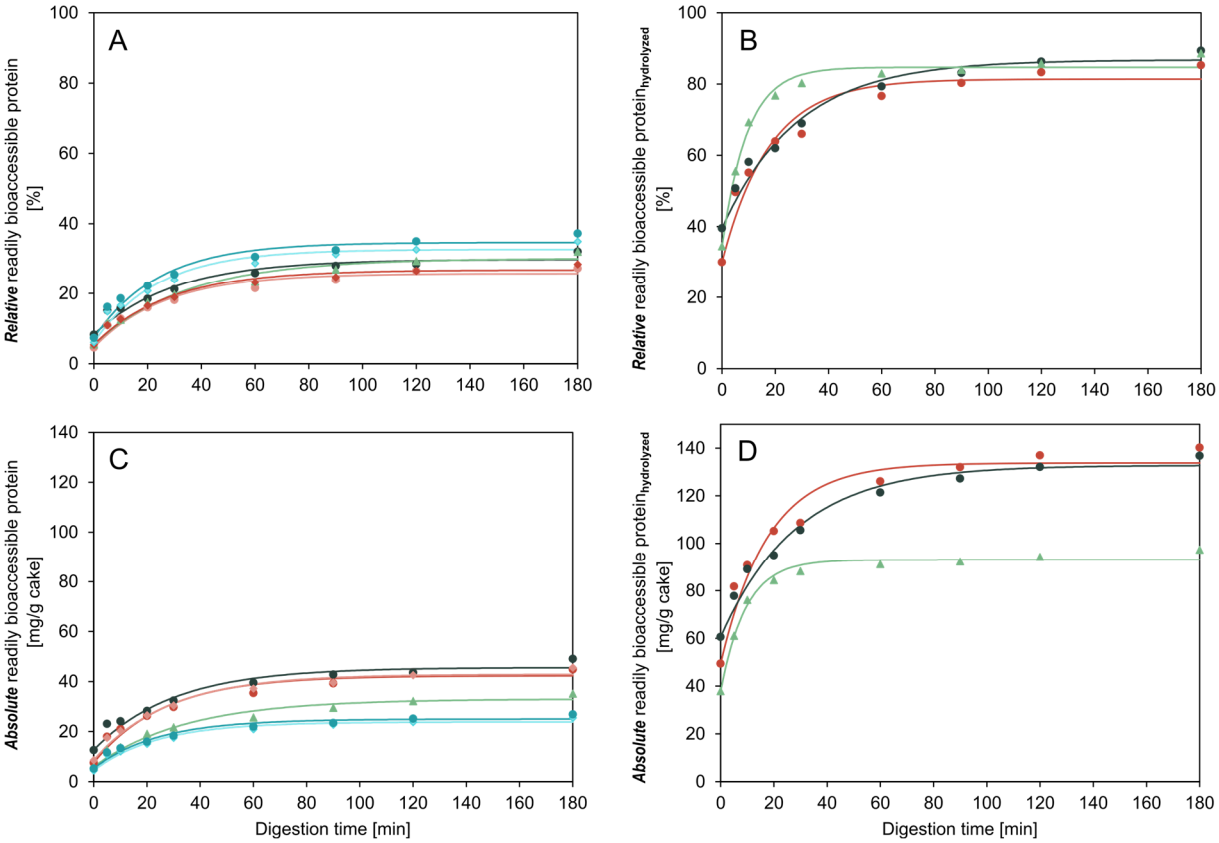

Figure 75. *In vitro* protein digestion kinetics of sponge cakesbased on wheat flour (WFC ▲), pea flour (PFC ●), pea starch and pea protein (PSPPIC ●), maize starch and pea protein (MSPPIC ◆), pea starch (PSC ●) and maize starch (MSC ◆) during small intestinal digestion. Digested protein is expressed as (A) relative readily bioaccessible protein [%], (B) relative readily bioaccessible protein$_{hydrolysed}$ [%], (C) absolute readily bioaccessible protein [mg/g cake] and (D) absolute readily bioaccessible protein$_{hydrolysed}$ [mg/g cake]. Symbols indicate experimental values, lines represent values predicted by the fractional conversion model according to **Eq. 1**.

protein. In the latter case, the only source of protein was egg.

The lipid content was similar in all the cakes, which implied that the lipids mainly derived from the egg and sunflower oil, while the contribution of the flour/fraction to the total lipid content was negligible.

In addition, the cakes formulated with pea starch as a purified ingredient or as part of pea flour (PFC, PSPPIC, PSC) contained the highest resistant starch contents of all the cakes. These formulations were also characterised by higher total as well as apparent amylose contents. Since the difference between the two values corresponded to the content in amylose complexed with lipids, it was concluded that the proportion of these amylose-lipid complexes was higher in PFC, PSPPIC and PSC. By contrast, the cakes based on wheat and maize (WFC, MSPPIC, MSC) contained significantly less total amylose but higher levels of amylose-lipid complexes.

3.2 Protein digestion

3.2.1 Digestion kinetics of readily bioaccessible protein

Figure 75 illustrates the kinetics of intestinal protein digestion for the different cakes. The first point of the curve corresponds not only to the start of the intestinal phase but also to the end of the gastric phase. Therefore, the quantities of RBP measured at this point reflected the *α*-amino groups (i.e. free amino acids, small oligopeptides) released by the action of pepsin during the gastric phase. For the different cakes, the relative RBP amounts determined at t_{0min} ranged from 5% to

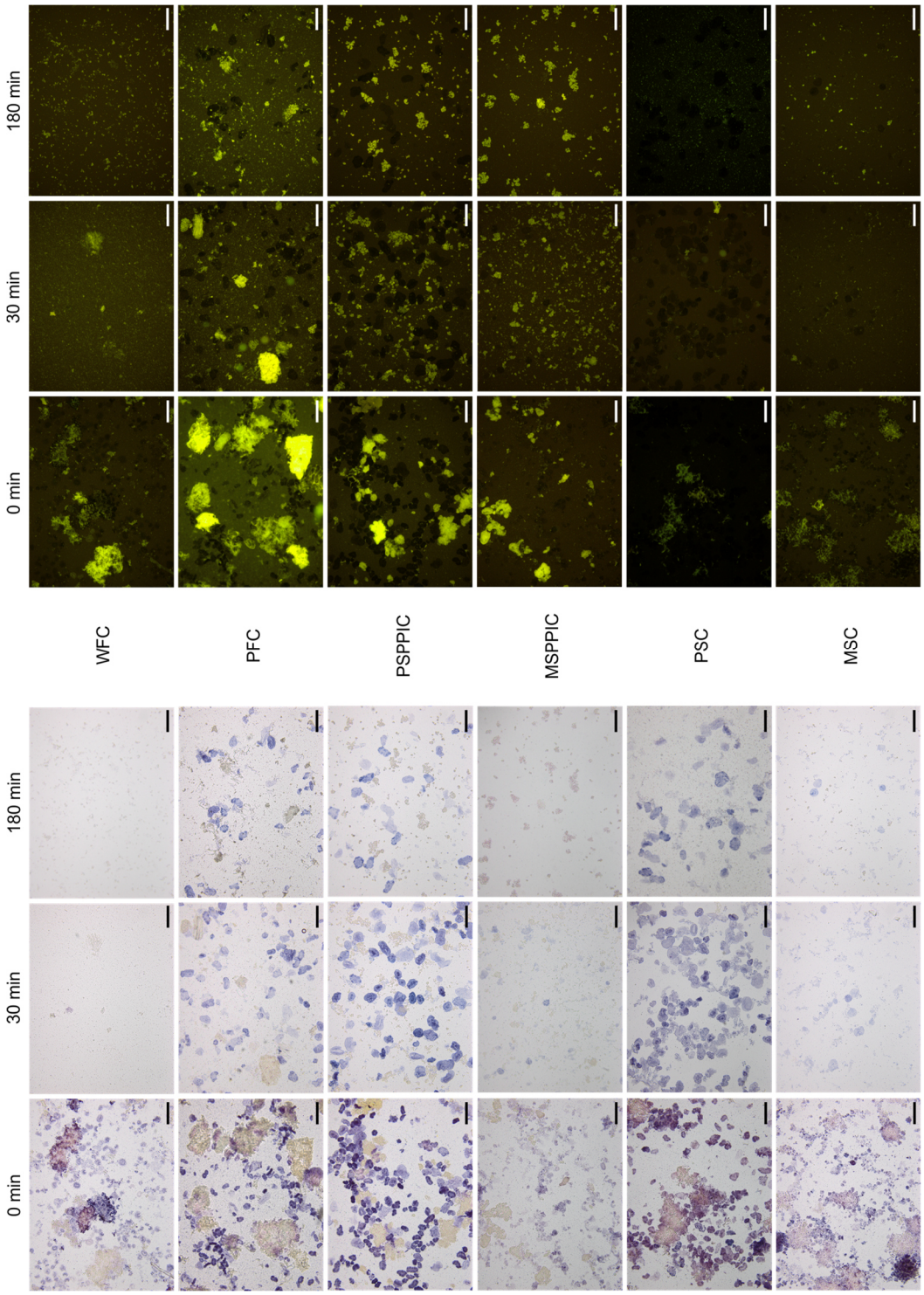

Figure 76. Microscopic images (10×) of digested pellets collected after 0 min, 30 min and 180 min small intestinal digestion of sponge cakes. Starch granules were visualized after staining with Lugol's solution under the light microscope (left side) and proteins under the fluorescence microscope (right side). Scale bar: 200 µm.

8%, increasing subsequently during intestinal digestion to reach a plateau at 27-37% (**Figure 75A**). As indicated in **Table 52**, the estimated reaction rate constants did not differ significantly between the cakes. The highest relative RBP amounts at the end of the intestinal phase were measured with the starch cakes (PSC, MSC), thus indicating digestion of a higher percentage of protein, which only derived from egg.

This was confirmed by the qualitative assessment of microscopic images of the corresponding pellets. As can be seen in **Figure 76**, the size of green fluorescing protein fractions decreased in all cakes as intestinal digestion

Table 39. Kinetic parameters [estimates ± standard error] of in vitro protein intestinal digestion of sponge cakesbased on wheat flour (WFC), pea flour (PFC) and a combination of pea starch and pea protein (PSPPIC). Parameters were estimated by a fractional conversion model (**Eq. 1**).

Cake	*k*	Relative $RBP_{hydrolysed}$			Absolute $RBP_{hydrolysed}$			R^2_{adj}
		Protein$_f$	Initial reaction rate	RMSE	*Protein*$_f$	Initial reaction rate	RMSE	
	[1/min]	[%]	[%/min]		[mg/g]	[mg/(g·min)]		
WFC	0.106 ± 0.010[a]	84.70 ± 1.09[a,b]	5.34 ± 0.53[a]	2.343	92.99 ± 1.20[b]	5.86 ± 0.58[a]	2.573	0.98
PFC	0.035 ± 0.005[b]	86.78 ± 1.88[a]	1.67 ± 0.22[b]	2.911	132.82 ± 2.88[a]	2.55 ± 0.34[b]	4.456	0.98
PSPPIC	0.056 ± 0.010[b]	81.35 ± 2.42[b]	2.89 ± 0.52[b]	4.522	133.81 ± 3.99[a]	4.76 ± 0.86[a]	7.438	0.95

Different letters within a column indicate significantly different means (p<0.05).

progressed, reflecting gradual proteolysis. While some protein fractions in the composite cakes (PSPPIC, MSPPIC) and PFC remained undigested even after 180 min, almost no fluorescence was visible in the pellets of the starch cakes and also WFC.

However, these latter cakes had lower protein contents than PFC, PSPPIC and MSPPIC (**Table 38**). In order to correct for the different protein levels in the six cakes, the relative RBP amounts were translated into absolute values [mg RBP/g cake] and plotted against digestion time. **Figure 75C** shows that despite lower relative amounts, the final absolute RBP values were significantly higher in PFC and the composite cakes.

3.2.2 DIGESTION KINETICS OF READILY BIOACCESSIBLE $PROTEIN_{HYDROLYSED}$

Account must be taken of the fact that the RBP fraction typically comprises both peptides and amino acids. In order to better compare the digestion of different protein sources, acid hydrolysis of the RBP fraction could be an attractive approach, enabling the determination of RBP as *α*-amino groups released by the combined action of proteolytic enzymes. Due to the higher absolute RBP quantities in cakes containing pea protein, either in the form of flour (PFC) or isolated protein (PSPPIC) compared to WFC (**Figure 75C**), these three cakes were selected to undergo acid hydrolysis. The resulting relative and absolute amounts of $RBP_{hydrolysed}$ are presented in **Figure 75B** and **Figure 75D**, respectively. In all three cakes, these values were remarkably higher than before acid hydrolysis, implying that enzymatic proteolysis during both gastric and intestinal digestion produced large quantities of peptides. WFC differed from the pea-based cakes in having a much more rapid increase in the relative quantity of $RBP_{hydrolysed}$ at the start of intestinal digestion compared to PFC and PSPPIC. Accordingly, the initial reaction rate of protein hydrolysis of the WFC, calculated on basis of the corresponding estimated kinetic parameters, was significantly higher than that of PFC and PSPPIC (**Table 39**). This suggested the rapid hydrolysis of proteins originating from egg and wheat flour by proteolytic enzymes, which was also visible from the microscopic images in **Figure 76**. This might be due to the higher ratio of enzyme to protein. Moreover, it could be imagined that anti-nutritional factors such as tannins and phytates, which have been found at elevated levels in pea ingredients and may survive the baking process, could have retarded protein digestion in the pea-based cakes (Febles *et al.*, 2002; Fernández-Quintela *et al.*, 1997; Sarwar Gilani *et al.*, 2012). Nevertheless, the absolute amounts of $RBP_{hydrolysed}$ released from PFC and PSPPIC clearly exceeded those of WFC within the first 30 min (**Figure 75D**).

As the intestinal digestion progressed, all three curves asymptotically approached a relative $RBP_{hydrolysed}$ amount of 85-89%, indicating that most of the proteins could be hydrolysed. Owing to the higher protein content in the pea-based cakes (**Table 38**), the absolute amounts of

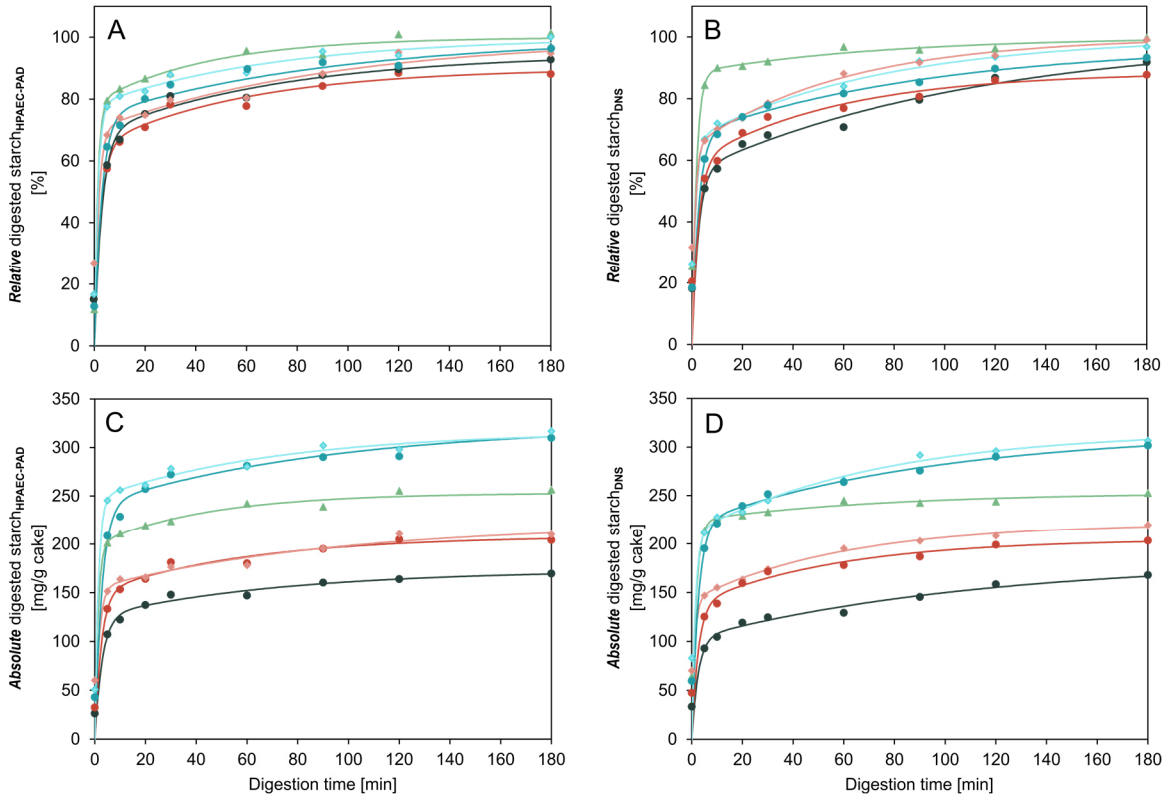

Figure 77. *In vitro* starch digestion kinetics of sponge cakes based on wheat flour (WFC ▲), pea flour (PFC ●), pea starch and pea protein (PSPPIC ●), maize starch and pea protein (MSPPIC ◆), pea starch (PSC ●) and maize starch (MSC ◆) during small intestinal digestion. Graphs on the left side (A, C) are based on HPAEC-PAD determination of multiple starch digestion products, those on the right side (B, D) on the DNS determination of reducing sugars. Digested protein is expressed as (A, B) relative digested starch [%] or (C, D) absolute digested starch [mg/g cake]. Symbols indicate experimental values, lines represent values predicted by the biphasic model according to **Eq. 2**.

$RBP_{hydrolysed}$ were thus considerably higher (137-140 mg/g) compared to WFC (97 mg/g).

These findings therefore revealed the high potential of pea proteins as substitutes for traditionally used wheat proteins in the design of food products with higher level of protein. Closer inspection of the digestion patterns of pea proteins from either pea flour (PFC) or refined pea protein isolate (PSPPIC) at comparable concentrations revealed similar digestion kinetics, implying that the protein isolation process was not linked to any impairment of proteolysis. By contrast, higher absolute initial reaction rates suggested a slight improvement in hydrolysis (**Table 39**) which might be associated with conformational changes during protein isolation or the presence of other constituents such as fibres that could affect enzyme-substrate interaction (Rodríguez *et al.*, 2020).

3.3 STARCH DIGESTION

3.3.1 DIGESTION KINETICS OF STARCH

Figure 77 compares the kinetics of intestinal starch digestion for the six cakes, which were measured either chromatographically as the sum of the different starch digestion products (HPAEC-PAD method, **Figure 77A+C**) or spectrophotometrically as reducing sugars (DNS method, **Figure 77B+D**). Both methods converged to reach the same conclusion: starch was instantly hydrolysed at the start of intestinal digestion in all samples, followed by a rapid levelling off at relative starch digestion values within the range 88-100%.

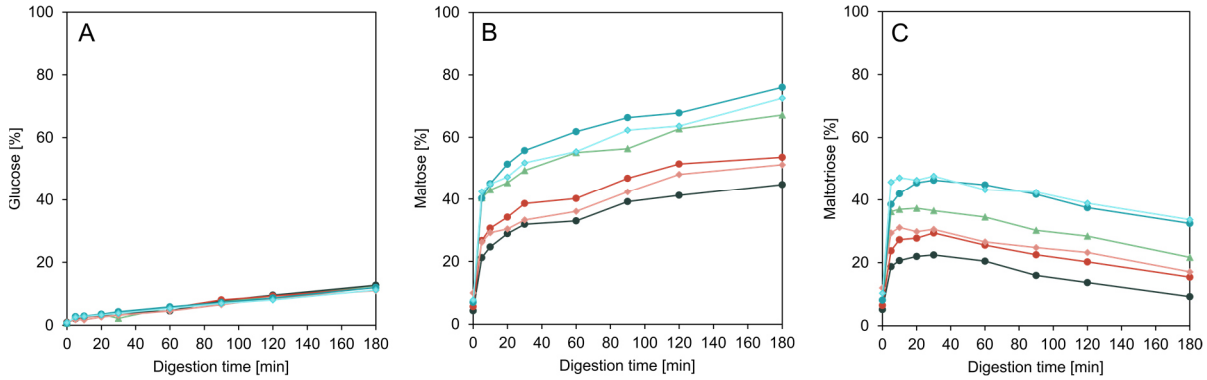

Figure 78. Time-dependent release of starch degradation products [%] from sponge cakes based on wheat flour (WFC ▲), pea flour (PFC ●), pea starch and pea protein (PSPPIC ●), maize starch and pea protein (MSPPIC ◆), pea starch (PSC ●) and maize starch (MSC ◆) during small intestinal digestion. The by HPAEC-PAD determined (A) glucose, (B), maltose and (C) maltotriose concentrations were related to the starch content of the cakes.

Interestingly, high starch digestion values were determined for all cakes at t_{0min}, which could not be related to enzymatic activity due to the absence of amylases in the simulated oral and gastric phases. In order to exclude any adulterating contribution of sugars present in the undigested cakes, a chromatographic sugar analysis of the cakes was carried out. However, only negligible inherent sugar levels were detected, which suggested not only that the raw materials contained barely any free sugars but also that development of the cakes did not lead to the hydrolysis of starch or sucrose and hence to any measurable amounts of glucose, fructose, maltose or maltotriose (data not shown). Moreover, it could be verified that the pH conditions in the gastric phase (pH 3) did not trigger the inversion of sucrose into glucose and fructose, as evidenced by the lack of fructose in the samples collected at t_{0min} (data not shown). It was therefore was assumed that the high initial starch digestion values were a consequence of the only partially effective heat shock applied to the samples at t_{0min}. Unlike the other samples, at t_{0min} these samples were heated for 5 min at 100 °C before the enzyme solution was added, on completion of which, the enzyme solution (which had been kept at a low temperature) was added and heated at 100 °C for a further 5 min. The aim was to suppress enzymatic activity by instant heat shock. However, it is conceivable that the open structure of the cakes allowed rapid diffusion of the enzyme solution into the cake, thereby already cleaving some easily accessible starch before being completely thermally inactivated. A correction of these initial values was therefore achieved by modelling starch digestion through zero. The estimated kinetic parameters are listed in **Table 53**.

The high susceptibility of starch to hydrolysis was further corroborated by the vast increase in the proportion of digested starch within 10 min of *in vitro* intestinal digestion. As can be seen from **Figure 77A** and **Figure 77B**, this short period was sufficient to hydrolyse a high proportion of starch, especially in WFC and the maize-based cakes (MSC, MSPPIC). In line with *in vitro* and *in vivo* studies on starch digestion reported in the literature, the main starch degradation products were maltose and maltotriose (**Figure 78**) (Gwala *et al.*, 2019; Quezada-Calvillo *et al.*, 2007).

In the case of the wheat flour and maize starch-containing cakes, starch digestion slowed down after already 5 min, while it was slightly delayed for PFC, PSC and PSPPIC (after around 8 min) (**Table 53**). At this point, it was suggested that the medium was highly enriched with starch degradation products, thus reducing the likelihood of contact between the enzyme and undigested

starch. It might be also possible that the complex network of the cakes could have slowed down enzyme diffusion to the starch in less available regions (Edwards *et al.*, 2014). We therefore believe that the enzymes began to hydrolyse larger starch breakdown products (e.g. maltotriose) into smaller molecules as well as starch in less accessible parts of the cake but at a slower reaction rate (**Table 53**). This is reflected in the gradual decrease in maltotriose and concurrent increase in maltose and glucose (**Figure 78**). Based on the chromatographically determined maltose and maltotriose concentrations, it could be determined that around 50-60% of the maltose released in this slower hydrolysis phase derived from the conversion of maltotriose, whereas the remaining 40-50% emerged from the hydrolysis of larger polymers. After 180 min, starch was completely digested.

Light microscopic imaging of the pellets confirmed the successive degradation of starch granules with continuing intestinal digestion for all cakes (**Figure 76**). Interestingly, starch was almost completely hydrolysed after only 30 min in WFC, MSC and MSPPIC, thus corroborating the generally greater susceptibility of the starch contained therein to enzymatic hydrolysis.

The high level of amylolysis in all the cakes within a short time after exposure to *α*-amylase in the intestinal phase indicated that the open cake structure allowed easy access of the enzymes to the starch. In this context, it should be remembered that in the present study the exclusion of the salivary *α*-amylase from the oral phase in the static *in vitro* digestion protocol was chosen. This can be explained by its expected rapid inactivation upon entry into the gastric phase due to the instant drop of the pH from 7 to 3, which thus means only a short residence time and activity of the enzyme during the oral phase. Based on the immediate enzyme-substrate interactions demonstrated in the present work, however, it can be imagined that even under the chosen static *in vitro* digestion conditions the use of salivary *α*-amylase could contribute significantly to the starch hydrolysis. The us of salivary *α*-amylase and its effect on the overall starch hydrolysis should thus be considered and evaluated in future studies of these type of food systems.

3.3.2 CORRELATION BETWEEN CHEMICAL AND STRUCTURAL PROPERTIES OF THE CAKES AND STARCH DIGESTION

Throughout intestinal digestion, the cakes made with pea starch (either purified or as part of pea flour) exhibited slightly lower relative amounts of digested starch compared to the other cakes. This might be linked to their elevated contents of both amylose and resistant starch (**Table 38**). According to the literature, pea starch is characterized by a C-type crystalline polymorph that comprises more resistant starch than the A-type polymorphs characteristic of wheat or maize (Bede *et al.*, 2021; Raigond *et al.*, 2015; Singh *et al.*, 2010). This may be due to the presence of longer amylopectin chains that are able to crystallize at double helix formation, leading to an enlarged crystal lattice with greater resistance to depolymerization (Jeong *et al.*, 2019; Singh *et al.*, 2010). Moreover, a higher amylose content is considered to contribute to the formation of resistant starch because of the organization of long amylose chains into double helices and the high potential to retrograde during product storage (Ashwar *et al.*, 2016; Raigond *et al.*, 2015). These characteristics might thus give substantiated explanations for the higher resistance of the cakes containing pea starch to enzymatic digestion.

The increased stability of pea starch against amylolysis could also be correlated with its reduced tendency to collapse during baking. As shown in **Figure 76**, some starch granules in the pea cakes at t_{0min} seemed to maintain certain structural features after baking, while most of the starch granules in cakes made using wheat flour or maize starch were disassembled, leaving a high proportion of granule ghosts, which are fragmented starch remnants after structural collapse that leach out of internal amylose and amylopectin (Atkin *et al.*, 1998). Release of these polymers might have facilitated enzymatic attack, thus causing the wheat and maize starch to be

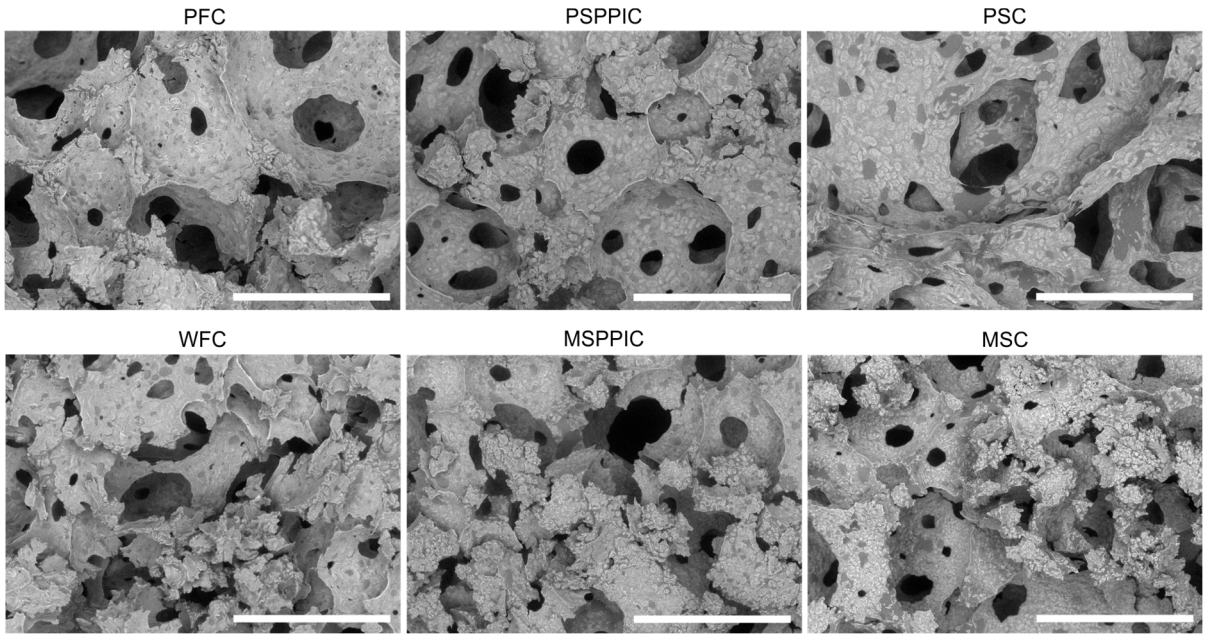

Figure 79. Scanning electron microscopy images (100×) of undigested crumbs of sponge cakesbased on pea flour (PFC), pea starch and pea protein (PSPPIC), pea starch (PSC), wheat flour (WFC), maize starch and pea protein (MSPPIC) and maize starch (MSC). Scale bar: 500 µm.

degraded more rapidly. Slightly larger quantities of damaged starch in wheat flour (5%) and maize starch (5%) compared to pea flour (2%) and pea starch (3%) might have further contributed to their structural lability.

Moreover, the swollen starch granules in WFC, MSPPIC and MSC were visibly smaller than those in the pea cakes, implying larger specific surfaces that simplified enzyme adsorption (Colonna *et al.*, 1992; Dhital *et al.*, 2010b; Li *et al.*, 2020).

Apart from the structural characteristics of the granules, the overall cake crumb structure was evaluated by SEM imaging. From **Figure 79** it emerges that the different cakes contained a protein gel in which the starch granules were entrapped. While the network in the pea cakes formed large and continuous planes, that in WFC, MSPPIC and MSC cakes tended to be characterized by multi-layered fractions. The latter could have favoured amylolysis due to the facilitation of enzyme diffusion through the numerous cavities and hollows, as well as provision of several points for enzymatic attack.

3.3.3 HYDROLYSIS INDEX AND NUTRITIONAL QUALITY

In order to estimate the impact of the different starch sources on the *in vivo* glycaemic response, their hydrolysis index was calculated by integrating their area under the curve from t_{0min} to t_{90min} and relating it to that of WFC, which was the most digestible cake (**Eq. 3**) (Aleixandre *et al.*, 2019; Fernandes *et al.*, 2020; Goñi *et al.*, 1997) The values obtained are listed in **Table 53**. Interestingly, the replacement of wheat flour with pea flour or a combination of pea starch and pea proteins led to a considerable reduction in HI from 100 (WFC) to 80-81 (PFC and PSPPIC). This implied that the use of pea ingredients could slow down starch hydrolysis and might thus be reflected in a decelerated glucose response.

This became especially clear when the relative starch digestion values were converted into absolute amounts. Compared to WFC and the starch formulations, PSPPIC and PFC contained considerably fewer starch digestion products (**Figure 77C** and **Figure 77D**). This might be due to the general lower starch content of the pea-based products compared to WFC as well as the distinct chemical and structural characteristics

discussed in **§ 3.3.2**. When comparing the digestive patterns of PFC and PSPPIC, it emerged that PFC had lower absolute starch digestion values, attributable to its slightly lower levels of starch (**Table 38**). Moreover, it could be imagined that fibres in the whole pea flour (which are typically removed during protein and starch purification) might have limited starch degradation to a certain extent. As discussed in the literature, dietary fibres can increase the viscosity of the digesta and bind to the starch granule surface, thereby hindering enzyme accessibility and attenuating glucose diffusion (Fabek *et al.*, 2014; Schuchardt *et al.*, 2016; Singh *et al.*, 2010). In addition, they can also bind directly to *α*-amylase and thus impede catalysis (Duijsens *et al.*, 2021).

4 CONCLUSION

The complete replacement of wheat flour with either pea flour or a combination of isolated pea protein and purified starch resulted in cakes with attractive nutritional characteristics due to their elevated levels of protein and reduced levels of starch.

Despite retarded protein hydrolysis in the pea-based cakes at the start of initial digestion when compared to the wheat references, the absolute fraction of readily bioaccessible protein was markedly higher (by around 40%). Similar digestion kinetics could be determined for the pea proteins from either whole flour or the purified protein isolate, suggesting that both raw materials could be considered as attractive ingredients for new high-protein food products.

On the other hand, the use of pea starch in a purified form or as part of pea flour led to a delayed release of sugars compared to both wheat flour and maize starch. This was reflected in a lower hydrolysis index, which might be used to estimate the *in vivo* glycaemic response. The greater resistance of pea starch to enzymatic depolymerisation could be attributed to a high content in amylose and resistant starch as well as the particular structural properties of the final pea cakes. As was concluded for pea proteins, purified pea starch and pea starch contained in whole pea flour had similar digestive patterns. Accordingly, the fractionation of pea flour into proteins and starch had no relevant influence on their digestibility.

Starch digestion was assessed by measuring either the accurate sum of individual starch digestion products (HPAEC-PAD method) or by evaluating the content in reducing sugars (DNS method), both of which appeared to be appropriate when describing starch digestion kinetics in complex food matrices.

Overall, we were able to show that pea ingredients have great potential for use as alternative plant-based food ingredients. From a sustainability point of view, pea flour in particular could be considered an emerging ingredient for the development of high-quality products with appealing nutritional value because of its low degree of refining when compared to purified pea ingredients.

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5 ADDITIONAL RESULTS

5.1 SUGAR CONTENTS IN UNDIGESTED CAKES AND ASSESSMENT OF SUCROSE INVERSION

As discussed in the article, the analysis of the sugars contained in the undigested cake was an important step to ensure that the baking process did not lead to the inversion of sucrose or hydrolysis of starch, and thus to the release of sugars (i.e. fructose, glucose, maltose and maltotriose) that could falsify the measured starch digestion kinetics. As detailed in **§ 4.3.5 of Part IV**, the sugars were extracted from the cakes using either UDMSO (stirred for 7 days) or water (either 2 min ultraturrax or 60 s vortex). All samples were diluted with water to an equal dilution factor of 1:5000 and then analyzed by the HPAEC-PAD method described. Unfortunately, however, no sugars apart from sucrose were detected in the samples, which was not a surprise due to its large portion (25% w/w) in the formulations. In order to measure the other sugars, it was therefore necessary to use more concentrated solutions. Since the measured sucrose concentrations were very similar in all samples, it was decided that exemplarily the UDMSO samples, which were closest to the mean value, were analyzed again at a less dilute concentration (1:500). As indicated in **Table 40**, it was now possible to detect glucose and, in the case of WFC also maltose. Nevertheless, these quantities were minimal (~1 mg/g cake) and, when converted to starch equivalents, corresponded to less than 1% starch digestion. To further ensure that the samples were not too diluted to detect the starch degradation products, another set of samples was prepared that was even less diluted (1:10), however using the water samples and not those prepared with UDMSO to prevent potential damage to the column. The chromatograms obtained showed a clear saturation of the sucrose peak, which resulted in the coelution with glucose that eluted shortly before sucrose. Consistently, glucose could not be quantified. Moreover, neither maltotriose nor maltose were detected (except in WFC: 0.7 mg maltose/g cake as in the 1:500 sample), which confirmed the absence of starch degradation products in the undigested cakes and thus no hydrolysis of starch during baking. In addition, no fructose could be measured, which suggested that sucrose had not been converted into glucose and fructose during baking.

Table 40. Concentration of glucose, maltose, maltotriose and fructose in undigested sponge cakes.

Cake	Glucose [mg/g cake]	Maltose [mg/g cake]	Maltotriose [mg/g cake]	Digested starch [%]	Fructose [mg/g cake]
WFC	1.12 ± 0.0035	0.730 ± 0.029	n.d.	0.6844 ± 0.0073	n.d.
PFC	1.003 ± 0.027	n.d.	n.d.	0.584 ± 0.016	n.d.
PSPPIC	1.016 ± 0.047	n.d.	n.d.	0.464 ± 0.021	n.d.
MSPPIC	0.893 ± 0.033	n.d.	n.d.	0.298 ± 0.011	n.d.
PSC	1.077 ± 0.084	n.d.	n.d.	0.520 ± 0.041	n.d.
MSC	0.86 ± 0.42	n.d.	n.d.	0.30 ± 0.14	n.d.

The latter could be verified by digestion of a special cake that did not contain any starch component but only sucrose as relevant source of sugar. As in the original recipe, this special cake (“PPI cake”) contained 45% w/w egg, 25% w/w sucrose and 5% w/w oil. The 25% w/w flour component was completely replaced by pea protein isolate. This resulted in a more cookie-like product, but with a dry matter content comparable to that of the other cakes (85.870 ± 0.071 g/100g). After the *in vitro* digestion

was carried out (as described in **§ 7.1 of Part IV**), the quantities of glucose and fructose were measured using the HPAEC-PAD method described in **§ 7.2.2 of Part IV**.

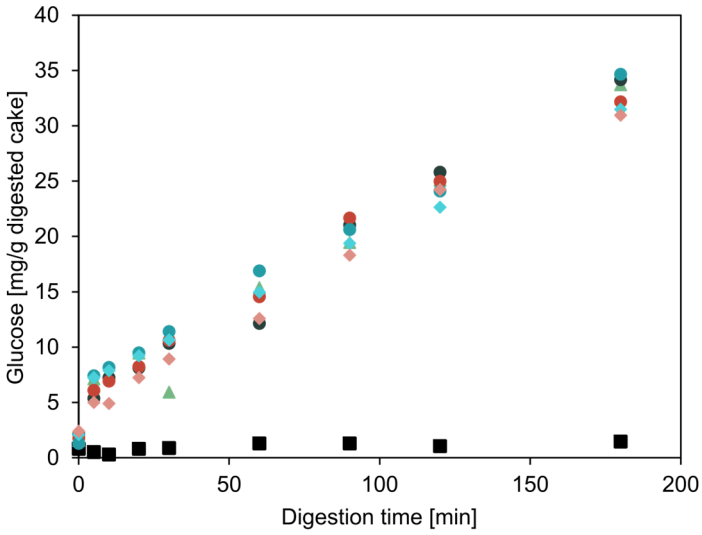

Figure 80. Concentration of glucose [mg/g digested cake] released during intestinal digestion of cakesbased on wheat flour (WFC ▲), pea flour (PFC ●), pea starch and pea protein (PSPPIC ●), maize starch and pea protein (MSPPIC ◆), pea starch (PSC ●), maize starch (MSC ◆) and pea protein isolate (PPI ■).

As illustrated in **Figure 80**, the concentration of glucose released from the PPI cake during the gastric phase (t_{0min}) was well below 2 mg/g digested cake and remained nearly constant during intestinal digestion. This implied that sucrose was not converted into glucose and fructose throughout the gastrointestinal digestion. This was further confirmed by the fact that no fructose could be measured. As mentioned earlier, the latter was also true for the other cakes (see **Table 40**), however an increase in the glucose concentration was detected, which was attributed to starch hydrolysis.

6 FINAL CONCLUSION

This study successfully demonstrated that the use of pea ingredients as only flour component in sponge cakes can lead to appealing products that not only have an attractive color and texture, but also good nutritional quality. By means of the chosen analysis strategy it could be shown that during the *in vitro* digestion of cakes made with pea flour or a combination of isolated proteins and starch not only more readily bioaccessible protein but also less starch breakdown products were released compared to the cereal-based references. Interestingly, the differences between pea flour and purified fractions were limited. From a sustainability point of view, pea flour should thus particularly be considered a new ingredient for protein-rich and gluten-free bakery products.

Moreover, the experimental approach allowed to conclude about the factors most likely affecting cake digestibility, which included both particular chemical characteristics and structural properties of the raw materials and cakes. This emphasized that the linking of different aspects of the same product (i.e. structural, nutritional and chemical aspects) is an interesting approach to obtain a holistic picture of its global product quality.

PART VI

CONCLUSION & OUTLOOK

The increasing interest in plant-based raw materials as an alternative to ingredients from animals, wheat and soy requires intensive research on the applicability of novel plant-based ingredients in foods. Among the potential sources, legumes have gained considerable popularity because of their nutritional and functional benefits, abundant availability, low cost, hypo-allergenicity and non-GMO status. Based on these characteristics, legumes are considered to be attractive ingredients for the formulation of baked goods that traditionally depend on the use of wheat flour, which however can cause a range of clinical disorders in sensitive populations. Nevertheless, replacing wheat completely with legume ingredients might lead to changes in quality-determining product criteria such as aroma, texture, appearance and digestibility due to their differing composition. The focus of this dissertation was therefore to assess the influence of different legume ingredients on reactivity during the development of sponge cakes as an example of a widely consumed bakery product and its effect on the above-mentioned aspects as well as their interdependence. The legume ingredients used included whole flours made from lentils, lupins, chickpeas, green peas and yellow peas as well as purified pea proteins and pea starch. These are the major conclusions and correlated perspectives:

Legumes exhibit a greater ability than wheat to generate volatile processed-induced compounds with probable impact on cake odor.

By developing a HS-SPME/GC-MS quantitative method based on isotopic dilution, it was possible to follow the generation and the amounts of VOCs at each key step of sponge cake making (from raw materials to batter beating to cake baking), which revealed that whole legume flours had a higher potential for entering into chemical reactions than wheat (**Figure 50**). During the preparation of the legume batters, numerous oxidation markers (alcohols, aldehydes, ketones) were formed, which could be attributed to a high LOX activity and elevated levels of its substrates linoleic and linolenic acid (**Table 25**). Based on the individual VOCs identified, it was concluded that 13- and 10-LOX were the main catalysts that oxidized the fatty acids during batter beating. The reactivity potential followed the order: wheat < lupin << chickpea < lentil < green pea < yellow pea < yellow pea produced under nitrogen. This could be further confirmed by the analysis of intermediates of lipid oxidation, namely conjugated dienes and hydroperoxides by spectrophotometric and titrimetric methods (**Figure 52**). The presence of thermal reaction markers in lupin flour (i.e. pyrazines, furanic compounds) implied that a thermal treatment had taken place, which presumably led to the inactivation of LOX, and thus explained its markedly reduced ability to form VOCs compared to the other legume flours. Yellow pea flour that had been produced and stored under nitrogen was highly susceptible to oxidation despite LOX activities and fatty acid profiles similar to the common yellow pea flour, suggesting that the precursors had been protected during production, so that exposure to atmospheric oxygen caused an instant reaction during cake production.

Baking triggered caramelization and the Maillard reaction, which led to the generation of pyrazines, furanic compounds and Strecker aldehydes, in particular with the legume products (**Figure 50**). With the exception of lupin, the legume cakes also contained higher levels of volatiles from lipid oxidation than the wheat reference, the majority of which was assumed to be residues of batter beating. By calculating the odor activity values of the identified volatile compounds on the basis of their quantities and threshold values reported in the literature, it was possible to identify key components which presumably had a significant influence on the overall aroma quality of the cakes. These were then subjected to a PCA, which revealed that the oxidation-derived VOCs with typical green, fatty odors (e.g.

hexanal) were mainly related to cakes made with lentil, chickpea, yellow pea and especially green pea flour (**Figure 56**). Moreover, Strecker aldehydes (e.g. 3-methylbutanal with malty odor) were assumed to be of substantial sensory importance in all the cakes, in particular in those based on wheat or the pea flours. Lentil cakes contained a high quantity of pyrazines (probably due to the presence of relevant amino acids at elevated amounts), among which 2-ethyl-3,5-dimethylpyrazine appeared to be an important odorant (earthy, nutty aroma). Lupin cakes were closer to wheat than to the other legume cakes in terms of the VOC profile.

PERSPECTIVES

The findings of this study emphasized that the choice of the raw material as well as how it is produced and stored can exert a considerable impact on the reactivity potential during the different steps of cake making. This allows to design products to the desired sensory attributes. If this idea is developed further, an interesting alternative approach could be to combine different legume flours with one another at varying ratios as it could broaden the possibilities for the creation of complex aroma profiles.

Since the calculation of the odor activity values in this study was based on odor threshold values that were determined in water, which corresponds to a matrix that is far from the cake matrix studied here, it is advisable to determine the key aroma components of the individual cakes by means of further and precise methods. One promising technique could be gas chromatography coupled with olfactometry (GC-O), which makes it possible to determine the most odor-active compounds in the legume-based applications. Moreover, sensory evaluations should be performed in order to conclude about the perceived sensory characteristics of the cakes.

The degree of oxidation depends on the local distribution and size of reactive ingredients in the batter and exposure time of LOX to its substrates, which are affected by the beating process.

This conclusion was elaborated by measuring the change in the concentration of primary and secondary oxidation markers as well as the microstructure of yellow pea batters as a function of the batter mixing time. By prolonging the batter beating after the addition of the yellow pea flour, neither the quantities of intermediate hydroperoxides (**Table 31**) nor those of final VOCs (**Table 29**) changed significantly, which could be explained by the uneven distribution and high proportion of large air bubbles and lipid droplets in the batter, impeding their probability of contact (**Figure 59**). However, when the mixing time after the incorporation of stripped sunflower oil was extended, a significantly higher concentration of hydroperoxides was detected. This indicated intensified lipid oxidation that was assigned to a more homogenous partitioning of lipids, proteins and air bubbles as well as a reduction in the size of air cells and lipid droplets, being correlated with a larger reaction interface. Nevertheless, this beating time was insufficient to convert the hydroperoxides into VOCs. This was only the case when the mixing times after the addition of both flour and oil were extended sequentially, which was due to the improved homogeneity and larger reaction interface as well as to the longer exposure time of LOX to its substrates.

Interestingly, the batter density increased with extended beating time, suggesting successive loss of air, and thus oxygen that is necessary for oxidative processes (**Table 30**). It was therefore concluded that the oxygen solubilized in the batter matrix was sufficient to trigger lipid oxidation. This was confirmed by the analysis of the densities of batters prepared for other studies, for example using different legume flours, because yellow pea batter was as dense as the lupin batter but accumulated significantly more

VOCs, while it was denser than the green pea batter but contained similar VOC concentrations (**Table 25**, **Figure 50**).

PERSPECTIVES

The knowledge obtained through this study helps to understand the factors that may play a crucial role in the generation of VOCs with potential odor impact during the development of complex products. The evaluation of the sensory quality of the cakes prepared with different beating times should be the next step. This may give insight into how significant these differences are in the final products. For this reason, we have started another project that deals with this question. In addition to changing the mixing parameters during batter making, the baking temperature will also be varied in order to obtain a holistic picture of the influence of various process parameters on reactivity and finally the sensory perception of the cakes. However, the focus should not only be on the assessment of the aroma but also on other quality parameters of the cakes, including safety, texture and color, which will be assessed using instrumental methods as well as sensory studies.

Pea flour fractionation yields purified pea ingredients which are less susceptible to lipid oxidation but more prone to Maillard reaction during cake development.

This result was obtained by following the amounts of VOCs by HS-SPME/GC-MS during the production of cakes based on either whole pea flour or a binary mixture of the purified constituents derived thereof, namely pea protein isolate and pea starch. The reduced ability of the pea fractions in the composite batter to generate not only final but also intermediate markers of oxidation (i.e. conjugated dienes and hydroperoxides) (**Figure 62**, **Figure 65**) could be attributed to the inactivity of LOX, which was probably a consequence of protein isolation. Compared to wheat, however, this composite batter contained higher amounts of oxidation-related VOCs, which might be due to the high proportion of linoleic and linolenic acids that can be easily oxidized as well as the removal of antioxidants during the industrial purification process. When a batter was prepared on the basis of pea starch only, hardly any oxidation markers were detected, suggesting, on the one hand, that the fatty acids contained in the pea flour remained in the pea protein isolate and not in the pea starch after fractionation (**Table 47**) and, on the other hand, that the fatty acids in the oil and egg were less susceptible to autoxidation. The same could be concluded when pea starch was replaced with purified maize starch. Both batters were also characterized by the lowest densities and consistencies (**Table 32**), thus confirming that these structural properties had no evident influence on the degree of oxidation.

The considerable differences in the amounts of oxidation-derived volatiles observed between the batters made with either pea flour or a combination of pea proteins and starch were much less pronounced after their transformation into cakes (**Figure 62**). Nevertheless, it was found that the VOC profile of the pea flour cakes was still determined by oxidation markers, whereas the composite cakes contained higher levels of molecules that originated from the heat-induced degradation of amino acids and sugars (i.e. Strecker aldehydes, furanic compounds and pyrazines). This was also the case with the cake formulated with pea starch alone. Apparently, the fractionation of pea flour yielded ingredients that were not only less prone to lipid oxidation but at the same time more available to participate in caramelization and the Maillard reaction during baking. A PCA implied that the oxidation-related volatiles with typical green-grassy odors could be of sensory importance in the pea flour cake, while pyrazines (nutty, roasted notes)

and, in particular, Strecker aldehydes (malty notes) were forecasted to be important contributors to the overall aroma of the composite and pure starch cakes (**Figure 66**). As for the wheat reference, it became evident that it had the lowest reaction potential during baking and thus a less complex profile of VOCs, not only compared to the pea flour cake but also the composite cake. Interestingly, similar VOC profiles were detected when replacing pea starch with maize starch in products based on starch alone or in combination with pea proteins. It is worth noting that these four formulations, however, led to the release of detectable amounts of the processed-induced compound furan, which has been classified as possibly carcinogenic to humans. This corroborated that the purified starch ingredients were more likely to undergo heat-induced reactions.

PERSPECTIVES

The higher potential of purified ingredients to produce process-induced compounds such as furan through mainly caramelization and the Maillard reaction raised the question of the health risk associated with the use of such ingredients. This study provided first insights into the ability of peas to form volatile process-induced compounds compared to wheat, using a global approach with focus on the detection of volatile markers. However, in order to clarify whether these reactive ingredients actually raise safety concerns, more precise quantification methods should be implemented in order to determine accurately the levels of the furanic compounds in the cake (e.g. HS trap as discussed by Cepeda-Vázquez et al. (2017)). Moreover, when assessing the risk linked to the transformation of new ingredients, other targeted analytical approaches (e.g. UHPLC-MS or UHPLC-DAD as discussed by Lee et al. (2021)) should be considered for the determination of less volatile contaminants such as acrylamide and HMF.

Another important step is to identify the key odor-active compounds in all the cakes. Despite the close correlation of pea flour cake with oxidation-derived VOCs that have been associated in the literature with undesriable green-beany odors, their sensory relevance needs to be clarified. This is particularly important because the pea flour also had a high potential to form thermal markers through the Maillard reaction and caramelization, many of which are known to possess low odor thresholds. It could therefore be possible that these heat-induced molecules might mask the undesirable aroma linked to the oxidation markers. For this reason, we decided to start another project which aims at identifying the key odor-active compounds using GC-O and at assessing the sensory quality of the different cakes using descriptive sensory evaluation methods. The experiments are ongoing and the results are planned to be published soon.

This study was also able to show that the inactivation of the LOX enzyme as a result of protein isolation led to a considerable reduction in lipid oxidation. However, new strategies should be developed to mildly inactivate the enzyme in whole legume flours without compromising functionalities as well as nutritional and sensory aspects. One possibility that could be tested would be the cold treatment of the raw materials, which may induce protein denaturation and thus LOX inactivation. First attempts to denature pea proteins at low temperatures (- 20 °C) have recently been published by Helmick et al. (2021), however using purified pea proteins in ethanol.

Legume flours and purified ingredients produce cakes with appealing characteristics.

The complete substitution of wheat flour with different legume ingredients led to attractive cakes as evidenced by their appealing volume and color properties. The cakes based on legume flours were generally characterized by significantly lower densities compared to the wheat reference (**Figure 51**), which was assigned to their elevated levels of proteins that could exert their important emulsifying and foaming properties (**Table 26**). An exception was lupin flour, with which a significant decrease in the capacity for gas retention was observed, leading to a dense cake, probably due to its high starch-to-protein ratio and elevated fiber content (**Table 26**). By contrast, the use of purified pea proteins in combination with pea starch resulted in cakes with higher volumes than those obtained with whole pea flour and might be assigned to the lack of fiber (**Figure 64**, **Table 47**). When pea starch was used without additional protein, the most voluminous cakes were produced, attributable to the high starch content that might have increased viscosity and thus air retention during baking. Moreover, it could be imagined that the egg proteins could have developed their full emulsifying and foaming potentials in the absence of plant-based proteins. Interestingly, replacing pea starch with maize starch was not associated with any significant change in density, from which it was deduced that pea starch could be an alternative for use in foods.

Further attempts to elaborate correlations between structural properties and chemical composition were made by performing further analyses on cake crumbs, including compression tests and image analysis of the cellular structure. As these were only partially representative, they could only be used to describe general trends. Accordingly, it appeared that the high density of the wheat cake was related to an increased cell wall stiffness (**Table 35**, **Table 36**), probably due to the high proportion of proteins with sulfur-containing amino acids, which can form rigid disulfide bridges. This inflexibility could have also been responsible for the observed formation of large air bubbles with thick cell walls (**Figure 72**). By contrast, the crumbs based on pea flour or a combination of pea protein and starch were smaller and had thinner cell walls. This could be due to the increased content of proteins with potentially higher agility and surface activity. The use of pea flour also resulted in an increased crumb firmness, that was not the case with the purified pea ingredients and might thus be due to an elevated fiber content absorbing water and contributing to a rigid network.

Moreover, it was demonstrated that the natural coloring of the legume ingredients played a role in the final color properties of the cakes developed. While the color of the crust was comparable to the wheat reference, the colors of the crumbs could vary (**Figure 51**, **Figure 64**). For instance, less colored raw materials such as chickpea flour, yellow pea flour and pea fractions did not induce any relevant color change, whereas green pea flour, lentil flour and lupin flour could lead to greenish, grayish and yellowish hues.

PERSPECTIVES

Based on these results, legume ingredients, both unrefined and refined, were found to be good functional ingredients in cakes. To get deeper insights into the structural differences of these legume- and wheat-based products, which could affect cake quality, further research should be carried out. Although first attempts were made, it was found that the methodological approach depended on the cake dimensions used and should thus be adapted accordingly. Moreover, non-destructive techniques such as X-ray microtomography might be promising tools to better understand the complexity of the cell structure in the cakes. Ultimately, the instrumental results obtained should be complemented with sensory studied to correlate the

experimental data with sensory attributes, such as mouthfeel and texture. This allows to obtain a global view on the applicability of new pulse ingredients in foods. We have performed some first descriptive sensory studies, which could confirm the trends described: cakes made with a mix of pea proteins and pea starch were described as soft and airy, whereas the whole pea flour cake was rather sticky and humid (Martínez-Noguera et al., 2021). These results are planned to be published soon. Consumer studies could also be performed in order to clarify whether the color changes induced by more colored legume flours would be a reason for rejection. These studies could be enlarged by assessing the global liking and acceptability of legume-based cakes, taking into consideration diverse aspects such as texture, taste and sustainability criteria.

*Another very interesting aspect, which is linked to the structure and composition of the cakes and which has not been dealt with in this work, concerns the interaction of flavor molecules with the cake matrix at the molecular level. It is known that odorous substances can interact with food components, i.e. proteins, lipids and carbohydrates, which can influence their release into the gas phase and therefore sensory perception. In a study on wheat-based sponge cake, Pozo-Bayon et al. (2008) were able to show that several odorous compounds can form complexes with the amylose contained in the starch and can thus entrap them in the matrix. How this interaction can change depending on the formulation is therefore an approach that should be investigated in future experiments. This is particularly interesting with regard to pea ingredients because we have been able to show in the present thesis that these products form less amylose-lipid complexes than cakes made with wheat flour or maize starch (**Table 38**), and thus indicates a different affinity for the formation of complexes, which however may be ligand specific and therefore needs to be elucidated.*

Whole pea flour and purified pea ingredients showed similar susceptibility to proteolysis and amylolysis during simulated gastrointestinal *in vitro* digestion.

The INFOGEST method used was a good tool to gain insight into the *in vitro* digestibility of proteins and starch in complex food systems. Similar protein digestion kinetics between the wheat reference and the cakes, which were made with either pea flour or a combination of pea proteins and pea starch, could be determined (**Table 52**, **Figure 75**) by quantifying the released amino groups and small peptides throughout intestinal digestion, which corresponded to the protein fraction that is readily bioaccessible. By acid hydrolysis of this fraction, a conversion of the small peptides into amino acids could be achieved, whereby a better comparison between the cakes was made possible. It turned out that proteolysis was retarded in the cakes containing pea proteins (from either flour or isolate) compared to the wheat reference, however yielded a higher concentration of readily bioaccessible protein hydrolyzed (**Figure 75**). Moreover, the digestion patters of the pea proteins contained in the flour or the isolate were comparable, which implied that the fractionation process did not impair protein digestibility.

By implementing two analysis methods based on the spectrophotometric measurement of reducing sugars (DNS method) and chromatographic quantification of individual starch hydrolysis products (HPAEC-PAD method), respectively, a similar biphasic starch digestion behavior was detected for all the cakes that was adequately modeled by combining two first order equations. This observation was

attributed to the immediate digestion of complex starch as soon as the amylolytic enzymes were added, followed by the hydrolysis of starch breakdown products (e.g. maltotriose) into maltose and glucose (**Figure 78**). Starch in cakes made with pea flour, pea starch or pea starch combined with pea proteins were less prone to amylolysis than those containing wheat flour, maize starch or maize starch combined with pea proteins (**Figure 77**). This was confirmed by light microscopic imaging of the digestive pellets due to the absence of starch granules in the wheat- and maize-based cakes after a considerably shorter time (**Figure 76**). The possible reasons for this could be the higher content of amylose and resistant starch (**Table 38**), the maintenance of certain granular structures after baking (**Figure 76**) and the bigger granule sizes in the pea cakes (**Figure 68**), which could have decelerated enzymatic attack. Moreover, scanning electron microscopy revealed a more continuous protein-starch network in the pea-based cakes compared to the multi-layered wheat and maize cakes, suggesting easier enzyme-substrate interaction in the latter cases (**Figure 79**). On closer inspection of the digestibility patterns of pea starch in its purified form or as part of the whole flour, it was found that both ingredients showed a similar susceptibility to amylolysis. Consequently, it was concluded that the fractionation process did not lead to a relevant modification in starch digestibility as already reported for the pea proteins.

PERSPECTIVES

Static in vitro digestion methods are simple tools to simulate in vivo food digestion. However, these methods do not accurately reflect physiological conditions because they do not consider interactions with the host, the gradual emptying from the stomach into the small intestines as well as changes in the pH, concentration of electrolytes and enzymes. In order to provide more physiologically relevant data, it would be interesting to use dynamic in vitro protocols such as those described by Ménard et al. (2014) and Mulet-Cabero et al. (2020) which take into account changes in the pH value, digestive secretion and gastric and intestinal transit times. In this context, the addition of an α-amylase in the oral phase, which had been excluded from the protocol on the suggestion of other researchers due to short residence times and instant inactivation in the gastric phase during static in vitro digestion, should be considered. When applying semi-dynamic in vitro digestion methods, in particular, the importance of α-amylase in the oral phase should be clarified as its activity may remain during the initial phase of gastric digestion. This is especially interesting because amylolysis appeared to have been initiated directly on addition of enzyme, which implied high accessibility of enzymes to its substrate starch. Another important aspect that should not be overlooked is the change in the viscosity of the chyme during digestion, which depends on the composition of the product and can thus influence the enzymatic catalysis. This should therefore be born in mind in future experiments when reformulating products.

One step further would be the performance of in vivo studies in order to confirm the results reported in this work and establish the actual postprandial metabolic effects. Moreover, the assessment of the bioaccessibility of minor compounds that are beneficial for the human health could be carried out to further elaborate differences between pea (refined or unrefined) and cereal ingredients.

From a global perspective, the methodological strategy to link reactivity with essential product criteria was a valuable and innovative formulation engineering approach to assess the applicability of new ingredients in complex formulated foods. It could be demonstrated that the evaluation of the potential of new raw materials as functional food ingredients should not be limited to the analysis of their properties as pure raw materials, but should be expanded in combination with other ingredients in real food systems. In this way, important product limitations can be disclosed (sensory, texture, appearance) and the relevance and suitability of raw material processing strategies, such as thermal treatment, processing under nitrogen or fractionation of flour, can be evaluated.

Regarding the applicability of legume ingredients in sponge cake applications, it could be concluded that there is great potential for product innovation, since various attractive products with interesting structure, aroma, appearance and digestibility characteristics can be produced depending on the combination raw material/processing.

APPENDIX

SUPPLEMENTARY TABLE OF ARTICLE 1 AND 3 – RETENTION INDICES

Table 41. Overview of all tentatively identified compounds in all products (raw materials, batters, cakes) and their retention times, CAS numbers, selected ions, calculated retention indices ($RI_{calculated}$) and reference retention indices ($RI_{reference}$) reported in the literature.

RT [min]	Tentative identification	CAS	Ion [m/z]	$RI_{calculated}$	Literature			
					$RI_{reference}$	Column type	Column length	
1.41	Pentane	109-66-0	72	500	500	-	-	
1.52	Hexane	110-54-3	57	600	600	-	-	
1.76	Heptane	142-82-5	71	700	700	-	-	
1.80	Acetaldehyde	75-07-0	44	708	716	HP-Innovax	30 × 0.32 × 0.50	[1]
2.04	Dimethyl sulfide	75-18-3	62	754	754	DB-Wax	60 × 0.53 × 1	[2]
2.10	3-Ethylhexane	619-99-8	43	765	-	-	-	
2.31	Octane	111-65-9	43	800	800	-	-	
2.39	Furan	110-00-9	68	808	802	Supelcowax-10	30 × 0.25 × 0.25	[3]
2.50	2-Methylpropanal	78-84-2	58	819	812	DB-Wax	60 × 0.25 × 0.25	[4]
2.55	Acetone	67-64-1	58	824	814	DB-Wax	60 × 0.25 × 0.25	[4]
3.08	Tetrahydrofuran	109-99-9	72	869	861	DB-Wax	60 × 0.25	[5]
3.24	2-Methylfuran	534-22-5	82	881	888	ZB-Wax	60 × 0.32 × 0.50	[6]
3.32	Butanal	123-72-8	72	887	895	HP-Innovax	30 × 0.32 × 0.50	[1]
3.36	Ethylcyclohexane	1678-91-7	83	890	885	Supelcowax-10	60 × 0.25 × 0.25	[7]
3.51	Ethyl acetate	141-78-6	43	900	887	DB-Wax	60 × 0.25 × 0.25	[4]
3.51	Nonane	111-84-2	43	900	900			
3.66	3-Methylfuran	930-27-8	82	908	901	Supelcowax-10	60 × 0.25 × 0.25	[8]
3.73	2-Butanone	78-93-3	72	911	900	DB-Wax	60 × 0.25 × 0.25	[4]
3.97	2-Methylbutanal	96-17-3	58	922	914	Supelcowax-10	60 × 0.25 × 0.25	[8]
4.08	3-Methylbutanal	590-86-3	58	927	925	DB-Wax	30 × 0.25 × 0.25	[9]
4.20	2,2,6-Trimethyloctane	62016-28-8	57	932	-	-	-	
4.31	2-Propanol	67-63-0	45	937	931	DB-Wax	60 × 0.25 × 0.25	[10]
4.49	Ethanol	64-17-5	45	944	942	DB-Wax	60 × 0.25 × 0.5	[11]
4.72	2,2,4,6,6-Pentamethylheptane	13475-82-6	112	953	954	Supelcowax-10	60 × 0.25 × 0.25	[12]
5.05	2-Ethylfuran	3208-16-0	96	959	957	DB-Wax	60 × 0.25 × 0.25	[13]
5.70	2-Pentanone	107-87-9	86	984	983	ZB-Wax	30 × 0.32 × 0.25	[14]
5.70	3-Methyl-2-butanone	563-80-4	43	984	989	Supelcowax-10	60 × 0.25 × 0.25	[8]
5.71	3-Pentanone	96-22-0	86	985	986	DB-Wax	60 × 0.32 × 0.5	[15]
5.78	Pentanal	110-62-3	58	987	984	DB-Wax	60 × 0.25 × 0.25	[13]
5.87	2,3-Butanedione	431-03-8	86	991	993	DB-Wax	60 × 0.53 × 1	[2]
6.37	2,2,4,4,6,8,8-Heptamethylnonane	4390-04-9	57	1007	-	-	-	
6.64	4-Methyl-2-pentanone	108-10-1	43	1015	1010	Supelcowax-10	60 × 0.25 × 0.25	[16]
6.68	Acetonitrile	75-05-8	41	1016	1013	Supelcowax-10	50 × 0.25 × 0.25	[17]
7.29	2-Butanol	78-92-2	45	1033	1034	DB-Wax	60 × 0.32 × 0.25	[18]
7.51	2-Butyl-1-octanol	3913-02-8	57	1039	-	-	-	
7.58	2,3-Dimethylcyclohexanol	1502-24-5	95	1041	-	-	-	
7.78	Toluene	108-88-3	91	1047	1049	DB-Wax	60 × 0.25 × 0.25	[19]
7.80	1-Propanol	71-23-8	59	1047	1049	DB-Wax	30 × 0.25 × 0.25	[20]
8.72	2,3-Pentanedione	600-14-6	57	1073	1066	DB-Wax	60 × 0.32 × 0.5	[21]
9.01	Dimethyl disulfide	624-92-0	94	1081	1086	DB-Wax	30 × 0.25 × 0.25	[22]
9.22	**Hexanal-d12**	**1219803-74-3**	**64**	**-**	**-**	**-**	**-**	
9.36	Hexanal	66-25-1	56	1091	1091	DB-Wax	30 × 0.25 × 0.5	[23]
9.76	2-Methyl-1-propanol	78-83-1	41	1102	1110	DB-Wax	60 × 0.25 × 0.25	[13]
9.95	β-Pinene	127-91-3	93	1106	1100	DB-Wax	30 × 0.32 × 0.25	[24]
10.34	3-Pentanol	584-02-1	59	1117	1111	ZB-Wax	30 × 0.25 × 0.15	[16]
10.80	2-Pentanol	6032-29-7	45	1128	1127	DB-Wax	30 × 0.25	[25]
10.97	Ethylbenzene	100-41-4	91	1133	1066	DB-Wax	60 × 0.32 × 0.5	[21]
11.14	2-Pentenal	1576-87-0	84	1137	1132	Supelcowax-10	60 × 0.25 × 0.25	[26]
11.65	3-Carene	13466-78-9	93	1151	1149	DB-Wax	30 × 0.232 × 0.5	[27]
11.73	1-Butanol	71-36-3	56	1153	1147	ZB-Wax	30 × 0.25 × 0.15	[16]
12.37	1-Penten-3-ol	616-25-1	57	1169	1165	DB-Wax	30 × 0.25	[25]

12.42	Myrcene	123-35-3	93	1170	1168	DB-Wax	30 × 0.32 × 0.5	[28]
13.15	2-Heptanone	110-43-0	58	1189	1185	DB-Wax	60 × 0.25 × 0.25	[19]
13.27	Heptanal	111-71-7	70	1192	1192	DB-Wax	30 × 0.25 × 0.25	[20]
13.44	Dodecane	112-40-3	57	1197	1200	-	-	
13.58	Limonene	138-86-3	93	1201	1202	DB-Wax	60 × 0.32 × 0.5	[21]
14.07	2-Methy-1-butanol	137-32-6	70	1214	1210	DB-Wax	30 × 0.32 × 0.5	[29]
14.07	3-Methyl-1-butanol	123-51-3	70	1214	1210	DB-Wax	30 × 0.32 × 0.5	[29]
14.25	Pyrazine	290-37-9	80	1219	1223	DB-Wax	30 × 0.32 × 0.5	[30]
14.51	2-Hexenal	505-57-7	98	1226	1225	DB-Wax	30 × 0.25 × 0.25	[31]
15.01	2-Pentylfuran	3777-69-3	138	1240	1235	DB-Wax	60 × 0.32 × 0.25	[32]
15.66	1-Pentanol	71-41-0	70	1257	1255	DB-Wax	30 × 0.32 × 0.25	[14]
15.81	3-Octanone	106-68-3	99	1261	1261	DB-Wax	30 × 0.25 × 0.25	[33]
15.93	Styrene	100-42-5	104	1265	1259	DB-Wax	60 × 0.25 × 0.25	[34]
16.18	**2-Methylpyrazine-d6**	**1219804-84-8**	**100**	**-**	**-**	**-**	**-**	
16.20	2-Methylpyrazine	109-08-0	94	1272	1276	DB-Wax	30 × 0.32 × 0.5	[30]
16.94	2-Octanone	111-13-7	58	1292	1297	DB-Wax	30 × 0.25 × 0.25	[20]
16.98	3-Hydroxy-2-butanone	513-86-0	45	1293	1301	DB-Wax	60 × 0.32 × 0.5	[21]
17.04	Cyclohexanone	108-94-1	55	1295	1296	Supelcowax-10	60 × 0.25 × 0.25	[35]
17.10	Octanal	124-13-0	84	1296	1293	DB-Wax	30 × 0.32 × 0.5	[30]
17.11	Tridecane	629-50-5	57	1297	1300	-	-	
17.57	1-Hydroxy-2-propanone	116-09-6	43	1310	1290	DB-Wax	30 × 0.25 × 0.5	[36]
17.58	1-Octen-3-one	4312-99-6	70	1310	1310	DB-Wax	30 × 0.25 × 0.5	[36]
18.16	2,5-Dimethylpyrazine	123-32-0	108	1327	1323	DB-Wax	30 × 0.25 × 0.5	[36]
18.22	2-Penten-1-ol	1576-96-1	57	1328	1327	DB-Wax	30 × 0.25 × 0.1	[37]
18.33	2-Heptenal	18829-55-5	83	1332	1332	DB-Wax	60 × 0.32 × 0.25	[38]
18.63	2-Ethylpyrazine	13925-00-3	107	1340	1344	DB-Wax	30 × 0.32 × 0.5	[30]
18.84	6-Methyl-5-hepten-2-one	110-93-0	108	1346	1352	DB-Wax	60 × 0.32 × 0.5	[21]
18.88	2-Hexenyl acetate	2497-18-9	67	1347	1345	DB-Wax	30 × 0.25 × 0.25	[39]
19.02	2,3-Dimethylpyrazine	5910-89-4	108	1351	1346	DB-Wax	30 × 0.25 × 0.5	[36]
19.31	1-Hexanol	111-27-3	69	1360	1360	DB-Wax	30 × 0.25 × 0.25	[33]
19.64	2,4,6-Trimethylpyridine	108-75-8	121	1369	1365	Supelcowax-10	60 × 0.25 × 0.25	[40]
20.42	3-Hexen-1-ol	928-96-1	82	1392	1394	DB-Wax	30 × 0.25 × 0.5	[36]
20.45	2-Ethyl-3-methylpyrazine	15707-23-0	122	1393	1402	DB-Wax	60 × 0.25 × 0.25	[41]
20.59	2-Nonanone	821-55-6	58	1397	1390	DB-Wax	30 × 0.25 × 0.5	[36]
20.70	2-Ethyl-6-methylpyrazine	13925-03-6	122	1400	1395	DB-Wax	30 × 0.32 × 0.5	[30]
20.72	Nonanal	124-19-6	57	1400	1396	DB-Wax	30 × 0.25 × 0.5	[36]
20.96	Trimethylpyrazine	14667-55-1	122	1408	1408	DB-Wax	30 × 0.25 × 0.5	[36]
21.01	Cyclohexanol	108-93-0	82	1409	1410	DB-Wax	60 × 0.25 × 0.25	[13]
21.16	2-Hexen-1-ol	928-95-0	57	1414	1414	DB-Wax	30 × 0.32 × 0.25	[24]
21.20	3-Octen-2-one	1669-44-9	111	1415	1416	DB-Wax	30 × 0.32 × 0.25	[42]
21.93	2-Octenal	2548-87-0	70	1437	1437	DB-Wax	30 × 0.25 × 0.25	[33]
22.36	2-Ethyl-3,5-dimethylpyrazine	13925-07-0	136	1450	1455	DB-Wax	30 × 0.32 × 0.5	[30]
22.39	3-Ethyl-2,5-dimethylpyrazine	13360-65-1	136	1451	1450	DB-Wax	30 × 0.25 × 0.5	[36]
22.59	1-Octen-3-ol	3391-86-4	99	1457	1458	DB-Wax	30 × 0.25 × 0.25	[33]
22.78	1-Heptanol	111-70-6	70	1463	1461	DB-Wax	60 × 0.25 × 0.25	[4]
22.90	3-Methylthiopropanal	3268-49-3	48	1467	1465	DB-Wax	30 × 0.32 × 0.5	[43]
23.02	6-Methyl-5-hepten-2-ol	1569-60-4	95	1471	1468	DB-Wax	30 × 0.32 × 0.5	[44]
23.19	Furfural	98-01-1	96	1476	1476	DB-Wax	30 × 0.32 × 0.5	[45]
23.23	**Furfural-d4**	**1219803-80-1**	**100**	**-**	**-**	**-**	**-**	
23.51	Acetic acid	64-19-7	60	1486	1484	DB-Wax	15 × 0.53 × 1	[46]
23.86	2-Ethyl-1-hexanol	104-76-7	57	1496	1499	DB-Wax	30 × 0.25 × 0.5	[36]
23.99	2-Decanone	693-54-9	58	1500	1495	DB-Wax	30 × 0.25 × 0.5	[36]
24.08	2,4-Heptadienal	4313-03-5	81	1503	1508	DB-Wax	60 × 0.32 × 0.5	[47]
24.56	2-Hepten-1-ol	33467-76-4	57	1519	1517	DB-Wax	30 × 0.25 × 0.25	[48]
24.86	1H-Pyrrole	109-97-7	67	1529	1521	DB-Wax	30 × 0.32 × 0.5	[30]
25.33	2-Nonenal	18829-56-6	83	1545	1545	DB-Wax	30 × 0.32 × 0.5	[43]
25.70	Linalool	78-70-6	71	1557	1552	DB-Wax	60 × 0.25 × 0.25	[4]
25.98	1-Octanol	111-87-5	56	1567	1569	DB-Wax	30 × 0.25 × 0.5	[36]
26.18	Dimethylsulfoxide	67-68-5	78	1573	1563	DB-Wax	60 × 0.25 × 0.25	[49]
26.48	3,5-Octadien-2-one	30086-02-3	95	1583	1569	DB-Wax	30 × 0.25 × 0.5	[36]
26.59	5-Methyl-2-furancarboxaldehyde	620-02-0	110	1587	1582	DB-Wax	60 × 0.25	[50]

26.64	3-Methyl-1H-pyrrole	616-43-3	81	1589	1569	DB-Wax	60 × 0.25 × 0.25	[13]
26.77	2,3-Butanediole	513-85-9	45	1593	1583	DB-Wax	30 × 0.32 × 0.5	[23]
27.68	2(3H)-Dihydro-5-methyllfuranone	108-29-2	85	1623	1619	DB-Wax	60 × 0.25 × 0.25	[51]
27.75	2-Octen-1-ol	18409-17-1	57	1626	1622	DB-Wax	60 × 0.25 × 0.25	[51]
27.82	2(3H)-Dihydro-4-methylfuranone	1679-49-8	56	1628	-	-	-	
28.21	2-Acetylpyrazine	22047-25-2	122	1641	1646	DB-Wax	60 × 0.32 × 0.5	[21]
28.23	Dihydro-2(3H)-furanone	96-48-0	86	1642	1643	DB-Wax	60 × 0.25 × 0.25	[51]
28.71	Phenylacetaldehyde	122-78-1	91	1658	1658	DB-Wax	30 × 0.32 × 0.5	[43]
28.83	2-Acetylthiazole	24295-03-2	127	1662	1662	DB-Wax	60 × 0.32 × 0.5	[21]
29.11	1-Nonanol	143-08-8	55	1671	1668	DB-Wax	30 × 0.32 × 0.5	[24]
29.17	2-Furanmethanol	98-00-0	98	1673	1667	DB-Wax	60 × 0.25 × 0.25	[19]
29.84	5-Acetyl-2-methylpyrazine	22047-27-4	136	1696	1679	DB-Wax	30 × 0.25 × 0.5	[36]
30.11	2-Acetyl-3-methylpyrazine	23787-80-6	136	1693	1640	DB-Wax	60 × 0.32 × 0.5	[21]
30.21	3-Methylbutanoic acid	503-74-2	60	1705	1687	DB-Wax	30 × 0.32 × 0.5	[30]
30.34	2,4-Nonadienal	5910-87-2	81	1707	1705	DB-Wax	30 × 0.32 × 0.5	[52]
30.35	5-Ethyldihydro-2(3H)-furanone	695-06-7	85	1703	1694	DB-Wax	30 × 0.25 × 0.5	[36]
30.75	2-Ethyl-4-methyl-1H-pyrrole	69687-77-0	94	1718	1711	DB-Wax	60 × 0.25 × 0.25	[13]
30.91	Pyrazinamid	98-96-4	123	1717	1740	DB-Wax	60 × 0.25 × 0.5	[53]
31.42	Naphthalene	91-20-3	128	1727	1719	Supelcowax-10	30 × 0.25 × 0.25	[54]
33.34	2,4-Decadienal	25152-84-5	81	1762	1763	DB-Wax	30 × 0.25 × 0.5	[23]
35.12	Hexanoic acid	142-62-1	60	1795	1803	ZB-Wax	60 × 0.32 × 0.5	[32]
35.78	Dimethyl sulfone	67-71-0	79	1915	1912	DB-Wax	60 × 0.25 × 0.25	[51]
36.01	Benzyl alcohol	60-12-8	122	1924	1924	DB-Wax	30 × 0.32 × 0.5	[43]
37.41	Maltol	118-71-8	126	1979	1977	DB-Wax	15 × 0.32 × 0.25	[55]
37.57	1-(1H-Pyrrol-2-yl)ethanone	1072-83-9	109	1985	1980	DB-Wax	60 × 0.25 × 0.25	[56]
39.98	2(3H)-Dihydro-5-pentylfuranone	104-61-0	85	2083	2063	DB-Wax	30 × 0.32 × 0.5	[57]
44.53	Dibenzofuran	132-64-9	168	2257	2270	TC-Wax	60 × 0.25	[58]
44.65	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	28564-83-2	144	-	-	-	-	

[1] Héberger *et al.* (1999), [2] Ott *et al.* (1997), [3] Bianchi *et al.* (2007), [4] Tatsuka *et al.* (1990), [5] Umano *et al.* (1994), [6] Marin *et al.* (2008), [7] Elmore *et al.* (2005), [8] Girard *et al.* (2000), [9] Carunchia Whetstine *et al.* (2005), [10] Mattheis *et al.* (1992), [11] Chida *et al.* (2004), [12] Tanchotikul *et al.* (1989), 1[3] Shimoda *et al.* (1996a), [14] Wu *et al.* (2007), [15] Piveteau *et al.* (2000), [16] Ledauphin *et al.* (2004), [17] Soria *et al.* (2003), [18] Beck *et al.* (2008), [19] Shiratsuchi *et al.* (1994), [20] Nielsen *et al.* (2004a), [21] Le Guen *et al.* (2000a), [22] Nielsen *et al.* (2004b), [23] Selli *et al.* (2006), [24] Rega *et al.* (2003), [25] Iwaoka *et al.* (1994), [26] Chung *et al.* (2001), [27] Le Quere *et al.* (1990), [28] Rega *et al.* (2004), [29] Bureau *et al.* (2000), [30] López-Galilea *et al.* (2006), [31] Lee *et al.* (2005), [32] Brunton *et al.* (2002), [33] Cho *et al.* (2008), [34] Shimoda *et al.* (1996b), [35] Vejaphan *et al.* (1988), [36] Pozo-Bayón *et al.* (2007), [37] Kato *et al.* (2001), [38] Chyau *et al.* (2001), [39] Froehlich *et al.* (1989), [40] Chung (2000), [41] Kim (2001), [42] Fan *et al.* (2006), [43] Mahattanatawee *et al.* (2007), [44] Wirth *et al.* (2001), [45] Gürbüz *et al.* (2006), [46] Peng (2000), [47] Le Guen *et al.* (2000b), [48] Weckerle *et al.* (2001), [49] Chung (1999), [50] Shimoda *et al.* (1990), [51] Cho *et al.* (2006), [52] Högnadóttir *et al.* (2003), [53] Moon *et al.* (2009), [54] Riu-Aumatell *et al.* (2005), [55] Kishimoto *et al.* (2006), [56] Shimoda *et al.* (1995), [57] Culleré *et al.* (2004), [58] Miyazawa *et al.* (2003)

Table 42. Concentration of alcohols [µg/g sample] identified in the flours, batters and cakes.

Compounds	Flours							Batters							Cakes						
	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN
Alcohols																					
Benzyl alcohol		0.14		0.87																	
Ethanol	173.47	6.26	497.25	466.76	1138.04	2046.62	2042.35	4.33	1.19	47.22	16.58	281.45	530.73	1071.56	2.20	0.36	2.04	5.49	6.76	1.06	0.29
1-Propanol		0.61		39.82				0.04	0.02	2.07	0.63										
1-Butanol	5.20	0.41	7.51	5.44						1.57		3.42	3.97	7.65		0.054		0.069	0.23	0.12	0.21
1-Pentanol	7.52	1.09	19.95	4.41	9.37	8.95	6.98	0.08	0.04	24.25	6.49	44.65	46.37	80.99	0.030		0.27	0.29	0.65	0.40	0.55
1-Hexanol	15.02	1.81	37.15	87.07	60.47	55.46	49.14	0.13	0.08	107.20	178.91	297.01	241.81	435.64	0.050	0.065	2.00	12.10	1.78	1.60	1.01
1-Octanol												1.15	1.12	1.98				0.05	0.12		0.11
1-Octen-3-ol	0.17									1.17	0.43	3.03	1.95	7.14	0.015		0.13	0.14	0.29	0.18	0.34
1-Penten-3-ol		0.74		12.79				0.16	0.04	62.69	24.10	147.19	197.16	475.42		0.094	0.93	1.30	1.52	1.66	1.02
2-Propanol	7.90	17.24	180.38	36.66	90.89	116.77	188.35		0.20			5.90									
2-Butanol		0.62	55.39	14.26	72.96							5.65									
2-Pentanol	7.37	0.56	10.51	5.83	8.70	16.52	19.28	0.09				2.49	4.74	9.55							
2-Penten-1-ol										0.91	1.81	1.08		2.16							
2-Hexen-1-ol				6.94													0.10	0.13	0.17	0.14	0.14
2-Hepten-1-ol										0.41		3.61		2.45							
2-Octen-1-ol										3.27	0.61										
2-Methyl-1-propanol	3.44	0.41	12.78	14.09																	
2-Methyl-1-butanol		0.78	14.25	17.66	6.84	10.30	11.45						8.33	5.57							
2-Ethyl-1-hexanol						25.56															
2-Butyl-1-octanol	6.94																				
3-Pentanol		0.27		9.38						2.62		53.36	165.86	135.43							
3-Hexen-1-ol				6.91							2.61	2.28	1.58	3.10							
3-Methyl-1-butanol	7.94									1.78		4.66				0.020			0.16		
Total	234.97	30.94	835.17	728.90	1387.27	2280.18	2317.55	4.83	1.58	255.16	232.17	856.92	1203.62	2238.62	2.30	0.59	5.48	19.56	11.68	5.15	3.67

Table 43. Concentration of aldehydes [µg/g sample] identified in the flours, batters and cakes.

Compounds	Flours							Batters							Cakes						
	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN
Aldehydes																					
Acetaldehyde															0.12	0.11	0.16		0.26	0.21	0.19
Butanal																	0.075	0.088	0.11	0.12	0.12
Pentanal											2.75				0.23	0.17	1.95	1.20	2.98	2.58	2.79
Hexanal	5.84	1.41		9.89	6.87	14.54	13.37	0.47	0.12	80.26	160.58	44.52	33.94	147.61	1.47	0.82	11.89	12.04	23.70	15.76	23.81
Heptanal								0.03		0.59	0.37				0.074	0.052	0.37	0.35	0.42	0.36	0.47
Octanal															0.016	0.020	0.093	0.079	0.11	0.083	0.12
Nonanal	0.35								0.01		0.14				0.034	0.049	0.16	0.17	0.20	0.15	0.22
Phenylacetaldehyde															0.064	0.11					
2-Pentenal										1.57	1.58		2.11	4.28							
2-Hexenal				3.05							3.51							0.077	0.090		0.082
2-Heptenal										4.99	2.17	3.18	1.68	5.27			0.065	0.061	0.17	0.084	0.21
2-Octenal										1.24	0.55	0.80		1.98			0.10	0.10	0.22	0.13	0.28
2-Nonenal											0.13										
2,4-Heptadienal											0.26										
2,4-Nonadienal										0.53	0.25										
2,4-Decadienal																	0.082	0.085	0.14	0.088	0.12
2-Methylpropanal															0.11		0.52	0.27			
2-Methylbutanal		0.37													1.67	1.40	1.82	1.06	1.96	2.23	1.69
3-Methylbutanal		0.27						0.03			0.19				2.05	1.63	1.79	1.16	2.11	2.32	1.71
Total	6.19	2.05	n.d.	12.94	6.87	14.54	13.37	0.53	0.13	89.17	172.48	48.50	37.72	159.14	5.84	4.37	19.06	16.74	32.47	24.11	31.80

Table 44. Concentration of ketones and furanic compounds [µg/g sample] identified in the flours, batters and cakes.

Compounds	Flours							Batters							Cakes						
	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN
Ketones																					
Acetone	12.08	26.56	60.24	77.41	87.85	105.09	125.52		0.58												
1-Hydroxy-2-propanone															0.72	0.69	1.45	1.25	1.67	1.44	1.57
1-Octen-3-one													0.94								
2-Butanone	1.88	1.25	9.55	18.02	123.49	19.22	26.73		0.020						0.059	0.080	0.10	0.08	0.12	0.10	0.11
2-Pentanone	1.29			7.14	6.39																
2-Heptanone		0.20								0.78			1.34	3.74	0.012		0.16	0.21	0.20	0.18	0.24
2-Octanone													1.35	2.17							
2-Decanone																			0.057		
2,3-Butanedione		0.33													0.081						
2,3-Pentanedione		0.46													0.23	0.27	0.25	0.26	0.14	0.25	0.18
3-Pentanone										7.62	6.38	16.71	22.59	36.36							
3-Octanone				2.81						0.88		4.11	2.59	8.14							
3-Octen-2-one																	0.051	0.071	0.12		0.11
3-Hydroxy-2-butanone		0.35									1.06				0.040	0.13		0.13	0.23	0.21	0.18
3-Methyl-2-butanone		0.32																			
6-Methyl-5-hepten-2-one	0.96	0.09																			
Total	16.21	29.56	69.79	105.38	217.73	124.32	152.25	n.d.	0.60	9.28	7.43	20.81	28.81	50.41	1.14	1.17	2.01	2.00	2.53	2.18	2.39
Furanic compounds																					
2(3H)-Dihydrofuranone	14.85	106.90	2.30	4.20											0.0048	0.032	0.0060	0.010	0.0060	0.0057	0.0057
2(3H)-Dihydro-4-methylfuranone		18.49																			
2(3H)-Dihydro-5-methyllfuranone		12.63	1.53																		
2(3H)-Dihydro-5-ethyllfuranone	17.34	39.11	7.37	5.81		4.58	3.69														
2(3H)-Dihydro-5-pentylfuranone												12.86	21.02	21.38							
2-Methylfuran		43.23		5.84																	
2-Ethylfuran			7.39	16.03			17.79								0.014	0.028	0.074	0.30	0.042	0.054	0.054
2-Pentylfuran	12.81	23.96	3.51	6.47	5.02	4.09	2.72								0.059	0.031	0.34	0.29	0.22	0.23	0.38
3-Methylfuran		38.19	5.28	106.25		5.98	8.39														
Furfural		8.61													0.10	0.35	0.26	0.17	0.17	0.12	0.15
2-Furanmethanol															0.015	0.016	0.019				
5-Methyl-2-furancarboxaldehyde																0.013	0.009	0.008			
Total	45.00	291.12	27.38	144.60	5.02	14.66	32.59	n.d.	n.d.	n.d.	n.d.	12.86	21.02	21.38	0.20	0.47	0.71	0.77	0.43	0.41	0.59

Table 45. Concentration of nitrogenous compounds, pyrones, acids/esters and sulfurous compounds [µg/g sample] identified in the flours, batters and cakes.

Compounds	Flours							Batters							Cakes						
	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN
Nitrogenous compounds																					
Pyrazine															0.022		0.013	0.012	0.015	0.012	0.014
2-Methylpyrazine		0.017													0.18	0.22	0.21	0.27	0.22	0.20	0.22
2-Ethylpyrazine															0.015	0.032	0.026	0.044	0.030	0.026	0.032
2,3-Dimethylpyrazine															0.017	0.015	0.017	0.018		0.017	0.020
2,5-Dimethylpyrazine		0.027													0.49	0.45	0.79	0.97	0.85	0.77	0.90
2-Ethyl-6-methylpyrazine															0.014	0.038	0.042	0.066	0.038	0.037	0.045
2-Ethyl-3,5-dimethylpyrazine															0.014	0.021	0.031	0.048	0.031	0.035	0.040
Trimethylpyrazine		0.0052													0.044	0.055	0.083	0.066	0.080	0.071	0.089
Pyrazinamid																					
2,4,6-Trimethylpyridine	0.013		0.018	0.022	0.0090	0.0077	0.012									0.0037	0.0029	0.0030	0.0020		
1H-Pyrrole																		0.19			
1-(1H-Pyrrol-2-yl)ethanone															0.0013	0.0070		0.0046	0.0025		
Total	0.013	0.060	0.018	0.022	0.0090	0.0077	0.012	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.79	0.84	1.21	1.69	1.26	1.17	1.36
Pyrones																					
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one																0.0054					
Maltol																	0.11				
Total	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0054	0.11	n.d.	n.d.	n.d.	n.d.
Acids & Esters																					
Acetic acid		9.44																			
Hexanoic acid		0.29															0.16	0.24			
3-Methylbutanoic acid		0.12			13.05	30.64	41.18												0.15	0.11	0.14
Ethyl acetate		1.60			29.90																
Total	n.d.	11.45	n.d.	n.d.	42.95	30.64	41.18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.16	0.24	0.15	0.11	0.14
Sulfurous compounds																					
Dimethyl sulfide			113.01	21.62			14.99														
Dimethylsulfoxide			6.25	1.63																	
Dimethyl sulfone			6.93																		
Dimethyl disulfide				5.78																	
Total	n.d.	n.d.	126.20	29.03	n.d.	n.d.	14.99	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 46. Concentration of terpenes and alkanes [µg/g sample] identified in the flours, batters and cakes.

Compounds	Flours							Batters							Cakes						
	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN	W	LU	CP	LE	GP	YP	YPN
Terpenes																					
β-Pinene	2.13	0.10	22.12	6.53	46.50																
Mycrene	6.58			4.06	24.64																
Limonene	371.46	0.22	7.36	154.72	686.60	21.73	22.88														
Linalool	0.40																				
3-Carene		0.22	28.29	8.31	163.97																
Total	380.56	0.54	57.77	173.63	921.71	21.73	22.88	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Alkanes																					
Pentane	1.08	0.12	16.63	8.25	14.56	7.09	34.06	0.014		2.88	0.17	7.56	9.21	48.27	0.014		0.21	0.092	0.32	0.24	0.19
Hexane		0.16			10.10	29.74	59.99														
Heptane	11.49	12.66	2036.97	62.67	873.38	8268.86	7994.03	0.60	3.85	6.61	0.67	514.24	237.56	1020.42	0.72	0.19	1.55	2.89	4.88	3.99	5.07
Octane	0.65	1.78		15.05	61.58	228.49	285.26								0.15	0.18	1.44	1.07	1.63	1.70	2.52
Nonane	6.41		18.95	16.76		46.00	33.03														
Dodecane	5.17	0.20	6.97	11.56	6.31	34.75															
Tridecane	3.78			9.55		13.77															
2,2,6-Trimethyloctane		0.26				48.01															
2,2,4,6,6-Pentamethylheptane	27.03	2.16	18.99	55.00	71.55	1222.03	55.99								0.24	0.69	0.33	0.38	0.47	1.31	0.55
2,2,4,4,6,8,8-Heptamethylnonane	46.81	4.22	27.95	103.24	104.67	2341.15	161.98									0.10					
3-Ethylhexane		0.51																			
Ethylcyclohexane		0.17					85.36														
Toluene	32.96	0.76	45.90		3803.81							77.65			0.34	0.20	0.46	0.70	3.67	0.47	0.55
Ethylbenzene	5.82	0.09	23.33	6.64	40.00																
Styrene	6.37			4.54			10.66														
Naphthalene	12.59																				
Total	160.16	23.09	2195.69	293.26	4985.97	12239.88	8720.37	0.62	3.85	9.49	0.84	599.45	246.78	1068.69	1.46	1.35	3.99	5.13	10.98	7.73	8.87

SUPPLEMENTARY TABLE OF ARTICLE 3 – COMPOSITION OF RAW MATERIALS

Table 47. Chemical composition and lipoxygenase (LOX) activity of the raw materials.

Composition	Pea flour	Wheat flour	Pea protein isolate	Pea starch	Maize starch
Moisture [%]	8.800 ± 0.085	12.820 ± 0.042	5.195 ± 0.064	10.220 ± 0.071	13.180 ± 0.028
Starch [%]	47.6 ± 5.1	75.99 ± 0.65	<0.3	88.9 ± 1.9	86.8 ± 2.7
Protein [%]	23.81 ± 0.71	9.68 ± 0.36	81.7 ± 2.5	<0.5	<0.6
Dietary fiber [%]	8.2 ± 1.9	3.4 ± 1.3	n.d.	<1.7	<0.5
Fat [%]	1.989 ± 0.089	1.560 ± 0.059	1.171 ± 0.028	<0.1	<0.1
Ash [%]	3.53 ± 0.40	0.86 ± 0.40	6.02 ± 0.40	<0.5	<0.4
LOX activity [nkat/g]	5379 ± 114	42.3 ± 1.2	<1	n.d.	n.d.

Table 48. Concentration of alcohols [µg/g sample] identified in the flours, batters and cakes.

Compound	Flours/Fractions					Batters						Cakes					
	PF	WF	PPI	PS	MS	PFB	WFB	PSPPIB	MSPPIB	PSB	MSB	PFC	WFC	PSPPIC	MSPPIC	PSC	MSC
Alcohols																	
Ethanol	1960.83	179.62	18.68	3.60	5.22	321.97	3.17	27.83	28.17	3.52	9.36	2.46	2.40	0.67	2.60	1.60	0.72
2-Phenylethanol		0.17															
1-Propanol					0.50	14.93	0.78	1.70	1.72	0.97	1.53						
1-Butanol		14.23		0.13	0.16	3.96	0.23	0.44	0.45	0.18	0.41	0.17	0.055	0.07	0.083	0.060	0.048
1-Pentanol	8.80	11.36	0.96	0.10	0.068	47.17	0.13	0.59	0.60			0.44	0.057	0.10	0.11	0.051	0.046
1-Hexanol	38.26	24.55			0.030	162.82	0.22	0.12	0.12			1.84	0.10	0.045	0.050	0.029	0.024
1-Heptanol		1.44	0.11			2.77						0.39	0.070	0.10	0.12	0.081	0.071
1-Octanol			0.051			2.59						0.08	0.017	0.027	0.035	0.020	0.018
1-Nonanol		1.17															
1-Penten-3-ol		12.58	6.53			168.00	0.39	2.51	2.54			3.37	0.49	1.27	1.25	0.52	0.49
1-Octen-3-ol						4.31						0.16			0.034	0.030	0.030
2-Propanol	323.99	45.53		0.44	0.35	14.98	1.18	2.21	2.23	1.80	2.65						
2-Pentanol	27.39					2.53											
2-Methyl-1-propanol	27.05	5.73															
2-Ethyl-1-hexanol	88.11					1.63						0.026					
2-Butyl-1-octanol		14.91	3.67														
2-Penten-1-ol			0.46			2.83						0.26	0.032	0.073	0.077	0.040	0.036
2-Octen-1-ol						5.42						0.060				0.015	0.012
2,3-Butanediole	311.11																
3-Pentanol						18.55											
3-Hexen-1-ol		1.72				4.82											
3-Methyl-1-butanol	23.41	10.07				4.98	0.083					0.045					
Cyclohexanol	6.94																
2,3-Dimethylcyclohexanol												0.050					
Total	2815.89	323.09	30.47	4.26	6.32	784.27	6.18	35.39	35.82	6.46	13.96	9.36	3.23	2.35	4.36	2.45	1.50

Table 49. Concentration of aldehydes [µg/g sample] identified in the flours, batters and cakes.

Compound	Flours/Fractions					Batters						Cakes					
	PF	WF	PPI	PS	MS	PFB	WFB	PSPPIB	MSPPIB	PSB	MSB	PFC	WFC	PSPPIC	MSPPIC	PSC	MSC
Aldehydes																	
Acetaldehyde						25.45											
2-Methylpropanal												0.81	0.64	1.29	1.22	0.91	0.75
2-Methylbutanal		1.34	0.21	0.056	0.022		0.092					3.47	3.44	6.11	6.05	4.52	4.14
3-Methylbutanal		1.42	0.24	0.11	0.31	2.74	0.16	0.14	0.14		0.14	3.67	4.58	6.43	6.26	5.56	5.30
Phenylacetaldehyde					0.20							0.18	0.14	0.11	0.10	0.077	0.11
Butanal			1.06		0.024			0.41	0.41			0.19	0.027	0.13	0.12	0.043	0.043
Pentanal				0.12	0.20			1.59	1.61			4.12	0.45	1.38	1.25	0.62	0.69
Hexanal	10.30	15.96	37.52	1.03	4.60	78.24	1.05	11.21	11.34	0.98	1.99	15.61	2.10	6.36	5.96	2.19	2.82
Heptanal			0.70		0.52		0.08	0.20	0.20			0.47	0.17	0.24	0.22	0.16	0.20
Octanal			0.68		0.042							0.086	0.021	0.054	0.057	0.037	0.042
Nonanal		0.65	0.27	0.11	0.20			0.19	0.20		0.13	0.23	0.072	0.14	0.13	0.10	0.13
2-Pentenal						1.83											
2-Hexenal												0.070			0.021		
2-Heptenal						1.95											
2-Octenal					0.041	4.86						0.17	0.02		0.024	0.017	
2-Nonenal					0.034												
2,4-Decadienal												0.18					
Total	10.30	19.37	40.69	1.42	6.19	115.06	1.38	13.73	13.90	0.98	2.26	29.24	11.65	22.26	21.40	14.23	14.22

Table 50. Concentration of ketones and furanic compounds [µg/g sample] identified in the flours, batters and cakes.

Compound	Flours/Fractions					Batters						Cakes					
	PF	WF	PPI	PS	MS	PFB	WFB	PSPPIB	MSPPIB	PSB	MSB	PFC	WFC	PSPPIC	MSPPIC	PSC	MSC
Ketones																	
1-Hydroxy-2-propanone												2.13	1.54	1.39	1.67	2.36	2.08
Acetone	203.16	22.88	30.35	0.54	1.05	6.40	0.17	2.54	2.82	0.35	0.29	1.14	0.19	0.70	0.69	0.29	0.22
2-Butanone	37.41	2.04	8.70	0.11	0.040	1.63		1.05	1.06	0.15	0.11	0.18	0.10	0.39	0.36	0.17	0.15
2-Heptanone		2.75	5.80		0.17	2.78			0.42			0.18		0.13	0.13	0.014	0.015
2-Octanone						1.70		1.19	1.21			0.088		0.055		0.037	
2-Nonanone			1.22						0.15								
2-Decanone			0.67									0.071		0.072	0.076	0.023	0.023
2,3-Butanedione													0.19	0.13	0.14	0.22	0.20
2,3-Pentanedione								1.08	1.09			0.64	0.41	0.61	0.51	0.55	0.50
3-Pentanone						36.50											
3-Octanone						3.49											
3-Octen-2-one			0.70		0.022							0.075					
3,5-Octadien-2-one			2.60											0.20	0.21		
3-Hydroxy-2-butanone	19.43					2.54						0.37	0.11	0.14	0.22	0.19	0.11
4-Methyl-2-pentanone					0.14												
6-Methyl-5-hepten-2-one					0.045												
Cyclohexanone			1.79														
Total	259.99	27.68	51.82	0.65	1.47	55.04	0.17	5.86	6.75	0.50	0.40	4.87	2.53	3.82	4.01	3.85	3.31
Furanic compounds																	
Furfural					0.065							0.17	0.092	0.15	0.26	0.18	0.20
2-Furanmethanol													0.013	0.0072	0.0083	0.017	0.014
Furan														0.0075	0.012	0.013	0.0077
2-Ethylfuran												0.064	0.021	0.094	0.11	0.024	0.026
2-Methylfuran												0.018		0.020	0.021	0.011	0.0084
2-Pentylfuran			4.14		0.083	2.47			0.26			0.16	0.050	0.14	0.19	0.050	0.06
Tetrahydrofuran					0.037												
Dibenzofuran					0.51												
5-Ethyldihydro-2(3H)-furanone	8.43	1.48										0.015	0.0044	0.0046			0.0051
2(3H)-Dihydrofuranone												0.0058					
Total	8.43	1.48	4.14	n.d.	0.70	2.47	n.d.	n.d.	0.26	n.d.	n.d.	0.43	0.18	0.42	0.60	0.30	0.32

Table 51. Concentration of nitrogenous compounds, sulfurous compounds and esters [µg/g sample] identified in the flours, batters and cakes.

Compound	Flours/Fractions					Batters						Cakes					
	PF	WF	PPI	PS	MS	PFB	WFB	PSPPIB	MSPPIB	PSB	MSB	PFC	WFC	PSPPIC	MSPPIC	PSC	MSC
Nitrogenous compounds																	
2,4,6-Trimethylpyridine			0.35						0.62								
1H-Pyrrole												0.0075			0.0092	0.013	0.010
Acetylpyrrole												0.0030	0.0011			0.0010	
3-Methyl-1H-pyrrole														0.0025	0.0024		
2-Ethyl-4-methyl-1H-pyrrole													0.0031	0.0042	0.0043	0.0053	0.0053
Pyrazine												0.014	0.029	0.025	0.026	0.058	0.049
2-Methylpyrazine												0.17	0.17	0.20	0.22	0.28	0.23
2,3-Dimethylpyrazine												0.013	0.015	0.016	0.017	0.025	0.021
2,5-Dimethylpyrazine												0.53	0.44	0.82	0.97	0.98	0.79
Trimethylpyrazine												0.052	0.035	0.085	0.093	0.074	0.064
2-Ethylpyrazine												0.022	0.014	0.012	0.013	0.022	0.017
2-Ethyl-3,5-dimethylpyrazine												0.022					
2-Ethyl-3-methylpyrazine												0.0040					
2-Ethyl-5-methylpyrazine												0.026	0.012	0.024	0.026	0.026	0.021
3-Ethyl-2,5-dimethylpyrazine													0.012	0.037	0.038	0.032	0.026
2-Acetyl-3-methylpyrazine														0.0012	0.0013	0.0011	
2-Acetylpyrazine													0.0028	0.0046	0.0050	0.0054	0.0057
5-Acetyl-2-methylpyrazine														0.0018	0.0017	0.0015	
Pyrazinamide												0.0020					
Total	n.d.	n.d.	0.35	n.d.	n.d.	n.d.	n.d.	n.d.	0.62	n.d.	n.d.	0.86	0.73	1.24	1.42	1.52	1.24
Sulfurous compounds																	
Dimethyl disulfide			0.32						0.36			0.10			0.10	0.11	0.69
Dimethyl sulfone												0.037	0.019			0.017	0.024
2-Acetylthiazole																0.015	0.015
3-Methylthiopropanal													0.059	0.051	0.048	0.041	0.065
Total	n.d.	n.d.	0.32	n.d.	n.d.	n.d.	n.d.	n.d.	0.36	n.d.	n.d.	0.13	0.0775	0.0506	0.14	0.18	0.79
Esters																	
Ethyl acetate														0.093		0.064	
2-Hexenyl acetate												0.030					
Total	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.030	n.d.	0.093	n.d.	0.064	n.d.

SUPPLEMENTARY TABLE OF ARTICLE 4 – READILY BIOACCESSIBLE PROTEIN

Table 52. Kinetic parameters [estimates ± standard error] of *in vitro* protein intestinal digestion of sponge cakesbased on wheat flour (WFC), pea flour (PFC) and a combination of pea starch and pea protein (PSPPIC). Parameters were estimated by a fractional conversion model (**Eq. 1 of Article 4**).

Cake	*k*	Relative RBP		Absolute RBP		R^2_{adj}
		Protein$_f$	Initial reaction rate	*Protein*$_f$	Initial reaction rate	
	[1/min]	[%]	[%/min]	[mg/g cake]	[mg/(g·min)]	
WFC	0.029 ± 0.005^{a}	30.08 ± 1.39^{b,c}	0.71 ± 0.13^{a}	33.03 ± 1.52^{b}	0.78 ± 0.13^{b}	0.96
PFC	0.034 ± 0.006^{a}	29.80 ± 1.30^{b,c}	0.72 ± 0.15^{a}	45.61 ± 1.98^{a}	1.11 ± 0.22^{a,b}	0.95
PSPPIC	0.038 ± 0.007^{a}	25.76 ± 1.11^{d}	0.80 ± 0.15^{a}	42.37 ± 1.82^{a}	1.32 ± 0.24^{a}	0.96
MSPPIC	0.036 ± 0.005^{a}	26.78 ± 0.86^{c,d}	0.77 ± 0.10^{a}	42.89 ± 1.37^{a}	1.23 ± 0.17^{a,b}	0.98
MSC	0.042 ± 0.007^{a}	32.66 ± 1.25^{a,b}	1.12 ± 0.19^{a}	23.85 ± 0.92^{c}	0.82 ± 0.14^{b}	0.96
PSC	0.042 ± 0.007^{a}	34.67 ± 1.39^{a}	1.14 ± 0.20^{a}	25.01 ± 1.00^{c}	0.82 ± 0.15^{b}	0.95

Table 53. Kinetic parameters [estimates ± standard error] of *in vitro* starch intestinal digestion of sponge cakes based on wheat flour (WFC), pea flour (PFC), pea starch and pea protein (PSPPIC), maize starch and pea protein (MSPPIC), pea starch (PSC) and maize starch (MSC) combination of pea starch and pea protein (PSPPIC). Parameters were estimated by a fractional conversion model (**Eq. 2 of Article 4**). Estimations are based on experimental data acquired by HPAEC-PAD or DNS method.

Cake	k_1	k_2	Time of phase transition	Relative digested starch [%]				Absolute digested starch [mg/g cake]			R^2_{adj}
				Starch$_1$	*Starch*$_2$	HI	Initial reaction rate	*Starch*$_1$	*Starch*$_2$	Initial reaction rate	
	[1/min]	[1/min]	[min]	[%]	[%]		[%/min]	[mg/g cake]	[mg/g cake]	[mg/(g·min)]	
HPAEC-PAD method											
WFC	0.79 ± 0.40^{a}	0.022 ± 0.006^{a}	5.2	78 ± 2^{a}	100 ± 0^{a}	100	62 ± 33^{a}	199 ± 8^{b}	253 ± 0^{b}	158 ± 83^{a,b}	1.00
PFC	0.34 ± 0.07^{a}	0.014 ± 0.009^{a}	9.2	68 ± 4^{b,c}	95 ± 6^{a,b}	86	23 ± 5^{a}	126 ± 8^{d}	173 ± 10^{d}	43 ± 9^{b}	0.99
PSPPIC	0.38 ± 0.09^{a}	0.017 ± 0.009^{a}	7.9	64 ± 5^{c}	90 ± 4^{b}	84	25 ± 6^{a}	150 ± 11^{c}	208 ± 10^{c}	59 ± 14^{a,b}	0.99
MSPPIC	0.62 ± 0.23^{a}	0.011 ± 0.002^{a}	5.9	70 ± 3^{a,b,c}	100 ± 0^{a}	89	44 ± 17^{a}	155 ± 6^{c}	222 ± 0^{c}	97 ± 37^{a,b}	0.99
PSC	0.35 ± 0.06^{a}	0.011 ± 0.003^{a}	9.6	74 ± 3^{a,b}	100 ± 0^{a}	90	27 ± 5^{a}	241 ± 11^{a}	323 ± 0^{a}	85 ± 16^{a,b}	0.99
MSC	0.72 ± 0.28^{a}	0.014 ± 0.003^{a}	5.5	78 ± 2^{a,b}	100 ± 0^{a}	96	57 ± 23^{a}	247 ± 8^{a}	317 ± 0^{a}	180 ± 74^{a}	0.99
DNS method											
WFC	0.58 ± 0.08^{a}	0.013 ± 0.003^{a}	7.5	88 ± 1^{a}	100 ± 0^{a}	100	52 ± 7^{a,b}	224 ± 5^{a}	253 ± 7^{b}	131 ± 20^{a,b}	1.00
PFC	0.39 ± 0.09^{a}	0.009 ± 0.001^{a}	7.6	56 ± 3^{d}	100 ± 0^{a}	75	23 ± 5^{b}	103 ± 5^{c}	183 ± 0^{d}	41 ± 9^{c}	0.99
PSPPIC	0.39 ± 0.10^{a}	0.018 ± 0.008^{a}	7.2	59 ± 4^{c,d}	89 ± 4^{b}	79	24 ± 6^{a,b}	137 ± 10^{b}	205 ± 8^{c}	55 ± 14^{a,b,c}	0.99
MSPPIC	0.88 ± 0.37^{a}	0.017 ± 0.001^{a}	3.8	64 ± 1^{b,c}	100 ± 0^{a}	88	57 ± 25^{a}	142 ± 3^{b}	222 ± 0^{c}	126 ± 55^{a,b,c}	1.00
PSC	0.39 ± 0.04^{a}	0.011 ± 0.003^{a}	8.5	68 ± 2^{b}	97 ± 0^{a}	84	27 ± 3^{a,b}	221 ± 6^{a}	315 ± 12^{a}	87 ± 9^{b,c}	1.00
MSC	0.72 ± 0.21^{a}	0.014 ± 0.001^{a}	4.8	67 ± 2^{b}	100 ± 0^{a}	87	48 ± 14^{a,b}	211 ± 5^{a}	317 ± 0^{a}	153 ± 45^{a}	1.00

SUPPLEMENTARY TABLE – CHEMICAL COMPOSITION OF INGREDIENTS USED

Table 54. Chemical composition of the ingredients used.

Raw material	Moisture [%]	Lipids [%]	Proteins [%]	Starch [%]	Sugars [%]	Fibers [%]	Ash [%]
Wheat flour (T55)	12.8 ± 0.0	1.1 ± 0.0	8.7 ± 0.4	70.2 ± 7.4	1.7 ± 0.9	2.9 ± 1.2	0.8 ± 0.4
Wheat flour (T65)	12.1 ± 0.0	1.6 ± 0.1	9.7 ± 0.4	76.0 ± 0.7	2.3 ± 1.5	3.4 ± 1.3	0.9 ± 0.4
Green pea flour	9.9 ± 0.0	1.7 ± 0.1	18.7 ± 0.6	51.9 ± 5.5	2.9 ± 0.9	8.2 ± 1.9	2.7 ± 0.4
Lentil flour	8.9 ± 0.0	1.7 ± 0.0	27.6 ± 0.8	40.7 ± 4.4	0.8 ± 0.5	8.9 ± 2.0	3.8 ± 0.4
Lupin flour	7.1 ± 0.1	11.9 ± 0.1	42.4 ± 1.3	<0.4	3.4 ± 0.9	26.7 ± 3.3	4.8 ± 0.4
Chickpea flour	8.2 ± 0.0	6.4 ± 0.0	22.2 ± 0.7	39.3 ± 4.3	2.7 ± 0.8	13.6 ± 2.4	3.6 ± 0.4
Yellow pea flour (I)	9.9 ± 0.2	2.1 ± 0.0	23.0 ± 0.1	48.8 ± 5.2	3.0 ± 1.4	8.8 ± 2.0	2.7 ± 0.0
Yellow pea flour (N_2)	9.9 ± 0.1	2.0 ± 0.0	23.0 ± 0.1	48.6 ± 5.2	2.3 ± 0.8	8.1 ± 1.9	2.7 ± 0.0
Yellow pea flour (II)	8.8 ± 0.1	2.0 ± 0.1	23.8 ± 0.7	47.6 ± 5.1	2.3 ± 0.8	8.2 ± 1.9	3.5 ± 0.4
Yellow pea protein	5.2 ± 0.1	1.2 ± 0.0	81.7 ± 2.5	n.d.	<0.2	n.d.	6.0 ± 0.4
Yellow pea starch	10.2 ± 0.1	<0.1	<0.5	88.9 ± 1.9	0.3 ± 0.3	0.9 ± 0.8	<0.5
Maize starch	13.2 ± 0.0	<0.1	<0.6	86.8 ± 1.1	<0.2	<0.5	<0.4
Egg (whole, liquid)	76.2 ± 0.1	9.2 ± 0.1	11.7 ± 0.4	n.d.	0.2 ± 0.1	n.d.	1.3 ± 0.1

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ÉCOLE DOCTORALE
Agriculture, alimentation, biologie, environnement, santé (ABIES)

Titre : Evaluation de la réactivité d'ingrédients à base de légumineuses et de son impact sur la qualité des produits transformés de type cake dans une démarche d'ingénierie de produits

Mots clés : composés volatils odorants, structure, digestibilité *in vitro*, farines de légumineuses, pois, isolat protéique

Résumé : Les légumineuses ont gagné en popularité en raison de leurs avantages nutritionnels, fonctionnels et écologiques. Cependant, leur utilisation pour remplacer des ingrédients comme le blé reste compliquée en raison de leur composition différente qui pourrait affecter la réactivité au cours de la fabrication des aliments et donc leur qualité (arôme, texture, digestibilité). Cette thèse a donc examiné l'impact des ingrédients produits à partir des légumineuses (farines diverses ; isolat protéique et amidon de pois) sur la qualité des aliments transformés (gâteaux de type génoise), en couplant l'analyse de marqueurs de réaction pouvant avoir un impact sensoriel sur le produit, avec des caractérisations de structure et de digestibilité *in vitro* des protéines et de l'amidon. Il est apparu que les farines étaient sujettes à des réactions oxydatives et thermiques, conduisant à un profil en composés volatils – et donc une activité odorante probable – plus complexe que le blé. L'ampleur de l'oxydation dépend de l'activité de la lipoxygénase et des paramètres de mélange de la pâte, ces derniers influençant la distribution des ingrédients et le temps d'exposition de l'enzyme à ses substrats. Le fractionnement de la farine de pois produit un amidon et des protéines purifiés qui forment moins de marqueurs d'oxydation, mais plus de marqueurs de dégradation thermique, ce qui peut être plutôt attribué à leur composition particulière qu'à leur effet sur la structure du produit. Cependant, les gâteaux à base d'ingrédients purifiés ou de farine entière présentent une cinétique de digestion *in vitro* similaire. Par comparaison avec le blé, les gâteaux à base de pois libèrent plus de protéines facilement accessibles et moins de produits de digestion d'amidon. Ceci pourrait être corrélé aux caractéristiques chimiques et aux propriétés structurelles spécifiques. Globalement, l'utilisation d'ingrédients à base de légumineuses a permis d'obtenir des produits à l'aspect et à la texture attrayants, ce qui souligne leur grand potentiel d'application dans des aliments.

Title: A product engineering approach to assess the reactivity of legume-based ingredients and its impact on the quality of processed foods such as cake

Keywords: odorous volatile compounds, structure, *in vitro* digestibility, legume flours, pea, protein isolate

Abstract: Legumes have gained popularity due to their nutritional, functional and ecological benefits. However, their use to replace ingredients like wheat is challenging because of their differing composition that might affect reactivity during food development and hence product quality (aroma, texture, digestibility). This dissertation therefore examined the impact of legume ingredients (diverse flours; purified pea proteins and starch) on the quality of processed foods, namely sponge cakes, by coupling the analysis of reaction markers, which can affect product sensory, with structural and *in vitro* protein and starch digestibility characterizations. It emerged that legume flours were prone to oxidative and thermal reactions, leading to a more complex profile of volatiles with probable odor activity than wheat. The extent of oxidation depended on the lipoxygenase activity and the batter beating process, which influenced the distribution of ingredients and the exposure time of lipoxygenase to its substrates. The fractionation of pea flour produced purified starch and protein that formed less oxidation but more thermal markers, which was rather assigned to their composition than to their effect on product structure. However, cakes based on the purified pea ingredients or whole pea flour had similar *in vitro* gastrointestinal digestion patterns. Compared to wheat, the pea-based cakes released more readily bioaccessible protein and less starch digestion products, attributable to their distinct chemical characteristics and structural properties. Globally, the use of legume ingredients resulted in appealing products with an attractive appearance and texture, which emphasizes their great application potential to create high-quality food.

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