Understanding of the mechanisms at the origin of functional and flavor properties in protein-rich fava bean ingredients

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Compréhension des mécanismes à l'origine des propriétés fonctionnelles et de la flaveur d'ingrédients riches en protéines issus de féveroles

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A. Résumé de Thèse

Titre : Compréhension des mécanismes à l'origine des propriétés fonctionnelles et de la flaveur d'ingrédients riches en protéines issus de féveroles

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Contexte et Enjeu

Afin de rendre les régimes alimentaires occidentaux plus durables, un **changement d'alimentation** s'impose [1], [2]. Parmi **les sources végétales** intéressantes pour leur teneur en protéines, **la fèverole** (*Vicia faba* L., famille des *Fabaceae*), consommée en tant que telle en Afrique du Nord et au Moyen-Orient [3], [4], s'avère très prometteuse pour produire des ingrédients à fort potentiel **agronomique**, **nutritionnel** et **fonctionnel** dans la formulation de produits alimentaires [3], [5]. La fèverole est une légumineuse à grain, capable de germer en saison froide à des températures du sol pouvant atteindre 12,5 °C. Sa culture peut fixer de grandes quantités d'azote résiduel biodisponible (jusqu'à 100-200 kg N/Ha), solubiliser le phosphore insoluble et augmenter l'activité microbienne dans le sol, améliorant ainsi les propriétés physiques du sol (densité apparente et porosité) et sa teneur en matières organiques. D'un point de vue nutritionnel, la fèverole est considérée encore plus intéressante que d'autres légumineuses (pois, pois chiche) grâce à son rapport

protéines/glucides plus élevé [5]. Elle est **riche en protéines** (23-41% p/p sur base sèche) et fournit ainsi une source **d'acides aminés essentiels** et **peptides bioactifs**. Elle est également riche en fibres, en vitamines et minéraux (fer, zinc, magnésium, folates) et contient des micro-constituants présentant des propriétés antioxydantes intéressantes comme des composés phénoliques et des saponines [6], [7]. Dans le cadre d'un régime alimentaire équilibré, un apport en féveroles associé à un apport en céréales en quantités adéquates permet de répondre aux besoins journaliers en acides aminés essentiels. En effet, les céréales sont riches en cystéine et méthionine et limitantes en lysine, alors que les légumineuses sont **riches en lysine** et pauvres en cystéine et méthionine [8], [9]. La féverole présente également des propriétés fonctionnelles intéressantes, notamment liées à la présence de ses protéines majoritaires, **les globulines (légumine, viciline, conviciline).** Ainsi, les ingrédients issus de fèverole présentent un intérêt fort dans la formulation des produits alimentaires, en particulier pour leurs **propriétés moussantes et émulsifiantes** impliquées dans différents types d'applications alimentaires et de boissons [6], [10], comme la crème glacée, le pudding, les mousses, etc [11]–[14].

Malgré le fort potentiel nutritionnel, fonctionnel et agronomique de la féverole, **son utilisation** comme ingrédient alimentaire sur le marché alimentaire **n'est que de 2,4%** par rapport aux ingrédients de légumineuses [15]. La fèverole présente en effet des freins liés à des **problématiques nutritionnelles et sensorielles**, qui peuvent influer sur son utilisation pour l'alimentation humaine [5]. **La digestibilité** et la **biodisponibilité des protéines** de fèverole, ainsi que la biodisponibilité des minéraux, peuvent être affectées par la présence de **facteurs antinutritionnels** tels que des saponines, des glycosides, des tannins, l'acide phytique ou des lectines [3]. La fèverole contient en particulier des glycosides de pyrimidine (**vicine et convicine**), trouvés exclusivement dans le genre *Vicia*, qui peuvent provoquer une maladie mortelle caractérisée par une anémie hémolytique, **le favisme** [16]–[18]. **L'acceptabilité** par les consommateurs des produits à base de légumineuses, dont la féverole, est par ailleurs diminuée du fait de la présence de **certaines notes aromatiques** ou de **mauvais goûts indésirables**, en particulier **l'amertume** et la perception **« beany »** [19]. **La couleur**, par exemple pour les graines avec une coque foncée, apparaît également comme un facteur limitant [19]. Afin d'améliorer l'acceptabilité des fèveroles par les consommateurs et augmenter leur utilisation sur le marché alimentaire, il est donc nécessaire de mieux comprendre ces facteurs limitants et de proposer des solutions pour les limiter [5].

Afin **d'améliorer l'acceptabilité des ingrédients à base de féverole**, les itinéraires technologiques mis en jeu lors de leur production et de leur fonctionnalisation pourraient constituer un potentiel prometteur. Les graines de fèverole entières peuvent être transformées en ingrédients tels que des **farines**, des **concentrâts** et des **isolats**. Ces ingrédients sont obtenus par exemple par séchage à l'air, décorticage, réduction de la taille

des graines, extraction des protéines, qui, à leur tour, peuvent subir des étapes de modifications supplémentaires par des procédés et des conditions appropriés. On distinguera donc les étapes dites de « **fabrication d'ingrédients** » et les étapes de « **modification d'ingrédients** ». Les ingrédients obtenus, non-modifiés ou modifiés par des processus ultérieurs, peuvent être utilisés dans différentes applications alimentaires. Ces différentes étapes peuvent avoir un impact sur les propriétés nutritionnelles (d'intérêt ou facteurs antinutritionnels), fonctionnelles et organoleptiques des ingrédients. Il est donc nécessaire de comprendre les mécanismes à l'origine de ces propriétés afin, par exemple, de pouvoir optimiser en conséquence les conditions de fabrication des ingrédients [3], [5], [6], [20]–[22].

Dans ce contexte, l'objectif du travail de thèse était de comprendre l'**impact des conditions de traitement d'ingrédients riches en protéines issus de féverole sur leurs propriétés fonctionnelles et leur flaveur**, toutes deux essentielles pour envisager des applications industrielles dans le domaine des boissons. L'exemple d'application proposé dans ce travail est **une boisson à base de légumineuse**, qui serait servie comme un cappuccino végétal ou végétalien. La plupart des analogues du lait sont généralement des dispersions colloïdales constituées de gouttelettes lipidiques, de protéines, de fibres alimentaires et de fragments de matrice végétale, en suspension dans un milieu aqueux contenant des sucres, des fibres solubles et des sels [42]. Dans une matrice colloïdale végétale de ce type, les ingrédients fonctionnalisés de féverole devraient jouer un rôle d'agent fonctionnel pour produire et stabiliser la dispersion colloïdale et/ou la mousse en surface du cappuccino végétal. Les caractéristiques de l'émulsion (ici huile-dans-eau) et de la mousse (avec interface air-dans-eau) ont un rôle clé dans ce type d'applications. Les protéines de féverole, présentant différentes structures et conformations, seraient essentielles pour produire et stabiliser ces systèmes et seraient ainsi à l'origine de la fonctionnalité des ingrédients [20,45,46]. D'autres constituants non protéiques également présents dans la féverole, notamment les lipides, l'amidon et les fibres, pourraient également influencer l'expression de cette fonctionnalité [18,20]. Comme évoqué précédemment, la formulation de telles matrices végétales, analogues du lait, pourrait cependant être freinée par les défauts sensoriels de cette légumineuse : une odeur et un goût désagréables, souvent liés à un arôme vert, herbacé ou de haricot (notamment attribué à des aldéhydes, alcools et cétones), mais aussi des saveurs amère et astringente potentiellement apportées par des composés sapides tels que des saponines et des composés phénoliques (isoflavones, flavonols, acides hydroxycinnamiques, etc.) [47-49]. Ainsi, identifier des itinéraires technologiques pour produire des ingrédients à partir de féverole et les utiliser dans la formulation d'aliments à base de légumineuses acceptables sensoriellement, comme des cappuccinos végétaux, conduit à relever plusieurs défis. Cette étude, menée dans **le cadre du projet FOODENGINE** et financée par le programme européen **Horizon 2020 – Action Marie Curie ITN**, avait pour

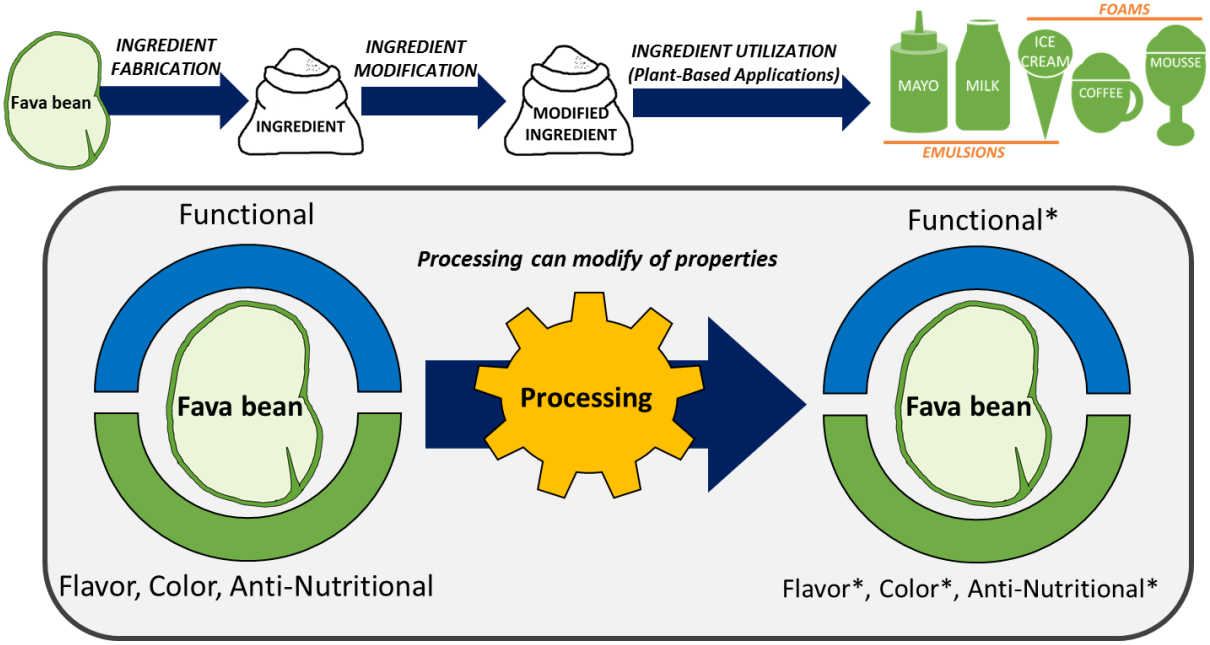
objectif d'étudier et comprendre le rôle des conditions de transformation d'ingrédients riches en protéines issus de fèveroles sur leurs propriétés fonctionnelles et leur flaveur. **Une approche multidimensionnelle** originale a été mise en œuvre pour comprendre les mécanismes biochimiques et physico-chimiques sous-jacents des propriétés de ces ingrédients, identifier **un compromis satisfaisan**t entre ces différentes propriétés et accroître ainsi l'utilisation de ces ingrédients dans le développement d'aliments sains et plus durables.

Après une **introduction générale (partie I)**, un **état de l'art (partie II)** est dressé à partir des nombreuses publications scientifiques disponibles dans la littérature sur la transformation des fèveroles et ses effets sur différents aspects fonctionnels et gustatifs A l'issue de cette partie, **l'approche méthodologie (partie III)** mise en place est décrite, de même que les matériels et méthodes utilisés pour évaluer les différentes propriétés d'intérêt et les molécules impliquées dans leur construction. L'étude a été menée sur un concentrât de fèveroles, considéré comme un ingrédient légèrement transformé, auquel on fait subir une étape de modification supplémentaire par le choix de conditions de transformation industriellement pertinentes, telles que le pH (2, 4, 6,4 et 11), la température (55, 75 et 95 °C) et la durée du traitement (30 et 360 min). La première partie des résultats **(partie IV)** tente de clarifier **l'interaction entre les réactions associées aux protéines de fèveroles, les propriétés physico-chimiques des protéines et les propriétés fonctionnelles**. Des approches statistiques ont été utilisées pour faciliter l'interprétation et l'évaluation des interrelations fonctionnelles et physico-chimiques, et établir un modèle de corrélation permettant un aperçu de leur relation complexe en fonction des conditions de procédés. Le comportement des protéines au cours de la modification des ingrédients a ensuite été étudié (agrégation, hydrolyse), ainsi que leurs propriétés physico-chimiques (charge, solubilité, fluorescence intrinsèque et intégrité thermique) et leurs propriétés fonctionnelles (capacité et stabilité de mousse et d'émulsion) dans des conditions d'utilisation. La seconde partie des réultats **(partie V)** est consacrée à **la perception de l'odeur des différents ingrédients générés par une analyse sensorielle qualitative et quantitative ainsi qu'à l'identification des composés volatils libérés dans l'espace de tête**, dans des conditions proches de l'application de boissons. Des relations ont été établies afin de comprendre l'interaction entre la composition en composés d'arômes, leur libération et les conditions de procédés auxquels ont été soumis les ingrédients protéiques. À la suite de cette étape, diverses applications ont pu être imaginées au regard des propriétés développées (fonctionnelles et sensorielles) dans ces ingrédients modifiés. Enfin, la dernière partie des résultats **(partie VI)** a porté sur **l'étude des molécules non volatiles (composés phénoliques et saponines)** extraites de quelques-uns des ingrédients générés dans les phases précédentes de l'étude, légèrement ou fortement modifiés, avec un accent particulier mis sur le traitement sans ajustement du pH en raison de sa pertinence industrielle. A l'issue

de cette étape, des hypothèses ont été établies quant aux conséquences sur les limitations de la fèverole vis-à-vis de leurs propriétés antioxydantes, gustatives et anti-nutritionnelles. Enfin, une **conclusion (partie VII)** reprend les principaux résultats marquants et intègre l'effet du traitement sur les différentes propriétés étudiées afin de proposer des compromis.

Etude Bibliographique

Une étude bibliographique a été réalisée pour comprendre les différentes manières dont la féverole peut être transformée en ingrédients et si les conditions de procédé ont une influence sur les propriétés fonctionnelles, la flaveur et la couleur des ingrédients produits.
La fèverole peut être utilisée sous différents formes, notamment des farines, des concentrâts et des isolats, potentiellement fonctionnalisées par des étapes de transformation ultérieures, et intéressantes pour diverses applications alimentaires industrielles. La modification des propriétés fonctionnelles de ces ingrédients sont induites par des modifications de la composition biochimique globale et/ou des modifications de la structure et de la conformation des protéines et des autres constituants présents. Les modifications des protéines peuvent être induites par des processus physiques, chimiques et biologiques. Elles influencent la solubilité des protéines, la distribution des charges et la structure propre des protéines, impactant ainsi leurs propriétés moussantes et émulsifiantes [11], [23]. Certaines



études ont montré que les protéines de fèverole étaient modifiées par la température et le pH [24], [25], les traitements mécaniques [26], les traitements par ultrasons de haute intensité [27], la succinylation [28], l'acétylation [29] et des traitements enzymatiques [30]. De plus, l'effet de différents traitements sur la structure des protéines a été étudié dans de nombreux travaux de recherche, en particulier en s'attachant aux phénomènes d'agrégation protéine-protéine et d'hydrolyse des protéines, tous deux bien connus pour influencer les

fonctionnalités des protéines [27], [29]–[31]. En revanche, les études disponibles dans la littérature montrent qu'il n'existe actuellement pas de compréhension fine et claire de toutes les réactions susceptibles de se produire pendant la fabrication et la modification des ingrédients de fèverole, et qui sont à l'origine des modifications des propriétés physico-chimiques et des propriétés fonctionnelles des protéines. Par ailleurs, si de nombreuses données existent sur la structure et/ou les propriétés des protéines de féverole, acquises à l'aide d'une grande diversité de méthodes analytiques, les analyses proposées visent avant tout à expliquer un phénomène en particulier. Elles ne portent pas sur l'établissement de liens entre les différents phénomènes observés. Ainsi, l'intégration de l'ensemble des résultats acquis au cours de ce projet permettrait d'apporter une meilleure compréhension de la relation entre les propriétés et les fonctionnalités d'identifier les phénomènes clés, , et aiderait ainsi à proposer des solutions d'amélioration de ces ingrédients et à favoriser leur utilisation dans des formulations alimentaires.

Les composés responsables de la flaveur des ingrédients issus des légumineuses dépendraient de l'origine génétique des plantes et/ou des conditions plus ou moins favorables à leur développement (disponibilité des précurseurs de certaines réactions, disponibilité des enzymes, paramètres mis en œuvre lors des opérations de transformation, etc.) [32]. Les molécules impliquées sont principalement des composés issus de la dégradation des lipides, mais également des composés générés à partir d'acides aminés, de glucides et de caroténoïdes, par le biais de réactions enzymatiques et/ou non enzymatiques [5], [32], [33] se produisant au cours des différentes étapes de transformation et/ou d'utilisation des ingrédients, c'est-à-dire de la récolte des fèveroles jusqu'à l'application alimentaire finale [5], [19]. Dans la littérature, la flaveur de pois a été largement étudiée, montrant que c'était la combinaison de différents produits d'oxydation des lipides (aldéhydes, cétones, alcools, furanoïdes) qui donnait une note verte, haricot, terre et foin. [19], [34]. Ces réactions ont également été mises en évidence dans la féverole : les acides gras insaturés, sous forme libres ou estérifiés, subiraient une oxydation enzymatique par la lipoxygénase et/ou une auto-oxydation liée à la présence d'initiateurs (par exemple des ions métalliques) et/ou à la température [5]. D'autres réactions, en particulier la dégradation des acides aminés et des sucres, sont également possibles, ainsi que leur réarrangement par la dégradation de Strecker et la réaction de Maillard [32], [35]. Quelques données existent sur l'effet des conditions de transformation sur la flaveur des graines de féverole. Les graines traitées par micro-ondes (950 W pendant 1,5 min) ou par traitement thermique (>70 °C, > 2 min) présentent ainsi une flaveur modifiée par rapport aux fèverole fraîches en raison de l'inactivation de la lipoxygénasique endogène [36]–[38]. En revanche, les farines issues de graines décortiquées et moulues contiennent une activité lipoxygénasique très élevée, suggérant ainsi des possibilités d'oxydation des lipides par voie enzymatique [39]. L'effet du pH a également été mis en évidence sur des isolats de protéines de fèverole. Une flaveur de

pois séché prédomine à pH neutre alors qu'une flaveur fruitée se développe à pH acide [40]. Malgré ces quelques études, les phénomènes chimiques et enzymatiques à l'origine de la flaveur des fèveroles et de leurs ingrédients ne sont pas complètement élucidés et il n'existe pas de relation entre les conditions de procédés utilisées et la perception sensorielle des ingrédients produits. Une meilleure connaissance de ces phénomènes aiderait l'industrie alimentaire à faire de meilleurs choix sur les conditions de transformation et à utiliser la connaissance acquise pour cibler une flaveur particulière en lien avec une application alimentaire donnée.

Les composants non volatils présents dans les fèveroles, notamment les composés phénoliques et les saponines, joueraient également un rôle important en lien avec les propriétés nutritionnelles et sensorielles des ingrédients [3], [5], [41]–[44]. Les composés phénoliques mis en évidence sont des flavonoïdes (flavan-3-ols, flavones, flavonols, flavononols, isoflavones, proanthocyanidines) et des acides phénoliques (hydroxybenzoïques et hydroxycinnamiques) [45], [46]. Ils peuvent prévenir divers stress oxydatifs et lutter contre les maladies liées au mode de vie telles que certains cancers [41], [42]. Les saponines, quant à elles, existent sous différentes formes, dont le soyasapogenol B, la soyasaponine βg, la soyasaponine Bb et l'ayukisaponine IV [5], [47]–[49]. Elles abaisseraient les concentrations plasmatiques en cholestérol et contribueraient ainsi à réduire le risque de certaines maladies cardiovasculaires. En revanche, ces deux grandes familles de composés agissent aussi comme facteurs antinutritionnels, au même titre que l'acide phytique, des lectines ou des glycosides pyrimidiques (vicine et convicine). Ceci suscite des inquiétudes quant à la sécurité des ingrédients issus de fèverole [3], [5], [50]. Par ailleurs, les composés phénoliques et les saponines interviennent également dans la saveur perçue (amertume et astringence en particulier), qui s'explique par la dispersion de ces composés dans la salive suivie de la stimulation de récepteurs spécifiques dans la cavité orale [19], [32], [51], [52]. Les composés phénoliques et leurs produits de réaction enzymatique et non enzymatique interviennent également dans la couleur des ingrédients, qui constitue un élément essentiel pour leur acceptabilité sensorielle [5], [53]–[55]. Les étapes de traitement des fèveroles (décorticage, fraisage et traitement thermique) qui interviennent avant la production des ingrédients, réduisent considérablement les teneurs en tannins, acide phytique et saponines [56], [57]. Par exemple, le stockage des légumineuses au-dessus de 30 °C, leur trempage, leur germination à un pH légèrement acide et leur traitement dans des solvants alcooliques (éthanol, méthanol) ont tous abouti à des dérivés des saponines originales, présentant une amertume plus faible [47], [48], [58]. Les teneurs en saponines sont également réduites par fraisage, décorticage et/ou cuisson [57], [59]. Ainsi, la transformation semble induire des changements au niveau des composés non volatils de la fèverole, même si la plupart des études ont jusqu'à présent porté sur les graines elles-mêmes ou les ingrédients qui en sont issus, mais pas sur la fonctionnalisation (modification) des ingrédients ou leur utilisation pour

des applications alimentaires finales. De nombreuses voies permettant de produire et modifier ces ingrédients. Ainsi, le comportement des composés non volatils doit être étudié afin de permettre aux industriels de l'agro-alimentaire de limiter les freins qui leur sont associés et de produire des ingrédients plus acceptables pour le marché alimentaire.
Cette étude de la littérature a permis de montrer que la compréhension des phénomènes, bien que partiellement disponible, n'était pas complète et se centrait avant tout sur quelques phénomènes, sans en intégrer la nature multidimensionnelle. Ainsi, pour un même type d'ingrédient, il n'existe pas d'approche transversale permettant de comprendre l'impact des transformations sur les différentes composantes de la qualité des ingrédients produits, limitant les industries alimentaires dans leurs choix pour développer des produits à base de légumineuses. En outre, dans chacune des études menées, les méthodes et/ou les conditions d'investigation sont différentes, malgré des objectifs similaires pour la plupart. Ainsi, ce projet de thèse s'est proposé de travailler sur un ingrédient de fèverole peu transformé initialement (concentrât commercial), riche en protéines, et de le modifier dans des conditions de procédés douces et pertinentes à une échelle industrielle. Il propose d'étudier les propriétés fonctionnelles et sensorielles des ingrédients ayant subi différentes modifications et de comprendre les phénomènes qui en sont à l'origine, dans l'objectif de suggérer un compromis entre les différentes propriétés et proposer des ingrédients d'intérêt pour tendre vers un alimentation saine, durable et appréciée.

L'Approche Experimentale

L'impact des conditions de transformation, choisies pour être réalistes sur le plan industriel, a été étudié en utilisant une approche multidimensionnelle et trouver ainsi un compromis favorable à l'expression des propriétés des différents ingrédients. Plus précisément, le partenaire industriel de cette thèse (Döhler GmbH) s'est procuré auprès d'un de ses fournisseurs, un concentrât de fèverole riche en protéines, traité selon un procédé de transformation doux à l'échelle industrielle. Il a été fabriqué à partir de fèveroles séchées et décortiquées, par broyage et « classification par air » (turboséparation). Ce concentrât a ensuite été modifié selon différentes conditions opératoires, qui ont été sélectionnées parmi les nombreuses voies de transformation possibles afin de répondre à des exigences industrielles en terme de coût énergétique et de faisabilité. Ainsi, l'utilisation des variables pH, température et durée de traitement, sont apparues pertinentes pour représenter les modifications possibles. Les gammes de valeurs choisies pour chacun de ces paramètres ont volontairement été élargies par rapport aux conditions probables d'utilisation, ceci afin de favoriser la mise en évidence de phénomènes potentiellement plus extrêmes et ainsi faciliter la compréhension des mécanismes mis en jeu et la mise en relation entre les mécanismes et les propriétés ciblées des ingrédients.

Le concentrât de fèverole a ainsi été modifié selon différentes conditions de transformation : pH de 2, 4, 6,4 ou 11 ; température de 55, 75 ou 95 °C ; durée du traitement de 30 ou 360 minutes. Vingt-quatre ingrédients différents ont ainsi été produits (trente-six en tenant compte des ingrédients produits trois fois afin de tester la répétabilité de la production). L'ensemble de ces ingrédients (concentrât initial non modifié et concentrâts modifiés dans les différentes conditions décrites ci-dessus) a ensuite été utilisé à deux pH différents, 4 et 7, dans des systèmes modèles proches d'applications de type boissons. Au cours de l'utilisation de ces ingrédients, la fonctionnalité des boissons (propriétés moussantes et émulsifiantes), la perception olfactive des produits et la composition en composés volatils et non volatils ont été étudiées.

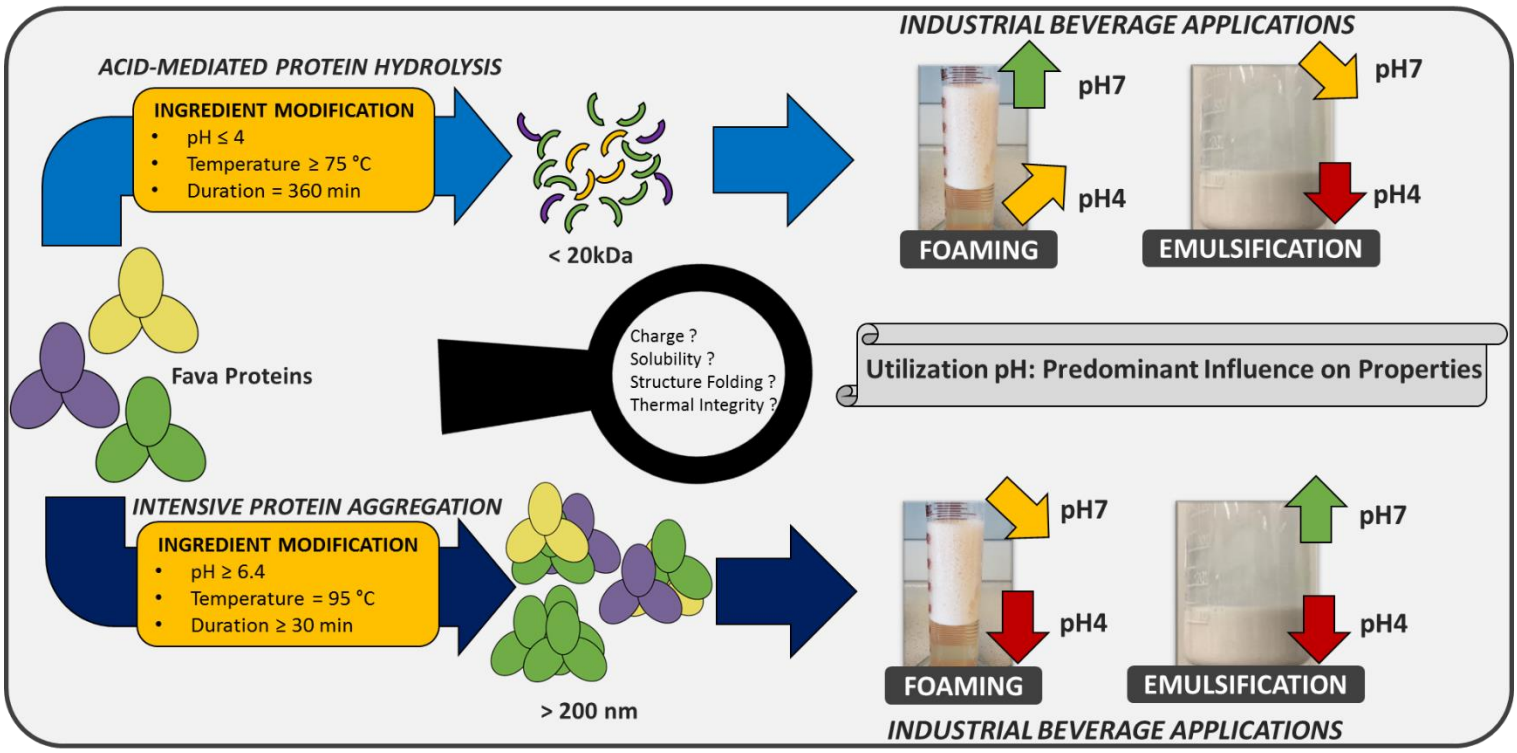
Compréhension des mécanismes à l'origine des propriétés fonctionnelles

Comme indiqué précédemment, le concentrât initial de fèverole a été modifié par le pH, la température et la durée du traitement, puis les ingrédients produits ont été utilisés à deux pH dans des systèmes proches des applications de boissons. Au cours de leur utilisation, leurs propriétés physico-chimiques et fonctionnelles ont été évaluées. Les propriétés physico-chimiques associées aux protéines étudiées et présentées dans cette partie, étaient la charge des protéines, la solubilité et les signaux de fluorescence intrinsèques, tandis que les paramètres fonctionnels étaient le pouvoir moussant, la stabilité de la mousse formée, la capacité émulsifiante et la stabilité de l'émulsion. De plus, certains signaux de fluorescence non associés aux protéines ont également été analysés, en relation avec des paramètres fonctionnels, ceci afin d'étudier si les propriétés fonctionnelles étaient influencées par les propriétés des protéines uniquement, ou par d'autres paramètres également.
Dans la première phase de cette étude, l'objectif était d'établir des liens entre les différentes propriétés des ingrédients, par le biais d'une analyse rapide intégrant un grand nombre de données, obtenues sur 37 ingrédients (1 concentrât de fèverole initial + 36 concentrâts modifiés), à travers 26 variables différentes (propriétés des ingrédients). Les deux méthodes statistiques utilisées étaient l'analyse en composantes principales (ACP) et la corrélation de Pearson. La deuxième phase du travail visait à expliquer les propriétés de la mousse et de l'émulsion à travers l'étude de différentes caractéristiques des protéines, et à établir la relation entre les deux. Au cours de la modification des ingrédients, des modifications structurelles des protéines, c'est-à-dire leur hydrolyse et leur agrégation, ont été observées. En outre, au cours de l'utilisation, la charge protéique, la solubilité et les signaux de fluorescence intrinsèques ont été suivis, mais avec un aperçu supplémentaire de l'intégrité thermique des protéines présentes dans les ingrédients plus doux ou plus vigoureusement transformés.
Les résultats montrent que les conditions de transformation utilisées sont capables de moduler les propriétés fonctionnelles du concentrât de fèveroles, l'analyse étant renforcée

par le biais de différents modèles statistiques. Les propriétés des mousses et des émulsions sont principalement gouvernées par le pH d'utilisation des ingrédients. Un pH proche du point isoélectrique des protéines de fèverole (pH 4) n'est favorable ni à la stabilité de la mousse, ni au pouvoir d'émulsification ou à la stabilité de l'émulsion.

Les propriétés moussantes et émulsifiantes sont régies par des mécanismes associés aux protéines, distincts cependant compte tenu des différences dans les phases dispersées. Ces fonctionnalités ne sont donc pas corrélées entre elles. La capacité et la stabilité de la mousse et de l'émulsion sont associées à différentes caractéristiques protéiques et non protéiques du concentrât de fèverole, suggérant la complexité d'utiliser une matrice complexe comme ingrédient. Des corrélations entre les propriétés fonctionnelles et les propriétés physico-chimiques ont été mises en évidence et s'expliquent par les propriétés des protéines. De fortes corrélations entre les propriétés fonctionnelles et physico-chimiques ont ainsi été observées par la charge protéique, la solubilité et la fluorescence intrinsèque. Leur comportement en réponse au pH appliqué lors de l'utilisation et de la modification était cohérent. Les outils statistiques utilisés, l'ACP et la corrélation de Pearson, permettent une compréhension globale de l'impact des conditions de transformation sur les différentes propriétés mesurées, ainsi que leurs interrelations. Une analyse rapide de grands ensembles de données peut être réalisée grâce à cette approche, la rendant ainsi très utile pour la recherche industrielle sur les ingrédients d'origine végétale.

La modification des ingrédients du concentrât de fèverole en fonction du pH, de la température et de la durée du traitement, a entraîné deux modifications structurelles principales : l'hydrolyse des protéines à des pH acides et l'agrégation des protéines. Ces réactions ont eu un impact sur les fonctionnalités, mais seulement dans une certaine mesure car l'effet du pH d'utilisation était toujours prédominant. L'hydrolyse acide des protéines a légèrement amélioré les propriétés moussantes, uniquement à un pH d'utilisation neutre, mais son rôle par rapport à l'émulsification est moins clair. L'agrégation n'a pas amélioré les propriétés moussantes des protéines mais a permis de conserver la stabilité de l'émulsion à pH neutre. D'autres phénomènes pourraient également avoir provoqué des modifications structurelles des protéines de fèverole, comme observé par calorimétrie différentielle à balayage (DSC). Si l'effet du pH d'utilisation est clairement mis en évidence et compris, ce n'est pas le cas des conditions de pH et de température utilisées pour modifier les ingrédients. Ainsi, la faible stabilité de plusieurs des mousses produites s'est par exemple avérée dépendante des conditions utilisées pour modifier l'ingrédient, mais les raisons de leur déstabilisation n'ont pas pu être explicitées. Plus largement, les conditions de modification des ingrédients n'étaient pas particulièrement reflétées dans les propriétés physico-chimiques, et les propriétés dépendaient encore une fois en grande partie du pH d'utilisation.



Compréhension des mécanismes à l'origine des propriétés de la flaveur

Selon une approche multidimensionnelle similaire à celle utilisée pour les propriétés fonctionnelles, la flaveur du concentrât initial et des concentrâts modifiés a été étudiée. Dans cet objectif, les mêmes ingrédients modifiés que précédemment ont été utilisés dans deux modèles distincts d'application de boissons (pH 4 et pH 7). La perception des odeurs et la composition et la libération des molécules volatiles de l'espace de tête ont été évaluées pendant l'utilisation des ingrédients. Cette étude va au-delà de l'étude de la flaveur des ingrédients modifiés pour piloter les propriétés fonctionnelles des ingrédients. Elle tente d'apporter des éléments de compréhension sur la façon dont les conditions de procédé déterminent la perception des odeurs, et si l'olfaction peut être corrélée aux composés volatils. A l'aide de la littérature, les réactions à l'origine de la formation des composés d'arômes ont été identifiées et des hypothèses relatives à la libération d'arômes en fonction du type de matrice « boisson » ont été proposées.

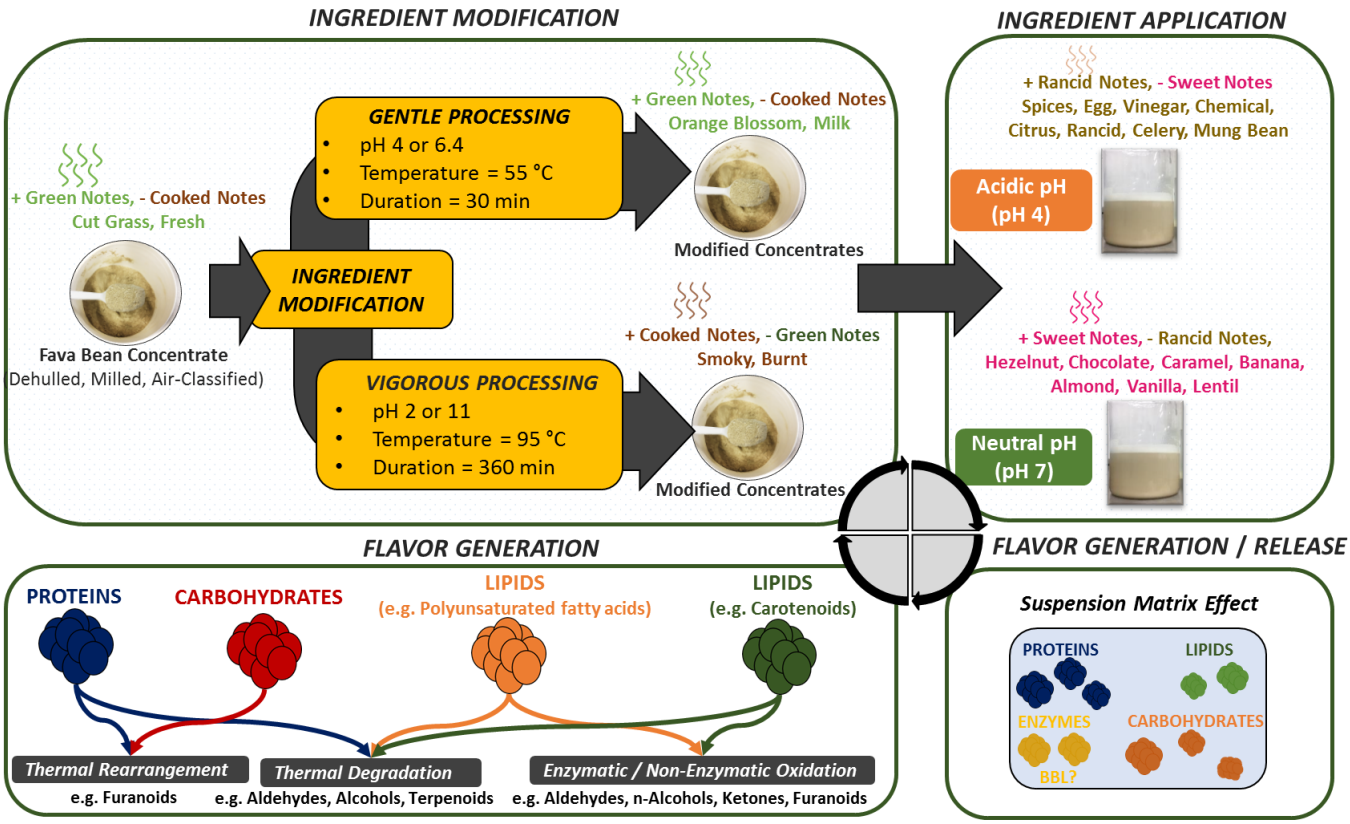
Pour l'évaluation des odeurs, un panel sensoriel de 21 panélistes a été recruté. Il a d'abord été entraîné à mémoriser 36 attributs olfactifs différents à l'aide de références. Ensuite, une méthode qualitative appelée test Check-All-That-Apply (CATA) a été réalisée, et les panélistes ont ainsi sélectionné les attributs caractérisant les suspensions fabriquées à partir de chacun des ingrédients produits. Au cours des discussions, 4 attributs olfactifs différents (vert, cuit, « doux » et rance) ont été évalués pour décrire quantitativement les intensités

perçues des différents ingrédients en suspension. Les composés volatils présents dans l'espace de tête (headspace, HS) des différentes suspensions d'ingrédients ont par ailleurs été suivis par piégeage sur des fibres SPME et analysés par chromatographie en phase gazeuse couplée à la spectrométrie de masse (HS-SPME-GC-MS). Les molécules volatiles détectées ont été regroupées selon différentes familles chimiques et analysées individuellement par ACP. Pour finir, les relations entre les attributs d'odeur, les substances volatiles de l'espace de tête et les conditions de procédé ont été établies pour comprendre les possibles interactions.

Il ressort de cette étude que l'odeur est fortement influencée par les conditions de modification et d'utilisation des ingrédients, en particulier par le pH.

Les différentes suspensions d'ingrédients évaluées dans cette étude apparaissent significativement distinctes dans leurs profils olfactifs. Les suspensions présentent différentes intensités de notes vertes, cuites, « douces » et brûlées. Les conditions du procédé, en particulier le pH d'utilisation, paraissent déterminantes sur la perception des odeurs. Cet effet était important pour le concentrât initial de fèverole. Pour les suspensions d'ingrédients non-modifiés et modifiés, une note rance plus intense ressort à pH 4 alors qu'une note plus « douce » semble prédominante à pH 7. L'utilisation des ingrédients à pH 4 met en évidence des descripteurs tels que vinaigre, viande, œuf, chimique, rance, brûlé, citron, vin rouge, épices, alors qu'à pH 7, des notes chocolat, amande, noisette, banane, vanille, café, caramel, frais, lait, lentille et bois sont perçues. Les conditions opératoires utilisées lors de la modification des ingrédients ont également un impact sur les odeurs perçues. Ainsi, le concentrât non modifié et les ingrédients traités dans des conditions douces sont perçus avec des notes vertes plus fortes, alors que dans des conditions plus « vigoureuses », des notes « cuites » apparaissent.

L'analyse des composés volatils de l'espace de tête a mis en évidence 88 composés différents, appartenant à différentes familles chimiques : aldéhydes, alcools, cétones, furanoïdes, terpénoïdes, alcanes, alcènes, acides organiques, esters et quelques composés chlorés et soufrés. Avec le concentrât initial de fèverole, un effet prédominant du pH d'utilisation est mis en évidence pour expliquer les différents profils chromatographiques. Cet effet est moins marqué pour les concentrâts modifiés mis en suspension, s'expliquant peut-être par des modifications de la matrice, entraînant une libération modifiée des composés volatils. Les conditions de procédés utilisées lors de la modification des ingrédients ont généré des composés d'arômes provenant principalement de l'oxydation des lipides, mais aussi de la dégradation des caroténoïdes, de la réaction de Maillard et de réactions de dégradation des protéines et des sucres.



Le processus peut-il influencer l'impact sensoriel et nutritionnel des composés non volatils ?

Les propriétés physico-chimiques et sensorielles des composés à l'origine du potentiel antioxydant, du goût (amertume et astringence), de la couleur et des effets antinutritionnels ont également été étudiés. L'objectif de cette étude était d'étudier les micro-constituants non volatils et leur évolution en fonction des conditions du procédé, ainsi que de suggérer si ces modifications avaient un impact potentiel sur les caractéristiques des ingrédients (flaveur, couleur, propriétés nutritionnelles) pour des applications alimentaires industrielles.

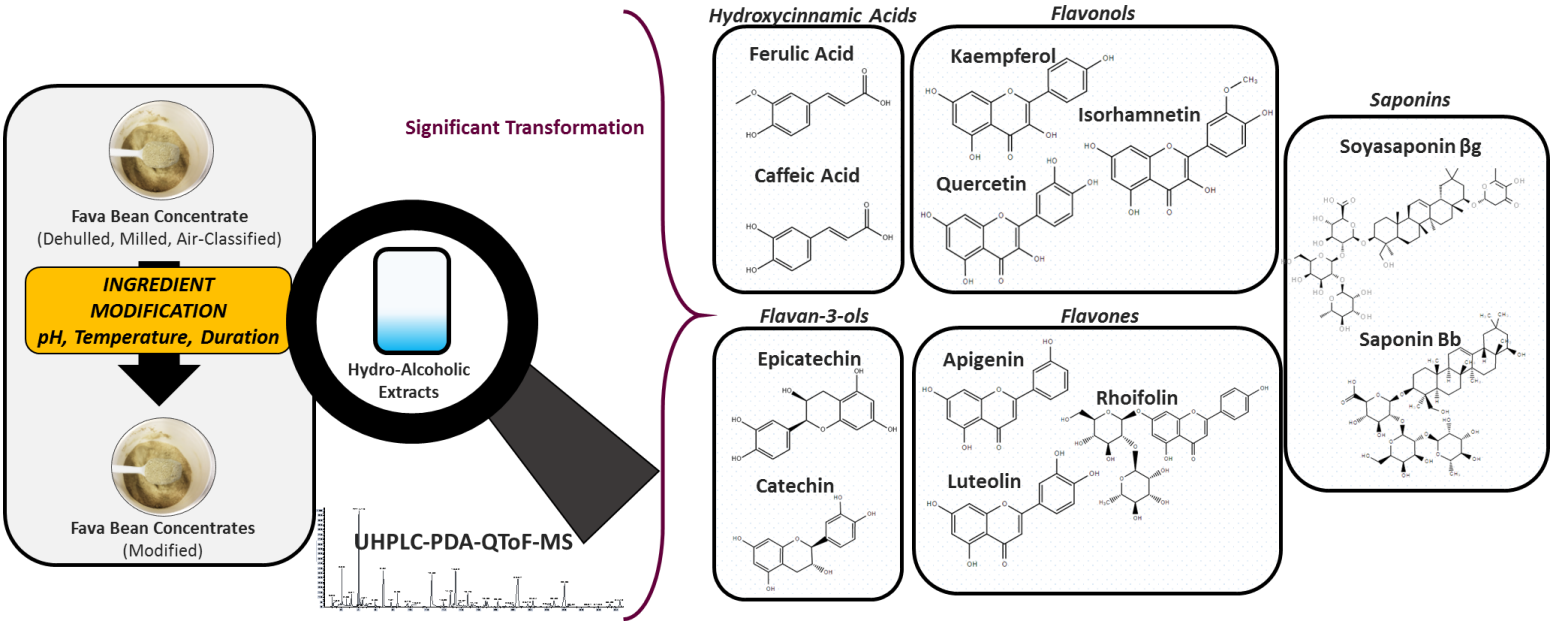
Une partie des échantillons étudiés précédemment pour leurs propriétés fonctionnelles et leur potentiel olfactif a été sélectionnée pour mener cette étude, correspondant à des conditions de modifications « douces » et plus « vigoureuses ». Ainsi, pour les procédés acides (pH 2 et 4) et alcalins (pH 11), deux conditions de traitement thermique ont été retenues : 55°C_Low (traitement doux) et 95°C_High (traitement plus vigoureux). De plus, une série de suspensions produites avec des modifications de pH (« pH naturel » 6,4) a été étudiée, avec prise en compte des ingrédients modifiés à 55, 75 et 95 °C, pendant 30 ou 360 minutes. Les composés non volatils des différents ingrédients sélectionnés ont été extraits à l'aide d'un mélange hydroalcoolique et analysés par chromatographie liquide ultra haute

performance couplée à un détecteur UV-Visible à barrette de diodes et à un spectromètre de masse simple quadripôle à temps de vol (UHPLC-PDA-QToF-MS). Il s'agissait ainsi de détecter les familles de molécules présentes dans les différents ingrédients et de proposer une analyse semi-quantitative. A l'aide de modèles statistiques, les molécules ont ensuite été regroupées en différentes familles afin de comprendre leur évolution en fonction des traitements associés. Des hypothèses sur les phénomènes mis en jeu au cours de la modification ont été proposées à l'aide des données disponibles dans la littérature.

L'analyse par UHPLC-PDA-QToF-MS a ainsi permis de mettre en évidence 39 composés phénoliques et 2 saponines dans les différents échantillons étudiés. Les composés phénoliques sont principalement des flavonoïdes (3 flavan-3-ols, 8 flavones et 26 flavonols), mais on retrouve également 2 acides hydroxycinnamiques. Les saponines identifiées sont la soyasaponine β et la saponine Bb.

Les résultats ont ainsi montré que les différentes conditions de traitement appliquées au concentrât initial conduisaient à des profils chromatographiques en composés phénoliques et saponines différents. Ces deux familles sont significativement impactées par le pH de modification. Les conditions acides (pH 2 et 4) et alcalines (pH 11) conduisent globalement à une diminution plus importante des teneurs en composés phénoliques que le pH « naturel » (pH 6,4). Les évolutions des composés phénoliques avec la température (degré et durée) semblent plus complexes. Les changements pourraient être une combinaison entre une meilleure extraction des composés due à une évolution des matrices dont ils sont extraits (ingrédients plus ou moins dénaturés) et une dégradation des molécules dans les conditions du procédé. Parmi les saponines, la soyasaponine β, connue pour sa forte amertume, était prédominante dans le concentrât initial alors que la saponine Bb, moins amère, augmentait dans les ingrédients modifiés, en particulier ceux traités sans aucun ajustement de pH. L'effet de la température sur l'évolution des saponines semble là encore complexe, montrant probablement un double effet réactivité et extractibilité.

Ainsi, ce travail a montré que l'itinéraire technologique mis en œuvre jouait un rôle important dans la transformation des composés phénoliques et des saponines. Ceci aura probablement un impact sur le goût, la couleur, le profil antioxydant et la qualité nutritionnelle (facteurs antinutritionnels ici) des ingrédients.



Conclusion

La fèverole est une source végétale qui présente **un potentiel élevé pour de nombreuses applications alimentaires industrielles**, notamment **à travers des propriétés nutritionnelles et fonctionnelles** des ingrédients produits à partir de ces graines. Il existe différentes manières de produire et de modifier ces ingrédients avec des conséquences importantes sur leurs propriétés fonctionnelles et leur flaveur, souvent indésirable pour les consommateurs. Dans ce travail de thèse, **un concentrât obtenu à partir de fèveroles décortiquées et moulues puis par un processus d'extraction doux (65% p/p, base sèche), a été utilisé comme matière de départ**. Ce matériau était riche en protéines et contenait également d'autres composants, à savoir 2% p/p de glucides, 3% de lipides, plus de 17% de fibres alimentaires et près de 8% de cendres (p/p, matière sèche). **Ce concentrât a ensuite été modifié par diverses conditions de procédés**, pertinentes sur le plan industriel et permettant d'obtenir toute une gamme d'ingrédients modifiés. **Puis ces derniers ont été utilisés dans deux conditions d'application différentes**.

L'originalité de ce travail de thèse était de mieux comprendre les mécanismes à l'origine des propriétés fonctionnelles et sensorielle. Pour cela, **une approche multidimensionnelle a été mise en œuvre** avec l'objectif final de proposer le meilleur compromis entre les différentes propriétés des ingrédients en fonction des cibles de produits souhaités. Dans un premier temps, les propriétés fonctionnelles (moussante et émulsifiante) de ces ingrédients ont été étudiées. Afin de comprendre les mécanismes à l'origine des modifications de propriétés fonctionnelles induites par les procédés de modifications de ces ingrédients, différentes caractéristiques des protéines ont été étudiées (hydrolyse des protéines à médiation acide et agrégation protéine-protéine) ainsi que lors de leur utilisation (solubilité, charge, repliement structurel et intégrité thermodynamique). Afin d'étudier les molécules

clés à l'origine des odeurs de fèverole, la perception olfactive des ingrédients a été étudiée et les principales notes identifiées ont été corrélées aux composés volatiles libérés lors de l'utilisation de ces ingrédients. De plus, la composition en molécules non volatiles a été étudiée pour une partie des ingrédients produits, afin de comprendre leurs impacts potentiels sur les changements de goût, de couleur, mais également certaines propriétés antioxydantes et antinutritionnels.

Les résultats de ces travaux ont ainsi montré que **les propriétés moussantes et émulsifiantes sont principalement gouvernées par le pH de modification et d'utilisation** de ces ingrédients et peuvent être expliquées par les propriétés des protéines. Par ailleurs, **les perceptions olfactives peuvent fortement varier en fonction des conditions de transformation appliquées**, allant de notes vertes à cuites ou rances, et dues à la présence de combinaisons de composés volatils spécifiques. Enfin, les propriétés physico-chimiques et sensorielles **des composés à l'origine du potentiel antioxydant, des saveurs (amertume et astringence), de la couleur et des effets antinutritionnels ont également été étudiées, confirmant le fort effet du pH sur ces ingrédients**.

Ce projet a ouvert la voie vers de nouvelles recherches sur les itinéraires technologiques à suivre pour proposer des ingrédients peu transformés, dans un contexte réaliste pour un développement industriel. Il s'agit de trouver, par une approche multi-dimensionnelle, un compromis satisfaisant entre les fonctionnalités intéressantes à exploiter, la limitation des facteurs antinutritionnels et la présence de notes olfactives ou de couleurs indésirables pour les consommateurs. Ce type de projet permet ainsi de contribuer à la transition alimentaire mondiale pour nourrir les 820 millions de personnes avec des aliments durables, sains et appréciés.

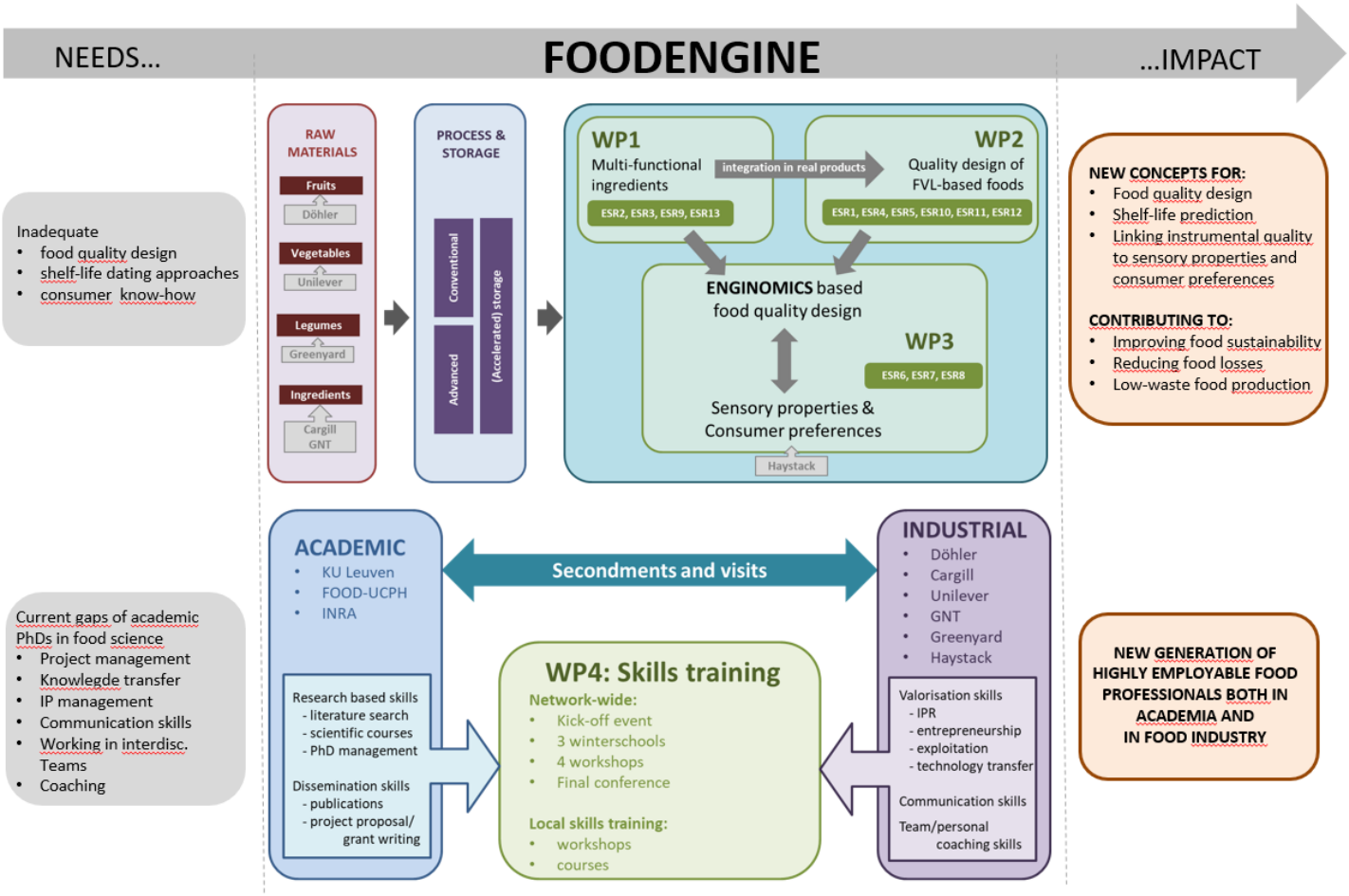
B. Preface

This PhD work is a part of a project that has received funding from the European Union's EU Framework Programme for Research and Innovation Horizon 2020 under Grant Agreement No 765415 (acronym FOODENGINE). FOODENGINE (www.foodengine.eu) is part of the Marie-Skłodowska Curie Action (MSCA) – Innovative Training Network (ITN) which is one of the many European Union's flagship funding programs for doctoral education and postdoctoral training of researchers [60]. Researchers who were recruited in this framework were called Early Stage Researchers (ESRs). The grant agreement for FOODENGINE was signed in 2017 by several partner institutions, including Institut National de la Recherche Agronomique et Environment (INRAE, UMR SayFood), France (academic partner), Kobenhavns Universitet or University of Copenhagen (UCPH), Denmark (academic partner) and Döhler GmbH, Germany (industrial partner). The project has been coordinated by the Katholieke Universiteit Leuven (KU Leuven), Belgium (academic partner).

The FOODENGINE Network: Academic and industrial partners along with the Early Stage Researchers (ESRs) recruited for doctoral education (www.foodengine.eu).

FOODENGINE consisted of several work packages (WPs), three of which were research-based. The WP1 focused on multi-functional ingredients for high quality food development. The objective here was to create clean label foods from these ingredients by process optimization of different types of sources and their use in liquid and low-moisture food systems. The WP2 focused more on quality design of fruits, vegetables and legume-based

(FVL) foods – using real and model food systems. This involved the use of different promising technologies that could transform food quality. The WP3 was finally designed to link the sensory properties and consumer acceptability and preference of the FVL-based foods. More emphasis on linking sensory quality and perception with instrumental methods was given on this work package. The other packages were more focused on giving research-based training to the ESRs involving both academic and industrial partners; offering specialized courses to build a complementary skill set by lecture, tutorials and workshops; exposure to both academic and industrial sectors via secondments (up to 30% of the contractual period); and finally conferences and events to showcase achievements of the training network.



FOODENGINE Work Packages (WPs): Overview of the research and training framework of FOODENGINE with integration of the different projects (www.foodengine.eu).

In the network, 12 out of 14 ESRs were recruited for 3-year PhD contracts. II was recruited for the position called ESR-12 within a 3-year contract from November 2018 until November 2021. The project was mainly hosted by Döhler GmbH, and the doctoral training along with the doctoral direction was provided by AgroParisTech/ INRAE to obtain the PhD-degree from the Paris-Saclay University and attached to the Doctoral School ABIES. The industrial partner of this ESR-12 position was Döhler GmbH, which is a global producer, marketer and

provider of technology-driven natural ingredients, ingredient systems and integrated solutions for the food and beverage industry (www.doehler.com). One of the main portfolios of Döhler is the production and transformation of cereal and pulse ingredients for plant-based solutions in the food market. Döhler plays an important role in many aspects of the food supply chain, including raw material procurement, ingredient production, ingredient modification, and business-to-business (B2B) and business-to-consumer (B2C) product applications. Döhler takes part in knowing the present market trends in foods as well as it looks at what kind of ingredients would be the future demands of the food market. Thus, combining the market need for plant-based ingredient solutions and the different research questions outlined by FOODENGINE, the ESR-12 project focused on the transformation of quality of plant-based ingredients in food applications. Therefore, the project was more streamlined with a focus on promising pulse fava bean and its functional potential and also its sensory and safety limitations. The overall objective of this project was to investigate ingredient production and/ or transformation on functional and sensorial quality in beverage systems. In addition to knowledge on mechanisms and protein properties at the origin of functional and sensory properties, this project search to highlight the most suitable ingredient processing conditions for highly acceptable beverage applications.

Communications & Valorisations

Conferences

1. Sharan, S.; Stadtmüller, J.; Bonerz, D.; Aschoff, J.; Orlien, V.; Olsen, K.; Rinnan, Å.; Maillard, M. N., Saint-Eve, A.; Zotzel, J. Functional robustness of a Vicia faba ingredient after processing: a light on foamability for food applications. NIZO Plant Protein Functionality Conference, Online, 21-22 October 2020. ***(poster presentation)***

2. Zanghelini, G.; Sharan, S.; Descharles, N.; Zotzel, J.; Bonerz, D.; Aschoff, J.; Saint-Eve, A.; Maillard, M. N. How can the composition in volatile compounds issued from different reaction pathways explain the diversity on the odour perception of various pulse ingredients? NIZO Plant Protein Functionality Conference, Online, 21-22 October 2020. ***(poster presentation)***

3. Sharan, S.; Stadtmüller, J.; Zotzel, J.; Bonerz, D.; Aschoff, J.; Maillard, M. N., Saint-Eve, A. Role of protein fractions in the functional potential of a *Vicia faba* ingredient. EFFOST Conference, Online, 10-12 November 2020. ***(poster presentation)***

4. Sharan, S.; Zotzel, J.; Manickam I.; Zanghelini, G.; Bonerz, D.; Aschoff, J.; Orlien, V.; Olsen, K.; Rinnan, Å.; Saint-Eve, A.; Maillard, M. N. Cross-disciplinary compromise between functional and sensory properties of modified fava bean ingredients for

industrial food applications. EFFOST Conference, Switzerland, 1-4 November 2021. ***(poster presentation)***

Publications

1. **Review Article:** Sharan, S.; Zanghelini, G.; Zotzel, J.; Bonerz, D.; Aschoff, J.; Saint-Eve, A.; Maillard, M. N. Fava Bean (*Vicia faba* L.) for food applications: From seed to ingredient processing and its effect on functional properties, antinutritional factors, flavor, and color. Comprehensive Reviews on Food Science and Food Safety. 2021, 20 (1), 401–428. https://doi.org/10.1111/1541-4337.12687.

2. **Research Paper (Communication):** Sharan, S.; Zotzel, J.; Stadtmüller, J.; Bonerz, D.; Aschoff, J.; Saint-Eve, A.; Maillard M-N.; Olsen, K.; Rinnan Å.; Orlien, V. Two statistical tools for assessing functionality and protein characteristics of different fava bean (*Vicia faba* L.) Ingredients. Foods. 2021, 10(10), 2489. https://doi.org/10.3390/foods10102489

3. **Research Paper – Manuscript Submitted (October 2021):** Sharan, S.; Stadtmüller, J.; Bonerz, D.; Aschoff, J.; Orlien, V.; Olsen, K.; Rinnan, Å.; Maillard, M. N., Saint-Eve, A.; Zotzel, J. Process conditions govern fava bean (*Vicia faba* L.) functionality: Emphasis on the interplay between protein modifications and physico-chemical properties, foaming and emulsification. Food Hydrocolloids.

4. **Research Paper – Manuscript Submitted (January 2022):** Sharan, S.; Zanghelini, G.; Descharles, N.; Pernin, A.; Zotzel, J.; Bonerz, D.; Aschoff, J.; Saint-Eve, A.; Maillard, M. N. Flavor of fava bean (Vicia faba L.) ingredients: flavor of fava bean (*Vicia faba* L.) ingredients: the interplay between odor perception & headspace volatile chemistry along with processing & application conditions.

5. **Research Paper – Manuscript in Preparation:** Siddharth Sharan, Even Le-Roux, Gabriela Zhanghelini, Jens Zotzel, Daniel Bonerz, Julian Aschoff, Anne Saint-Eve, Marie-Noëlle Maillard. Process conditions modify non-volatile components in fava bean (*Vicia faba* L.) ingredients: New insights into the interplay between process conditions, phenolic compounds and saponins.

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D. Table of Contents

E. List of Tables

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G.List of Symbols and Abbreviations

A/W	Air-in-water
ANF	Anti-nutritional factors
ANOVA	Analysis of variance
BBI	Bowman-Birk inhibitors
BBL	Broad bean lipoxygenase
CA	Correspondence analysis
CAS	Chemical abstracts service
CATA	Check-all-that-apply
DDA	Data dependent acquisition
DDMP	2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one
DSC	Differential scanning calorimetry
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
EC	Emulsion capacity
ES	Emulsion stability
ESI	Electrospray ionization source
FBIC	Fava bean initial concentrate
FC	Foaming capacity
FS	Foam stability
G6PD	Glucose 6-phosphate dehydrogenase
GC	Gas chromatography
HIUS	High intensity ultrasound
HS	Headspace
MS	Mass spectrometry
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
O/W	Oil-in-water
PARAFAC	Parallel factor analysis
PDA	Photodiode array detector
PMM	Protein micellar mass
PSD	Particle size distribution
PUFA	Polyunsaturated fatty acid
QToF	Quadrupole-time-of-flight
RBC	Red blood cells
RI	Retention index
SDS	Sodium dodecylsulphate
SM-FTN	Sample manager flow-through needle
SNK	Newman-Keuls
SPME	Solid phase micro extraction
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
UHPLC	Ultra-high performance liquid chromatography

I

General Introduction

I. General Introduction

In 2019, scientists reported that more than 820 million people have insufficient food, and a much higher population consumes micronutrient-deficient diet leading to a higher risk of diet-related diseases such as obesity, coronary heart diseases and stroke [61]. This calls for a need to change, and therefore, a global transformation is required, comprising more healthy and environmentally sustainable foods. It is essential for the food system to feed nearly 10 billion people [61], [62]. Consideration of plant-based diet is a part of this global transformation. The popularity of plant based diet has been growing, amongst groups of consumers, researchers and food companies owing to benefits regarding health, agronomy and sustainability of resources on earth for the rising population [1], [2]. Production of protein-rich plant-based foods requires the least energy and resources, and releases lower levels of greenhouse gas emissions (GHGEs), when compared to those from animal sources [63], [64]. Reducing meat intake by even 25% and transitioning towards flexitarian and vegetarian foods would consequently minimize the impact on agricultural land ecosystems, biodiversity and carbon dioxide emissions [64]. In addition, a diet comprising legumes, whole grains, vegetables, fruits, nuts and seeds is associated with the prevention and management of diseases such as obesity, type 2 diabetes, hypertension, hyperlipidemia and cancer [65]–[67]. Such diets trigger mechanisms that promote insulin resistance, a healthy body weight and food microbiome interactions, and decrease the intake of saturated fats, advanced glycation end products, nitrosamines and heme iron [1], [65], [68]–[70].

Figure 1 – Fava bean (*Vicia faba* L.). Fresh fava bean seeds (Döhler GmbH)

Amongst plant-based foods, pulses are a category of nitrogen-fixing legumes (*Leguminosae* family) that refer to dried fruits or pods containing seeds [8]. Some examples of pulses are beans, lentils, peas, chickpeas, and these have been consumed for over 10,000 years and are largely consumed in the world [7], [71], [72]. Amongst pulses, fava bean (*Vicia faba* L.) belongs to the *Fabaceae* family and is known to exist as staple dietary food in cultures from

North Africa and Middle East [3], [4]. It is drawing attention due to its high agronomic, nutritional and functional potential for its use for human consumption [3], [5]. fava bean is a cool-season grain legume crop which germinates at low soil temperatures as low as 12.5 °C. Its crop can fixate high amounts of nitrogen (up to 100-200 kg N/ Ha), solubilize insoluble phosphorus and also increase microbial activity in the soil, thereby improving soil physical properties (bulk density and porosity) and soil organic matter content. This pulse crop can usually grow without irrigation, especially in regions of cold and rainy seasons [73], [74]. Fresh fava bean seeds are generally present inside pods (**Figure 1**), which are taken out and usually cooked before consumption or dried for future use [75], [76].

Nutritionally speaking, fava bean has shown to be superior to the other pulses like pea and chickpea thanks to its higher protein/ carbohydrate ratio [5]. It is rich in fibers, vitamins, minerals (iron, zinc, folate, and magnesium), and contains important phytochemicals such as phenolic compounds and saponins which possess antioxidant properties. Most importantly, it rich in proteins (23-41% w/w dry basis) and thus provides a source of certain essential amino acids and bioactive peptides [6], [7]. Its intake along with cereals in suitable quantities fulfils daily requirement of essential amino acids. While cereals are rich in cysteine and methionine and limiting in lysine, pulses are rich in lysine and poor in cysteine and methionine [8], [9]. In addition to nutritional benefits of pulses, fava bean ingredients can also be used as functional agents in different types of food and beverage applications [6], [10]. Speaking of proteins, there are various types found in fava bean, majorly globulins (legumin, vicilin, convicilin) existing in different conformations. These different structures play distinct roles in protein-associated functional properties [3], [77], [78], such as foaming and emulsification, which play a key role in beverage applications as ice-cream, pudding, mousse, etc. [11]–[14].

Despite the high nutritional, functional and agronomic potential of fava bean, its utilization as a food ingredient and as a protein-rich functional ingredient is respectively 2.4% and 0.5% in the food market [15]. Therefore, in addition to its various potentials, fava bean has also several safety (anti-nutritional factors) and sensory (flavor and color perception) limitations which may affect its utilization as a source of proteins for the human diet [5]. Concerning protein digestibility, fava proteins seem to be affected by the presence of anti-nutritional factors (ANF) such as saponins, glycosides, tannins, alkaloids, phytic acid conjugates and lectins, which can reduce the bioavailability of proteins and minerals. Additionally, fava bean possesses pyrimidine glycosides (vicine and convicine), found exclusively in *Vicia* genus, which can cause favism – a fatal disease characterized by hemolytic anemia [16]–[18]. Furthermore, sensory properties (odor, taste and color) can play an important limiting role in determining acceptability of fava bean ingredients and their utilization in food applications. Indeed, consumer acceptance of pulse-based products is hampered by the presence of undesirable flavors or off-flavors, particularly bitterness and beany odor [19].

Color is also an important factor for acceptability. For instance, seeds with dark brown hull color are associated with a poor acceptability in the market. To improve fava bean acceptability and increase their use in the food market, its potential as well as its limitations need to be taken into consideration [5].

In the quest for improving acceptability, acting on the processing steps of fava bean into ingredients could serve as a promising medium. Prior to its use, the whole fava bean must be processed into ingredients such as flours, concentrates and isolates, which may also be further modified by industrial processing. The steps from bean treatment (e.g. air drying and dehulling) to the production of ingredients (size reduction and subsequent protein extraction) can be denoted as "ingredient fabrication". Ingredients produced could also be further modified using process conditions and this is denoted as "ingredient modification". Either the unmodified or modified ingredients can be utilized in different food applications (ingredient utilization). The role of ingredient modification is generally to improve their functional properties – to render them more suitable for food applications [5], [6]. Ingredient fabrication, modification, utilization and all sections of ingredient processing may impact ingredient nutritional, functional, organoleptic and anti-nutritional properties (**Figure 2**), and it is necessary to understand the mechanisms at the origin of these properties to finally optimize the appropriate process conditions and their levels along with suitable assessment tools [3], [5], [6], [20]–[22].

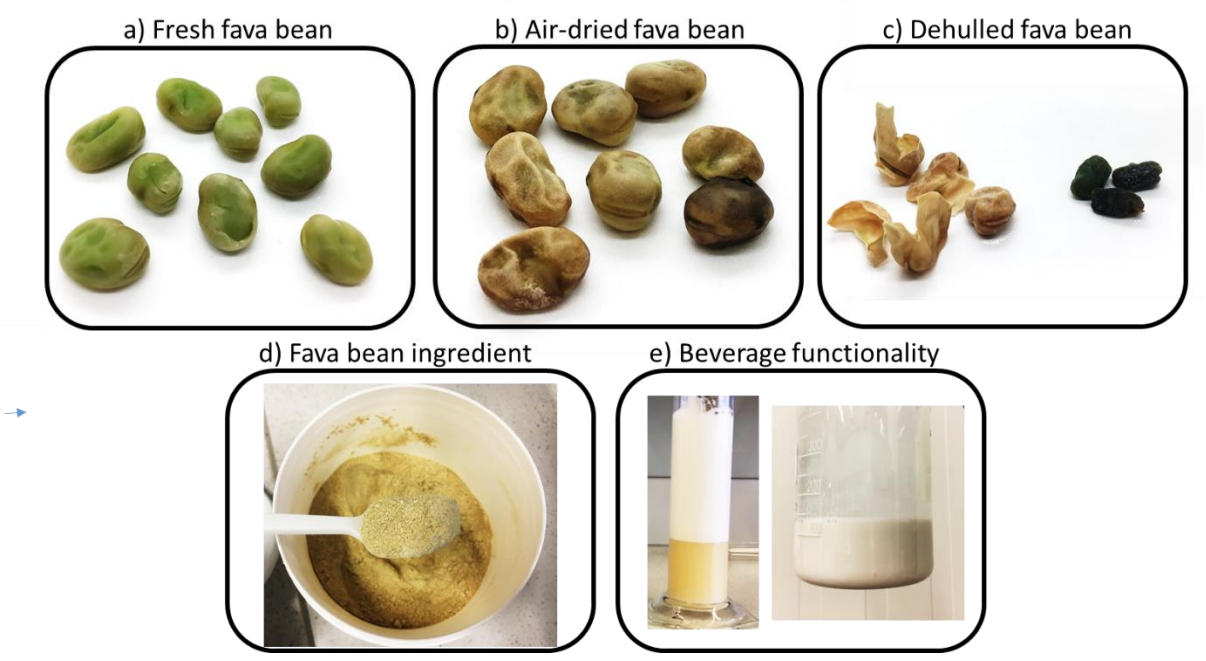

Figure 2 – Visual effect of Fava bean processing: Illustration of some examples of fava bean processing steps (Döhler GmbH)

I.1. PhD Context

In this context, the **focus of this PhD work** was to understand the impact of processing conditions on fava bean protein-rich ingredient properties – in particular, functional properties and flavor used for industrial beverage applications. To help understanding this research objective, various examples of beverage applications can be imagined. Generally, most of the plant-based beverages are colloidal dispersions consisting of oil bodies, fat droplets, proteins, dietary fibers, plant-matrix fragments; all suspended in an aqueous medium containing dissolved sugars, soluble fibers and salts [79]. These colloidal dispersions are produced by the wet-based disruption and filtration of plant-based sources such as soy beans, coconut flesh, oats, hazelnuts, almonds, rice; and then homogenization of the wet dispersions with functional agents such as plant-derived proteins (e.g. protein-rich ingredients from soy, fava, pea, etc.), polysaccharides (e.g. pectin, locust bean gum, starch, etc.) and phospholipids (e.g. soy lecithin) for improved colloidal stability and added functionality [79]–[81]. Taking an arbitrary colloidal matrix (e.g. coconut drink) that needs functionalization, fava bean protein-rich ingredient can act as a functional agent either for colloidal homogenization/ emulsification and/or for foam production which are both necessary for a stable coconut-based beverage. Foam and emulsion properties play a key role in these type of beverage applications. While foams are formed from adsorbed air-in-water (A/W) interfaces, most food emulsions are produced from that of oil-in-water (O/W). In fava bean, various proteins in different conformations play an important role in determining the functionality of the ingredient [5], [24], [82]. In addition, various non-protein constituents, lipids, starch, and dietary fibers, are also present in fava bean and can influence functionality in a food matrix [3], [5]. Furthermore, taking organoleptic properties into account, if such milk analog applications are prepared, introduction of pulse ingredients such as fava or pea ingredients may introduce unpleasant off-flavor, which could often be linked to green, grassy or beany aroma which can notably be attributed to aldehydes, alcohols and ketones. Additionally, bitter and astringent taste may be contributed by sapid compounds such as saponins and phenolic compounds (isoflavones, flavonols, hydroxycinnamic acids, etc.) [35], [52], [83]. Flavor perception arises from a combination of interactions between volatile odorant compounds and non-volatile taste molecules with olfactory and sapid receptors [19]. During perception process, odor is one of the first key indications of flavor in foods, reflecting its quality and at the origin of acceptability [51], [84]–[86]. Accordingly, several aspects are implied in the properties of a plant-based foods and beverages, and by consequences the choice of used plant-ingredient impacts its acceptability. Thus, the aim of this study was to understand the mechanisms underlying functional and flavor properties through physico-chemical and statistical analyses. This type of study, i.e. a cross-dimensional or multi-dimensional study, was performed in simple aqueous systems, based model for real food systems, so as to extrapolate interpretations and conclusions for other types of

beverage applications as well. Furthermore, along with this multi-dimensional approach, the objective was to arrive at a compromise between different properties – to evaluate which type of process condition is suitable for a particular industrial beverage application.

Attempts to process fava bean ingredients have been undertaken before, but not necessarily with a cross-dimensional approach. Taking functional properties into consideration, protein modifications by physical, chemical and biological process techniques permits to better understand the development of foams and emulsions by influencing protein solubility, charge distribution and protein folding [11], [23]. Literature showed that fava proteins have previously been modified by temperature and pH [24], [25], mechanolysis [26], high-intensity ultrasound treatment [27], succinylation [28], acetylation [29], and enzymatic treatments [30]. The effect of any treatments on protein structure and the related impact on functionalities has been first established with the study of protein-protein aggregation and protein hydrolysis which are well known to influence ingredient functionalities [27], [29]–[31]. Protein aggregation and hydrolysis can be of different types and extent that result in a variety of effects on functional properties [27], [30], [87]. In addition, fava bean not only contains proteins but also various non-protein constituents, including starch, dietary fibers, lipids along with certain anti-nutritional factors [3], [5]. Hence, the reactions occurring during ingredient processing may be a result of proteins and/ or non-protein constituents [88], [89]. For now, there is no clear overview of all the possible reactions occurring during processing of fava ingredients at the origin of the changes in functional and physico-chemical properties. Different methodological tools could be used to evaluate ingredients, and various instrumental analyses can be used to measure the physico-chemical protein properties and ingredient functionalities, resulting in a myriad of results. These data can be examined by dataset and provide an in-depth knowledge of each individual aspect. Connecting all multidimensional results may give a complementary insight into relationship between properties and functionalities, and thus helps understand mechanisms underlying changes in functional properties.

Regarding flavor, the intrinsic aroma of plants depends on its genetic makeup, but also on the availability of precursors, distribution of enzymes and presence of favorable conditions for the reactions to take place [32]. Majority of fava bean flavor derives itself from degradation of lipids, amino acids, carbohydrates and carotenoids through enzymatic and/ or non-enzymatic reactions [5], [32], [33]. These phenomena are impacted throughout the ingredient processing steps, i.e. from bean harvest until final food application [5], [19]. Lipid oxidation is the primary cause of flavor in pulses. For instance in literature, pea flavor has extensively been studied, where different lipid oxidation products such as aldehydes, ketones, alcohols and furanoids give a combination of green, beany, earthy, and hay-like perception [19], [34]. Reactions at the origin of fava bean flavor have been investigated too,

but to a lesser extent and not particularly in direct correlation with sensory perception. In fava bean, free or esterified unsaturated fatty acids undergo enzymatic oxidation by broad bean lipoxygenase (BBL), and can also undergo auto-oxidation due to the presence of initiators (e.g. metal ions) and/ or temperature [5]. Other reactions including amino acids and sugars degradation are also possible, along with their rearrangement by Strecker's degradation and Maillard reaction, causing additional flavor development [32], [35]. Process conditions, thus, influence the extent and possibility of flavor-associated reactions and govern flavor formation. For fava bean, the impact of process conditions on the bean flavor itself has been studied to a certain extent. Fava bean seeds under microwave treatment (950 W for 1.5 min) or heat treatment (>70 °C, > 2 min) give modified flavor compared to fresh beans due to the inactivation of the BBL [36]–[38]. Fava ingredients, including flours that are dehulled and milled, contain high BBL activity, thus suggesting possibilities of enzymatic lipid oxidation [39]. The effect of pH has also been tested on fava protein isolates for flavor modification – where dried pea-like flavor predominated at neutral pH, and unpleasant fruity flavor developed at acidic pH [40]. Despite some understanding on fava flavor, there is not yet a complete, comprehensive knowledge of the chemistry of fava bean flavor with process conditions in relation to sensory perception. Understanding these aspects would help the food industry make better choices for appropriate process conditions to use while targeting specific food application with a particular flavor perception – thus a step further towards acceptability of the use of fava bean as promising food ingredients.

In the pursuit for higher fava bean acceptability, non-volatile components including phenolic compounds and saponins play an important role as they are linked to different properties defining nutritional, functional and sensory properties of the ingredients [3], [5], [41]–[44]. Phenolic compounds in fava bean can be either flavonoids, i.e. flavan-3-ols, flavones, flavonols, flavononols, isoflavones, proanthocyanidins, or phenolic acids, i.e. hydroxybenzoic acids or hydroxycinnamic acids [45], [46]. Phenolic compounds have been drawing attention owing to their antioxidant potential, where these can prevent various oxidative stress and fight lifestyle diseases such as cancer [41], [42]. In fava bean, saponins exist in many forms, including soyasapogenol B, soyasaponin βg, soyasaponin Bb and ayukisaponin IV [5], [47]–[49]. Saponins also have been shown to lower plasma cholesterol concentrations and thereby help reduce the risk of heart disease. Anti-nutritional factors comprise certain phenolic compounds, saponins, phytic acid conjugates, lectins and favism-inducing pyrimidine glycosides (vicine and convicine). These cause concerns for safety of fava ingredients [3], [5], [50]. In particular, taste perception (e.g. bitterness and astringency) is related to the dissolution of non-volatile, sapid compounds including phenolic compounds and saponins in the saliva followed by the stimulation of specific receptors in the oral cavity [19], [32], [51], [52]. Color perception is also an essential part of sensory acceptability, and is associated with non-volatile compounds, including phenolic compounds and products of enzymatic and

non-enzymatic reactions [5], [53]–[55]. Pulse ingredient processing transforms both phenolic compounds and saponins. Bean treatment (dehulling, soaking and heat treatment) prior to ingredient production reduces tannins, phytic acids and saponins to a considerable extent [56], [57]. Precisely, storage of pulses above 30 °C, pulse soaking, germination at slightly acidic pH, and treatment in ethanol or methanol solvents all have resulted in saponin variants with lower bitterness [47], [48], [58]. In fava beans, saponins are lowered by soaking, dehulling, cooking and/ or germination before ingredient production [57], [59]. Thus, processing seems to impart changes in the non-volatile aspects that related to all the fava limitations, but most of the treatment studied until now have been on bean processing and not primarily on ingredient processing for final food applications. As there are many ways of producing and modifying fava ingredients, evolution of non-volatiles by ingredient processing needs to be studied in depth. In this way, food industries could target these limitations and their chemical origins, and monitor their changes due to processing so that more acceptable fava ingredients are generated for the food market.

I.2. PhD Outline

Thus, in a nutshell, there is a lot of literature on fava bean processing and its effects on different functional and flavor aspects. To outline all that is present in the literature, **Chapter II** of the present manuscript proposes an overview of bibliographic findings and interpretations from different studies. Nevertheless, it was found that the bibliographic understanding is not complete/ comprehensive, nor is it multi-dimensional in nature. For a single type of ingredient, there exists no cross-dimensional overview to understand the impact of different processing on various properties and to help food industry to make appropriate choices to develop plant-based products. Also, methods or conditions of their investigations of each study are different although the objectives of most studies are similar. Thus, this PhD attempted to investigate a potential fava bean protein-rich ingredient, to modify it with simple yet industrially relevant process conditions, and to study several functional and flavor properties on these modified ingredients in order to highlight a multi-dimensional compromise of different properties owing to their chemical origins.

In this manner, **Chapter III** outlines the used approach and makes rationale of the different materials and methods that were chosen for this study, along with certain bibliographic evidences. In brief, a fava bean concentrate, produced by air-classification and thus considered as a gently processed ingredient [90], was further modified by process conditions. The impact of industrially relevant process conditions such as pH (2, 4, 6.4 and 11), temperature (55, 75 and 95 °C) and duration of treatment (30 and 360 min), was established in this work.

The first part of the investigation is outlined as the **Chapter IV**, which attempts to clarify the interplay between fava protein-associated reactions, protein physico-chemical properties and functional properties in two sub-chapters. Thus, the first half of chapter IV used statistical approaches (Pearson's correlation analysis and Principal Component Analysis (PCA)) to facilitate interpretation and assessment of the functional and physico-chemical interrelationships, and to establish a globally estimated correlation model with insight into their complex relationship as a function of process conditions. The second part, then went deeper into the details of protein modifications during ingredient modification and brought forth the ambiguities in the relationship between them. Precisely, fava protein aggregation and hydrolysis during ingredient modification, and physico-chemical properties of fava proteins (charge, solubility, intrinsic fluorescence and thermal integrity) were tested along with their functional properties (foam and emulsion capacity and stability) at utilization conditions simulating beverage applications.

Going beyond functional properties, chapters V and VI focused on studying flavor and anti-nutritional limitations of fava bean with process conditions. **Chapter V** focused on odor perception: qualitative and quantitative sensory properties; and headspace release of volatiles in conditions close to beverage application. Relationships were established to understand the interplay between processing, flavor release and volatile chemistry of different ingredients with diverse flavor profiles. As a result of these insights, diverse applications could be imagined by using process conditions. Finally, **Chapter VI** examined non-volatile molecules (phenolic compounds and saponins) extracted from selected ingredients which were mildly or vigorously modified, with a special focus on non-pH adjusted processing due to its industrial relevance. With the non-volatile evolution, changes in fava limitations concerning their antioxidant, taste and anti-nutritional properties were speculated.

At the end, concluding remarks were made (**Chapter VII**), where the effect of processing on all the different properties were integrated to bring a perspective of compromise between these properties. An example of plant-based cappuccino was adopted to propose how far this PhD study has been able to help understand the different mechanisms underlying functional and flavor properties. Insights on further investigations and different scientific approaches were also discussed so that a more thorough scientific knowledge of plant-based applications can be gained.

II

Bibliographic Study

II. Bibliographic Study

Fava Bean (*Vicia faba* L.) for Food Applications: From Seed to Ingredient Processing and its Effect on Functional Properties, Anti-Nutritional Factors, Flavor and Color

Review Article Published – Comprehensive Reviews in Food Science and Food Safety (https://doi.org/10.1111/1541-4337.12687)

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II.1. Introduction

Interest in plant-based nutrition has been steadily growing within the last years, along with the rising concern within groups of consumers, scientists and organizations regarding health and nutritional aspects of sustainable diets [1], [2]. Plant-based diets containing legumes, whole grains, vegetables, fruits, nuts and seeds are associated with the prevention and management of diseases such as obesity, type 2 diabetes, hypertension, hyperlipidemia and cancer [65]–[67]. Indeed, such diets trigger mechanisms that promote insulin resistance, a healthy body weight and food microbiome interactions while decreasing the intake of saturated fats, advanced glycation end products, nitrosamines and heme iron [1], [65], [68]–[70]. In addition to dietary benefits, the production of plant proteins requires less energy and resources when compared to that of animal proteins (**Figure 3**). For instance, if the consumption of meat continues to be at the same rate, phosphorus reserves could get completely depleted by the use of fertilizers within the next 50-100 years [1].

Fava bean, also known as faba bean, field bean, horse bean or broad bean, belongs to the *Fabaceae* family and is cultivated as a staple dietary food in cultures from North Africa and Middle East [3], [4]. This pulse crop can usually grow without irrigation, especially in regions of cold and rainy seasons. The most commonly grown genotypes of fava bean are: (a) *Vicia faba* var. *major*, with large seeds, (b) *Vicia faba* var. *equine,* with medium-sized seeds and (c) *Vicia faba* var. *minor,* with small seeds [74], [91].

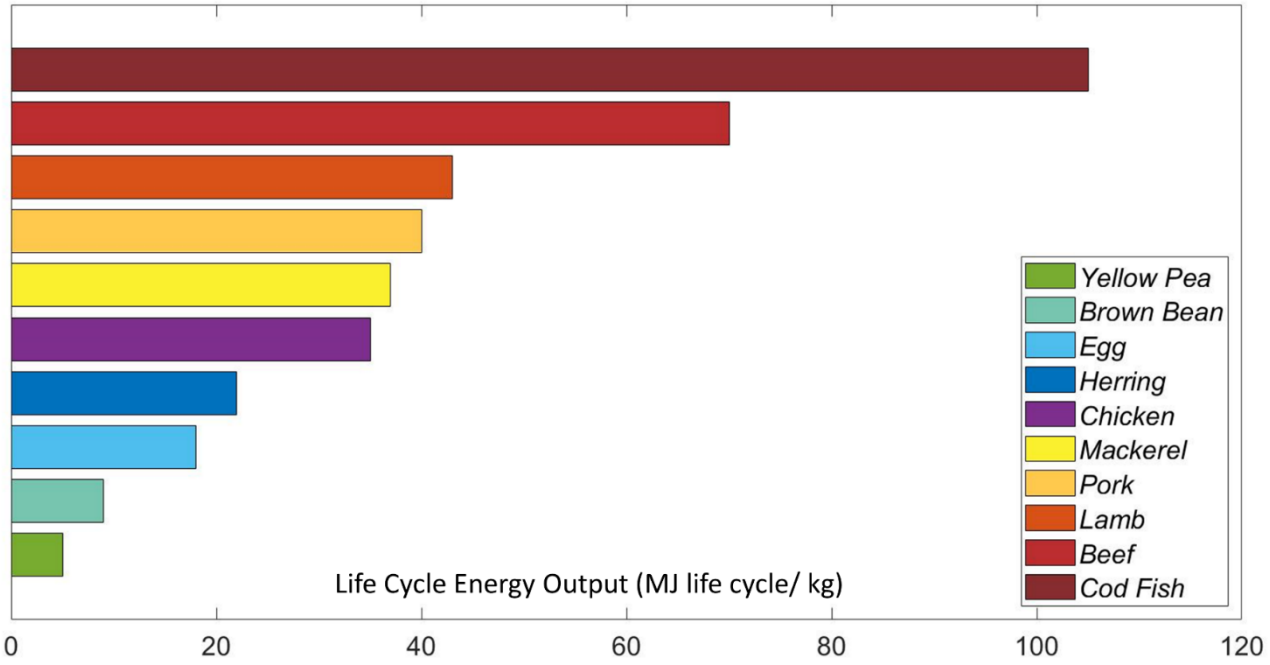


Figure 3 – Sustainability of Plant-Based Sources: Life cycle energy outputs for ready to eat proteinaceous foods represented in mega-joules life cycle per kilogram of that food category [63].

Fava bean is a sustainable protein source with a great potential in nutritional and functional properties [3]. Given the vast sphere of knowledge available in the literature on the nutritional potential of fava bean and its impact by processing, we stress in this review rather on the potential of fava bean proteins which determine functional properties of ingredients (e.g. flours, concentrates, isolates) that are relevant for industrial food applications. We draw particular attention to the impact of production and functionalization of such ingredients on their functional properties. Further attention is given to flavor and color, which play a key role in the acceptability of fava bean and its ingredients, as well as anti-nutritional factors (ANF) that are specific to fava bean and are determinant to the safety of its ingredients.

II.2. Fava Bean : A Potential Protein Source for Human Consumption

II.2.1.1. Agronomy

Fava bean is a cool-season grain legume crop, which germinates at soil temperatures as low as 12.5 °C. The crop fixates nitrogen (up to 100-200 kg·N·ha^{-1}), solubilizes insoluble phosphorus and increases microbial activity in the soil, thus improving soil properties such as organic matter content, bulk density, porosity and field capacity. In the last two decades, dry fava bean global production has increased from 3.7 to 4.9 million tons. In 2018 (latest year reported), China was the greatest producer of dry fava bean, followed by Ethiopia and United Kingdom (**Figure 4**). A noteworthy agronomic benefit of fava bean is its high yield

per harvest area. In 2018, for instance, fava bean crops had the lowest requirement in harvest area when compared to other pulse crops for a similar or higher yield (**Figure 4**). While the yield of green pea (*Pisum sativum*) matches that of fava bean, the area of its harvest of green pea is five times as much compared to that of fava bean (**Figure 4**).

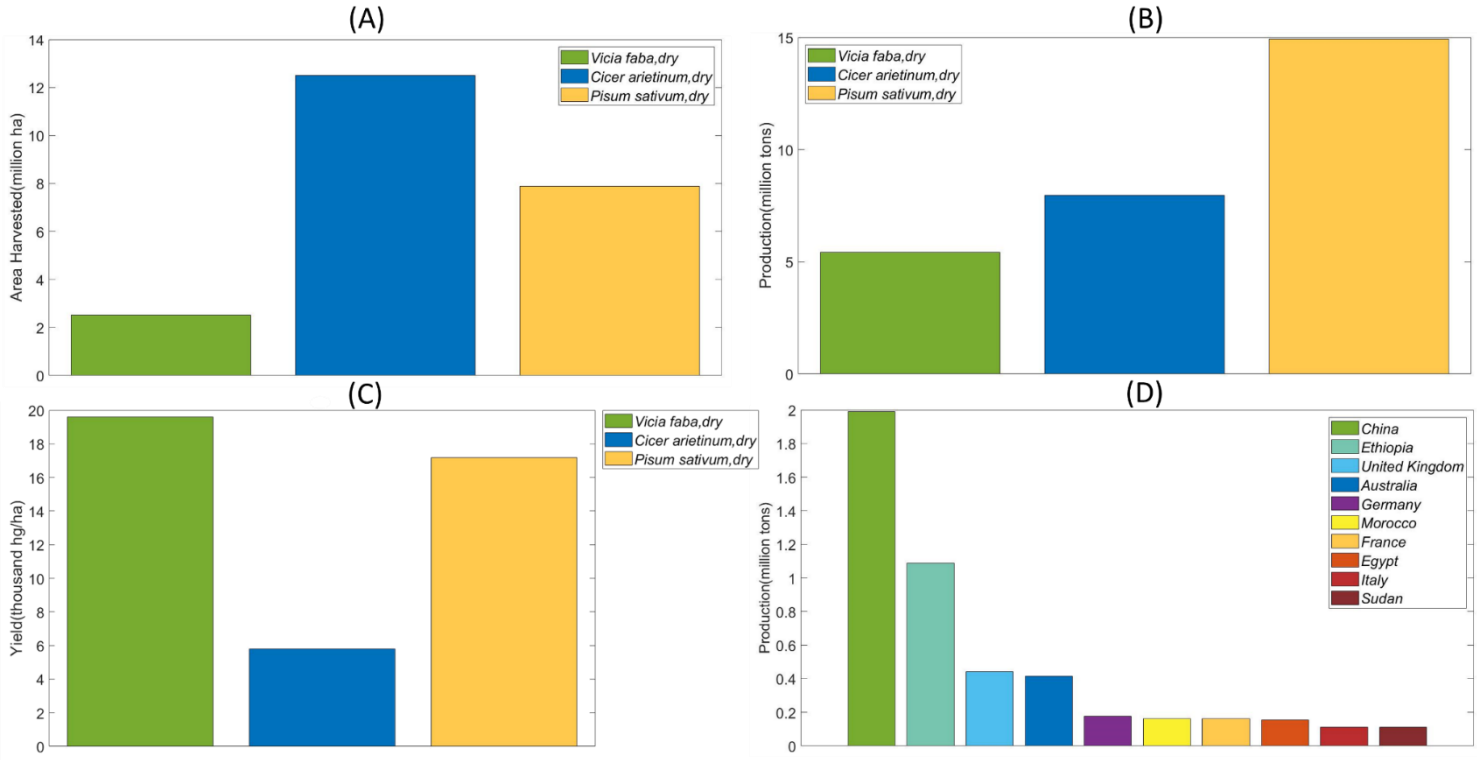


Figure 4 – Agronomic Benefit of Fava Bean: 2017 statistics of some key pulses including fava bean (*Vicia faba*), pea (*Pisum sativum*) and chickpea (*Cicer arietinum*) on the basis of area utilized for crop harvest (A), crop production (B), and dry crop yield (C) along with fava bean production in top producers in the world (D) [92].

II.2.1.2. Nutrition

Pulses, including fava bean, are considered to be a major source of proteins, fibers, vitamins, minerals and compounds possessing antioxidant and anti-carcinogenic properties [7]. Fava bean is nutritionally beneficial owing to its high protein-to-carbohydrate ratio when compared to other pulses (**Figure 5**), as well as its amino acid profile compared to the adults' requirements for essential amino acids (**Table 1**).

Table 1 – Relative Amino Acid Levels in fava bean seeds and globulins.

Amino Acids	Whole Seed[1]*	Whole Seed[2]¥	Legumin[3]*	Vicilin[3]*	Adult Daily Requirement[4]¥
Tyrosine	3.5	3.67 - 4.27	2.61	2.59	
Tryptophan	nd	nd	nd	nd	3.8
Phenylalanine	4.5	3.58 - 5.25	3.56	5.20	
Methionine	0.9	0.79 - 1.10	0.59	0.31	2.2
Cysteine	nd	1.10 - 1.42	0.80	0.31	
Lysine	7.1	5.80 - 8.56	4.57	7.13	4.5
Histidine	2.8	2.70 - 4.15	2.44	1.95	1.5
Threonine	4.2	3.76 - 4.39	4.28	3.27	2.3
Valine	5.1	3.75 - 5.64	4.91	4.90	3.9
Isoleucine	4.5	3.29 - 4.64	3.98	5.12	3.0
Leucine	8.4	6.60 - 8.27	7.84	9.21	5.9
Arginine	9.8	8.80 - 12.10	7.95	5.59	
Glycine	5.1	4.15 - 4.93	7.40	5.00	
Alanine	6.6	3.43 - 3.53	6.10	4.87	
Proline	4.7	4.42 - 6.29			
Serine	6.1	4.68 - 6.29	6.50	6.59	
Glutamic acid	14.9	14.20 - 15.89	16.40	15.30	
Aspartic acid	12.0	9.67 - 10.98	10.6	11.6	

*- all the values have been reported as % of total amino acid residues

¥ - all the values have been reported as g amino acid /100g protein

nd - not determined

Note: Tryptophan was not determined due to analytical challenges and low quantities. In any case, legumin monomer before post translational modification consists of four tryptophan residues whereas vicilin monomer pro-polypeptide has none [93]–[95].

1 = Hove et al., 1978, 2 = Makkar et al., 1997, 3 = Jackson, Boutler, & Thurman, 1969, 4 = FAO/WHO, 2007

While cereals are rich in cysteine and methionine and limiting in lysine, pulses are rich in lysine and poor in cysteine and methionine, and their dietary intake along with cereals in suitable quantities fulfills the daily requirement of essential amino acids [8], [9]. Fava bean seeds contain 23-41 % proteins on dry weight basis [96], [100], [101]. About 80 % by weight of the total seed proteins constitute enzymatically inactive seed storage proteins present in seed cotyledons supplying nutrients to help the seed germinate into a seedling [78], [102]. The storage proteins exist as protein bodies that surround larger starch granules inside individual cells within the microstructure of the cotyledon (**Figure 6**). In particular, nutritional quality of fava bean proteins has been studied extensively in the literature [103]–[106].

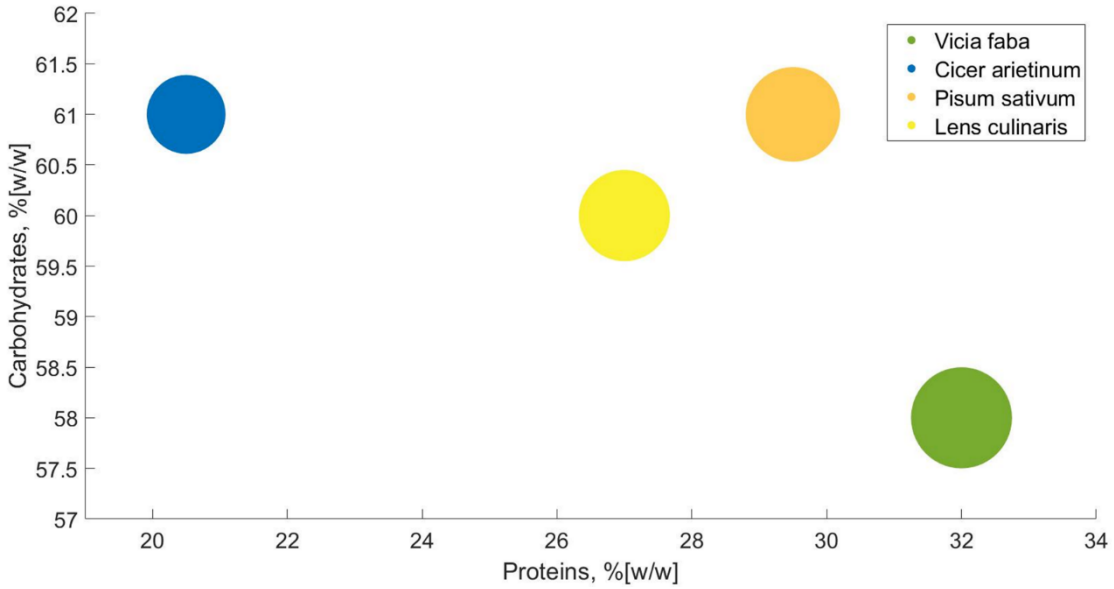


Figure 5 – Nutritional Significance of Fava Bean: Bubble plot of proteins %[w/w] dry weight as a function of carbohydrates %[w/w] dry weight indicating a higher protein to carbohydrate ratio in fava bean (*Vicia faba*) [96], [100], [101] compared to chickpea (*Cicer arietinum*) (Bramsnaes & Olsen, 1979; FAO/WHO, 1991; Patterson et al., 2005; Boye et al., 2010), pea (*Pisum sativum*) [96], [100] and lentil (*Lens culinaris*) [100], [108], [110]. Dietary intake of higher proportion of proteins compared to carbohydrates has favorable health benefits [111].

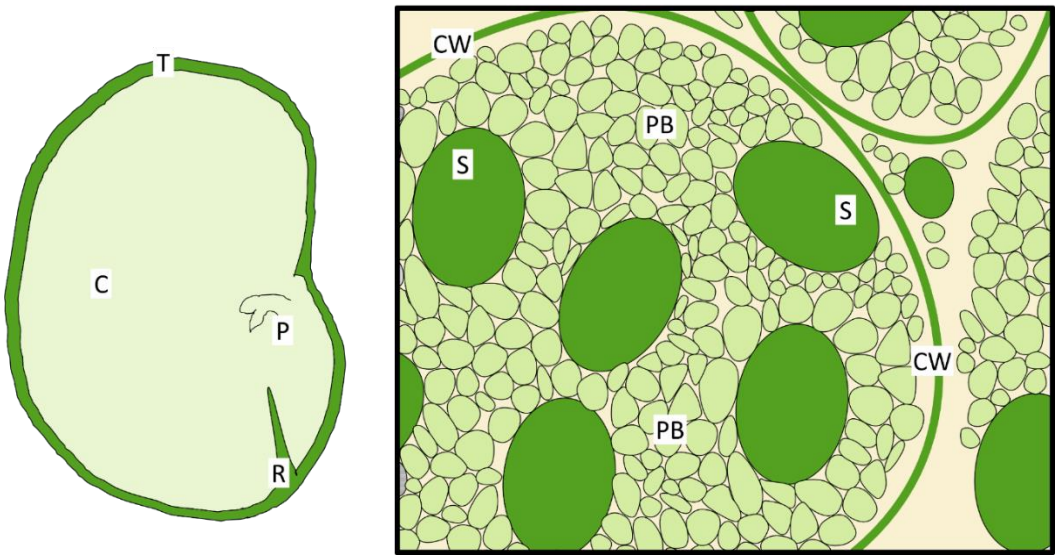


Figure 6 – Protein Localization in Fava Bean Seeds: Illustration of a seed of fava bean L. *(Left)* consisting of testa or the seed coat (T), cotyledon (C) containing food reserves mainly starch and protein bodies, plumule or the embryonic shoot (P) and radicle or the embryonic root (R); along with the illustration of the microstructure of the cotyledon comprising of large starch granules (S) of size ranging between 18-23 µm surrounded by smaller protein bodies of size 1-10 µm, together embedded with a cell wall (CW) structure [112]–[116]

II.2.1.3. Functional Properties

Functional properties are a result of physico-chemical phenomena occurring during ingredient or food product storage, processing and consumption. They are driven mainly by protein properties: their hydration in fluids, their surface activity (hydrophobicity, charge distribution) and their structure (primary, secondary, tertiary and quaternary), which together contribute to solubility, wettability, aggregation, interfacial adsorption in colloids (foams and emulsions) and rheological characteristics (viscosity, elasticity, adhesiveness and gelation). Since food products exhibit a multicomponent character, their functionalities should be considered as a result of the interaction of proteins with other constituents, including macro-constituents (lipids, polysaccharides) and micro-constituents (phenolic compounds, phytic acid, saponins, etc.) (Schwenke, 2001; Alu'datt et al., 2013; Mirmoghtadaie, Shojaee Aliabadi, & Hosseini, 2016). These properties can be modified during ingredient production (due to change in composition and the use of process conditions) or by ingredient post-processing (due to the use of process conditions). We henceforth refer to modification of functional properties of ingredients as 'functionalization'. Functional food ingredients from fava bean are potential foaming, emulsifying and gelling agents which can be used for producing dairy and meat alternatives (Boye, Zare, & Pletch, 2010; Multari et al., 2015; Singhal, Karaca, Tyler, & Nickerson, 2016). Fava bean proteins have superior functional properties when compared to animal proteins, and even in comparison to other pulses sources [119], [120]. These functional properties of fava bean ingredients depend on bean variety [121], protein structural conformation [26], [78], [122]–[124], interactions with other macromolecules [125], [126] and processing (Sosulski & McCurdy, 1987; Cepeda, Villarán, & Aranguiz, 1998; Luo & Xie, 2013; Jiang, Wang, & Xiong, 2018; Yang, Liu, Zeng, & Chen, 2018). The effect of processing on functional properties are discussed more in depth in further sections.

II.2.1.4. Protein Diversity

Fava bean proteins comprise many types of proteins that can be classified based on their solubility in different solvents, namely albumins, globulins, glutelins and prolamins [102].

II.2.1.4.a. Globulin Proteins

Amongst seed storage proteins, approximately 85 % by weight consist of salt-soluble proteins called globulins which are rich in aspartic acid, glutamic acid, leucine and arginine [128], [129]. Globulins are classified based on their sedimentation coefficients ($S_{20.w}$) into 7S proteins (vicilin and convicilin in fava bean, chickpea, green pea and lentil; conglycinin in soybean (*Glycine max)* and β-conglutin in lupin (*Lupinus spp*.)) and 11S proteins (legumin in fava bean, chickpea, green pea and lentil; glycinin in soybean and α-conglutin in lupin), each type being conserved in all other species of legumes [10], [102], [130], [131].

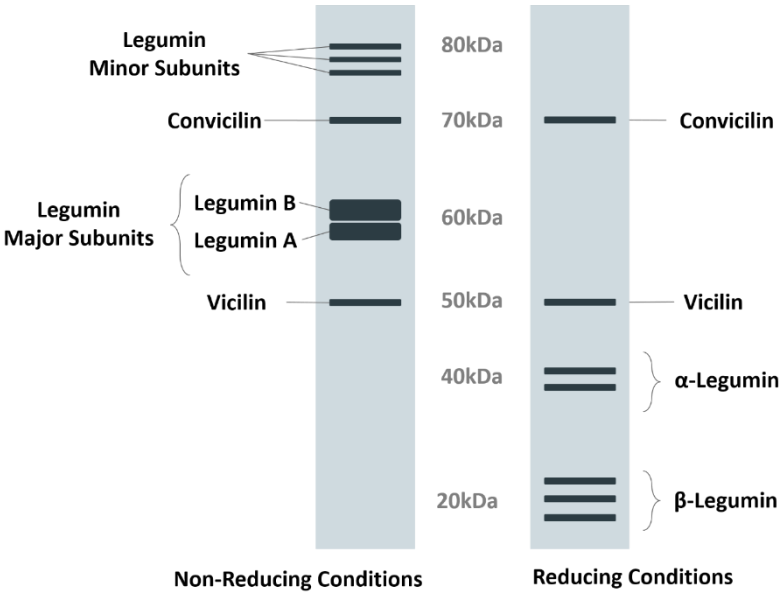


Figure 7 – Subunit Heterogeneity of Fava Bean Globulins: Representation of subunit heterogeneity in the globulins of fava bean through 1-dimensional polyacrylamide gel electrophoresis in non-reducing and reducing conditions [132], [133].

In fava bean, legumin (11S globulin) is a hexameric holoprotein, whereas vicilin (7S globulin) exists as trimers, both made of polymorphic subunits encoded by multigene families. With isoelectric points at pH 4.8 and pH 5.5 respectively, legumin and vicilin can be separated using isoelectric precipitation. In mature fava beans, legumin accounts for 55 % of the total seed protein. Major subunits of fava legumin are of two types: A and B. Legumin A contains methionine residues, whereas type B is methionine-free. The subunits of legumin exhibit heterogeneity in electrophoresis and in ion-exchange chromatography. Four major 60 kDa subunits have been isolated from legumin using ion exchange chromatography in 6 mol/L urea. Two other legumin subunits with 75 kDa and 80 kDa have also been identified (**Figure 7**). All these subunits comprise α-chains (MW 40 kDa) and β-chains (MW 20 kDa) that are linked by a disulfide bridge. This disulfide bond is formed before the post-translational processing of the αβ precursor chains, and therefore legumin A α-chain would always be exclusively linked to the legumin A β-chain. The formation of the hexameric state is a statistical mixture of different subunits (minor and major subunits) that arrange between themselves to form a functional legumin (**Figure 8**) and thus possess different chromatographic profiles with heterogeneous molecular weights [128], [134], [135]. Vicilin represents about 30 % of the storage proteins and convicilin corresponds to 3.2 % of the total seed protein content in fava bean. Vicilin and convicilin polypeptides comprise MW 50 kDa and MW 70 kDa subunits, respectively. These subunits are both cysteine-free and are not linked by disulfide bridges. At pH values below 3 or above 11, vicilin dissociates into two 3S molecules [132], [133].

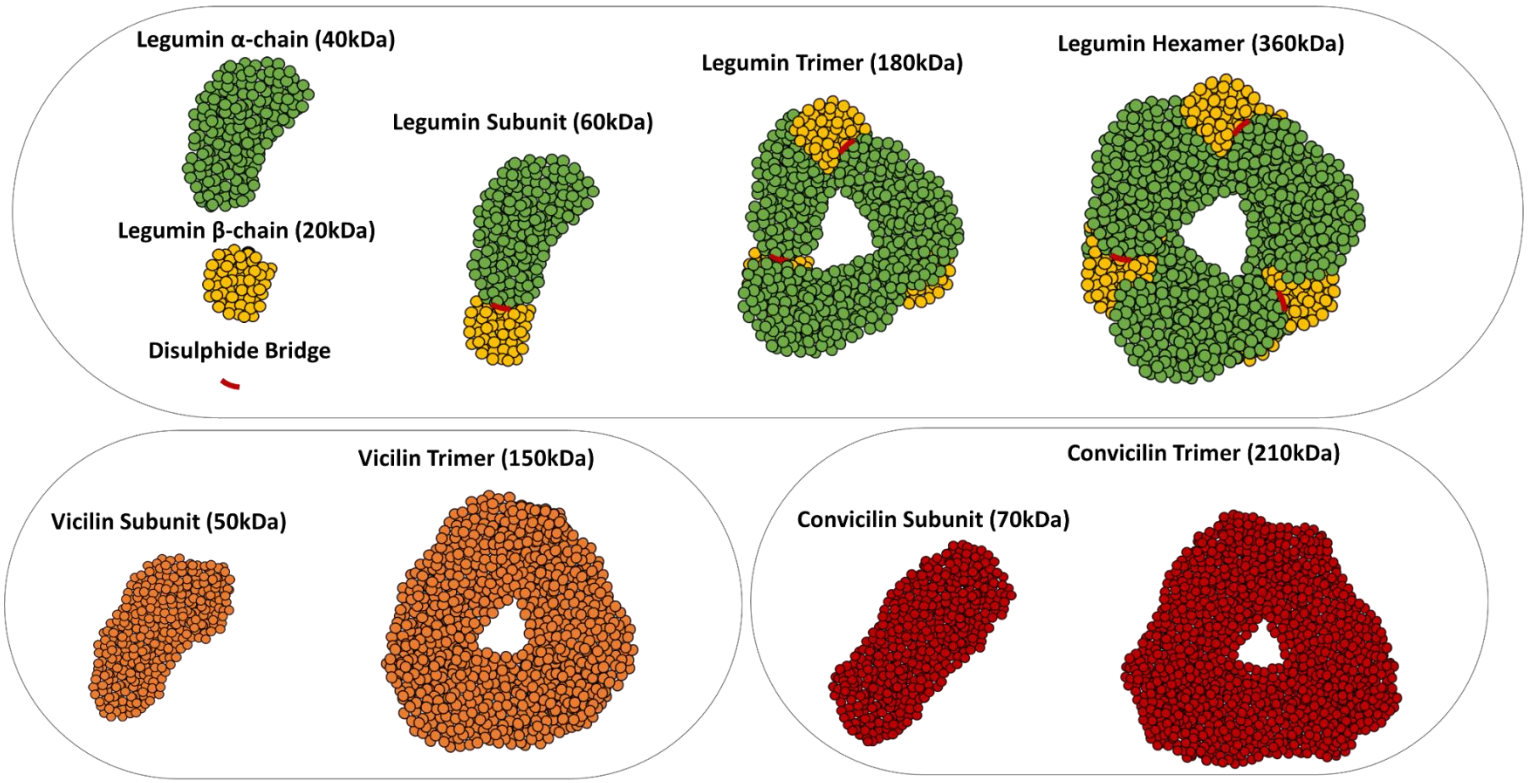


Figure 8 – Native Subunit Arrangement of Fava Bean Globulins: Legumin (MW 360 kDa) forming a hexamer whereas vicilin (MW 150 kDa) and convicilin (MW 210 kDa) forming trimers in their native quaternary conformations. This is hand-drawn from homology modelled protein sequences of fava bean to illustrate the protein subunits arrangement. X-ray crystallographic data is yet to be obtained for fava bean proteins [136], [137].

II.2.1.4.b. Non-Globulin Proteins

Another group of seed proteins, prolamins, are alcohol-soluble proteins devoid of lysine and tryptophan, but rich in leucine, proline and glutamic acid [3]. They can be solubilized in alcohol/water mixtures (ethanol/water 60/40 or 70/30 [v/v], or propan-1-ol/water 50/50 [v/v]) and contain high levels of glutamine and proline [102]. Glutelins on the other hand, are soluble in sodium hydroxide and show an amino acid profile similar to those of prolamins, but with higher levels of glycine, methionine and histidine [3]. Albumins contain higher amounts of sulfur-containing amino acids than globulins or other seed proteins [138].

Seed albumins in fava bean are primarily metabolic proteins that may or may not have enzymatic functions, including protease inhibitors (*e.g.* trypsin inhibitor), lectins (*e.g.* phytolectin), albumin-2 (PA2), defensins 1 and 2 and Bowman-Birk inhibitors (BBI) [139], [140]. Enzymes in the seed regulate the synthesis, transport and storage of starch and proteins during every stage of seed development. Transcripts coding certain enzymes involved in carbohydrate metabolism have been identified in fava bean seeds, *viz*. sucrose

phosphate synthase (118 kDa), ADP glucose pyrophosphorylase (MW 200-240 kDa), invertase (MW 64 kDa) and glucan phosphorylase (MW 110 kDa) [141]–[143]. Sucrose-binding proteins (SBPs), which are homologous to vicilin, have also been found in fava bean seeds [128]. Active transport systems, *viz*. sucrose carriers (*VfSUT1*) and hexose carriers (*VfSTP1*), help in the transport of sugars in different parts of the bean [144]. Another enzyme, phosphoenolpyruvate carboxylase (PEPCase), is found in developing cotyledons that synthesize organic acids which are essential for amino acid synthesis [145]. Proton-coupled amino acid and peptide transporters in the cotyledons, such as amino acid permeases (*VfAAP1, VfAAP3* and *VfAAP4*) and peptide transporters (*VfPTR1* and *VfPTR2*), mobilize nitrogen during seed development [146], [147]. Bowman-Birk type serine proteinase inhibitor (MW 7 kDa) has been isolated and characterized in fava bean [148]. The aquaporin family, which includes tonoplast intrinsic protein (*Vf*TIP1, *Vf*TIP2, *Vf*TIP3), plasma membrane intrinsic proteins (*Vf*PIP2) and nodulin26-like intrinsic protein (*Vf*NIP1), ensures no loss of seed viability by transporting water during seed drying [149]. A recent study characterized certain fava bean proteins, including elongation factor Tu (43 kDa), citrate synthase (47 kDa), GroEL chaperonins (97 kDa and 52 kDa), phosphate ABC transporter periplasmic substrate-binding protein (36 kDa), electron transfer flavoprotein subunit alpha (31 kDa), alkyl hydroperoxide reductase C22 subunit (21 kDa), motA/TolQ/ExbB proton channel family protein (27 kDa), htlv-1 Gb21 ectodomain maltose-binding protein chimera (49 kDa) and putative sucrose-binding protein (47 kDa) [78].

II.3. Fava Bean: Limitations for Human Consumption

Despite the aforementioned potential of fava bean in agronomy, nutritional and functional properties, several aspects might hamper its utilization as a source of proteins for the human diet. Certain key safety (anti-nutritional) and sensory (flavor and color) limitations are discussed below. The effect of processing on these aspects are considered in further sections.

II.3.1.1. Anti-Nutritional Factors

Concerning protein digestibility and safety, fava bean proteins seem to be affected by the presence of ANF, which reduce the bioavailability of proteins and minerals.

II.3.1.1.a. Vicine & Convicine

Vicine (2,6-diamino-4,5-hydroxypyramidine-5-[β-D-glucopyranoside]) and convicine (2,4,5-trihydroxy-6-aminopyramidine-5-[β-D-glucopyranoside]) are pyramidine glycosides which are found in the genus *Vicia* and popular ANF associated with fava bean beans and ingredients. Dried, dehulled fava bean contains 0.73 %[w/w] vicine and 0.30 %[w/w] convicine [150]. Hydrolysis of β-glucosidic bonds transforms them into their respective aglycones *viz*. divicine (2,6-diamino-4,5-hydroxypyramidine) from vicine and isouramil (6-amino-2,4,5-trihydroxypyramidine) from convicine. The reaction occurs either by β-

glucosidase during the development of the seeds, or by microbial β-glucosidase during consumption and digestion in the large intestine and cecum (Rizzello et al., 2016). These aglycones cause favism, a fatal disease characterized by hemolytic anemia which is common in the Middle East and the Mediterranean basin [16]–[18]. Favism is a life-threatening disease for sensitive individuals having red blood cells (RBCs) with low-activity variants of glucose 6-phosphate dehydrogenase (G6PD). G6PD protects cells from oxidative stress by producing reduced nicotinamide adenine dinucleotide phosphate (NADPH) and regenerating reduced glutathione in the hexose monophosphate shunt. Oxidative stress and resulting phagocytosis are a result of G6PD deficiency. Thus, fava bean ingestion causes acute hemolysis as the aglycones produced from vicine and convicine foster oxidative damage in G6PD deficient individuals (Rizzello et al., 2016).

II.3.1.1.b. Other Anti-Nutritional Factors

Fava bean also comprises other factors commonly found in pulses including saponins, glycosides, tannins, alkaloids, phytic acid conjugates and lectins that either reduce digestibility of seeds and/or favor development of certain pathologies (Gupta, 1987; Gupta, Gangoliya, & Singh, 2015). Lectins, a class of glycoproteins, constitute about 2-10 % of total proteins in legume seeds (Gupta, 1987). They reversibly bind to specific sugars and glycoproteins on gut cellular surface and interfere with the digestion and absorption of nutrients, along with favoring development of food allergies [153]. Fava bean lectins called favins contain two chains, an α-chain (5.6 kDa) and a β-chain (20 kDa), which are linked to carbohydrate moieties [154]. Saponins, on the other hand, induce erythrocyte hemolysis, enzyme inhibition and affect cholesterol levels, nutrient absorption and growth [3], [59], [155]. Relationship between foaming property of saponins and bloating has been established in rumens [44]. Tannins are water soluble polyphenols which form precipitates with proteins and metal ions, thereby protecting plants against pathogens and rotting by depriving the organisms from metal ions and proteins [156]. Phytic acids are main storage forms of phosphorus in many plant tissues. They bind with proteins, minerals and starches, forming insoluble complexes and reducing their bioavailability [3], [157].

II.3.1.2. Flavor

Consumer acceptance of pulse-based products is hampered by the presence of undesirable flavors [19]. Flavor perception is the result of a multimodal combination of stimuli that arise mainly from the interaction of (i) volatile odorant compounds and (ii) non-volatile taste molecules with sapid and/ or olfactory receptors on the tongue and/ or in the nasal cavity [19]. The flavor of a plant is primarily dependent on its genetic and structural characteristics (*i.e.* the availability and distribution of enzymes and flavor precursors) and both environmental and cultivation aspects [32]. Flavor-contributing volatile and non-volatile

compounds are either inherent to the grain or produced during the food supply chain including harvesting, processing, and storage [19].

II.3.1.2.a. Volatile Odorant Compounds

Odorant compounds are organic molecules comprising alcohols, aldehydes, ketones, carboxylic acids, terpenes, sulfur-containing compounds, methoxypyrazines and aromatic hydrocarbons (Murray, Shipton, Whitfield, & Last, 1976; Jeleń & Gracka, 2016; Singh, 2017). Their typical low molecular mass (<300 Da) carbonic chains result in strong hydrophobicity, as well as the ability of volatilizing into the gas phase and reaching olfactory receptors in the nasal cavity (Jeleń & Gracka, 2016; Wang & Arntfield, 2017; Roland et al., 2017). While a minor number of odorant compounds are present in the natural state of legume grains, as is the case for the highly odorant 3-alkyl-2-methoxypyrazines (mainly isobutyl, isopropyl and *sec*-butyl) (Murray & Whitfield, 1975; Jakobsen, Hansen, Christensen, Brockhoff, & Olsen, 1998), the majority of odor-active volatiles arise from the degradation of non-volatile precursors such as lipids, amino acids, carbohydrates and carotenoids during harvesting, post-harvest processing and storage [19], [32], [35].

Lipid oxidation *via* enzymatic and/or non-enzymatic pathways is deemed the primary source of flavor-related molecules in pulse ingredients [32], [35], [162]. Enzymatic lipid oxidation involves the action of lipoxygenase (LOX) on free or esterified fatty acids, whereas non-enzymatic lipid oxidation comprises free-radical chain reactions that occur in the presence of molecular oxygen and initiators such as light, photosensitizers (*e.g.* chlorophylls), metallic ions (mainly Fe^{2+} and Cu^{2+}) and/or temperature [52], [162]–[165]. Fava bean contains roughly 1.3-3.2 g/100g of lipids, amongst which 30.7-56.0 % correspond to linoleic acid ($C_{18:2}$, *n*-6), a polyunsaturated fatty acid (PUFA) that is highly susceptible to undergo oxidative reactions [166]–[169].

The volatile compounds reported in fava bean consist mainly of aldehydes and alcohols, which represent over 60 % of the total composition in volatiles detected in the seeds [33], [169]. Alcohols and aldehydes are shown to be responsible for the green, grassy and beany notes associated with fava bean beans [35], [83], [170]. Other compounds that potentially play a role in fava bean flavor on account of their low odor thresholds are 3-isopropyl-2-methoxypyrazine (0.004 ppb in water), a naturally-occurring methoxypyrazine associated with pea-like and earthy aromas (Murray & Whitfield, 1975; Czerny et al., 2008), 2-pentylfuran (6 ppb in water), a furan which can impart green, beany and earthy notes [33], [162], [172], [173], and limonene (10 ppb in water), a terpenoid characterized by citrus, green and fruity odors [169], [170], [172]. A detailed listing of the volatile compounds detected in the seeds of fava bean and their theoretical odors is presented on **Table 2**.

II.3.1.2.b. Non-Volatile Taste Compounds

Non-volatile sapid molecules in legumes are developed essentially during the growing stage. Taste originates from an initial dissolution of non-volatile compounds in the saliva and the stimulation of specialized receptor cells on the tongue and throughout the oral cavity [19], [32], [51]. Pulses are mainly associated with bitter and astringent tastes, which could be related to the inherent presence of sapid glycosylated compounds such as saponins, isoflavones, flavonols and phenolic acids [19], [35], [52], [83]. Saponins are non-volatile triterpene glycosides composed of non-polar aglycone backbones with one or more sugar moieties [59], [174]. They are distributed in the cells of a wide variety of plants, being particularly noteworthy in pulses, in which they exert foaming properties in aqueous solutions and can impart bitter or metallic tastes and astringency [174]. Amongst the saponins identified in fava bean seeds are soyasapogenol B (0.020 $mg \cdot g^{-1}$), soyasaponin Bb (0.040 $mg \cdot g^{-1}$), soyasaponin βg and azukisaponin IV [59], [175], [176]. Soyasaponin βg differs from the Bb type in that it contains a 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) moiety attached to its C_{22}, imparting enhanced bitterness [48].

Polyphenols are also known to impart astringent sensations in the mouth by forming insoluble precipitates with salivary proteins [177], [178]. Certain phenolic compounds have additionally been reported to trigger bitter taste receptors, as is the case for the aglycones of quercetin and kaempferol, which are the major flavonols accounted for in pulses [19]. The main phenolic compounds detected in fava bean are flavonols (glycosylated derivates of quercetin, kaempferol, apigenin and myricetin), flavan-3-ols (catechin compounds), flavanonols, flavanones, isoflavones (genistein and daidzein), proanthocyanidins (prodelphynidins and procyanidins) and phenolic acids (caffeic, ferulic, p-coumaric, synaptic, fukiic and protocatechuic acids). They are mostly located in the cotyledons of the fava bean seeds [179]–[182].

Table 2 – Volatile Compounds in Fava Bean Seeds

Compounds	Theoretical odor[3]	CAS Registry
Alcohols		
1-heptanol[1,2]	leafy, coconut, herbal, strawberry, chemical, musty, sweet, woody, violet,	111-70-6
(E,E)-3,5-octadien-2-ol[2]	nd	126869-35-0
1-hexanol[1,2]	oil, alcoholic, ethereal, resin, fusel, sweet, fruity, flower, green	111-27-3
1-nonanol[1,2]	dusty, rose, fat, floral, green, clean, wet, orange, fresh, bitter, oily	143-08-8
1-octanol[1,2]	burnt, orange, rose, waxy, chemical, metal, aldehydic, mushroom, green	111-87-5
1-octen-3-ol[1,2]	raw, fishy, oily, earthy, fungal, chicken, mushroom, green	3391-86-4
1-pentanol[1,2]	oil, balsamic, vanilla, fusel, sweet, balsam	71-41-0
1-propanol[2]	alcoholic, fermented, alcohol, musty, fusel, pungent, peanut	109-78-4
2,3-butandiol[2]	nd	344750-80-7
2-butanol[2]	oily, sweet, wine, apricot	05-11-17
2-ethyl-1-hexanol[1]	mild, oily, sweet, slightly floral	104-76-7
2-methylbutanol[2]	roasted, onion, malt, wine, fruity	137-32-6
2-octen-1-ol[1]	oily, nutty, fatty	18409-17-1
2-pentanol[2]	waxy, stale creamy, chicken fatty	6032-29-7
2-phenylethanol[2]	lilac, rose, rose water, honey, rose flower, floral, spice, bitter, rose	60-12-8
2-phenylpropan-2-ol[1]	Hyacinth	617-94-7
2-propanol[2]	Alcoholic	67-63-0
3-methylbutanol[2]	oil, alcoholic, burnt, whiskey, malt, banana, fusel, fruity	123-51-3
3-octanol[2]	citrus, nut, moss, herbal, earthy, woody, melon, minty, mushroom, spicy	589-98-0
4-ethylcyclohexanol[2]	nd	19781-62-5
benzyl alcohol[2]	berry, balsamic, rose, floral, walnut, sweet, cherry, flower, grapefruit	100-51-6
ethanol[2]	alcoholic, ethereal, medical, sweet	1725-82-2
Aldehydes		
(E)-2-heptenal[1]	soap, vegetable, fat, fresh, fatty, pungent, almond, green	18829-55-5
(E)-2-nonenal[2]	fatty, orange peels	18829-56-6

(E)-2-octenal[1,2]	fatty, walnut, fruity, leaf, green	2548-87-0
2-methylbutanal[2]	cocoa, coffee	96-17-3
2-methylpropanal[2]	nd	78-84-2
3-methylbutanal[2]	peach, sour, chocolate, ethereal, malt, fatty, aldehydic	590-86-3
benzaldehyde[1,2]	cherry, almond, sweet, burnt sugar, sharp, strong, bitter	100-52-7
decanal[1,2]	citrus, soap, orange peel, tallow, waxy, floral, sweet, aldehydic	112-31-2
heptanal[1,2]	citrus, ozone, fat, herbal, fresh, wine, rancid, fatty, aldehydic, green	111-71-7
hexanal[1,2]	leafy, grass, sweaty, tallow, fat, fresh, fatty, fruity, aldehydic, green	66-25-2
nonanal[1,2]	citrus, lime, orange peel, rose, fat, green, fishy, waxy, fatty, grapefruit	124-19-6
octanal[1,2]	lemon, citrus, soap, orange peel, fat, waxy, fatty, aldehydic, green	124-13-0
pentanal[1]	bready, fermented, berry, malt, pungent, fruity, nutty, almond	110-62-3
phenyl acetaldehyde[2]	hyacinth, honey, clover, sweet, hawthorne, cocoa, grapefruit, green, peanut, floral, bitter	122-78-1
p-isopropylbenzaldehyde[2]	spicy, acid, herbal, sharp, oily, cumin, green	27246-91-9
Alkanes		
2,4-dimethyldodecane[2]	nd	6117-99-3
2,6-dimethyldecane[2]	nd	13150-81-7
3,7-dimethylnonane[2]	nd	17302-32-8
3-methyltridecane[2]	nd	6418-41-3
decane[2]	nd	124-18-5
dodecane[1,2]	nd	112-40-3
heptane[1,2]	ethereal, sweet	142-82-5
hexane[2]	nd	110-54-3
nonadecane[2]	nd	629-92-5
nonane[1,2]	nd	111-84-2
octane[1,2]	Gasoline	111-65-9
pentadecane[2]	nd	629-62-9
pentane[2]	nd	109-66-0
tetradecane[2]	nd	90622-46-1

tridecane[1,2]	nd	629-50-5
undecane[1]	nd	1120-21-4
Alkenes		
(E)-3-ethyl-2-methyl-1,3-hexadiene[1,2]	nd	61142-36-7
Aromatic hydrocarbons		
1,2,3-trimethylbenzene[2]	nd	526-73-8
ethylbenzene[1,2]	nd	100-41-4
isopropylbenzene[1]	nd	98-82-8
p-isopropyltoluene[2]	Citrus	13816-33-6
p-xylene[1]	nd	106-42-3
styrene[1]	balsam, gasoline, floral, sweet, plastic	100-42-5
toluene[1,2]	paint, sweet	108-88-3
Esters		
2-ethylhexyl acetate[2]	nd	103-09-3
methyl 3-methylbutanoate[2]	strong, pineapple, apple, fruity	556-24-1
δ-caprolactone[2]	coconut, cream, chocolate	502-44-3
Y-caprolactone[2]	coconut, tobacco, coumarin, herbal, sweet	502-44-3
Furans		
2-ethylfuran[1]	earthy, sweet, burnt, malty	3208-16-0
2-pentylfuran[1,2]	butter, green bean, vegetable, earthy, beany, fruity, metallic, green	3777-69-3
4-Methyl-4-vinyldihydro-2(3H)-furanone[2]	nd	nd
dihydro-2(3H)-furanone[2]	caramel, oily, fatty, sweet, creamy	96-48-0
Ketones		
(E,E)-3,5-octadien-2-one[1,2]	fat, fatty, fruit, fruity, grassy, mushroom, green	30086-02-3
(E,Z)-3,5-octadien-2-one[1]	nd	4173-41-5
2,3-octanedione[1]	"warmed-over"	585-25-1
2-butanone[1,2]	ethereal, ether, fruity, acetone, camphor	78-93-3
2-heptanone[1,2]	coconut, soap, herbal, sweet, woody, fruity, spicy, cinnamon	110-43-0
2-nonanone[2]	soap, herbal, fresh, fishy, hot milk, earthy, sweet, soapy, weedy, green	821-55-6
2-octanone[2]	natural, earthy, gasoline, weedy, herbal, woody, bitter, soap	111-13-7

3-hydroxy-2-butanone[2]	butter, cream, milky, fatty, creamy, sweet, dairy, buttery	513-86-0
3-octanone[2]	butter, herbal, resin, fresh, mushroom, sweet, lavender, herb	106-68-3
3-octen-2-one[1]	crushed bug, nut, herbal, earthy, hay, sweet, blueberry, mushroom, spicy	1669-44-9
6-methyl-5-hepten-2-one[1,2]	pepper, apple, mushroom, citrus, musty, rubber, nutty, green, hazelnut, bitter, lemongrass	110-93-0
acetone[1,2]	solvent, apple, pear, ethereal	107-87-9
acetophenone[2]	mimosa, hawthorn, sweet, acacia, almond, pungent, chemical, flower, bitter, must	98-86-2
Organic acids		
2-methylbutanoic acid[2]	sour, sweat, acid, strawberry, roquefort cheese, pungent, cheese	116-53-0
3-methylbutanoic acid[2]	sour, sweat, acid, stinky, sweaty, animal, rancid, tropical, feet, cheese	503-74-2
acetic acid[2]	sour, pungent, sharp, vinegar	64-19-7
hexanoic acid[2]	fatty, sour, sweat, cheese	142-62-1
Terpenes		
D-limonene[2]	mint, lemon, citrus, orange, fresh, sweet	5989-27-5

NOTE: nd – non-determined, [1] = Oomah et al., 2014; [2] = Akkad et al., 2019; [3] = Garg et al., 2017

II.3.1.3. Color

Pigmented pulses including fava bean, chickpea, lentil and kidney bean (*Phaseolus vulgaris*) are rich sources of phenolic compounds. Seed coats contain tannins as the main phenolic compounds with antioxidant activity [184]. In fava bean, tannins constitute 72-82 % of the total phenolics content in the hull of colored beans. 96 % of the seed tannins are comprised of proanthocyanidins [55], [185]. Proanthocyanidins in white- and brown- colored fava bean are 0 % and 6 % respectively in seed hulls [186]. Single recessive genes (*tan tan*) in fava bean have been identified responsible for the absence of tannins. Furthermore, zero-tannin lines are devoid of anthocyanin pigments in their flower petals and dark seed coats as a result of blocked flavonoid biosynthetic pathways [185], [187].

Seed color is thus an important factor as for instance, seeds with dark brown testa color are associated with poor acceptability of the seeds in the market. Depending on storage and post-harvest processing of the seeds, the color of freshly-harvested beige seed testa can develop into brown or dark brown color. Temperature, seed moisture content, light and oxygen can cause discoloration of fava bean and other beans [55], [185]. The effect of processing on fava bean color is discussed in further sections.

II.4. Processing of Fava Bean for Food Applications

Industrially relevant ingredients from fava bean - flours, concentrates and isolates, have been extensively studied in the literature. They can be prepared using a combination of various processing methods, along with alternatives to the classically utilized unit operations during the processing and extraction of protein-rich ingredients (**Figure 9**). These operations can be divided into ingredient production (including size reduction and protein extraction) followed by ingredient functionalization (occuring either during or after ingredient production) and finally ingredient application in foods.

II.4.1.1. Ingredient Production

II.4.1.1.a. Size Reduction

Before fava bean beans are milled, they may be air dried [188], soaked [189], hot-air or microwave treated [190]. Dehulling of the fava bean seeds is an optional step during processing. The pre-treated beans are milled to produce flours, which are optionally defatted, reducing up to 50 % [w/w] of total lipids in the fava bean flours (**Figure 9**). Industrially, a defatting step is discouraged due to the use of solvents including isopropanol and supercritical carbon dioxide [26], hexane [191] or petroleum ether [192].

II.4.1.1.b. Protein Extraction

Protein extraction from pulse flours yields either protein concentrates or protein isolates. Concentrates are prepared using dry or wet processing methods, whereas isolates derive solely from wet processes, all of which are based on unique protein properties of solubility, density, charge or size. By definition, protein concentrates contain 65-90 %[w/w] proteins that are water- or alcohol- soluble, along with sugars, fibers and flavor components [193], [194]. Isolates, on the other hand, contain at least 90 % proteins and are devoid of fibers [193], [194]. Fava bean protein concentrates are typically produced using air classification, which separates beans into fractions based on different particle sizes [195]. Air currents fed into a classifying chamber separate flour based on centrifugal and gravitational forces as a function of size and density, which then generates two main fractions: a fine protein-rich and a coarse starch-rich fraction [196]. Electrostatic separation is another dry extraction method relying on differences in dielectric properties of particles instead of their size and density. Based on the types and magnitudes of charges, an electric field can separate protein-rich and carbohydrate-rich particles [126], [197]. Another process removes sugars using an aqueous ethanol solution, resulting in protein-rich wet flakes that are desolvated and further dried to produce concentrates [198]. Flours and concentrates are then dispersed in a wet phase for the preparation of fava bean protein isolates. Fava bean protein isolates are produced with the help of alkaline extraction, isoelectric precipitation, ultrafiltration and salt extraction (**Figure 9**). Solubilized flours or concentrates can first be adjusted to an alkaline pH (pH 9-11) to remove insolubilized fibers and starch, then proteins are precipitated at their isoelectric point (pH 4-5), washed and reconstituted at neutral pH (pH 6.8) and further dried to yield powders (Schwenke, Anders, Junker, & Schneider, 1991; Krause, Buchheim, & Schwenke, 1996). Protein isolation has other alternatives that are based on conditions other than changing the pH. Membrane-based protein separation is one alternative to acid leaching process; it encompasses micro-filtration (0.1-5.0 µm), ultra-filtration (0.01-0.1 µm), nano-filtration (0.001 µm) and reverse osmosis, which separate and extract components based on molecular sizes [201]. Membrane processing provides many advantages over wet processing which modifies and denatures proteins based on pH and heat. Proteins of smaller sizes can be removed using such processes as well, along with phytates and lysinoalanine [202]**.** Salt extraction techniques also allow to extract proteins, especially globulins. Among salts, both ammonium sulfate and sodium chloride are commonly used in lab-scale pulse protein extraction. Industrially, proteins are clarified in sodium chloride solution (0.3-0.5 M) at neutral pH to remove insoluble material and precipitated by dilution or by dialysis to lower the ionic strength [203]. Removal of salts promotes formation of self-aggregated, non-covalent protein micelles, called the protein micellar mass (PMM) that grow in size and amount (Parades-López, Ordorica-Falomir, & Olivares-Vázquez, 1991; Sun & Arntfield, 2010). The 7S trimers and 11S fava bean hexamers are dissociated using 0.3-0.6 mol/L salt

solutions at slightly acidic pH as a soluble fraction, then concentrated and stripped off the salt to 0.2 mol/L to allow formation and precipitation of the PMM. Subsequently, PMM-protein concentration is increased by either ultra-filtration and/or centrifugation and neutralization followed by a drying method [206]–[208].

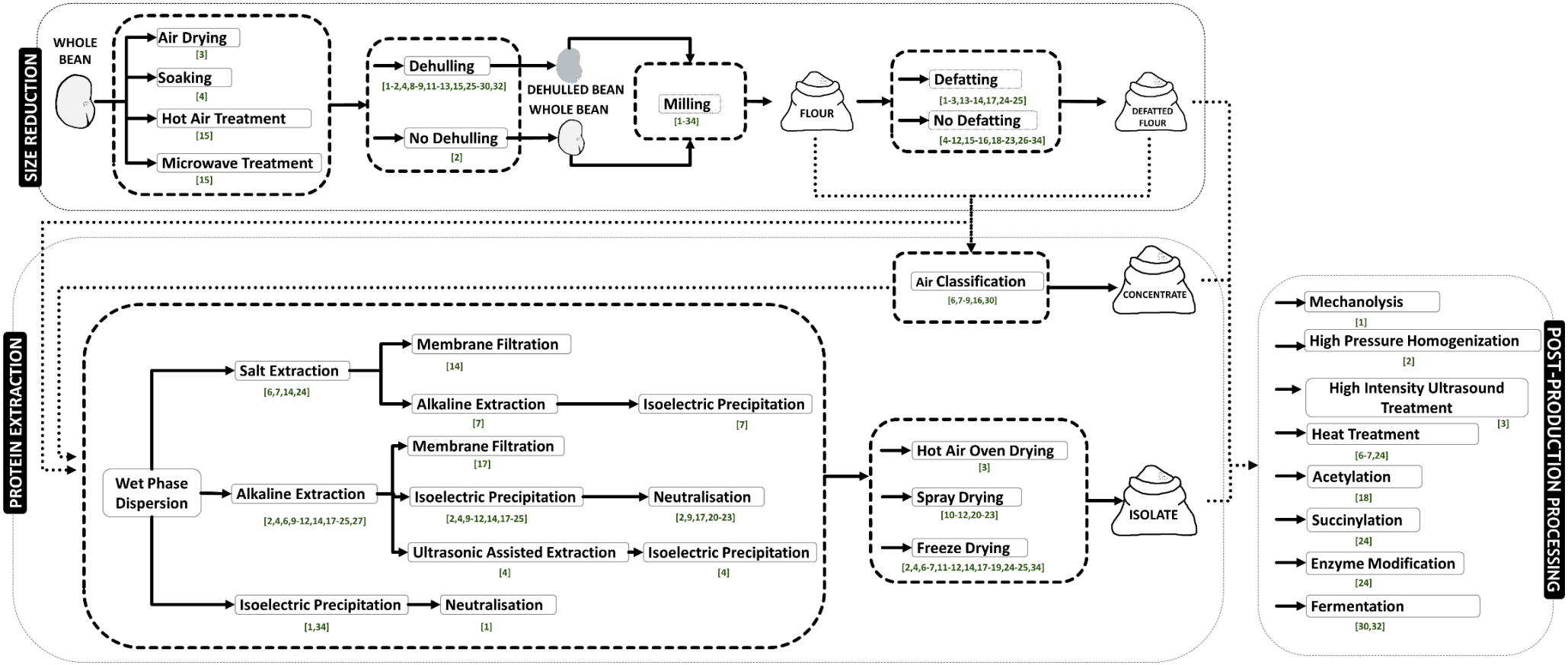


Figure 9 – Production and Processing of Fava Bean Ingredients: Illustration of various methods studied in literature for the production and post-processing of ingredients from fava bean. 1 = [26]; 2 = [87], 3 = [27]; 4 = [209]; 5 = [210]; 6 = [25]; 7 = [24]; 8 = [211]; 9 = [119]; 10 = [212]; 11 = [213]; 12 = [31]; 13 = [192]; 14 = [191]; 15 = [190]; 16 = [214]; 17 = [215]; 18 = [216]; 19 = [217]; 20 =[199]; 21 =[218]; 22 =[200]; 23 = [219]; 24 = [28]; 25 = [30]; 26 = [220]; 27 = [221]; 28 = [222]; 29 = [223]; 30 = [224]; 31 = [225]; 32 = [151] ; 33 = [226]; 34 = [14].

Isolate production steps require wet phase dispersion, with addition of salts and/or acids, and hence are followed by neutralization and drying techniques to obtain food-grade powders that are stored and utilized as ingredients for food applications. Drying improves ingredient preservation and transport properties [227] . Isolates produced by wet extraction are industrially spray-dried, freeze-dried or vacuum dried [206]. For fava bean isolates however, only spray-drying and freeze-drying have been noted in literature (**Figure 9**).

In general, wet processing also consumes large amounts of water and energy and generates acidic by-products, thus raising a concern from a sustainable point of view [228], [229]. Considering the disadvantages of wet extraction, dry extraction processes *viz.* air classification and electrostatic separation, which do not use chemical reagents, can serve as promising alternatives that preserve native structural and functional properties of the components [126], [197].

II.4.1.2. Ingredient Functionalization

II.4.1.2.a. During Ingredient Production

Functional modifications during changes in composition due to processing have been established for fava bean flours, concentrates and isolates (**Table 3**). Fava bean ingredients show a decrease in aqueous solubility with increasing degree of protein extraction, accompanied by an increase in oil and water holding capacities and oil emulsification capacity (**Table 4**). It must be noted that, in addition to the degree of protein extraction, there is also an influence of process conditions employed during the protein extraction and prior to extracting proteins, during pre-processing of the beans and flours on resultant ingredient properties. Alternatives to remove fat from fava bean have been explored, among which isopropanol defatting increases the presence of α-helices, possibly due to traces of solvent present. Supercritical carbon dioxide assisted defatting does not leave any trace of solvent but still reveals higher levels of α-helix, indicating possible protein modifications in both defatted flours. The latter improves foaming property by 200 % [26].

Table 3 – Proximate Composition of fava bean Flours, Concentrates and Isolates

Nutrients	Flour	Concentrate	Isolate
Crude Protein	21 – 35	53 – 71	80 – 95
Crude Fat	1 – 2	2 – 5	1 – 2
Crude Fiber	2 – 10	2 - 3	0 – 31
Carbohydrates	35 – 58	8 – 30	2 – 4
Total Ash	2 – 4	5 – 6	3 – 4

NOTE: All values are reported as % [w/w] dry weight basis [14], [105], [119], [213], [214], [230], [231].

Ultrafiltration to isolate fava bean proteins yields 94 %[w/w] of proteins [232], which results in foaming and emulsifying properties (**Table 5**) that are comparable to those from isoelectric precipitation, which yields 91 %[w/w] of proteins [232]. On the other hand, fava bean isolates from isoelectric precipitation containing 84 %[w/w] proteins have superior emulsifying property and protein solubility when compared to salt extracted isolate with similar protein content, i.e. 82 %[w/w] proteins [191], highlighting the role of process conditions in modifying functionalities.

Table 4 – Effect of Processing on Fava Bean Ingredient Functionalities

Study	Samples	Protein Solubility	Foaming Property	Emulsion Property	Water Holding Property	Oil Holding Property	Gelling Property
Degree of Protein Extraction[1]	Flour	C	C	C	C	C	C
	Concentrate	-	nd	+	+	+	nd
	Isolate	- -	nd	+	+ + + +	+ + + + + +	nd
Defatting[2]	Non-Defatted Isolate	C	C	C	C	C	C
	Isopropanol Defatted Isolate	nd	+	nd	nd	nd	nd
	Supercritical Carbon dioxide Defatted Isolate	nd	+ + + +	nd	nd	nd	nd
Drying[3,4]	Freeze Dried Isolate	C	C	C	C	C	C
	Spray Dried Isolate	nd	=	+ +	+	nd	nd
Salt Extraction[5]	Acid-Base Extracted Isolate	C	C	C	C	C	C
	Salt Extracted Isolate	-	nd	-	nd	nd	nd
Membrane Extraction[6]	Acid-Base Extracted Isolate	C	C	C	C	C	C
	Ultrafiltration	nd	+	=	nd	nd	nd
Mechanolysis[2]	Untreated Protein Isolate	C	C	C	C	C	C
	Mechanolyzed Isolate	nd	+ + + + +	nd	nd	nd	nd
High Pressure Homogenization[7]	Untreated Protein Isolate	C	C	C	C	C	C
	103MPa Treated Isolate	+ + + +	nd	-	nd	nd	nd
	207MPa Treated Isolate	+ + + +	nd	-	nd	nd	nd

High Intensity Ultrasound Treatment[8]	Untreated Protein Isolate	C	C	C	C	C	C
	High Intensity Ultrasound Treated Isolate	+ +	+ +	nd	nd	nd	nd
Acetylation[9]	Untreated Protein Isolate	C	C	C	C	C	C
	16 % Acetylated Isolate	nd	nd	+	nd	nd	nd
	42 % Acetylated Isolate	nd	nd	+	nd	nd	nd
	78 % Acetylated Isolate	nd	nd	+	nd	nd	nd
	97 % Acetylated Isolate	nd	nd	+ +	nd	nd	nd
Succinylation[10]	Untreated Isolate	C	C	C	C	C	C
	60 % Succinylated Isolate	nd	+	nd	nd	nd	nd
	83 % Succinylated Isolate	nd	+ +	nd	nd	nd	nd
	95 % Succinylated Isolate	nd	+ + + + + +	nd	nd	nd	nd
Enzyme Hydrolysis[11]	Non-Hydrolyzed Protein Isolate	C	C	C	C	C	C
	Pepsin Hydrolyzed Isolate	+ + + + +	+	-	nd	+	nd
	Trypsin Hydrolyzed Isolate	+ + + +	+	-	nd	+ + + +	nd
	Flavourzyme Hydrolyzed Isolate	+ + + +	+	- -	nd	=	nd
	Neutrase Hydrolyzed Isolate	+ + + +	+	-	nd	-	nd

NOTE: nd – non-determined, C – Control.
0 % Change in Property – ‘’=’’;
0-50 % Increase / Decrease in Property – ‘’+ / -’’;
50-100 % Increase / Decrease in Property – ‘’+ + / - -’’;
100-150 % Increase / Decrease in Property – ‘’+ + + / - - -’’;
150-200 % Increase / Decrease in Property – ‘’+ + + + / - - - -’’;
200-250 % Increase / Decrease in Property – ‘’+ + + + + / - - - - -’’;
250-300 % Increase / Decrease in Property – ‘’+ + + + + + / - - - - - -’’;
All % changes are calculated with respect to the control
[1] = Sosulski & McCurdy, 1987; [2] = Husband et al., 1994; [3] = Otegui et al., 1997; [4] = Cepeda et al., 1998; [5] = Karaca et al., 2011; [6] = Makri, Papalamprou, & Doxastakis, 2005; [7] = Yang, Liu, Zeng, & Chen, 2018; [8] = [27]; [9] = Schmandke et al., 1981; [10] = Schwenke, Rauschal, & Robowsky, 1983; [11] = Eckert et al., 2019

Processing conditions influence protein properties which then reflects in the ingredient functionalities. During pre-processing of beans, size reduction, protein extraction and ingredient preparation, the proteins, along with starch, lipids and other constituents, are exposed to changes in temperature, pH, pressure and salt concentrations (Schwenke, 2001). In fava bean, effects of process conditions on protein isolates have been established using thermal denaturation curves. The PMM isolated using sodium chloride and considered as 'native' gave an enthalpy change (ΔH) of 4.39 cal·g^{-1}. 10 %[w/w] aqueous dispersion of this isolate, denatured for 30 minutes at 80 °C (ΔH = 2.59 cal·g^{-1}), 90 °C (ΔH = 0.99 cal·g^{-1}) and 95 °C (ΔH = 0 cal·g^{-1}), showed the effect of temperature in heat treatment towards the extent of denaturation in fava bean globulins. A decrease in enthalpy change was hypothesized to be due to the increase in stability of hydrophobic interactions that unfold during denaturation of proteins [25]. A similar effect was presented in a heat treatment of 10 minutes at 75 °C, 80 °C and 95 °C, where complete denaturation was observed in DSC thermograms for 95 °C treated protein concentrate and protein isolate from fava bean [24]. Along with temperature, the effect of pH during protein isolation has been studied. Alkaline extraction at pH 12 followed by isoelectric precipitation at pH 4.5 and freeze-drying yielded isolates with ΔH = 0 cal·g^{-1}, whereas pH 8 alkaline-extracted isolate gave a thermogram with ΔH = 1.59 cal·g^{-1}. When heat treated (95 °C for 30 minutes), the latter showed complete denaturation with ΔH = 0 cal·g^{-1}. Isolation using sodium chloride is minimally invasive in the case of fava bean proteins (ΔH = 4.39 cal·g^{-1}) when compared to the pH-based protein isolation (ΔH = 0-1.59 cal·g^{-1}) [24], [25], [208]. The effect of pressure on fava bean proteins has not been studied to a great extent. Fava bean protein isolate, under high pressure homogenization of above 103 MPa presented enhanced foaming properties (**Table 4**).

In sum, the extraction of fava bean proteins through wet processing, which is based on salts, pH and/or drying, leads to protein denaturation and either loss or gain in functional property. Alternatives to downstream processing to remove water have been compared, in which the fava bean protein isolates produced by spray drying are superior to freeze drying in foaming and emulsifying properties (**Figure 9** and **Table 4**).

II.4.1.2.b. After Ingredient Production

Ingredients formed by conventional processing methods have been additionally treated by 'post-production processing' techniques, including physical, chemical, enzymatic or fermentative processes to assess changes in their functional properties and to evaluate their scope in food applications. Broadly speaking, chemical treatment is the most efficient treatment for functionalizing fava bean ingredients when compared to physical treatment. Protein solubility is better improved by high pressure homogenization and enzyme hydrolysis than by high intensity ultrasound (HIUS) treatment. Mechanolysis and succinylation improve foaming properties to the greatest extent compared to enzyme

hydrolysis or HUIS treatment. Only acetylation enhances emulsifying properties while high pressure homogenization and enzyme hydrolysis both impair emulsification (**Table 4**).

In addition to the type of post-production processing, the extent of processing also has an effect on functional properties. Increase of the extent of succinylation and acetylation increases foaming and emulsifying properties, respectively. Conversely, higher pressure during homogenization does not further impact functional properties (Yang, Liu, Zeng, & Chen, 2018). Interestingly, the type of enzyme during enzyme hydrolysis also has varying effects on functionalities. For instance, oil binding property can be greatly or subtly enhanced by trypsin and pepsin, respectively, while remaining unmodified by flavourzyme or being impaired by neutrase (**Table 4**). Enzymatic hydrolysis of purified legumin from fava bean enhances emulsion properties, increases creaming stability and decreases surface tension of leguminT (trypsin-hydrolyzed) (Schwenke, Staatz, Dudek, Krause, & Noack, 1995). Lastly, alcalase treatment of fava bean protein isolate can yield tripeptides with potential pre-biotic properties [234].

As shown in **Table 4**., there exists a wide range of post-production processing possibilities yet to be determined for all functionalities for fava bean ingredients. In addition to this, industrial relevance of post-processing also needs to be considered while studying novel or new methodologies.

II.4.1.3. Ingredient Food Application

With regard to the utilization of fava bean ingredients, the United States of America contributes the most to vegan/dairy-free market, followed by the United Kingdom and Canada. Amongst these products, fava bean ingredients that are rich in proteins have been utilized mainly in dairy and meat alternatives [15].

Table 5 – Replacement of Traditional Ingredients by Fava Bean Ingredients in Food Applications

Ingredient	Post-Processing	Application
Flour	-	20 %[w/w] meat replacement in sausage[1]
Protein micellar mass (PMM)	-	20 %[w/w] in snack food and meatball analog[2]
Flour	-	Up to 30 %[w/w] wheat flour replacement in noodles[3]
Flour	-	100 %[w/w] in pasta[4]
Flour	Air classification	100 %[w/w] semolina replacement in pasta[5]

	Fermentation(Lactic acid bacteria, 30 °C, 48h), freeze drying, milling	
Flour	-	Up to 100 %[w/w] wheat flour replacement in pasta[6]
Flour	Dough preparation, fermentation (*Lactobacillus plantarum*)	30 %[w/w] semolina replacement in pasta[7]
Flour	-	40 %[w/w] wheat flour replacement in crackers[8]
Protein Isolate	-	3 %[w/w] in mayonnaise[9]

Reports on the utilization potential of fava bean ingredients, with higher emphasis on the replacement of traditional ingredients (meat, wheat flour, egg, semolina) by fava bean ingredients.
1 Abo-Bakr, 1987; 2 = Youssef, 1988; 3 = Giménez et al., 2012; 4 = Laleg, Cassan, Barron, Prabhasankar, & Micard, 2016; 5 = Rosa-Sibakov et al., 2016; 6 = Laleg et al., 2017; 7 = Rizzello et al., 2017; 8 = Millar et al., 2017; 9 = Alu'datt et al., 2017.

The environmental and nutritional benefits, along with functional properties of fava bean as ingredients are yet to be completely translated into real time use in food applications. During the most recent decade, foods with vegan/dairy-free claims using fava bean flours accounted for 2.40 % of the products with similar claims that used legume and pulse flours as ingredients. During the same period, foods with vegan/dairy-free claims using fava bean proteins (concentrates and isolates) constituted only 0.45 % of total foods using plant-proteins as ingredients [15]. In the literature, fava bean ingredients have a great potential in food applications, notably in partially or completely substituting traditionally used ingredients in foods, *viz*. pastas, crackers, mayonnaise, sausages and meatball analogs. However, research insights are less on the application potential of fava bean ingredients. Only flours find a place in research, being primarily used to replace meat, wheat flour, semolina and eggs (**Table 5**). The application potential of isolates and concentrates along with nutritional, sensory and safety specifications of all ingredients during their utilization is yet to be understood. Regardless of the potential in functional properties stated in the previous sections, current market and research preference on fava bean applications is remarkably low. This low utilization could be related to various factors, including the limitation of fava bean as ingredients with regard to sensory and safety aspects. Flavor and color contributors present in fava bean seeds are also subjected to changes during production and processing of ingredients – either moving towards, or away from their consumer acceptability. Moreover, amount of ANFs which are one of the determinants of food safety in ingredients are influenced by process conditions too. Despite sensory and safety limitations of fava bean, there might be other factors accounting for low utilization of

fava bean ingredients that we are yet to decipher. Insights on these aspects might throw light on some of the reasons behind the gap between the potential and present state of fava bean ingredients (Nasar-Abbas et al., 2009; Boye, Zare, & Pletch, 2010; Pechey & Monsivais, 2016).

II.5. Impact of Fava Bean Processing on its Ingredient Value

II.5.1.1. Effect on Anti-Nutritional Factors

II.5.1.1.a. Vicine & Convicine

The glycoside precursors of favism can be reduced by processing techniques. They are generally unstable in acidic medium and degrade into their aglycones at higher temperatures. Convicine is more readily hydrolyzed than vicine. After a week at 30 °C, convicine reduces to 22 % and 96 % in 0.1 N and 1.0 N hydrochloric acid whereas vicine degrades to 17 % and 83 %, respectively [237]**.** Ingredients from pre-processed beans have varying glycoside contents depending on the type of bean processing. Dehulling of seeds increases vicine and convicine to 58 %[w/w] and 25 %[w/w], respectively [150]. Alternatively, bean roasting and cooking decreases the glycoside contents. Bean roasting at 120 °C for 10 min, which decreases 2-6 %[w/w] vicine and 0-10 %[w/w] convicine, is less effective than bean cooking at 121 °C for 20 min, which eliminates 12-40 %[w/w] vicine and 17-60 %[w/w] convicine. Gamma irradiation removes up to 38 % of glycoside content [210]. Hydrogen peroxide seed treatment lowers vicine by 91-93 %[w/w] and bean soaking and germination lowers vicine by 86 %[w/w]. Both these treatments completely remove convicine [238]. In addition to bean processing, selection of young, ripe or older seeds impacts the final ingredient glycoside content due to the changes in the level of β-glucosidase enzyme. Young and old seeds are low in enzyme activity while ripe seeds have the highest enzyme activity (Rizzello et al., 2016). The extent of glycoside removal also depends greatly on the bean variety [239].

Protein extraction, depending on wet or dry process, impacts the glycoside concentrations. Wet protein extraction method leaches the glycosides out, leaving behind isolates with 42 %[w/w] vicine and 9 %[w/w] convicine removal when compared to the whole seeds. Conversely, air classification (dry method) increases vicine and convicine concentrations by 53 % and 56 % respectively [150].

Ingredient post-processing, including fermentation of fava bean flour by *Lactobacillus plantarum* can remove more than 95 % of glucosides (Rizzello et al., 2016). Apart from fermentation, enzymatic processes eliminate upto 90 % glycosides. Microbial β-glucosidases from *Aspergillus oryzae*, *Fusarium graminearum* and lactic acid bacteria have been reported

for fava bean flours [240]. Frying of fava bean flour dough for bean cake application removes 56 % vicine and 34 % convicine [241].

II.5.1.1.b. Other Anti-Nutritional Factors

Bean pre-processing effect on other fava bean ANF has been most extensively studied. Dehulling and soaking beans prior to processing reduces upto 11 % phytic acid, 59 % overall tannins [56] and 26-29 % saponins [57] in fava bean beans. Trypsin inhibitors activity increases by dehulling as they are mainly located in the seed cotyledons [56]. Heat treatment along with soaking further reduces ANF. Maximum reduction of all ANF is observed in dehulled beans that are soaked and autoclaved. For instance, autoclaving of dehulled and soaked beans reduces up to 66 % overall tannin content [56], [57]. Favins are thermosensitive and hence heat treatment lowers their levels to a great extent. There are also some lectins that are partially heat stable and survive the passage through the gut, causing digestive disorders and diseases by their interaction with the gut epithelium [242]. Despite this property, lectins still remain in the seeds after dehulling and soaking. Even germination fails to remove lectins from the seeds [57]. During ingredient production and protein concentration, wet process leaches out upto 46 % phytic acid, 91 % tannins and even 2.5 % trypsin inhibitors [213]. ANF that are flavor and color contributors are discussed in their respective sections too.

II.5.1.2. Effect on Flavor

II.5.1.2.a. Volatile odorant compounds

Numerous studies have been carried out in recent years to better understand the formation and evolution of odorant volatile compounds in pulse ingredients during different stages of protein extraction [243] or due to thermal treatments [170], [244], [245] and storage [246], [247].

Enzyme-mediated degradations begin during harvesting or in early stages of processing that disrupt the physical barriers separating enzymes (*e.g.* lipases, lipoxygenases, lyases and dehydrogenases) from their respective substrates (*e.g.* esterified or free fatty acids, amino acids and glucosides). This enables the degradation of the substrates into highly odorant volatile compounds [35], [83], [248]. Additional formation of odor-active compounds can be induced by temperature and pH variations during processing and storage of the ingredients, usually involving the degradation and rearrangement of amino-acids and carbohydrates *via* the Strecker degradation and the Maillard reaction [32], [35], [164].

Dehulling and milling reportedly boost the formation of aldehydes in fava bean by increasing the total surface area and prompting the exposition of lipids to air [169]. Fava bean flour exhibits high LOX activity (0.219-0.330 $mmol \cdot min^{-1} g^{-1}$), which could increase the potential

for oxidative reactions and consequent formation of deleterious flavor notes in food preparations with high amounts of lipids (Yang, Piironen, & Lampi, 2017). Fava bean seeds have been reported to contain two type-II isoenzymes: broad bean lipoxygenase-1 (BBL-1), which produces ketodienes and both 9- and 13- hydroperoxides, and broad bean lipoxygenase-2 (BBL-2), originating predominantly 13-hydroperoxides [38], [249]. Both isoenzymes have shown oxidizing activity towards linoleic acid, methyl linoleate and trilinolein [38], [250], [251]. Several treatments have been proposed to inactivate the enzyme in fava bean seeds and thus control LOX-catalyzed lipid oxidation, including microwave heating at 950 W for 1.5 min [36], blanching at 70 °C for 2 min (Al-Obaidy & Siddiqi, 1981) and heat treatment at 75 °C for 2 min (Al-Obaidy & Siddiqi, 1981) or at 70 °C for 15 min [38].

II.5.1.2.b. Non-volatile taste compounds

The overall saponin content of fava bean seeds (13.70-39.30 $mg \cdot g^{-1}$) [59], [97] has been shown to decrease significantly upon soaking, dehulling, cooking and/or germination [57], [59]. Amongst saponins, soyasaponin βg can readily convert into soyasaponin Bb by releasing its DDMP moiety in the form of maltol either enzymatically or when exposed to temperatures above 30 °C, slightly acidic pH values and highly polar solvents [47]–[49]. Polyphenols have been shown to decrease upon boiling and autoclaving by leaching into the cooking broth [181], [252].

II.5.1.3. Effect on Color

The color of fava bean ingredients also have an impact in the acceptability of the beans as ingredients in foods [55].

Proanthocyanidins in fava bean get oxidized through phenolic reactions and, by this way, can also lead to the development of a dark coloration [253]. It has been shown that high tannin varieties of fava bean beans, containing more proanthocyanidins, darken more in the presence of air than low tannin varieties, whereas white-seeded varieties present no darkening in an oxygen-rich environment [185], [187].

In addition to the constituents responsible for color of fava bean ingredients, the degree of protein extraction is also an important factor for ingredient color. Dehulled and milled seeds from fava bean yield flours that are creamy-yellow in color, with air classification increasing the yellow coloration. Air classified concentrates show lighter color than the isolates produced by acid- or alkali- extraction, isoelectric precipitation and freeze drying [119]. Freeze drying of dehulled, milled, alkaline extracted and isoelectrically precipitated proteins contribute to dark coloration while the spray drying process have no darkening effect [31].

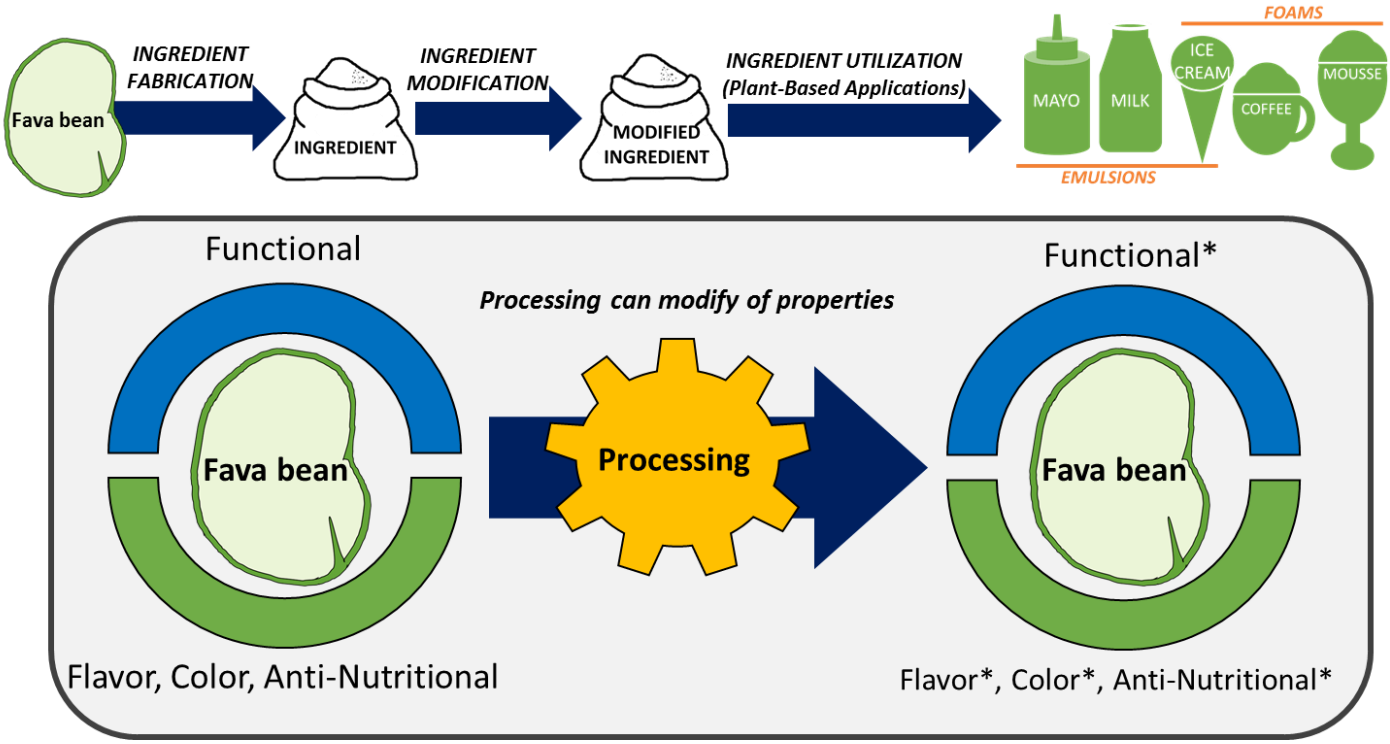
Several reactions can be hypothesized to form colors in ingredients. Browning reactions take place during production, processing and storage of foods. Browning reactions emerge

through several ways in foods, including phenolic compounds' oxidation, Maillard reaction, ascorbic acid oxidation, lipid oxidation and caramelization [254]. Maillard reaction in particular plays a role during high thermal conditions, and is popularly known to occur by reaction between amino acid and/or peptides along with carbohydrates. Amino acids are destroyed as a result of this reaction, losing nutritional significance and leading to formation of toxic and anti-nutritional end products that compromise food safety [35], [255]. These phenomena are yet to be studied in fava bean ingredients to assess their acceptability and safety.

II.6. Conclusions

Fava bean is an agronomically sustainable plant-based source, and its proteins have a great potential in nutritional and functional properties for food applications. There are various approaches for producing and processing industrially relevant ingredients that further favor or impair their functional properties based on the type of processing techniques. Protein denaturation has primarily been studied in the context of functional property of fava bean, but there exists a need to explore the interactions of lipids, polysaccharides as well as polyphenols and phytic acids that could modify functional properties too. Despite promising studies on functional aspects, insights on food applications and consumers' recognition and appreciation in the food market for fava bean ingredients are low. Moreover, fava bean ANF, flavor and color properties certainly a limit the acceptability and safety of its ingredients in foods. Although changes in ANF, flavor and color are likely to occur during production and processing of ingredients, more insight is needed on the ingredients' complete functional, nutritional, anti-nutritional, and organoleptic profile to increase their food market acceptability. Care must be taken in assessing these limitations as there might be different other factors playing a role in food market availability, some of which include bioavailability of essential amino acids, availability of raw material for human consumption, socio-economic limitations. A great deal of knowledge still remains unexplored. For instance, is fava bean concentrate produced from a dry protein extraction technique and further post-processed functionally significant for applications, nutritionally safe and sensorially pleasing for consumers and industrially relevant for the food market? As we cannot answer this now, we still have a long way to go in our scientific understanding to improve the use of fava bean as 'sustainable' ingredients in the food market.

II.7. Key Highlights



- Fava bean is a sustainable protein source with a great potential in nutritional and functional properties. Fava bean seeds contain 23-41 % proteins on dry weight basis, and thus are a rich source of proteins.

- Amongst seed storage proteins, approximately 85 % by weight consist of salt-soluble proteins called globulins. Amongst globulins,l (MW 360 kDa) forms a hexamer whereas vicilin (MW 150 kDa) and convicilin (MW 210 kDa) form trimers in their native quaternary conformations. They are attributed to different structural and functional properties.

- There is a vast sphere of knowledge available in the literature on the nutritional potential of fava bean and its impact by processing. Flours, concentrates and isolates are the main three types of fava bean ingredients. Flours and isolates have popularly been studied, compared to concentrates.

- The environmental, nutritional and functional benefits of fava bean as ingredients are undervalued in food applications. For instance, foods with vegan/dairy-free claims using fava bean proteins (concentrates and isolates) constituted only 0.45 % of total foods using plant-proteins as ingredients.

- Consumer acceptance of pulse-based products is hampered by the presence of undesirable flavors. Flavor perception is the result of a multimodal combination of stimuli that arise

mainly from the interaction of (i) volatile odorant compounds and (ii) non-volatile taste molecules with olfactory and/ or sapid receptors in the nasal and/ or on the tongue cavity.

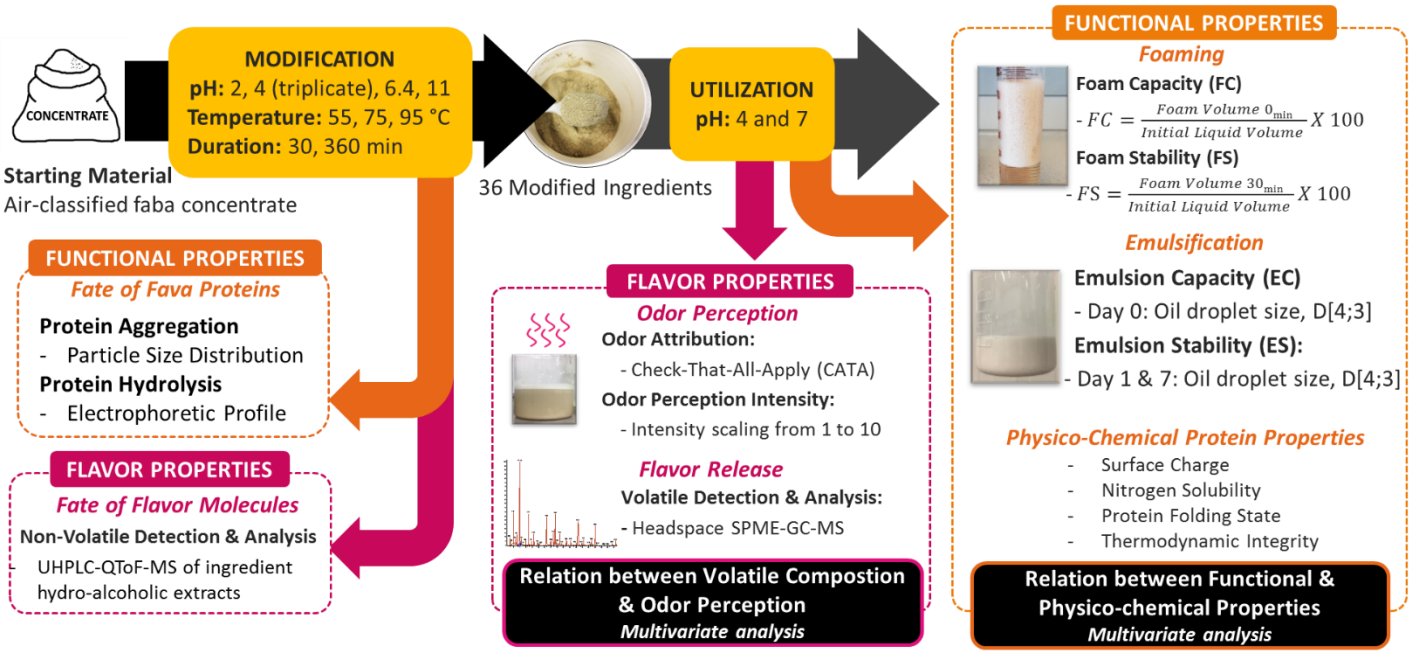
- Flavor-contributing volatile and non-volatile compounds are either inherent to the grain or produced during the food supply chain including harvesting, processing, and storage. Lipid oxidation *via* enzymatic and/or non-enzymatic pathways is deemed the primary source of flavor-related molecules in pulse ingredients, amongst several other reactions. Alcohols and aldehydes are shown to be responsible for the green, grassy and beany notes associated with fava bean beans. Pulses are mainly associated with bitter and astringent tastes, which could be related to the inherent presence of sapid glycosylated compounds such as saponins, isoflavones, flavonols and phenolic acids. Polyphenols are also known to impart astringent sensations in the mouth by forming insoluble precipitates with salivary proteins. Saponins exert foaming properties in aqueous solutions and can impart bitter or metallic tastes and astringency.

- Processing conditions influence protein properties which then reflects in the ingredient functionalities. Fava protein denaturation can start at temperatures above 70 °C. The glycoside precursors of favism (vicine and convicine) can be reduced by several processing techniques. Flavor and color transformation can also take place by different processes right from bean pretreatment to ingredient production and further during the storage. Temperature and pH seem to play an important role in all these properties.

III

Methodological Approach

III. Methodological Approach



This part presents the materials and protocols implemented during the different steps of the PhD project to meet their different objectives. The different sections represent different aspects of the research methodology, along with literature-based evidences on why these materials or methods were chosen, followed by a much precise explanation of the methods used in the study.

III.1. Fava Bean Ingredient Processing for Beverage Applications

III.1.1. Starting Material

Fava bean protein concentrate (**Table 6**) was procured by Döhler GmbH, which was obtained from dried and decorticated beans by milling and air classification (**Figure 10**). The fava bean variety has not been disclosed for reasons of confidentiality with the bean suppliers.

Table 6 – Proximate Composition of the fava bean concentrate used for this study (Döhler GmbH)

Nutrients	% (w/w) dry basis
Proteins	65
Lipids	3
Fibers	17
Carbohydrates	2
Ash	8

The fava bean concentrate, by virtue of its minimal processing, would theoretically contain proteins in their rather native form. Effects due to industrial decortication, milling and air-classification on proteins have been assumed to be minimal. This assumption was further reinforced by a preliminary measurement of its thermal integrity by Differential Scanning Calorimetry (DSC) which gave an average non-zero denaturation enthalpy of 6.15 J/g protein and denaturation temperature of 89.65 °C. Analysis of this type has been done before to determine and assess protein integrity in fava ingredients [24], [25]. Fava concentrates produced by densification or air-classification have been previously reported as "gently processed" since they contain minimally denatured proteins [90]. A recent study has also compared fava bean concentrates with isolates, where both showed considerably lower environmental impact compared to cow's milk. In this study, fava air-classified concentrates have shown to be much superior in foam and emulsion properties compared to fava isolates – thereby reinforcing the industrial interest to study this type of ingredient [256]. It was therefore rationalized that if the concentrate has minimally denatured proteins, it may also contain minimally degraded precursors or flavor such as lipids, sugars, carotenoids, or proteins themselves [90], [257]. The concentrate also contains micro-components including mineral salts and anti-nutritional factors (ANF). Interactions between every food component is inevitable, and hence it was anticipated

that the results would demonstrate fava concentrate as a promising ingredient but with a very complex character [257].

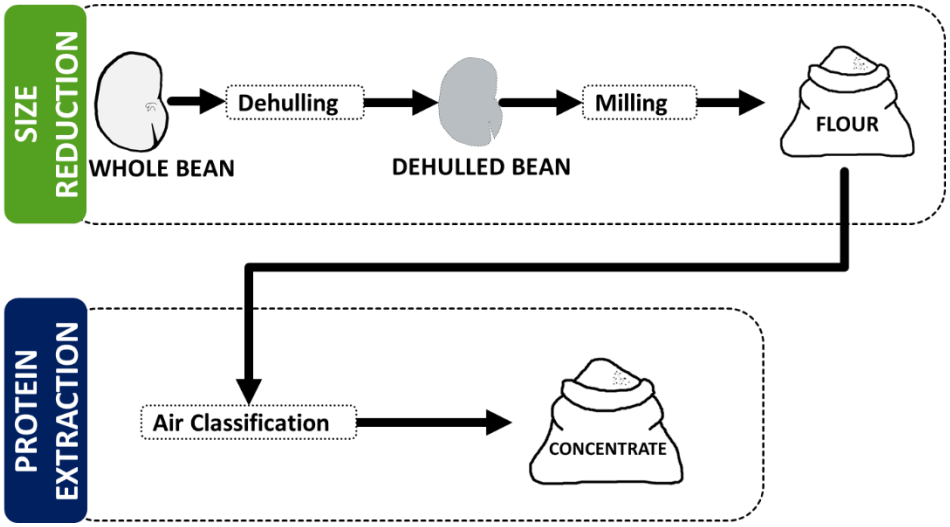


Figure 10 – Faba Bean Initial Concentrate (FBIC) Production. History of the protein extraction *via* air-classified fava bean concentrate (starting material).

III.1.2. Ingredient Processing

The starting material was considered to be further modified by process conditions. Given the variety of types of processing techniques in every step of the supply-chain – it is difficult to prioritize the processing techniques for the industry. There are many processes and therefore not all could be studied in this work, and several of them have in common the process of heat treatment for a certain duration and under certain pH conditions. We have therefore chosen to propose an approach to study process conditions: pH, temperature and treatment duration; and to widen the ranges of values so as to highlight potentially more extreme or exaggerated phenomena, which makes it easier to understand the underlying mechanisms for the ingredient properties. Based on this context, the PhD work was interested in the choice of these process conditions and their levels on protein-associated functionalities and properties: 1) functional potential: to investigate the fate of the proteins during ingredient modification; and 2) fava flavor limitation: to investigate if the process conditions during ingredient modification can drive modifications in flavor.

III.1.2.1. Ingredient Functionalization/ Modification

III.1.2.1.a. Explanation & Rationale

For the PhD project, the starting material was functionalized by three process conditions: pH, temperature and treatment duration, named as $pH_{process}$, $T_{process}$ and $t_{process}$ respectively. Different levels of $pH_{process}$ (2, 4, 6.4 and 11), $T_{process}$ (55, 75 and 95 °C) and $t_{process}$ (30 and 360 min) have been chosen for ingredient modification, based on literature knowledge around functional and flavor properties of fava bean (**Table 7**). Additionally, a series with the original suspension pH was considered (without any pH modification, ie. $pH_{process}$ 6.4) which was then heated at either of the temperature-time combinations. During the modification experiments, it was noticed that there was a very high difficulty in processing and handling at $pH_{process}$ 4 owing probably to the isoelectric point of majority of fava proteins [3], [5]. Therefore, all the treatments at $pH_{process}$ 4 were performed in triplicate in order to assess reproducibility of ingredient modification experiment in the most complex conditions. Thus, in total, 36 different modified suspensions were produced, which were freeze-dried and milled to produce ingredient powders that were stored at -20 °C before the analyses. A general flowchart illustrates different unit operations used to produce the different modified ingredients (**Figure 11**).

Table 7 – Choice of different process conditions for fava concentrate functionalization and utilization.

Process Conditions	Rationale: Functional Properties	Rationale: Flavor Properties
$pH_{process}$		
$pH_{process}$ 2	• Acidic conditions as low as pH2 cause fava protein denaturation [24], [25] • Possible indications of acid-hydrolysis of pulse proteins [258], [259]	• Lower hydrophobic interactions caused by protein hydrolysis can remove opportunity of flavor binding and enabling higher off-flavor release [32] • Degradation and rearrangement of amino acids and sugars reactions are pH-dependent reactions that yield flavor, e.g. formation of Schiff's base ≤ pH 7 from Amadori rearrangement products during Maillard reaction [32], [35], [169], [260], [261] • Acidic pH also inactivates certain enzymes, including LOX [162]
$pH_{process}$ 4	• Close to the isoelectric point of majority of fava proteins i.e. fava globulins [3], [5]	• pH 4 is below the pKa of ionizable groups of certain volatiles, thus an important factor for flavor release • Acidic pH also inactivates certain lipoxygenases [162].

$pH_{process}$ 6.4	• pH of natural suspension of fava concentrate in deionized water • Of industrial importance as this condition would be the least chemically intrusive for functional modifications (Döhler GmbH)	• Of industrial importance to see flavor modifications at non-adjusted pH (Döhler GmbH)
$pH_{process}$ 11	• Extreme alkaline conditions, shown to cause fava protein denaturation, with possible indications of alkaline hydrolysis of proteins [24], [262]	• Degradation and rearrangement of amino acids and sugars are pH-dependent reactions that yield flavor, e.g. formation of reductones ≥ pH7 from Amadori rearrangement products during Maillard reaction [32], [35], [169], [260], [261] • Alkaline pH can enable binding of proteins with flavor compounds [263] • Alkaline denaturation of proteins could impact flavor [263]
$T_{process}$		
55 °C	• Below the protein onset denaturation temperature, i.e. 70-80 °C for fava proteins [24], [25]	• Below the denaturation temperature of flavor contributing enzymes, including lipoxygenase [37], [38] • Enabling interactions between native proteins and flavor molecules [25], [32], [35]
75 °C	• Around the protein onset denaturation temperature, i.e. 70-80 °C for fava proteins [24], [25] • Below the peak denaturation temperature of fava proteins, i.e. 80-90 °C [24], [25]	• Around the denaturation temperature of flavor contributing enzymes, including lipoxygenase [37], [38] • Favorable condition for many flavor-associated reactions [32], [35], [260], [261]
95 °C	• Above the peak denaturation temperature of fava proteins i.e. 80-90 °C [24], [25]	• Complete denaturation of proteins could enable interactions with flavor molecules [25], [32], [35] • Above the denaturation temperature of flavor contributing enzymes [32], [35], [37], [38] • Extreme condition for many flavor-associated reactions [32], [35], [260], [261]
$t_{process}$		
30 min	• Extremely low duration, energy efficient for industrial processes (Döhler GmbH)	• Treatment > 70 °C for 15 min sufficient for inactivation of fava lipoxygenase [38]
360 min	• Extremely high duration, enabling protein-associated reactions, e.g. hydrolysis, to take place [258], [259]	• Complete fava protein hydrolysis at 75 °C observed at 360 min (Döhler GmbH) • Extremely high extent of flavor-associated reactions can be observed here [32], [35]
$pH_{utilization}$		
$pH_{utilization}$ 4	• Close to the isoelectric point of majority of fava proteins, i.e. fava globulins [3], [5]	• pH4 is below the pKa for certain volatiles – Thus an important factor for flavor release.

	• Key pH to assess functional properties during plant-based, acidic beverage applications including mayonnaise and spreads (Döhler GmbH)	• Acidic pH also inactivates certain LOX [162] • Key pH to assess flavor release and perception during plant-based, acidic beverage applications including mayonnaise and spreads (Döhler GmbH)
$pH_{utilization}$ 7	• Key pH to assess functional property during plant-based, neutral beverage applications, including cappuccinos and ice-creams (Döhler GmbH)	• Key pH to assess flavor property during plant-based, neutral beverage applications, including cappuccinos and ice-creams (Döhler GmbH)

III.1.2.1.b. Ingredient Modification (Chapter IV - VI)

a) **Modified Suspensions.** The FBIC was modified as follows: 20% (w/w) suspensions were prepared with deionized water and agitated for 30 min at 500 rpm (~30 g) using an overhead dissolver stirrer (IKA Works, Inc., Staufen, Germany), followed by pH adjustment ($pH_{process}$) to 2, 4 or 11 using 6 mol/L hydrochloric acid or 3 mol/L sodium hydroxide (Sigma Aldrich, Missouri, United States) and further stirred for 30 min at 500 rpm. Additionally, a series with the natural suspension pH was prepared ($pH_{process}$ 6.4) by stirring for 30 min at 500 rpm. The suspensions were heated ($T_{process}$) in a temperature-controlled bath (Lochner Labor+Technik GmBH, Germany) at 55, 75 or 95 °C and agitated at 700 rpm for a duration ($t_{process}$) of either 30 (Low) or 360 (High) min. The suspensions produced after these treatments are denoted as *modified suspensions*. All the treatments at $pH_{process}$ 4 were performed in triplicates in order to assess reproducibility.

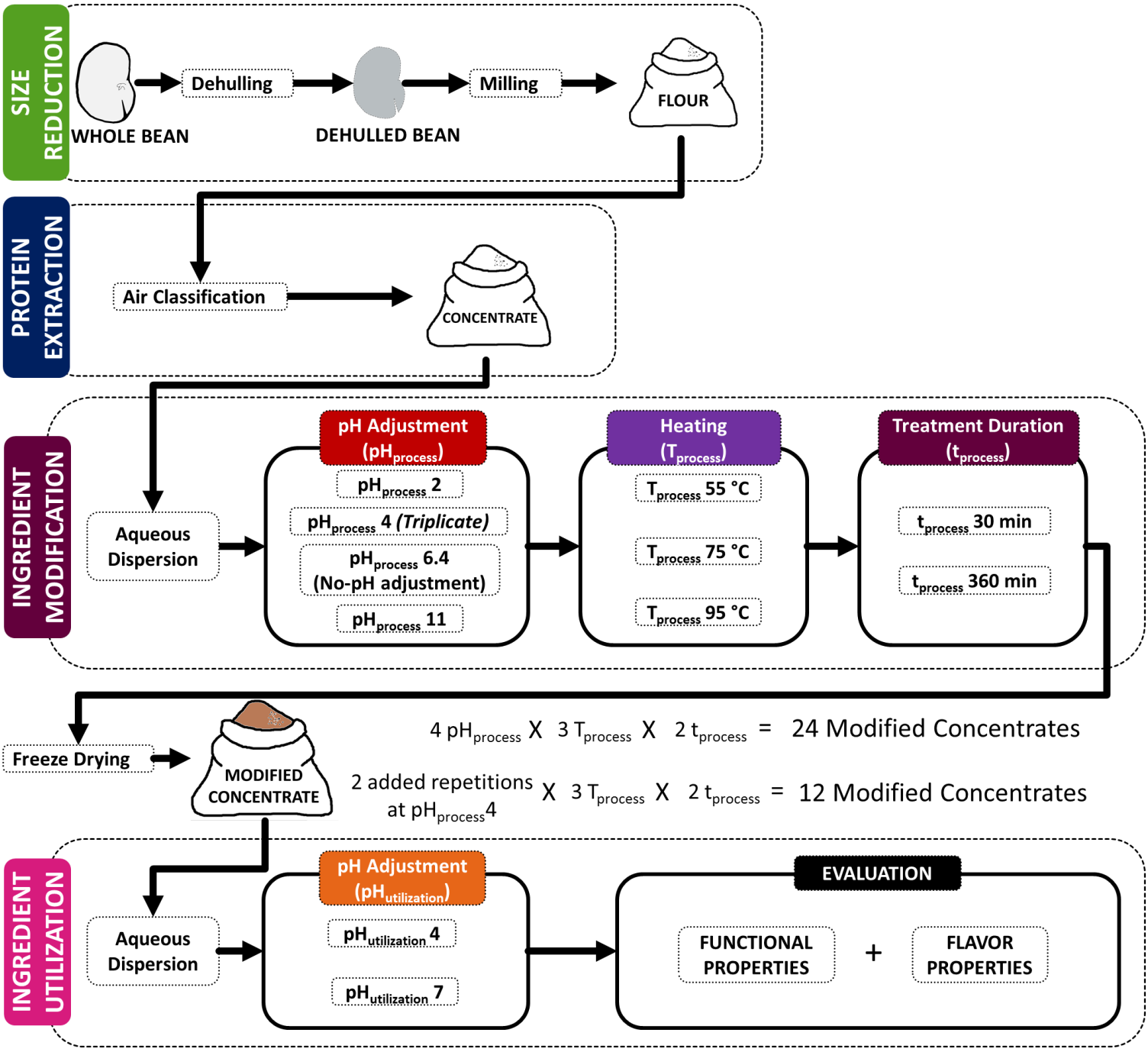


Figure 11 – Complete process flow from the bean to the modified concentrates, illustrating different process conditions i.e. pH (pHprocess), temperature (Tprocess) and treatment duration (tprocess) used to functionalize the starting material.

b) **Modified Ingredients.** The different *modified-suspensions* were frozen at −20 °C, followed by freeze-drying and milling to 0.08 mm mesh size by an ultra-centrifugal mill ZM 200 (Retsch GmbH, Germany). This resulted in different modified ingredient powders, which are named as $pH_{process}\_T_{process}\_t_{process}$ (e.g. pH2_55 °C_Low) based on the conditions used to modify them.

III.1.2.1.c. Ingredient Application/ Utilization

Including the starting material, a total of 37 ingredients were thus assessed. This assessment was done in conditions that mimicked industrial beverage applications, meaning that the ingredients were suspended in deionized water at ambient temperature and then brought to two different pH of utilization, i.e. $pH_{utilization}$ 4 and $pH_{utilization}$ 7. These two pH have been popularly studied while investigating ingredient properties [119], [120], [189]. In total, 37 * 2 = 74 samples were thus produced for each type of analysis, and each analysis was performed in triplicates. One important aspect sparsely found in the literature is the medium used for ingredient suspension. Buffer solutions have been popularly used to test functional properties [29], [120], [215], [264], [265]. But ingredient utilization close to industrial applications do not use buffer systems (Döhler GmbH). Thus, there was a need to test the properties in realistic conditions when it came to functional and flavor properties. However, for understanding of protein molecular behavior in aqueous systems, buffer systems were used where only the effects of the two utilization pH were studied [266].

Precise methods of evaluation of ingredient properties during ingredient utilization are discussed in sections below.

III.2. Understanding the Mechanisms of Functional Property Modifications

III.2.1. Functional Properties

As noted in the previous sections, discussions with the industrial partner of this project were done to examine beverage application potential of fava ingredients. Thus, foaming and emulsification were chosen as the most essential functional properties to explore in beverage applications apart from protein solubility [11]. These properties were analyzed at utilization conditions close to beverage applications. Foaming and emulsification were characterized by foam and emulsion capacity and stability (**Error! Reference source not f**

ound.). The choice and the description of the precise method used for exploring each kind of functional property will be discussed in **Chapter IV**.

III.2.1.1. Sample Preparation (Chapter IV)

III.2.1.1.a. Ingredient-1%-aqueous-suspensions

All ingredients were suspended in deionized water in triplicate to 1% (w/w) protein concentration and stirred for 30 min at ambient temperature at the two $pH_{utilization}$ (4 and 7) to prepare *ingredient-1%-aqueous-suspensions*. The pH were adjusted either using 6 mol/L hydrochloric acid or 3 mol/L sodium hydroxide. These systems were chosen as mimicking realistic beverage applications.

III.2.1.2. Evaluation of Functional Properties (Chapter IV)

III.2.1.2.a. Foamability

150 mL of the *ingredient-aqueous-suspension* was whipped mechanically at room temperature using a WMF Mechanical Frother (Württembergische Metallwarenfabrik GmbH, Geislingen, Germany) for 2.5 min and the foam was transferred to a graduated cylinder (inner diameter = 48.9 mm and height = 400 mm measured using a digital caliper). Foam height and liquid height were recorded manually to calculate the foam and final liquid volume respectively. Foaming capacity (FC, %) was calculated as the ratio of volume of foam generated after whipping and initial liquid volume. Foam stability (FS, %) was foam capacity measured after 30 min [82]. Foam was categorized unstable when FS was below 50%.

$$\textbf{FC (\%)} = \frac{\text{Foam Volume } 0_{min}}{\text{Initial Liquid Volume}} \text{X } 100 \text{ ; } \textbf{FS (\%)} = \frac{\text{Foam Volume } 30_{min}}{\text{Initial Liquid Volume}} \text{X } 100$$

III.2.1.2.b. Emulsification

The *ingredient-aqueous-suspensions* were added with palm oil medium chain triglycerides (90:10 w/w) and homogenized for 1 min at 8000 rpm using T-10 Basic ULTRA-TURRAX homogenizer (IKA Works, Germany) fitted with an S-10N-10G dispersing element. The coarse emulsions, thus formed, were passed twice through a Niro-Soavi NS 1001L Panda homogenizer (Gea Group, Germany) at 200 bars. The emulsions were pasteurized at 80 °C for 10 min just after the emulsion preparation to prevent microbial growth during storage. The pasteurized emulsions were stored at 4 °C to evaluate emulsion stability for seven days [191]. The emulsion oil droplet size at day 0, 1 and 7 was characterized using laser

light scattering by Mastersizer 3000 (Malvern Instruments Ltd., U.K.) with degassed, deionized water used as the dispersant. The particle size distribution from 0.005 to 5000 µm as a function of volume was recorded followed by the estimation of the volumetric mean diameter (D[4;3]), which were used to assess the emulsion capacity and stability [214], [215]. Contour plots of the D[4;3] values were generated by Minitab (Minitab Inc., Pennsylvania, United States) using distance method of interpolation.

III.2.2. Structural Modification of Fava Proteins

Changes in the protein-associated functionality are due to the variations in the dispersed phase and the evolution of protein properties, its interaction capability, and/or modifications in the protein during processing [11], [20], [267]. Therefore the used approach was to understand what modifications in proteins can explain changes in functional properties. In the two main steps in ingredient processing, i.e. ingredient modification and ingredient utilization, changes in proteins were expected and thus studied at every step of processing.

III.2.2.1. During Ingredient Modification

In this study, protein-associated reactions, *viz*. proteins hydrolysis and aggregation, were investigated by the study of samples collected at different times during ingredient modification.

III.2.2.1.a. Sample Preparation (Chapter IV)

a) ***Modified-Suspensions.*** The modified-suspensions were used to analyze protein-associated reactions. The samples were drawn during modification at 0, 30 and 360 min, then frozen at -20 °C to stop any further reaction to take place.

III.2.2.1.b. Evaluation of Protein Modifications (Chapter IV)

a) **Protein Aggregation.** Particle aggregation in the *modified-suspensions* (section II.1.1.2.c) was measured using laser light scattering by Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, U.K.) with degassed, deionized water used as the dispersant. The particle size distribution (PSD) from 0.005 to 5000 µm as a function of volume was recorded and the volumetric mean particle diameter, D[4;3], was used to compared the level of particle aggregation after the different ingredient modification treatments.

b) **Protein Hydrolysis.** The *modified-suspensions* were diluted to 2.25 mg protein/mL with Milli-Q water (Millipore, France) with a mixture containing 1% (w/v) 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris), 0.1% (w/v) sodium dodecylsulphate (SDS) and 1.4% (w/v) glycine, then submitted to sonication for 30 min and centrifugation at 10,000 g for 2 min to obtain a supernatant of dissolved polypeptides. Protein concentration of the supernatants were determined at this stage by Dumas method using Rapid MAX N Exceed (Elementar, Langenselbold, Germany). Aliquots of 22.5 µg of proteins were loaded along with peqGOLD protein marker II (VWR International, Pennsylvania, United States) into 12% (w/v) Bio-Rad Mini-PROTEAN®TGX™ gel (Bio-Rad Laboratories, California, United States) and run at 200 V for 45 min. The polypeptide bands were stained by 0.25% (w/v) coomassie brilliant blue dye. Electrophoresis was performed under non-reducing conditions. The resultant gel band-size intensities of larger (40–100 kDa) and smaller (< 40 kDa) subunit groups were analyzed by semi-quantitative comparison of their pixel intensities in the gel using GelAnalyzer [268]. The change in band-size intensity (%) was calculated in relation to the subunit groups found in FBIC.

III.2.2.2. During Ingredient Utilization

During ingredient utilization, physico-chemical properties of proteins (solubility, zeta potential, intrinsic fluorescence and protein thermal integrity) were examined. The two $pH_{utilization}$ (4 and 7) were considered while examining different protein properties. Finally, to establish the links between the interplay of process conditions, functional properties and physico-chemical properties, a multivariate approach was conducted (**Error! R eference source not found.**).

III.2.2.2.a. Sample Preparation (Chapter IV)

a) ***Ingredient-1%-buffer-suspensions.*** 1% (w/w) protein suspension of all ingredients (starting material + modified ingredients) was prepared in triplicate in citrate phosphate buffer (0.1 mol/L citric acid, 0.2 mol/L dibasic sodium phosphate) at two $pH_{utilization}$ (4 and 7) and stirred for 30 min at ambient temperature to produce *ingredient-1%-buffer-suspensions*.

b) ***Ingredient-0.1%-buffer-suspensions.*** 0.1% (w/w) protein suspension of all ingredients (starting material + modified ingredients) was prepared in triplicate in citrate phosphate buffer (0.1 mol/L citric acid, 0.2 mol/L dibasic sodium phosphate) at

two $pH_{utilization}$ (4 and 7) and stirred for 30 min at ambient temperature to produce *ingredient-0.1%buffer-suspensions*.

c) ***Ingredient-10%-aqueous-suspensions.*** 10% (w/w) aqueous suspension of the ingredients was prepared in triplicate using MilliQ water (Millipore, France).The mixture at such high concentration needed stirring overnight at 4 °C, followed by adjustment to pH 4 and 7 with another overnight stirring at 4 °C. Final concentration was brought to 6% (w/w) proteins.

III.2.2.2.b. Evaluation of Physico-Chemical Properties (Chapter IV)

a) **Nitrogen Solubility.** The soluble fractions of the *ingredient-1%-buffer-suspensions* were separated at 8,000 g for 20 min and its total nitrogen content was determined by the Dumas method using Rapid MAX N Exceed (Elementar, Langenselbold, Germany). The solubility (%) of proteins at each pH was presented as the ratio between the total nitrogen content of the supernatant and the total nitrogen content of the initial suspension.

b) **Surface charge.** Surface charge represented by the zeta potential of the undiluted soluble fractions of the *ingredient-1%-buffer-suspensions* was determined by dynamic light scattering in DTS1070 folded capillary cells equilibrated for 120 s at 25 °C using Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, U.K.).

c) **Intrinsic protein fluorescence.** Protein folding nature of the *ingredient-1%-buffer-suspensions* was analyzed by fluorescence using a FS 920 fluorescence spectrometer (Edinburgh Instruments Ltd., Livingston, United Kingdom). Additional experiments with 0.1% (w/w) protein concentration were conducted to observe any changes in fluorescence signals due to the dilution. The excitation-emission map of the protein region was developed by varying excitation wavelengths from 250 to 340 nm at 5 nm increments, and by varying emission wavelengths from 300 to 360 nm at 2 nm increments for a dwell time of 0.05 s, using excitation and emission slits of 5 nm. Fluorescence data was processed by parallel factor analysis (PARAFAC) [269]. The fluorescence landscapes were first pre-processed by removing the Rayleigh scatter according the procedure suggested by Thygesen, Rinnan, Barsberg, & Møller, 2004. This was then analyzed by PARAFAC into three matrices: score matrix, an excitation loading matrix and an emission loading matrix. The two suspensions at 0.1 and 1% (w/w) were analyzed separately, but both gave rise to a three-factor model. The

fluorescence landscapes were processed and analyzed in MATLAB (Mathworks, Massachusetts, United States).

d) **Protein thermal integrity (DSC).** *Ingredient-10%-aqueous-suspensions* of starting material, along with the modified ingredients treated very gently (pHX_55 °C_Low) and vigorously (pHX_95 °C_High) at different $pH_{process}$ were prepared to assess their protein integrity due to process conditions. Approximately 60 mg of these suspensions were transferred to a 120 µL medium pressure crucible and analyzed in a differential scanning calorimeter (Mettler Toledo, Ohio, United States). The crucible was heated from 50 to 120 °C at 5 °C/min, with an empty reference crucible. The denaturation temperature and enthalpy were determined using the DSC software package (STARe SW 16.00).

III.2.2.2.c. Relationship Between Functional & Physico-Chemical Properties (Chapter IV)

a) **Correlation Analysis.** Pearson's correlation matrix was generated between all the parameters determined for all the studied modified ingredients, using Minitab (Minitab Inc., United States).

b) **Multivariate analysis.** A PCA model of all the samples (ingredients evaluated at $pH_{utilization}$ 4 and 7) was created using latent variables from the parameters of the functional properties assessed (foaming and emulsification) along with ingredient protein parameters (nitrogen solubility, absolute zeta potential, protein and non-protein PARAFAC components) and was constructed using LatentiX2.12 (Latent5, Denmark, www.latentix.com).

III.3. Understanding the Mechanisms of Flavor Property Modifications

III.3.1. Evaluation of Odor Perception

Just as the evaluation of functional properties, perception of odor was chosen as a key criteria to evaluate the impact of modified ingredients at different conditions of utilization. Odor perception gives the first indication of flavor of foods, reflecting the food quality and evoking psychological, physiological and behavior responses in humans [51], [84]–[86]. Therefore the study of odor and its origins was kept of high importance in this study. Odor perception was evaluated by a trained panel. Both qualitative and quantitative

aspects of odor were studied. A large list of attributes was used proposed to panelists in regards to literature and previous studies performed in the lab. Two methods were used to describe odor of all the studied samples: odor Check-All-That-Apply (CATA) and intensity evaluation. The panel was first trained to memorize all possible odor notes associated to odor of plant-based food and then retrieve these attributes in the ingredient suspensions.

III.3.1.1. Sample Preparation **(Chapter V)**

III.3.1.1.a. Preparation of ingredient-5%-aqueous-suspensions

For the sensory evaluation, all ingredients (starting material and modified ingredients) were suspended to 5% (w/w) powder concentration in deionized water and stirred for 30 min at 20 °C. Furthermore, the pH of these suspensions was readjusted to $pH_{utilization}$ (4 or 7) using 6 mol/L hydrochloric acid or sodium hydroxide respectively. A total of 74 suspensions were produced from 37 ingredients, on the same day of the sensory analysis, and stored at 20 °C until the evaluation. The references and products suspensions were presented to the panelists in 80 ml plastic cups covered with a lid. The samples were labeled with random three-digit numbers.

III.3.1.2. Odor Perception Description (Chapter V)

III.3.1.2.a. Subjects

Twenty-one volunteer panelists (13 women and 8 men, 18-40 years in age) were recruited based on their ability and willingness to participate in this study. The panelists had previously different levels of experiences in sensory study participation. The overall aim of the experiment was communicated to them beforehand, where they gave their free and informed consent and additionally received for their participation. Prior to the sessions, the panelists were asked not to smoke or consume coffee, tea or other flavor-intense foods. All sessions were conducted and monitored by the authors. The experimentation was performed at UMR SayFood (Université Paris-Saclay, INRAE, AgroParisTech, on the sites of Grignon & Massy, France). All communication was done in French.

III.3.1.2.b. Panel Training

The panel training was conducted with three objectives: a) to memorize different odor attributes with the references provided; b) to reach a consensus between all the judges

on the choice of the attributes to use and their definition; and c) to train to evaluate the key odor by selection of the main notes characterizing the samples and evaluation of the perceived intensities of a reduced number of attributes. The complete list of attributes was selected after discussion with the panelists to describe all the perception of the ingredients and various reference products were proposed to subjects to help for recognition and learning of various sensations. After the training sessions, a total of 36 attributes was finally selected for the subsequent sessions (**Table 8**). for Check-All-That-Apply test (CATA test). Additionally, four different classes of attributes were identified during the discussions: green (notes vertes), "sweet" (notes sucrées), rancid (notes rances) and cooked notes (notes cuites). The lexicon "sweet" has been used to describe aroma of beverages (e.g. brewed coffee), where the term is associated with caramel/vanilla aroma notes [271]. The intensities of these four odor notes were evaluated for all the samples, following to CATA test.

Table 8 – Final list of attributes of the sensory odor profiling with the used references

Attributes in English	Attributes in French	Reference
Cut Grass	*Herbe coupée*	10% w/w *(Z)*-3-hexenol (CAS: 928-96-1) in ethanol
Celery	*Céleri*	Cubes of fresh cut celery
Hay	*Foin*	Horse hay from experimental farm (Grignon, France)
Lentil	*Lentille*	Liquid from canned lentils (Auchan, France)
Potato	*Pomme de terre*	Liquid from canned lentils (Auchan, France)
Mung Bean	*Haricot mungo*	Liquid from canned mung bean sprouts (Auchan, France)
Fresh	*Frais*	10% w/w L-menthol (CAS: 89-78-1) in ethanol
Wood	*Bois*	Bits of tree barks (Grignon, France)
Earthy	*Terre*	Moist soil (Grignon, France)
Spices	*Epice*	Four spices mix (Auchan, France)
Caramel	*Caramel*	Caramel sauce (Vahiné, France)
Rancid	*Rance*	Vegetable oil stored for several years (Grignon, France)
Grilled	*Grillé*	Grilled almonds (Auchan, France)
Burnt	*Brûlé*	Almonds grilled until black (Grignon, France)
Smoky	*Fumé*	Smoky barbecue sauce (Auchan, France)
Coffee	*Café*	Arabica coffee powder (Auchan, France)
Chocolate	*Chocolat*	Cacao powder (Auchan, France)

Hazelnut	*Noisette*	Whole hazelnuts (Auchan, France)
Coconut	*Coco*	Grated coconut (Auchan, France)
Orange Blossom	*Fleur d'oranger*	Orange Blossom Aroma (Fabster, France)
Vanilla	*Vanille*	10% w/w ethyl vanillin (CAS: 121-32-4) in ethanol
Banana	*Banane*	Banana concentrated aroma(Fabster, France)
Almond	*Amande*	Almond oil (Auchan, France)
Citrus	*Citron*	Fresh cut pieces of lemon
Red Wine	*Vin Rouge*	Red wine 'Les Fiefs de Lagrange' (Saint-Julien, France)
Vinegar	*Vinaigre*	Vinegar (Auchan, France)
Milk	*Lait*	Whole milk (Lactel, France)
Butter	*Beurre*	10% w/w 2,3-butanedione (CAS: 431-03-8) in ethanol
Cream	*Crème*	Whole milk fresh cream (Yoplait, France)
Egg	*Oeuf*	Hard boiled eggs, peeled and cut (Auchan, France)
Meat	*Viande*	Beef bouillon (Auchan, France)
Ammoniac	*Ammoniac*	10% w/w ammoniac (CAS: 7664-41-7) in ethanol
Soap	*Savon*	Unscented soap bar (Le Petit Marseillais, France)
Chemical	*Chimique*	10% w/w ethyl acetate (CAS: 141-78-6) in ethanol
Cigarette	*Cigarette*	Nil[§]
Petrol	*Pétrole*	Nil[§]

[§] - The attributes were well known by the panelists, and the references for these were avoided due to safety and sensory reasons [272]–[275].

III.3.1.2.c. Odor description & Intensity

Sample evaluation session was conducted after the training sessions, using the LimeSurvey platform (LimeSurvey GmbH). Each sample was evaluated by two methods: a) selection of most pertinent attributes for the sample using Check-All-That-Apply (CATA) method (**Table 8**); and b) perceived intensity scaling ranging from 0 (none or negligible perception) to 10 (very intense) of four principal notes identified: green, "sweet", rancid and cooked notes. The samples were presented to the judges in an order according to a Latin Square experimental design to account for possible carry-over effects. The tests were carried out in single replicate, where analysis of 12 out of 37 ingredients represented true triplicates (from production of ingredients to their odor analyses).

III.3.1.2.d. Data Analysis

Statistical sensory data analysis was conducted using XLSTAT 2021.1. (Addinsoft, France). A matrix of the selected attributes from CATA data across all samples and judges were obtained in a binary form (0 or 1). First, Coqran's Q test ($p \leq 0.05$) was performed to identify the attributes that significantly discriminated the ingredient samples, followed by the Critical difference (Sheskin) multiple pairwise comparison between the attributes. Correspondence analysis (CA) with Chi-square distancing was then conducted on these significant attributes, across all judges and samples. For the intensity evaluation, analysis of variance (ANOVA) with post-hoc treatment using Newman-Keuls (SNK) method ($p \leq 0.05$) was performed across all the samples and judges. Furthermore, means of the intensities noted across all judges for each ingredient suspension were determined and a Principal Component Analysis (PCA) by Pearson's correlation method was conducted on this matrix.

III.3.2. Fate of Odorant Volatile Compounds

In order to understand the molecules at the origin of odor perception and the impact of process condition on their evolution, analysis of volatile volatile odorant compounds was performed. Similar to the study of odor perception, the composition and generation of volatiles in the headspace were examined for all the ingredients suspended at two $pH_{utilization}$ for. For that, we chose to trap the volatiles in the headspace by a selected fibre. Volatiles were then separated by gas chromatography and their detected peak areas were considered for data analysis to study the effects of process conditions on individual volatile molecules as well as the volatile groups.

To comprehend the interplay between process conditions, odor perception and volatile compound generation and release, a multivariate statistical approach was used.

III.3.2.1. Sample Preparation (Chapter V)

III.3.2.1.a. Ingredient-10%-aqueous-suspensions

A 2 g mixture of 10% (w/w) ingredient suspensions, readjusted to pHutilization 4 or 7 and then introduced with 100 ng of d7-heptanol standard, were prepared using the following method: the ingredients (FBIC and modified ingredients) were suspended in triplicates in deionized water in 20 mL SPME vials which were immediately sealed with aluminum

polytetrafluoroethylene coated silicone septum caps in order to avoid loss of volatiles. The vials were agitated by IKA Vortex 2 (IKA Works, Inc., Staufen, Germany) for 30 min at 20 °C, followed by a pH adjustment of either pH 4 or 7 using 0.1 mol/L hydrochloric acid or 0.1 mol/L sodium hydroxide respectively (Sigma Aldrich, Missouri, United States). Additionally, 100 ng of d7-heptanol (Ref: D6920, Cluzeau Infor Labo C.I.L, France) from a 0.1 µg/mg ethanol stock solution was introduced into the vials after the pH adjustment. The required amounts of acid, base and standard stock were added into the vials using a 50 µL eVol™ syringe (Trajan Scientific and Medical, Australia).

III.3.2.2. Study of Volatile Chemistry (Chapter V)

III.3.2.2.a. Extraction of volatiles

The volatile compounds were extracted from the headspace of the samples by automated solid-phase micro-extraction (HS-SPME). Prior to extraction, each vial was incubated at 50 °C for 36 min under agitation (10/1 s on/off) to reach equilibrium between the matrix and the headspace. For the ensuing extraction, a gray-notched divinylbenzene/carboxen/polydimethylsiloxane DVB/CAR/PDMS fiber (2 cm, Supelco) was exposed to the headspace for 42 min at the same temperature. The fiber was then desorbed on a GC injection port, which was held at 250 °C, during 2 min. A 10 min fiber reconditioning procedure was performed at 270 °C between samples. The tri-phase fiber was selected to ensure an efficient extraction of a wide range of volatile compounds [170], [276], [277].

III.3.2.2.b. Detection of volatiles

The headspace extracts were analyzed by gas chromatography (GC) in a Trace GC Ultra system coupled to an ISQ single quadrupole mass spectrometer (MS, Thermo Scientific, Rodano, Italy). A non-polar ZB-5MSPLUS column (30 m x 0.25 mm x 0.25 µm, Zebron, Phenomenex, United States) was chosen for separation. Helium was used as carrier gas at a constant flow rate of 1.2 mL/min. The parameters were based on the method optimized by Cepeda-Vázquez (2017) in the same equipment, with slight adjustments following pre-tests to achieve a better chromatographic separation of the volatile compounds present in faba beans. Injections were done in splitless mode. The GC oven was programmed as follows: initial temperature 40 °C (held for 5 min), then raised at 1 °C/min until 90 °C and 15 °C/min until a final temperature of 240 °C (held for another 5 min). Mass spectrometry was carried out using electron impact at 70 eV as ionization mode. MS transfer line and

ion source temperatures were set to 250 °C and 200 °C, respectively. A standard solution of deionized water with 100 ng d7-heptanol was analyzed for every sequence of 10 runs to assure the steadiness of the system's response over time. Data acquisition was done in full scan mode from m/z 33 to 300. Each compound was identified and confirmed by means of the Wiley 8 and NIST 08 mass spectral libraries, calculation of normal alkane retention index (RI) and comparison to NIST Chemistry Web- Book Standard Reference Data Program 69 indices. The chromatographic peak areas for each compound were calculated by extracting the quantifier ions specific for that compound, and then integrated using the Quan browser of Xcalibur 2.1.0 (Thermo Fisher Scientific Inc., United States). The retention indices were calculated using the isothermal and non-isothermal formulae established by Kovatz (Eq. 1) and Van den Dool and Kratz (Eq. 2), respectfully (National Institute of Standards and Technology (NIST), 2008), based on the retention times of a series of alkanes (C5-C17, C19-C23) analyzed under the same conditions. Eq. 1 was only applied in the peaks eluted in the first and last 5 min of analysis, for which the oven temperature was held constant, while Eq. 2 was used for all additional peaks.

$$I_x = (100 * n) + 100 * \frac{(logt_x - logt_n)}{(logt_{n+1} - logt_n)} \quad \text{(Eq. 1)}$$

$$I_x = (100 * n) + 100 * \frac{(t_x - t_n)}{(t_{n+1} - t_n)} \quad \text{(Eq. 2)}$$

where Ix = retention index of the volatile, tx = retention time of the volatile, tn and tn+1 = retention times of the smaller and larger alkanes corresponding to the volatile.

The integrated volatile peak areas above the limits of quantification were selected and normalized with d7-heptanol peak areas of their respective chromatograms and the ingredient dry weights measured for each chromatographic analysis.

III.3.2.3. Relationship Between Odor Perception & Volatile Chemistry (Chapter V)

III.3.2.3.a. Multivariate Analysis

Statistical data analysis was conducted using XLSTAT 2021.1. (Addinsoft, France). The relationships between the composition, the odor characteristics and intensities, as well as the relative amounts of volatile constituents of different ingredient suspensions were examined using Multiple Factor Analysis (MFA). Four qualitative variables ($pH_{process}$, $T_{process}$,

$t_{process}$, $pH_{utilization}$), along with three quantitative matrices (CATA data, average odor intensities and normalized volatile peak areas) were used for the MFA. Summation of the binary CATA data for each sample, across all judges was prepared exclusively for this analysis.

III.3.3. Fate of Non-Volatile Compounds

Taste is an essential part of flavor perception. Due to safety constraints, we choose to study only the composition in non-volatile molecules of modified ingredients, and it was not possible to evaluate sensory taste perception. Indeed, the ingredients were modified by a variety of extreme acidic and alkaline conditions. Therefore a sensory analysis on taste perception would require a stringent examination and authorization of safety of each ingredient not possible during the PhD work. Nevertheless, an attempt to find taste indications was made through the analysis of non-volatiles in the ingredients. For this part of PhD work, only selected promising modification conditions were chosen – either very gently processed (55 °C for 30 min) or very vigorously processed (95 °C for 360 min) for each $pH_{process}$ type of modification. The least chemically intrusive processes i.e. without any pH adjustment or $pH_{process}$ 6.4 was studied in much greater detail, taking account all the levels of temperature ($T_{process}$) and time ($t_{process}$). Hydro-alcoholic mixtures were used to extract non-volatiles from the different ingredients, which were then separated by liquid chromatography. Relative peak areas of the detected non-volatile compounds were used for data analysis.

III.3.3.1. Sample Preparation (Chapter VI)

III.3.3.1.a. Ingredient-Hydro-alcoholic-Extracts

Non-volatile compounds were extracted according to a protocol adapted from Chaieb et al., 2015 and Love et al., 2020 [278], [279]. A suspension of each ingredient (FBIC or modified ingredients) was prepared in a 30 mL glass vial (VWR, Rosny sous bois, France) by adding to the 0.6g of ingredient, 4mL of a mixture composed of absolute ethanol (Carlo Erba, Val de Reuil, France) and Milli-Q water (Millipore, France) (70/30, v/v) and containing 1mg/L of added leucine enkephaline (CAS 81678-16-2; Waters, Milford, USA) for internal calibration. After sealing the vial by butyl/PTFE septum cap, the mixture was stirred in a multi-post magnetic agitator (2Mag MIXdrive 6 HT, Germany) at 350 rpm for 60 min at room temperature, then centrifuged (Thermo Fisher Scientific Heraeus Multifuge X3R) at 20 °C and 3600 g for 10 min. The supernatant, henceforth called hydro-alcoholic extract,

was then filtered through a 0.22 µm nylon filter (25 mm diameter, AIT, France) into a 2 mL HPLC vial closed with silicone/PTFE septum (AIT, France). Each sample was prepared in triplicate from three different ingredient suspensions.

III.3.3.2. Evaluation of Non-Volatile Compounds (Chapter VI)

III.3.3.2.a. Analysis of the Extracted Non-Volatiles

Analysis of the hydro-alcoholic extracts was performed by ultra high performance liquid chromatography coupled with a photodiode array detector and a quadrupole-time-of-flight hybrid mass spectrometer (UHPLC-PDA-QToF-MS). Analyses were performed on a Waters Acquity H-Class apparatus composed of a quaternary solvent manager pump (QSM), a refrigerated sample manager flow-through needle (SM-FTN) thermostated at 10 °C, and a column oven coupled to a photodiode array detector (PDA) and a high resolution quadrupole-time-of-flight (QToF) hybrid mass spectrometer Xevo G2-S QToF, equipped with an electrospray ionization source (ESI). 1µL of the filtered hydro-alcoholic extract was injected onto an Acquity Ethylene Bridged Hybrid (BEH) C18 column (100 x 2.1 mm, 1.7 µm particle diameter, 130 Å) thermostated at 30 °C. The mobile phase was composed of [A] water and [B] acetonitrile, both acidified with formic acid (Carlo Erba, Val de Reuil, France) at 0.1 % (v/v). An elution gradient was performed at a flow rate of 0.49 mL/min according to the following conditions: isocratic 10 % of [B] between 0 and 0.99 min; linear gradient from 10 to 20 % of [B] until 6.70 min; linear gradient from 20 to 100 % of [B] until 26 min; linear gradient between 100 and 10 % of [B] until 28 min; isocratic 10 % of [B] between 28 and 30 min. The MS full scan analysis was under negative polarity, using the resolution mode for a scan time of 0.5 s and a mass range from m/z 50 to 1500 acquired in centroid; The collision energy was fixed at 6 eV; The internal calibration of the QToF analyzer was performed every 20 s at a continuous flow of 5 µL/ min of leucine enkephalin (1 mg/L) for a total of 3 scans lasting 0.2 s each; The ESI parameters consisted of a capillary voltage of 0.5 kV, a sampling cone of 40 V with a cone nitrogen gas flow of 50 L/h, a source offset of 80 V kept at 120 °C and a desolvation gas (nitrogen) at 550 °C with a flow of 1200 L/h. The MS/MS analyses were performed on ions of interest by data dependent acquisition (DDA), switching from MS (noted MS^1) to MS/MS (noted MS^2) when the intensity of a candidate ion was above a threshold of 20 000 intensity/scan, and then performing a scan of the daughter ions for 0.5 sec of the selected precursor to confirm their identity, under the same MS conditions than the ones described above, at a constant collision energy of 30 eV; The internal calibration and the ESI parameters were also identical to those described above. The MS-analysis was performed by two successive steps in order to focus i) on major pseudo molecular ions (MS^1),

and ii) on products ions after fragmentation (MS^2 DDA mode, with fragmentation). Simultaneous acquisition was performed with the PDA detector, at 20Hz from 190 to 500 nm, with a resolution of 1.2 nm. The UV and mass spectra were acquired and treated by MassLynx software. The data treatment was extracted with open source software for mass spectrometry files mzMine 2 [280]. The peak areas obtained were normalized with the leucine enkephalin signal area and by the exact amount of ingredient used for preparing the sample, in dry weight.

III.3.3.3. Data Analysis (Chapter VI)

Statistical data analysis was conducted using XLSTAT 2021.1. (Addinsoft, France). A matrix of the normalized peak areas of all detected compounds by UHPLC-PDA-QToF-MS was obtained across all the hydro-alcoholic extracts. Three-way ANOVA ($pH_{process}$, $T_{process}$, $t_{process}$) with post-hoc treatment using Newman-Keuls (SNK) method ($p \leq 0.05$) and PCA using Pearson's correlation method were conducted on this matrix.

IV

What Mechanisms Explain Foam & Emulsion Properties?

IV. What Mechanisms Explain Foam & Emulsion Properties?

IV.1. General Introduction

This chapter brings together different aspects of fava bean protein properties and associated beverage functionalities as a function of process conditions – to understand mechanisms involved in the interplay between them.

The first part of this chapter is proposed with an article (recently published, https://doi.org/10.3390/ foods10102489), which tries to understand the relationships between different physico-chemical and functional properties of fava bean ingredients through a statistical approach. The objective of this study was to establish these links with a rapid and efficient analysis of a large data-set, obtained from 37 different ingredients (initial and modified fava bean concentrates) across 26 different variables (ingredient properties). The two statistical methods used were Principal Component Analysis (PCA) and Pearson's correlation. Precisely, the fava bean initial concentrate (FBIC) was modified by pH (2, 4, 6.4 and 11), temperature (55, 75 and 95 °C) and treatment duration (30 and 360 min). These were further utilized at two pH (4 and 7) in systems close to beverage applications. During their utilization, both physico-chemical and functional properties were evaluated. Protein-associated physico-chemical properties investigated here were protein charge, solubility and intrinsic fluorescence signals, whereas the functional parameters studied were foam and emulsion capacity and stability. Additionally, certain non-protein associated fluorescence signals were also analyzed in relation to functional parameters, to study if the functional properties are solely influenced by protein properties.

The second part of this chapter aims to explain the foam and emulsion properties through different protein characteristics, with a deepened investigation on the relationship between both. During ingredient modification, protein structural modifications, i.e. protein hydrolysis and aggregation, were monitored. Further during utilization, protein charge, solubility and intrinsic fluorescence signals were monitored, but with an additional insight of protein thermal integrity of gentler and vigorously processed ingredients.

IV.2. Two Statistical Tools For Assessing Functionality & Protein Characteristics of Different Fava Bean (Vicia Faba L.) Ingredients

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Abstract: Fava bean (*Vicia faba* L.) is a promising source of proteins that can be potentially used as nutritional and/or functional agents for industrial food applications. Fava ingredients are industrially produced, modified, and utilized for food applications. Their processing conditions influence physico-chemical protein properties that further impact ingredient functionality. To design a functionally suitable ingredient, an understanding of the interrelationships between different properties is essential. Hence, this work aimed to assess two statistical analytical tools, Pearson's correlation and Principal Component Analysis (PCA), for investigating the role of the process conditions of fava ingredients on their functional and protein properties. Fava concentrates were processed by pH (2, 4, 6.4 and 11), temperature (55, 75 and 95 °C) and treatment duration (30 and 360 min) into different modified ingredients. These were utilized under two application conditions (pH 4 and 7), and their foam and emulsion properties as well as their ingredient characteristics (charge, solubility, and intrinsic fluorescence) were measured. The results show that foam and emulsion properties are not correlated to each other. They are associated with different protein and non-protein attributes as fava concentrate is a multi-component matrix. Importantly, it is found that the results from the two statistical tools are not fully comparable but do complement each other. This highlights that both statistical analytical tools are equally important for a comprehensive understanding of the impact of process conditions on different properties and the interrelationships between them. Therefore, it is recommended to use Pearson's correlation and principal component analysis in future investigations of new plant-based proteins.

Keywords: PCA, Pearson's correlation, processing, foam, emulsion, beverage application

IV.2.1. Introduction

The popular and increasing demand for plant-based foods amongst consumers brings forth the need to understand plant-protein ingredients' properties, including their functionality for food applications. Fava bean is a promising pulse source of proteins for human consumption, but contains a mixture of non-protein constituents including lipids, starch, dietary fibers and anti-nutritional factors [3], [5]. Prior to its use, the whole fava bean must be processed into ingredients such as flours, concentrates, and isolates, which may be further modified through industrial processing. Ingredient fabrication and ingredient modification impact ingredient functional properties, and thus must be optimized using appropriate process conditions and levels along with suitable assessment tools.

The protein-associated functionalities, foaming and emulsification, play a key role in beverage applications. While foams are formed from adsorbed air-in-water (A/W) interfaces, most food emulsions are produced from oil-in-water (O/W) interfaces. Typically, they both need surfactants such as proteins to stabilize the two immiscible phases. However, differences may occur due to changes in the protein functionality and/or effectiveness due to variations in the dispersed phase, its interaction capability, and/or modifications in the protein during application [11], [20], [267]. In fava beans, various protein types exist in different conformations, and any changes in these conformations during ingredient fabrication, modification and utilization affect the functionality of the ingredient [2,6,7]. In addition, various non-protein constituents, lipids, starch, and dietary fibers are also present in the ingredients [3], [5] and may affect how the ingredient functions in a food matrix. Different methodological tools can be used to evaluate ingredients, and various instrumental analyses are used to measure the physico-chemical protein properties and ingredient functionalities, resulting in a myriad of results. These data can be examined individually and provide in-depth information of each individual aspect. Connecting all results may, on the other hand, give a complementary insight into the relationships between properties and functionalities. However, it is difficult to overview many results; thus, statistical data analysis may facilitate the interpretation and assessment of such interrelationships and establish models for choosing raw materials and ingredient processing conditions. This will rely on the reliability of the model; thus, it is essential to be able to correctly evaluate a large dataset. Consequently, a properly estimated correlation model will showcase the complex relationship between protein properties and ingredient functionalities.

This paper aims to compare two different data analytical tools, Pearson's correlation analysis and Principal Component Analysis (PCA), in their assessment of a large data set. These advanced and relevant statistical tools were chosen for their diverse nature in explaining relationships between variables: one through covariance (PCA) and the other through correlation. Despite being advanced, they can now be easily used through available software and thus are relevant to both industries and researchers working on large data sets [281], [282]. By virtue of these tools, the relationship between physico-chemical protein properties (solubility, zeta potential, and intrinsic fluorescence) and ingredient functionalities (foaming and emulsification) of fava bean concentrates is evaluated. The properties measured were modified by different ingredient process conditions (pH, temperature, and treatment duration), and the functionalities were evaluated at two different pH during utilization.

IV.2.2. Materials and Methods

IV.2.2.1. Ingredient Modification

Fava bean protein concentrate containing 65% proteins (*w/w* d.b.) was procured by Döhler GmbH by milling of dried and dehulled beans followed by air classification [214]. This initial concentrate was then modified by the following method: 20% (*w/w*) suspensions were prepared with deionized water and stirred for 30 min at 500 rpm using an overhead dissolver stirrer (IKA Works, Inc., Staufen, Germany), followed by pH adjustment ($pH_{process}$) to 2, 4 or 11 using 6 N hydrochloric acid or 3 N sodium hydroxide (Sigma Aldrich, St. Louis, MO, United States) and further stirred for 30 min at 500 rpm. Additionally, a series with the natural suspension pH was used ($pH_{process}$ 6.4), which was also stirred for 30 min at 500 rpm. The suspensions were then heated ($T_{process}$) in a temperature-controlled bath (Lochner Labor+Technik GmBH, Berching, Germany) maintained at 55, 75 or 95 °C and agitated at 700 rpm for a duration ($t_{process}$) of either 30 or 360 min. All the treatments at $pH_{process}$ 4 were performed in triplicates. In total, 36 different suspensions were produced and frozen at −20 °C, followed by freeze-drying and milling to 0.08 mm mesh size to produce ingredient powders.

IV.2.2.2. Foaming

All ingredients were, in triplicates, suspended to 1% (*w/w*) protein concentration at ambient temperature at two different pH of utilization: $pH_{utilization}$ 4 and 7. 150 mL of these suspensions were whipped mechanically at room temperature using a WMF Mechanical

Frother (Württembergische Metallwarenfabrik GmbH, Geislingen, Germany) for 2.5 min and the foam was gently transferred to a graduated cylinder (inner diameter = 48.9 mm and height = 400 mm measured using a digital caliper). Foam height and liquid height were recorded manually to calculate the foam and final liquid volumes. Foaming capacity or FC (%) was calculated as the ratio of volume of foam generated after whipping and initial liquid volume. Foam stability or FS (%) corresponded to the foam capacity measured after 30 min [82].

$$\text{FC (\%)} = \frac{\text{Foam Volume at 0 }_{\text{min}}}{\text{Initial Liquid Volume}} \times 100; \text{ FS (\%)} = \frac{\text{Foam Volume at 30 min}}{\text{Initial Liquid Volume}} \times 100$$

IV.2.2.3. Emulsification

All ingredients were suspended in triplicates to 1% (*w/w*) protein concentration at ambient temperature at $pH_{utilization}$ 4 and 7. These suspensions were added with palm oil medium chain triglycerides (90:10 *w/w*) and homogenized for 1 min at 8000 rpm using T-10 Basic ULTRA-TURRAX homogenizer (IKA Works, Inc., Staufen, Germany) fitted with an S-10N-10G dispersing element. These coarse emulsions were passed twice through a homogenizer (Niro-Soavi NS 1001L Panda, Gea Group, Düsseldorf, Germany) at 200 bars. To prevent microbial growth during storage, the emulsions were pasteurized at 80 °C for 10 min after preparation. The pasteurized emulsions were stored at 4 °C for seven days [191]. The emulsion oil droplet size at days 0, 1 and 7 was characterized using laser light scattering by Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK) with degassed, deionized water used as the dispersant. The particle size distribution from 0.005 to 5000 µm as a function of volume was recorded followed by the estimation of the volumetric mean diameter (D (4;3)), 97th percentile diameter (D_{97}) and median diameter (D_{50}). The different representations were significantly correlated (Pearson's correlation coefficient > 0.900, $\alpha = 0.05$), and therefore it was decided that it is sufficient to use only one popularly reported measure, D(4;3), to evaluate emulsion capacity and stability [214], [215].

IV.2.2.4. Nitrogen Solubility

A 1% (*w/w*) protein suspension of all ingredients was prepared in citrate phosphate buffer (0.1 mol/L citric acid, 0.2 mol/L dibasic sodium phosphate) at $pH_{utilization}$ 4 and 7 and stirred for 30 min at ambient temperature to produce modified-ingredient-buffer suspensions. The soluble fraction was separated at 8000 g for 20 min and its total nitrogen content was determined by the Dumas method using Rapid MAX-N Exceed (Elementar,

Langenselbold, Germany). The solubility of proteins was determined as the ratio (in %) between the total nitrogen estimated from soluble fraction and the suspension.

IV.2.2.5. Absolute Zeta Potential

Absolute value of the zeta potential of the soluble fractions from the modified-ingredient-buffer suspensions was determined by dynamic light scattering in DTS1070 folded capillary cells equilibrated for 120 s at 25 °C using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK).

IV.2.2.6. Intrinsic Fluorescence

The modified-ingredient-buffer suspensions (1% and 0.1% *w/w* protein suspensions) were characterized by fluorescence excitation-emission scans using FS-920 fluorescence spectrometer (Edinburgh Instruments Ltd., Livingston, United Kingdom) followed by a dimensionality reduction in the fluorescence map by parallel factor analysis (PARAFAC) [269]. PARAFAC is a rapid and efficient curve resolution tool that helps decompose the fluorescence signals into its individual fluorophores. The scores from the PARAFAC models conform to Beer's Law [283]. This combination of fluorescence and PARAFAC for explaining intrinsic fluorescence of protein and protein interactions is gaining popularity [15–17].

The spectral analysis was performed at both 0.1% (*w/w*) and 1% (*w/w*) protein concentrations separately. This was due to probable inner filter effects (physical interference) and quenching (chemical interference) observed at 1% concentration [284]. The fluorescence map was obtained by measuring the emission spectra at excitation wavelengths from 250 to 450 nm at 5 nm intervals. The emission spectra were recorded from 300 to 550 nm at 2 nm intervals, with a dwell time of 0.05 s/nm. Slit widths of 5 nm were used for both excitations and emissions, and the iris was set to 100. Rayleigh scattering was removed [270]. The three-way array spectral map obtained was further decomposed by PARAFAC in MATLAB 2017b (Mathworks, Natick, MA, United States) into three matrices: a score matrix, an excitation loading matrix and an emission loading matrix. The landscape was then divided into two areas: one in the protein region, ranging from 250 to 300 nm in excitation and 325 to 360 nm in emission [285], [286], and one for the higher region, with excitation between 305 and 450 nm and emission between 362 and 550 nm (**Figure 12**). A three-component PARAFAC model was sufficient for modelling the protein region (PR1-3). This region is in the range of amino acid residues in proteins

(tryptophan, tyrosine and phenylalanine) [285]–[287]. At the same time, it was also necessary for seven components of the secondary region, which hereafter is noted as the non-protein region (NPR1–7). The NPR7 component at 1% (*w/w*) protein suspension was removed as it only describes small changes in the spectral behavior of the NPR1 due to inner filter effects. This secondary region explains non-native protein signals from other fluorophores, including vitamins and flavonoids that are inherently present in fava bean [3], [75], [287], [288]. The NPR signals may also contain information on possible protein modifications from Maillard reactions and polyphenol interactions [289], [290] (**Figure 12**).

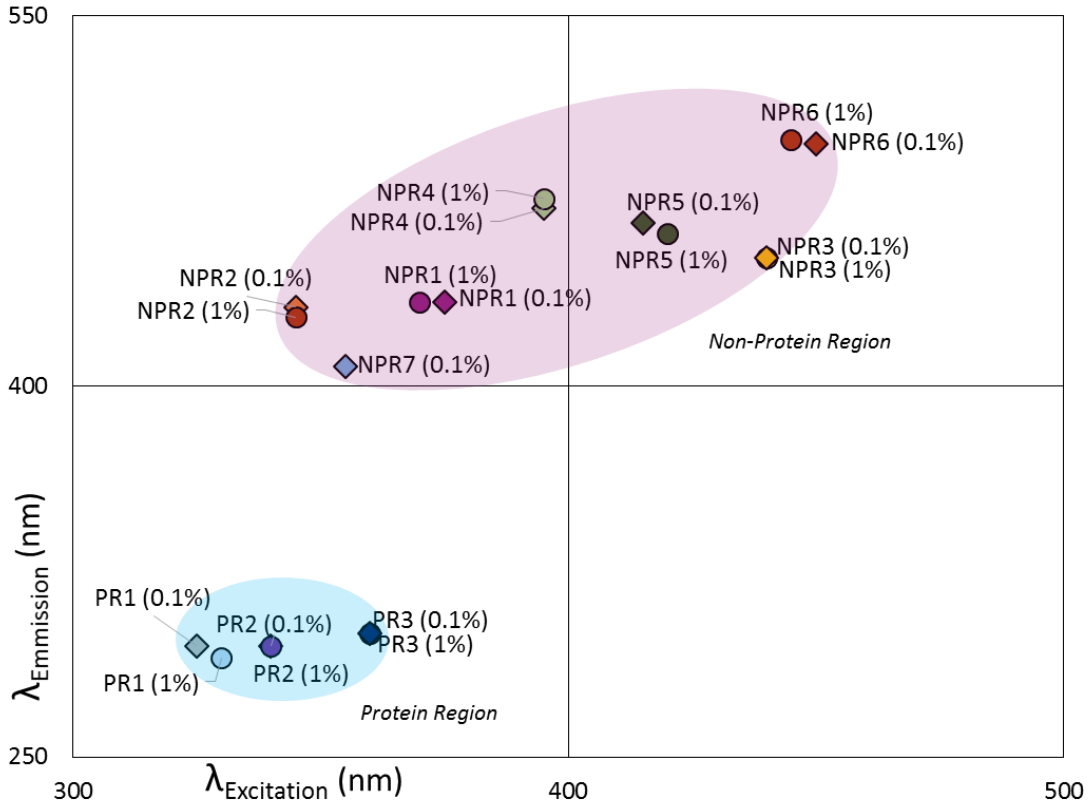

Figure 12 – Fluorescence/PARAFAC Components: Illustration of the separation of the PARAFAC components based on their maximum excitation and emission wavelengths.

IV.2.2.7. Correlation Analysis

Pearson's correlation matrix was generated between all the parameters analyzed for the ingredients in the study, which used Minitab 19.2 (Minitab Inc., State College, PA, United States) for the modified ingredients' properties.

IV.2.2.8. Principle Component Analysis (PCA)

A PCA model of all the ingredients evaluated at $pH_{utilization}$ 4 and 7 was created using latent variables from the parameters of the functional properties assessed (foaming and emulsification) along with ingredient protein parameters (nitrogen solubility, absolute zeta potential, protein and non-protein PARAFAC components). This was constructed using LatentiX2.12 (Latentix ApS, Frederiksberg, Denmark, www.latentix.com, accessed on: 10.03.2020).

IV.2.3. Results and Discussion

The foam and emulsion properties (capacity and stability) of the 37 ingredients (1 fava bean initial concentrate + 24 modified concentrates + 12 process replicates) at $pH_{utilization}$ 4 and 7 were evaluated against the following attributes: (i) nitrogen solubility, indicating the solubility of proteins; (ii) absolute zeta potential, representing the protein surface charge; (iii) fluorescence PARAFAC components derived from the protein region (PR1–3); and (iv) fluorescence PARAFAC components derived from the non-protein region (NPR1–7). This resulted in a data set of 74 observations by 26 variables.

First of all, it must be taken into consideration that in this study, both PR and NPR signals were considered. It is likely that both these types of signals contribute to the observed functional properties. In complex food systems containing a mixture of components, the modification of non-protein molecules and interactions between these molecules with proteins have been verified earlier [26, 27]. PARAFAC can be useful in explaining different chemical components in such systems. For example, cereal flours have been characterized by PARAFAC through a four-component model explaining proteins, vitamins and phenolic acids [288]. Similarly, the presence of phenolic compounds such as caffeic acid, kaempferol and quercetin and fluorophores related to sugar degradation, the Maillard reaction (hydroxymethylfurfural), and carotenoid and chlorophyll degradation have been detected and characterized by PARAFAC in other food systems [14, 15, 22, 24, 28]. As this study deals with protein-rich ingredients that have been modified by process conditions, possible interactions between protein and sugars and/or polyphenols could also be expected, leading to changes in protein conformations and availability for functional requirements. Complexes of pulse proteins with phenolic compounds including hydroxycinnamic acids, flavonols, flavones and flavan-3-ols have been presented in previous reports [15, 29]. The protein–tannin interaction in fava bean has also been reported to modify protein properties. Thus, the NPR signals can be important with

respect to functional properties. However, further studies on the chemical nature of these signals would offer stronger insight into the complexity of their interaction. Any relationship found between the signals and their functional properties should encourage a deeper understanding of their involvement in functional properties.

Foaming capacity (FC) and stability (FS) were evaluated against all the sample characteristics using Pearson's correlation coefficients (**Table 9**). They were significantly correlated to nitrogen solubility and absolute zeta potential. They, however, correlated differently to different protein and non-protein fluorescence signals measured at different protein concentrations. For instance, FC correlated strongly to PR1, 2 and 3 fluorescence signals at 0.1% protein suspensions, NPR3, 4 and 5 at 0.1% protein suspensions and NPR1, 3 and 5 at 1% protein suspension. On the other hand, FS correlated significantly to PR1, 2 and 3 at 1% protein suspension along with NPR2, 4, and 7 at 0.1% and NPR1, 4, and 6 at 1% protein suspensions. It is interesting to note that the protein region at the low-concentration (0.1%) suspension is more related to the foaming capacity, while the high-concentration (1%) suspension is more related to the foaming stability. The correlation between the NPR fluorescence components to foam capacity and stability suggests the possibility of non-protein components influencing foaming, as a function of the process conditions.

Table 9 – Pearson's correlation analysis between foam (FC and FS) and emulsion (D(4;3)) properties and protein and non-protein features.

	Foaming		**Emulsification**	
Properties	**FC**	**FS**	**$D(4;3)_{Day0}$**	**$D(4;3)_{Day7}$**
Nitrogen Solubility	**0.284 ***	**0.495 ***	**−0.291 ***	−0.271
Absolute Zeta Potential	**0.343 ***	**0.693 ***	**−0.357 ***	**−0.366 ***
PR1 (0.1%)	**0.288 ***	−0.004	**0.240 ***	0.227
PR2 (0.1%)	**−0.305 ***	0.035	**−0.199 ***	**−0.187 ***
PR3 (0.1%)	**0.367 ***	0.166	**0.339 ***	**0.312 ***
PR1 (1%)	0.203	**0.447 ***	**−0.277 ***	**−0.286 ***
PR2 (1%)	−0.137	**−0.271 ***	0.221	0.213
PR3 (1%)	0.223	**0.404 ***	**−0.436 ***	**−0.455 ***
NPR1 (0.1%)	−0.078	−0.040	−0.041	−0.030
NPR2 (0.1%)	0.197	**0.493 ***	**−0.370 ***	**−0.321 ***
NPR3 (0.1%)	**0.274 ***	0.149	0.058	0.066

NPR4 (0.1%)	**0.324 ***	**0.678 ***	**−0.515 ***	**−0.477 ***
NPR5 (0.1%)	**−0.228 ***	−0.096	**−0.428 ***	**−0.378 ***
NPR6 (0.1%)	0.093	−0.015	**−0.254 ***	**−0.233 ***
NPR7 (0.1%)	0.207	**0.329 ***	−0.184	−0.149
NPR1 (1%)	**−0.261 ***	**−0.543 ***	0.162	0.121
NPR2 (1%)	−0.015	−0.123	−0.066	−0.073
NPR3 (1%)	**0.339 ***	0.119	0.184	0.187
NPR4 (1%)	0.196	**0.526 ***	**−0.578 ***	**−0.543 ***
NPR5 (1%)	**−0.276 ***	−0.016	**−0.453 ***	**−0.394 ***
NPR6 (1%)	−0.170	**−0.309 ***	−0.009	−0.015

Significant differences are indicated by bold and * ($\alpha = 0.05$).

Emulsification was also tested against different protein and non-protein features. Emulsion oil droplet sizes obtained at three-time intervals (days 0, 1 and 7) were also evaluated against the sample characteristics using Pearson's analysis. The D(4;3)-value represented the extent of flocculation of oil droplets and possible protein aggregation in the emulsions, thus indicating inversely the capacity of the proteins to form emulsions (day 0) and their capability to stabilize the emulsions (day 7). The emulsion capacity ($D(4;3)_{Day0}$) was significantly correlated with nitrogen solubility and absolute zeta potential, but the $D(4;3)_{Day7}$ after storage was significantly correlated only to the absolute zeta potential (**Table 9**). A negative correlation between D(4;3) and the two properties indicates that higher protein solubility and absolute zeta potential resulted in decreased emulsion flocculation and protein aggregation. Other significant factors associated with $D(4;3)_{Day0}$ were the PR1, 2, and 3, and NPR2, 4, 5 and 6 for 0.1% suspensions and PR1 and 3, and NPR4 and 5 for 1% suspensions. Just as the case of protein solubility, the $D(4;3)_{Day7}$ after storage of the emulsions was no longer associated with PR1 at 0.1% protein concentration. In general, the set of correlation parameters associated with emulsification is similar, while the correlation parameters differ considerably for the foaming. This indicates that the emulsion capacity and stability are highly correlated, while the two foaming parameters behave differently.

Overall, different correlations between foam and emulsion properties with nitrogen solubility and absolute zeta potential were obtained by Pearson's analysis (**Table 9**). This indicates, that the two beverage functionalities work by different mechanism(s), as supported by the lack of correlation between them (**Table 10**). Differences in the dispersed phases between food foams (air) and emulsions (oil or water), and the

differences in the molecular mechanisms of interaction of proteins with these phases have been suggested in other works [3, 30, 31]. Studies on ingredients derived from chickpea, lupin, pea, lentil and fava beans have been performed where relationships between protein properties (surface charge, solubility, and intrinsic fluorescence) and foam and emulsion properties have been established to a certain degree [191], [292]–[294]. From the previous studies and the results presented, one could infer that it may not be a single property, but a combination of different associated properties that can better explain the underlying mechanism and properties of protein functionalities. For example, from the absolute zeta potential, it could be inferred that despite the protein surface charge representing the amphiphilic behavior of the proteins, further understanding is required to validate how this property helps the protein interact with the distinctive dispersed phases to enable different functional properties. However, it is not within the scope of the present paper to explain the specific physicochemical properties of the functionalities.

Table 10 – Pearson's correlation analysis between foaming and emulsification.

	Foam Capacity (FC)	**Foam Stability (FS)**
Emulsion Capacity, $D(4;3)_{Day0}$	0.165	−0.099
Emulsion Stability, $D(4;3)_{Day7}$	0.172	−0.054

The results of the principal component analysis are shown as a biplot of scores and loadings in **Figure 13**. The PCA scores were separated at two levels: primary separation by different $pH_{utilization}$ 4 and 7, and secondary separation by $pH_{process}$ (2, 4, 6.4 and 11) during ingredient modification. This indicates that both the pH during ingredient processing and application have an important effect and explain about 51% of variance between different properties. The first two PCA components explained the major variance in the data and, as seen in **Figure 13**, efficiently described the system with regard to both $pH_{utilization}$ and $pH_{process}$. As seen below, the functionalities did not correlate with each other, whereas the emulsion properties are more correlated than the foaming parameters. Furthermore, the $pH_{utilization}$ mostly influenced foaming, and in particular the FS. The

$pH_{process}$, on the other hand, has the largest impact on the differences in emulsion properties. This can be seen as the difference in the $pH_{utilization}$ is from first to third quadrant, with the foam properties mainly moving samples along the second principal component. The emulsion properties are all along the first principal component, the main direction of the difference between the $pH_{process}$ of the samples.

The PCA showed differences in characteristics associated with foam (FC and FS) and emulsion ($D(4;3)_{Day0,\ 1\ and\ 7}$) properties. However, comparing this PCA with the Pearson's correlation (**Table 9**), it is seen that they do not lead to the exact same conclusion, as the Pearson's correlation is a pair-wise comparison of variables, while the PCA takes into account all variables at the same time. Furthermore, the correlation pattern seen in the Pearson's results is not totally explained by the PCA, with the PCA describing around half of the variance (while the Pearson's correlation is based on all the variance in each of the pair-wise estimates). The PCA is a variance analysis and it clearly indicates that the main variability in the data is due to the two pH parameters (process and utilization). Therefore, it is necessary to investigate both the PCA and the Pearson's correlation for a successful interpretation.

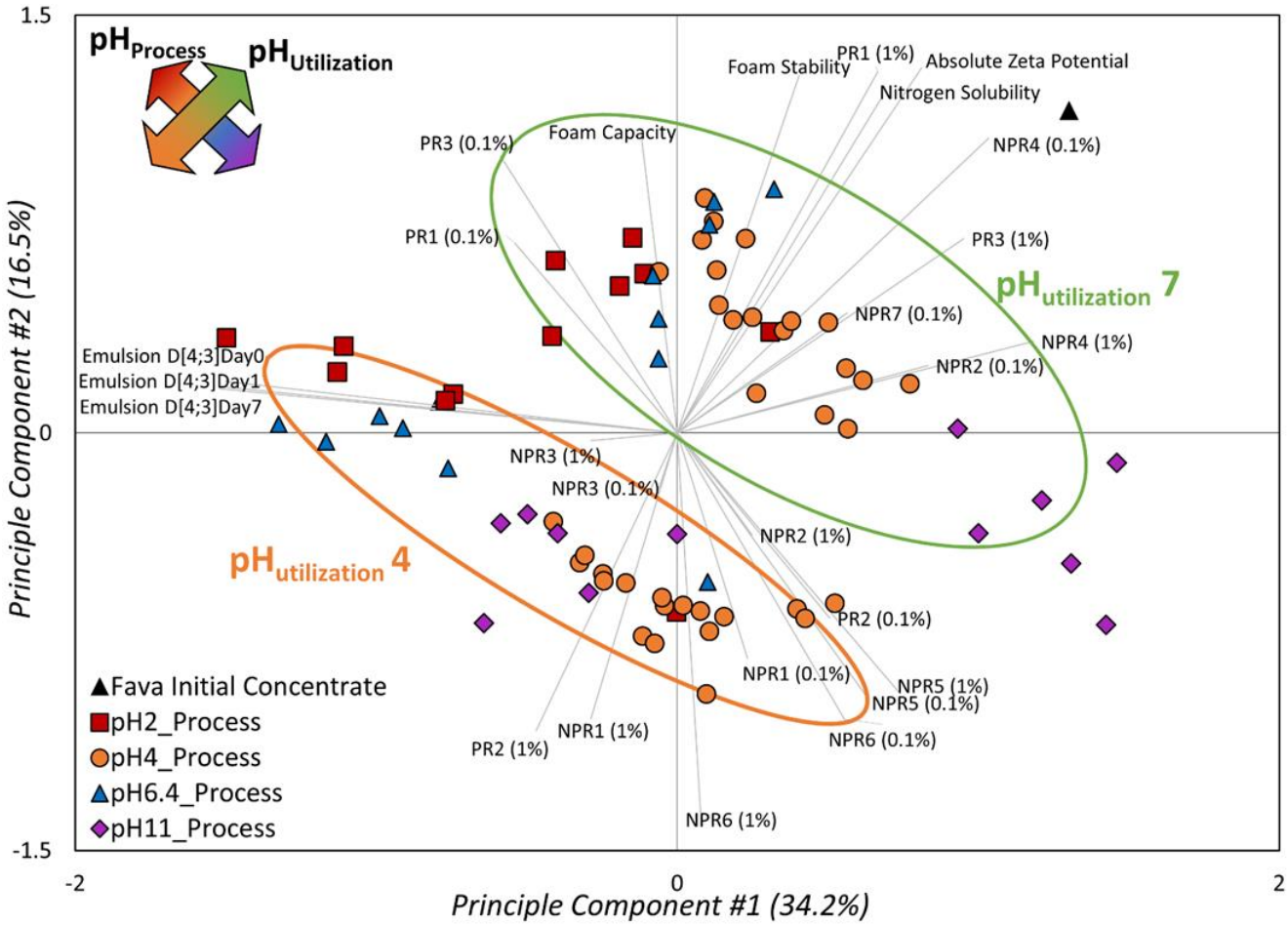

Figure 13 – Principal Component Analysis: PCA biplot of fava ingredients (1 fava bean initial concentrate + 36 modified concentrates) evaluated at two conditions (pH 4 and pH 7) as scores, with the foam and emulsion functionalities and other ingredient attributes as loadings. The effect of pH during modification is shown by different symbols. The pH during utilization process is indicated with confidence ellipses (α = 0.95). PR and NPR are the PARAFAC components (at 0.1% and 1%, **Table 9**) based on the protein and non-protein regions of the fluorescence landscape.

For example, the absolute zeta potential was significantly correlated to FC, FS, $D(4;3)_{Day0}$ and $D(4;3)_{Day7}$ (**Table 9**), while in the PCA biplot this association is not clear for the emulsion properties (**Figure 13**). However, through a closer look at the values in **Table 9**, it becomes clear that the highest correlation is between FC and the zeta potential, while the emulsion properties are negatively correlated (which also is seen in the PCA). On the other hand, the PCA shows a clear association between absolute zeta potential and the $pH_{utilization}$. Therefore, an overall interpretation from the two analyses suggests that foam

and emulsion properties are strongly correlated to the zeta potential and nitrogen solubility (**Table 9**), and they are all influenced by process conditions, especially pH during ingredient utilization (**Figure 13**). Relationships between charge and solubility, and foam and emulsion properties have been indicated in plant-based ingredients. In fact, the same negative correlation between higher surface charge and decreased emulsion droplet size has been noted for chickpea, fava, pea and lentil isolates [191]. A lower absolute charge is often related to a lower solubility, and the protein intrinsic fluorescence is often used to characterize protein hydrophobicity and the folded nature. Process conditions changing protein properties have been shown to modify foam and emulsion properties [6]. **Figure 13** and **Table 9** clearly illustrate these different relationships between process conditions, changes in protein and non-protein aspects, and thereby changes in foam and emulsion properties.

The two analyses associating functionalities to the fluorescence signals indicate that fava bean concentrate is a multi-component system containing different proteins and non-protein elements as well as protein modifications, which all seem to affect functional properties. These fluorescence signals (PR and NPR) were associated with functionalities (**Table 9**) and were highly impacted by the pH during ingredient modification and utilization (**Figure 13**). The two separate PARAFAC models of the fluorescence data (at 0.1% and 1% *w/w*) both gave three underlying components in the protein region, but different components (seven and six components at 0.1% and 1%, respectively) in the non-protein region. Additionally, these signals were highly affected by the dilution. For instance, the PR components at 0.1% and 1% protein suspensions were differently correlated to functionalities. This clearly indicates the possibility of inner filter effects in the fluorescence data, most probably more pronounced at the 1% suspension than at the 0.1% one. Despite this probable inner filter effect in the 1% suspension, it is of interest to note that the data from 1% suspension are more related to the foaming, and thus also to $pH_{utilization}$, while the data from 0.1% are more related to the emulsion properties, and thus also to $pH_{process}$.

IV.2.4. Conclusions

Statistical models facilitated a rapid comprehension of the large data set that represented functional and physico-chemical properties. Beverage functionality, as measured by the foam and emulsion properties of different fava bean ingredients modified by various process conditions, was correlated to their multi-component character. These two

beverage functionalities were first and foremost not correlated to each other. The associations between the ingredient characteristics and functionalities obtained by Pearson's correlation analysis and PCA were not fully comparable as one explained association and the other suggested causalities and effects. Where Pearson's correlation validated the associations between functionalities and physico-chemical properties, PCA suggested the impact of process conditions on ingredient properties along with some obvious associations between the properties. Despite certain breakthroughs in the critical understanding of research methods, we must note that further investigations are needed to identify and explain the underlying phenomena in the ingredient responsible for the functionality. In this respect, a paper focusing on the mechanistic understanding of the results presented in this paper is under preparation.

Author Contributions: Conceptualization, S.S., M.-N.M., A.S-E., and J.Z.; methodology, validation, formal analysis, investigation and data curation, S.S., Å.R., and J.S.; resources, J.Z., D.B., and J.A.; writing—original draft preparation, S.S.; writing—review and editing, all.; visualization, S.S.; supervision, Å.R., V.O., K.O., J.Z., A.S.-E. and M.-N.M.; project administration, M.-N.M.; funding acquisition, M.-N.M., D.B., and J.A. All authors have read and agreed to the published version of the manuscript.

IV.3. Process conditions govern fava bean (*Vicia faba* L.) functionality: Emphasis on the interplay between protein modifications and physico-chemical properties, foaming and emulsification.

Manuscript Submitted – Food Hydrocolloids

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Abstract

Fava bean (*Vicia faba* L.) is a promising source of proteins owing to its benefits on health and environmental sustainability. Thus, fava protein-rich ingredients have a great potential in industrial food applications since processing of such ingredients can modify proteins and their functional properties. This study shows that there is no straightforward relationship between fava protein-associated reactions (hydrolysis and aggregation), protein properties and functional properties. A high number of modified fava concentrates was produced for the study from an air-classified fava protein concentrate processed at different combinations of pH (2, 4, 6.4 and 11), temperature (55, 75 and 95 °C) and duration of treatment (30 and 360 min). It was found that during ingredient modification: (1) protein hydrolysis was favored by low pH and high temperature, while (2) protein aggregation occurred at high pH and temperature. These reactions influenced foam and emulsion properties differently, emphasizing the differences in their individual stabilizing mechanisms. Despite the modifications in fava proteins, their physico-chemical and functional properties in the processed ingredients were nevertheless primarily governed by the pH of beverage application. The surprising interplay shown between properties encourages the need to dive further into the different protein-associated interactions that can occur in fava concentrate.

Keywords: Protein functionality**,** plant-based, modification, hydrolysis, aggregation

IV.3.1. Introduction

Fava bean (*Vicia faba* L.) has a great potential for human consumption due to its nutritional, functional and agronomic aspects [3]. Fava bean can be processed to form ingredients (ingredient fabrication) and these ingredients can further be modified using process conditions (ingredient modification) and eventually be utilized in food applications (ingredient utilization) [5]. In fava bean, various protein types, majorly globulins (legumin, vicilin, convicilin) exist in different conformations. Any changes in these conformations during ingredient fabrication, modification and utilization affects the functional property of the ingredient [5], [24], [82]. Functionalities such as foaming and emulsification, play a key role in beverage applications such as ice-cream, pudding, mousse, etc. [11]–[14]. While foams are formed from adsorbed air-in-water (A/W) interfaces, most food emulsions are produced from that of oil-in-water (O/W). Generally, proteins are effective surfactants, and thus play an essential role in the foaming and emulsification properties of plant-based ingredients. Though foams and emulsions are based on the same structure-function relationship of proteins, differences may occur because of changes in the ingredient's effectiveness or functionality due to variances in the dispersed phase, its interactions with proteins, and/or modifications in the proteins themselves [11], [20], [267].

Protein modifications by physical, chemical and biological process techniques can facilitate foams and emulsions by influencing a balance between protein solubility, charge distribution and protein folding [11], [23]. During ingredient processing, fava proteins have been modified by temperature and pH [24], [25], mechanolysis [26], high-intensity ultrasound treatment [27], succinylation [28], acetylation [29], and enzymatic treatment [30]. The effect of any treatments on protein structure and the related effect on functionalities at application conditions is not well understood. Amongst different protein modifications, protein-protein aggregation and hydrolysis have shown to improve functionalities [27], [29]–[31]. Protein aggregation and hydrolysis can be of different types and extent that result in a variety of effects on functional properties [27], [30], [87]. In addition, fava bean contain not only proteins but also various non-protein constituents, including starch, dietary fibers, fats along with certain anti-nutritional factors [3], [5]. Hence the reactions occurring during ingredient processing may be a result of proteins and/ or non-protein constituents [88], [89]. For now, there is no clear overview of all the possible reactions occurring during processing of fava ingredients that can evidently explain the changes in functional and physico-chemical properties. This investigation

attempts to clarify the interplay between fava protein-associated reactions, protein properties and functional properties and brings forth the ambiguities in the relationship between them. The impact of industrially relevant process conditions such as pH, temperature and treatment duration on fava bean concentrate was evaluated in regards to: (1) fava protein aggregation and hydrolysis during ingredient modification, (2) physico-chemical properties of fava proteins at utilization conditions (charge, solubility, intrinsic fluorescence and thermal integrity), and (3) functional properties (foam and emulsion capacity and stability) at conditions simulating beverage applications.

IV.3.2. Materials & Methods

IV.3.2.1. Sample preparation

Starting material

Fava bean initial concentrate (FBIC) containing 65% (w/w d.b.) proteins was procured by Döhler GmbH (Darmstadt, Germany). The concentrate was produced by milling of dried and dehulled beans followed by air classification [214].

IV.3.2.1.b. Modified-Suspensions

The FBIC was modified as follows: 20% (w/w) suspensions were prepared with deionized water and agitated for 30 min at 500 rpm (~30 g) using an overhead dissolver stirrer (IKA Works, Inc., Staufen, Germany), followed by pH adjustment ($pH_{process}$) to 2, 4 or 11 using 6 mol/L hydrochloric acid or 3 mol/L sodium hydroxide (Sigma Aldrich, Missouri, United States) and further stirred for 30 min at 500 rpm. Additionally, a series with the natural suspension pH was prepared ($pH_{process}$ 6.4) by stirring for 30 min at 500 rpm. The suspensions were heated ($T_{process}$) in a temperature-controlled bath (Lochner Labor+Technik GmBH, Germany) at 55, 75 or 95 °C and agitated at 700 rpm for a duration ($t_{process}$) of either 30 (Low) or 360 (High) min. The suspensions produced after these treatments are denoted as *modified suspensions*. All the treatments at $pH_{process}$ 4 were performed in triplicates in order to assess reproducibility.

IV.3.2.1.c. Modified Ingredients

The different *modified-suspensions* were frozen at −20 °C, followed by freeze-drying and milling to 0.08 mm mesh size by an ultra-centrifugal mill ZM 200 (Retsch GmbH, Germany). This resulted in different modified ingredient powders, which are named as $pH_{process}\_T_{process}\_t_{process}$ (e.g. pH2_55 °C_Low) based on the conditions used to modify them.

IV.3.2.1.d. Ingredient-Aqueous-suspensions

All ingredients were suspended in deionized water in triplicates to 1% (w/w) protein concentration and stirred for 30 min at ambient temperature at the two $pH_{utilization}$ (4 and 7) to prepare *ingredient-aqueous-suspensions.* The pH were adjusted either using 6 mol/L hydrochloric acid or 6 mol/L sodium hydroxide. These systems were chosen as mimicking realistic beverage applications.

IV.3.2.1.e. Ingredient-buffered-suspensions

1% (w/w) protein suspension of all ingredients (FBIC + modified ingredients) were prepared in triplicates in citrate phosphate buffers (prepared from 0.1 mol/L citric acid, 0.2 mol/L dibasic sodium phosphate) at two $pH_{utilization}$ (4 and 7) and stirred for 30 min at ambient temperature to produce *ingredient-buffered-suspensions*. Ionic strength of the buffer solutions used was calculated by the formula $\sum C_i z_i^2/2$, where C_i is the molar concentration of the ion species 'i' and z_i is the net charge of that ion [295].

IV.3.2.2. Protein-associated reactions

IV.3.2.2.a. Protein aggregation

Particle aggregation in the *modified-suspensions* (section 1.1.2) was measured using laser light scattering by Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, U.K.) with degassed, deionized water used as the dispersant. The particle size distribution (PSD) from 0.005 to 5000 µm as a function of volume was recorded and the volumetric mean particle diameter, D[4;3], was used to compared the level of particle aggregation after the different ingredient modification treatments.

IV.3.2.2.b. Protein acid-hydrolysis (SDS-PAGE)

The *modified-suspensions* were diluted to 2.25 mg protein/ml with Milli-Q water (Millipore, France) with a mixture containing 1% (w/v) 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris), 0.1% (w/v) sodium dodecylsulphate (SDS) and 1.4% (w/v) glycine, then submitted to sonication for 30 min and centrifugation at 10,000 g for 2 min to obtain a supernatant of dissolved polypeptides. Protein concentration of the supernatants were determined at this stage by Dumas method using Rapid MAX N Exceed (Elementar, Langenselbold, Germany). Aliquots of 22.5 µg of proteins were loaded along with peqGOLD protein marker II (VWR International, Pennsylvania, United States) into 12% (w/v) Bio-Rad Mini-PROTEAN®TGX™ gel (Bio-Rad Laboratories, California, United States) and run at 200 V for 45 min. The polypeptide bands were stained by 0.25% (w/v) coomassie brilliant blue dye. Electrophoresis was performed under non-reducing conditions. The resultant gel

band-size intensities of larger (40–100 kDa) and smaller (< 40 kDa) subunit groups were analyzed by semi-quantitative comparison of their pixel intensities in the gel using GelAnalyzer [268]. The change in band-size intensity (%) was calculated in relation to the subunit groups found in FBIC.

IV.3.2.3. Physico-chemical properties

IV.3.2.3.a. Nitrogen solubility

The soluble fractions of the *ingredient-buffered-suspensions* (section 1.1.4) were separated at 8,000 g for 20 min and its total nitrogen content was determined by the Dumas method using Rapid MAX N Exceed (Elementar, Langenselbold, Germany). The solubility (%) of proteins at each pH was presented as the ratio between the total nitrogen content of the supernatant and the total nitrogen content of the initial suspension.

Surface charge

Surface charge represented by the zeta potential of the undiluted soluble fractions of the *ingredient-buffered-suspensions* was determined by dynamic light scattering in DTS1070 folded capillary cells equilibrated for 120 s at 25 °C using Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, U.K.).

IV.3.2.3.b. Intrinsic protein fluorescence

Protein folding nature of the *ingredient-buffered-suspensions* was analyzed by fluorescence using a FS 920 fluorescence spectrometer (Edinburgh Instruments Ltd., Livingston, United Kingdom). Additional experiments with 0.1% (w/w) protein concentration were conducted to observe any changes in fluorescence signals due to the dilution. The excitation-emission map of the protein region was developed by varying excitation wavelengths from 250 to 340 nm at 5 nm increments and by varying emission wavelengths from 300 to 360 nm at 2 nm increments for a dwell time of 0.05 s, using excitation and emission slits of 5 nm.

IV.3.2.3.c. Protein thermal integrity (DSC)

FBIC and the modified ingredients treated either very gently (pHX_55 °C_Low) or vigorously (pHX_95 °C_High) at different pH (noted pHX) were taken to assess their protein integrity due to process conditions. *Ingredient-aqueous-suspensions* of 10% (w/w) was prepared by stirring overnight at 4 °C, followed by adjustment to pH 4 and 7 and overnight stirring at 4 °C. The concentration was brought to 6% (w/w) with Milli-Q water (Millipore, France) and approximately 60 mg was transferred to a 120 µL medium pressure crucible and run in a differential scanning calorimeter (Mettler Toledo, Ohio, United

States). The crucible was heated from 50 to 120 °C at 5 °C/min, with an empty reference crucible. The denaturation temperature and enthalpy were determined using the DSC software package (STARe SW 16.00).

IV.3.2.4. Functional Properties

IV.3.2.4.a. Foaming

150 ml of the *ingredient-aqueous-suspension* was whipped mechanically at room temperature using a WMF Mechanical Frother (Württembergische Metallwarenfabrik GmbH, Geislingen, Germany) for 2.5 min and the foam was transferred to a graduated cylinder (inner diameter = 48.9 mm and height = 400 mm measured using a digital caliper). Foam height and liquid height were recorded manually to calculate the foam and liquid volume, respectively. Foaming capacity (FC, %) was calculated as the ratio of volume of foam generated after whipping and liquid volume. Foam stability (FS, %) was foam capacity measured after 30 min [82]. Foam was categorized unstable when FS was below 50%.

$$\textbf{FC (\%)} = \frac{\text{Foam Volume } 0_{min}}{\text{Liquid Volume}} \text{X } 100 \text{ ; } \textbf{FS (\%)} = \frac{\text{Foam Volume } 30_{min}}{\text{Liquid Volume}} \text{X } 100$$

IV.3.2.4.b. Emulsification

The *ingredient-aqueous-suspensions* were added with palm oil medium chain triglycerides (90:10 w/w) and homogenized for 1 min at 8000 rpm using T-10 Basic ULTRA-TURRAX homogenizer (IKA Works, Germany) fitted with an S-10N-10G dispersing element. The coarse emulsions formed were passed twice through a Niro-Soavi NS 1001L Panda homogenizer (Gea Group, Germany) at 200 bars. The emulsions were pasteurized at 80 °C for 10 min just after the emulsion preparation to prevent microbial growth during storage. The pasteurized emulsions were stored at 4 °C for seven days to evaluate emulsion stability [191]. The emulsion oil droplet size at day 0, 1 and 7 was measured using laser light scattering (Mastersizer 3000, Malvern Instruments Ltd., U.K.) with degassed, deionized water used as the dispersant. The particle size distribution from 0.005 to 5000 µm as a function of volume was recorded followed by the estimation of the volumetric mean diameter (D[4;3]), which were used to assess the emulsion capacity and stability [214], [215]. Contour plots of the D[4;3] values were generated by Minitab (Minitab Inc., Pennsylvania, United States) using distance method of interpolation.

IV.3.2.5. Statistical Analyses

IV.3.2.5.a. Fluorescence Data Analysis

Fluorescence data was processed by parallel factor analysis (PARAFAC) [269]. The fluorescence landscapes were first pre-processed by removing the Rayleigh scatter according the procedure suggested by Thygesen, Rinnan, Barsberg, & Møller, 2004. This was then analyzed by PARAFAC into three matrices: score matrix, an excitation loading matrix and an emission loading matrix. The two suspensions at 0.1 and 1% (w/w) were analyzed separately, but both gave rise to a three-factor model. The fluorescence landscapes were processed and analyzed in MATLAB (Mathworks, Massachusetts, United States).

Three-way analysis of variance (ANOVA) (pH, temperature, duration of treatment) was conducted using Minitab (Minitab Inc., Pennsylvania, United States). The threshold for statistical significance was $\alpha = 0.05$.

IV.3.3. Results & Discussion

IV.3.3.1. Effect of processing (modification conditions) on fava proteins

Protein associated reactions like protein aggregation and protein hydrolysis occurred during ingredient modification. The volumetric mean diameter extracted from the particle size distribution (PSD) of all the *modified-suspensions* showed different degrees of aggregation reactions as a function of the process conditions (**Figure 14**). In general, a gradual increase in the aggregate size as a function of temperature ($T_{process}$) and time ($t_{process}$) was observed for the modification at $pH_{process}$ 4, 6.4 and 11. As seen, intensive aggregation (> 200 nm) took place as a result of especially three ingredient modifications (orange bars) at 'High' $T_{process}$ (**Figure 14**A). The PSD of these special *modified-suspensions* (**Figure 14**B) confirmed that these contained large aggregates of different sizes (up to 1000 µm) indicating protein associated reactions. This was compared to the monomodal distribution of FBIC suspension, which was unmodified by the process conditions. This inference corresponded well with an earlier report on fava protein aggregation that yielded similar polymodal distribution of aggregates reaching sizes of 1000 µm [87], [256]. Interestingly, there was an indistinct trend of aggregation observed at $pH_{process}$ 2 showing

some extent of aggregation for all $T_{process}$ (**Figure 14**A). At $pH_{process}$ 4 and 6.4, the lower $T_{process}$ had only minor impact on protein aggregation.

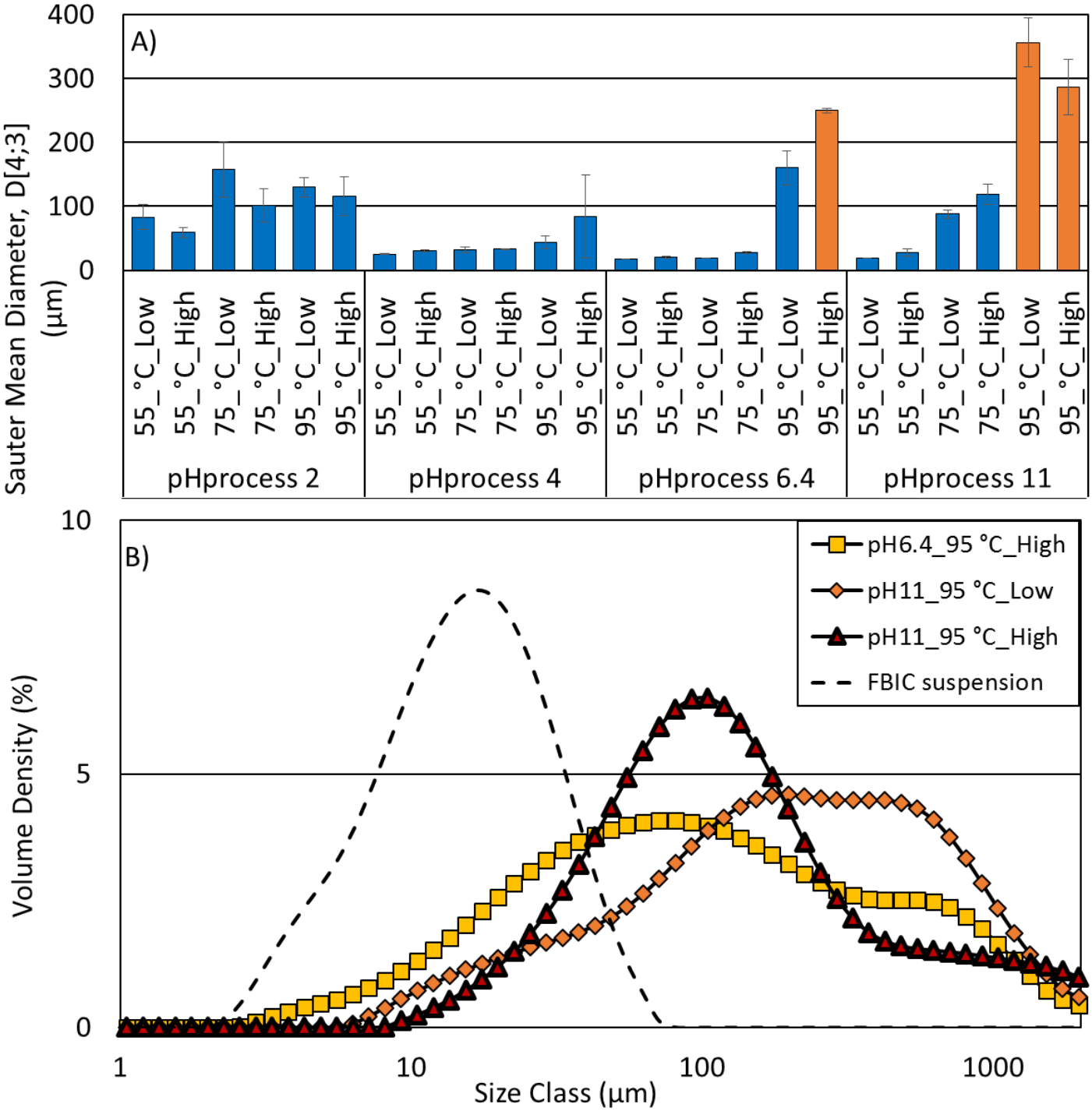

Figure 14 – Fava Bean Protein Aggregation. A) The volumetric mean particle diameter, D[4;3] of particles in *modified-suspensions*, B) PSD of the three *modified-*

suspensions with aggregation reactions. These were compared to the fava bean initial concentrate (FBIC) suspension at the same concentration.

The non-reduced SDS–PAGE analysis revealed changes in type and molecular weight distribution of the soluble proteins extracted from the *modified-suspensions*. The typical protein profile of fava bean (in FBIC) is seen in lane T0 (**Figure 15**) representing the globulins consisting of legumin minor subunit (80 kDa), convicilin subunit (70 kDa), legumin major subunit (60 kDa), vicilin subunit (50 kDa), and albumin (10-20 kDa) [132], [133], [296]. It is emphasized that in the SDS–PAGE analysis, the same total protein concentration is loaded in each lane, hence the electrophoretic result shows the relative distribution of the individual solubilized proteins in a comparable mode. **Figure 15** showed severe changes in the extracted protein fractions from the *modified-suspensions* owing to the differences in the band intensities obtained. Band-size of larger subunits (40–100 kDa) decreased by around 37% during modification at pH2_75 °C_High (marked in red in **Figure 15**A). A total band disappearance (> 96% decrease) of the large subunits occurred at acidic conditions (≤ pH 4), treated at 95 °C for 360 min (i.e. pH2_95 °C_High and pH4_95 °C_High, marked in red in **Figure 15**A and B). A simultaneous band-size increase (> 31% increase) in smaller peptides (< 40 kDa) indicated occurrence of protein hydrolysis to a certain extent (pH2_75 °C_High, marked in red in **Figure 15**A). Thus, acid-hydrolysis of fava proteins occurred at lower $pH_{process}$ (≤ 4), at higher $T_{process}$ (≥ 75 °C) and at 'High' $t_{process}$ (360 min) during modification. Modification at higher $pH_{process}$ (≥ 6.4) and at higher $T_{process}$ (95 °C) resulted in no visual band change of either larger subunits (> 82% decrease) or smaller subunits (> 8% decrease) due to protein aggregation (purple, **Figure 15**C and D) in agreement with results from PSD (**Figure 14**).

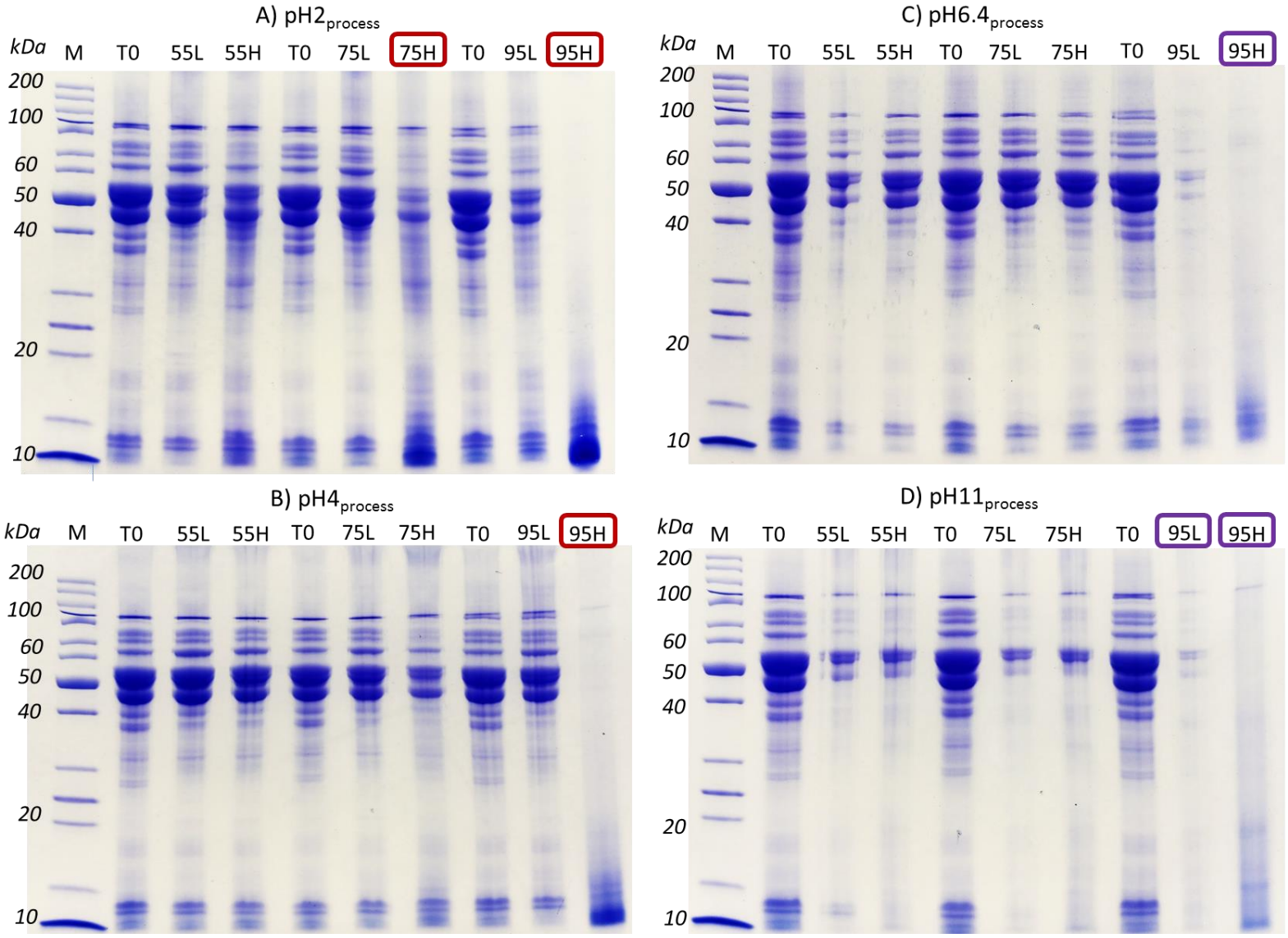

Figure 15 – Fava Bean Protein Acid-Hydrolysis. Non-Reduced SDS-PAGE of *modified-suspensions* at different $pH_{process}$: (A) 2, (B) 4, (C) 6.4, and (D) 11. Each gel column represents samples produced at different $T_{process}$ (55, 75 and 95 °C) and at different $t_{process}$, i.e. Low = 30 min (L) or High = 360 min (H) at a particular $pH_{process}$. Included are FBIC suspension (T0) as reference and protein marker (M).

IV.3.3.2. Effect of utilization conditions on fava proteins

The fava proteins after being modified by the process conditions showed further distinction in properties when suspended at two $pH_{utilization}$ (4 and 7). The highest ionic strength change (μ = 0.07) was at protein concentration 1% (w/w) due to changes in the $pH_{utilization}$. Functional properties of fava proteins are often favored at $\mu \leq 0.4$ ionic

strength. Thus, the change in ionic strength was concluded not to affect the functional properties [192].

The zeta potential, representing protein surface charge of fava bean proteins, was close to 0 (0.96 ± 0.53 mV) for FBIC at $pH_{utilization}$ 4, indicating that the overall isoelectric pH of fava proteins (predominantly legumin and vicilin) was close to pH 4 (**Figure 16**). However, at $pH_{utilization}$ 7 (neutral pH), the surface charge was highly negative (-8.24 ± 1.70 mV) due to effects of proteolytic active side residues. The charge of the modified ingredients at $pH_{utilization}$ 4 was significantly changed as a function of process conditions, i.e. $pH_{process}$ ($p = 0.036$, $\alpha = 0.95$) and $T_{process}$ ($p = 0.045$, $\alpha = 0.95$), but not $t_{process}$ ($p = 0.157$, $\alpha = 0.95$). There was an overall shift of charge at $pH_{utilization}$ 4 towards a more negative charge in the modified ingredients compared to FBIC, with an exception of the ingredients pH2_55 °C_High, pH6.4_55 °C_Low and pH6.4_55 °C_High. Comparing surface charges with the ingredients containing aggregated or hydrolyzed fava proteins, no specific trend was seen (**Figure 14**, **Figure 15** and **Figure 16**). With regard to $pH_{utilization}$ 7, the different treatment modifications did not affect the surface charge at neutral pH ($p > 0.050$, $\alpha = 0.95$). It would have been expected that the severe process conditions might have further unfolded the globular proteins, exposing previously hidden polar groups that lead to a changed surface charge. Interestingly, there seemed to be a difference in robustness in the property at two different $pH_{utilization}$: where significant effects of process conditions were observed at $pH_{utilization}$ 4, no such effect was observed at the $pH_{utilization}$ 7.

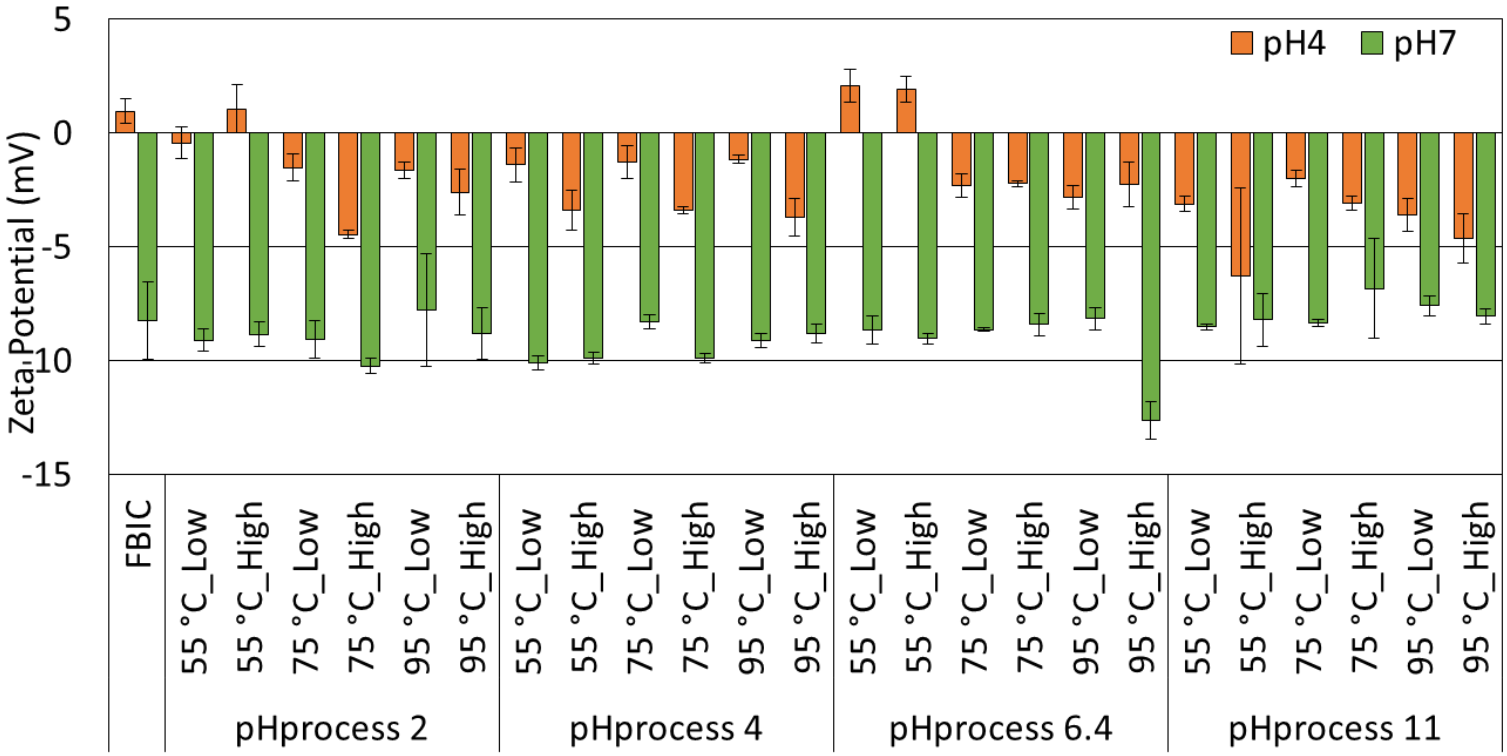

Figure 16 – Fava Bean Protein Charge. Zeta potential of FBIC and all *ingredient-buffered-suspensions* at $pH_{utilization}$ 4 and 7.

The nitrogen solubility representing the solubility of fava proteins at $pH_{utilization}$ 4 and 7 (**Figure 17**) showed that FBIC had a very low protein solubility at $pH_{utilization}$ 4 as expected due to an overall neutral charge (**Figure 16**), thus, disfavoring repulsion between residues. On the contrary, FBIC proteins were highly soluble (82 ± 4%) at neutral pH, which could be attributed to the higher overall negative charge and thus enhanced repulsion, hindering the precipitation and favoring solubility. The solubility of the modified ingredients did not change significantly at $pH_{utilization}$ 4 ($p > 0.050$, $\alpha = 0.95$) despite the presence of more negative charged proteins (**Figure 16**). On the other hand, protein solubility of the modified ingredients at $pH_{utilization}$ 7 decreased significantly compared to FBIC as a function of $pH_{process}$ ($p = 0.028$, $\alpha = 0.95$) and $T_{process}$ ($p = 0.000$, $\alpha = 0.95$), but not $t_{process}$ (Low or High) ($p = 0.753$, $\alpha = 0.95$).

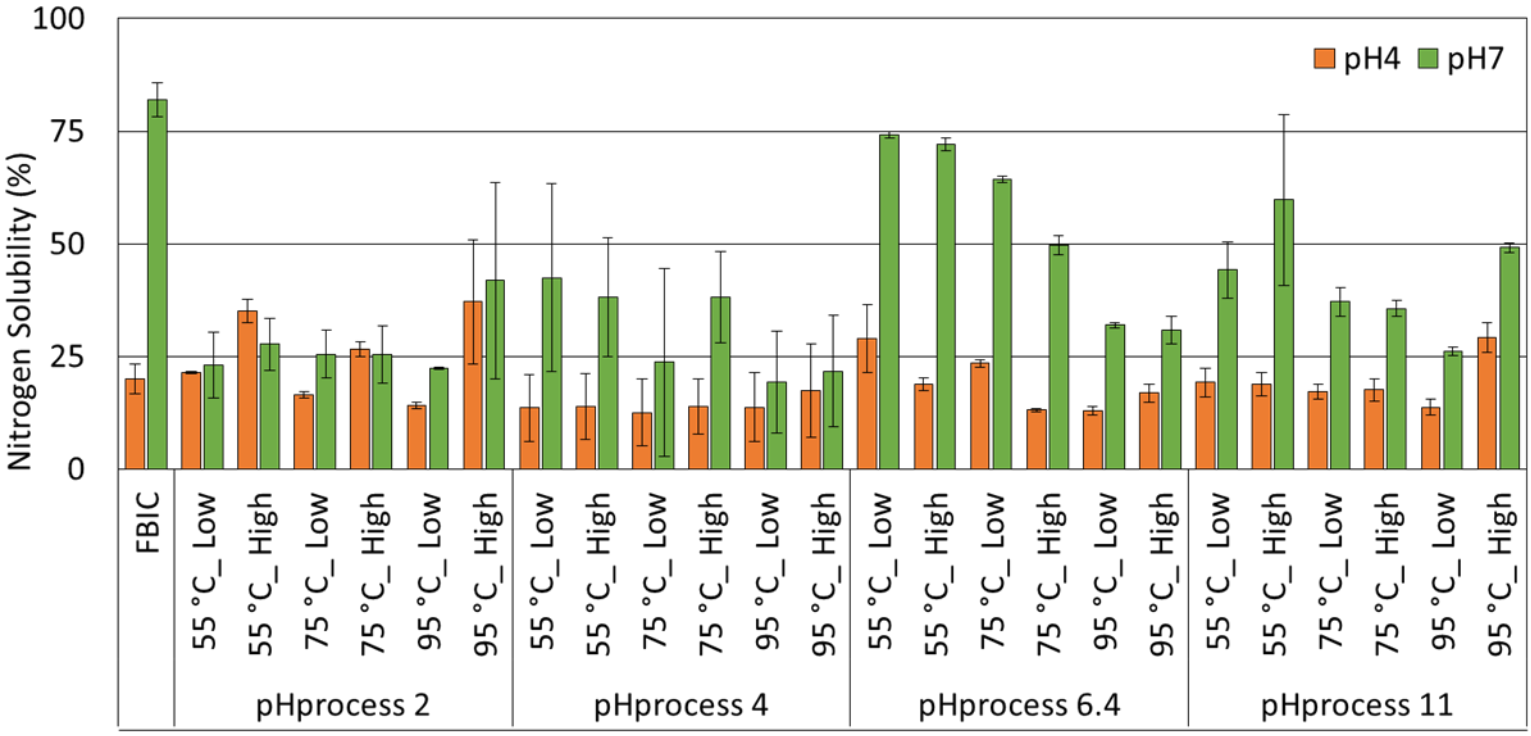

Figure 17 – Fava Bean Protein Solubility. Nitrogen solubility of FBIC and all *ingredient-buffered-suspensions* at $pH_{utilization}$ 4 and 7.

A markedly decrease in the $pH_{utilization}$ 7 solubility with increase in $T_{process}$ was observed for the ingredients modified at $pH_{process}$ 6.4 (**Figure 17**). However, for ingredients modified at $pH_{process}$ 2, 4 and 11, solubility changed differently as a function of $t_{process}$ for each $T_{process}$. Solubility of fava proteins was not consistent, hence robust, as the surface charge property at the two different $pH_{utilization}$, indicating that the ingredient modification process has more impact on this property. It is well known that the magnitude of the solubility is determined mostly by two opposing contributions: 1) structural changes exposing previously hidden polar groups in effect increasing protein/solvent interactions and facilitating solubility and/or 2) structural changes exposing reactive side chains, in effect increasing protein/protein association resulting in aggregation and reduced solubility [297]. Solubility and surface charge of proteins are often related [266], but as seen comparing results in **Figure 16** and **Figure 17**, the two properties were affected differently by the process conditions at the two $pH_{utilization}$. The surface charge and, thereby, any effect on ionizable side group in the proteins due to different molecular microenvironments (e.g. denaturation), did not completely explain the change in solubility.

Onto seek for explanation of the observed solubility, fava proteins from the modification and utilization conditions were characterized by their intrinsic fluorescence. This was first done at 1% (w/w) protein concentration, but possible inner filter effects were expected due to the physical nature of the suspensions. To give an example, all the 1% (w/w) protein suspensions were visually cloudy at $pH_{utilization}$ 4 due to the formation of protein precipitates. In addition, presence of quenchers at this concentration could also lead to attenuation of the fluorescence signals [284]. Thus, fluorescence at a dilution of 0.1% (w/w) protein suspensions was also considered to avoid obscurity in comprehending the results. Eventually, the PARAFAC model constructed from the fluorescence data yielded three components from each 0.1% and 1% (w/w) *ingredient-buffered-suspensions*.

PARAFAC is a rapid and efficient tool that helps in decomposition of fluorescence signals into components. PARAFAC models conform to the Beer's Law and has been well established for organic chemicals [283]. Models explaining intrinsic fluorescence of protein and protein interactions are gaining popularity [290], [298], [299]. The constructed PARAFAC model in this investigation consisted of a score matrix and a loadings matrix. The loadings contained suggested information on protein chemistry (**Table 11**) whereas the scores indicated varied intensities between the ingredients for every respective loading (**Figure 18**). The PARAFAC loadings representing excitation and emission wavelengths were comparable between the two concentrations. It was clear that PR1 and 2 of 0.1% (w/w) protein suspension corresponds to PR1 and 2 of 1% (w/w) protein suspension, suggesting a likelihood of information on tryptophan residues of a native (more buried) and denatured (more exposed) polypeptides, respectively [285], [286]. In addition, PR3 of 0.1% (w/w) and 1% (w/w) protein suspension may represent an additional complexity of polypeptides folding. As polypeptides also exist in some molten globule state and aggregated forms, there is a resultant, additional complexity in the state and behavior of tryptophan residues in the polypeptides of different systems [300], [301]. Furthermore, the process conditions during ingredient modifications add to an additional polypeptide complexity by virtue of several possible protein-associated reactions [10], [89], [302].

Table 11 – Intrinsic fluorescence of fava proteins with excitation and emission Loadings of *ingredient-buffered-suspensions* (0.1% and 1% (w/w) protein)

PARAFAC Component	Excitation Loading Peak (nm)	Emission Loading Peak (nm)	Protein Folded Complexity	Suggested Chemistry
0.1%(w/w) Protein Suspension				
PR1	295	325	I	Tryptophan, buried$^{\alpha}$
PR2	295	340	II	Tryptophan, exposed$^{\alpha}$
PR3	300	360	III	-
1%(w/w) Protein Suspension				
PR1	290	330	I	Tryptophan, buried$^{\beta}$
PR2	295	340	II	Tryptophan, exposed$^{\beta}$
PR3	300	360	III	-

PR1, 2 and 3 represent 1st, 2nd and 3rd protein-associated components detected by the PARAFAC loadings.

$^{\alpha}$*: Tryptophan excitation and emission peak without interference of quenching by other components*

$^{\beta}$*: Tryptophan excitation and emission peak with possible interference of quenching by other components*

Note: *Chemical hypothesis based on previous literature on tryptophan intrinsic fluorescence* [285], [286]

While the PARAFAC loadings represented complexities in protein polypeptide folding, the PARAFAC scores represented effects of modification and utilization conditions on the polypeptide folding. In brief, the scores between 0.1 and 1% protein concentrations explained different phenomena for the similar type of loadings (**Figure 18**). For FBIC (0.1%), the PR1 component, suggesting more buried tryptophan residues (**Table 11**), showed scores at $pH_{utilization}$ 7 10% higher than those at $pH_{utilization}$ 4 (**Figure 18**A). However, at 1% concentration, a 274% increase of score from $pH_{utilization}$ 4 to 7 for fava proteins in FBIC was obtained (**Figure 18**D). The PR2 component that suggests rather exposed tryptophan residues (**Table 11**), showed a 846% decrease in the scores at 1% (w/w) FBIC (**Figure 18**E), but only 25% decrease of the scores at 0.1% from $pH_{utilization}$ 4 to 7 (**Figure 18**B). Similarly for the PR3 component, suggesting additional protein complexity, the

difference between the scores of FBIC was more pronounced at 1% (48% difference, **Figure 18**F) than in 0.1% (3.5% difference, **Figure 18**C) between $pH_{utilization}$ 4 and 7. For the modified ingredients as well, the differences between the scores at $pH_{utilization}$ 4 and 7 were more distinct at 1% protein concentration (**Figure 18**D, E, F) compared to those at 0.1% protein concentration (**Figure 18**A, B, C). This difference between the two $pH_{utilization}$ at 1% (w/w) protein concentration was caused by the higher physical interferences by the cloudy solution due to aggregation/precipitation at the isoelectric pH (pH4) and absence of aggregates/precipitates at neutral pH (more translucent solutions). Hence, smaller differences between the two $pH_{utilization}$ at 0.1% (w/w) protein concentration indicated absence of physical interference. A re-burial of tryptophan residues due to aggregation/precipitation at their isoelectric pH would result in no detectable change in the fluorescence scores at $pH_{utilization}$ 4. However, it is noted that presence of quenchers and their concentrations may add to complexity and the interpretation of the results [284]. For the modified ingredients, the three-way ANOVA for 0.1% (w/w) protein suspensions showed that the protein folding complexity was impacted by process conditions, particularly $pH_{process}$ and $T_{process}$, but at different utilization conditions. At $pH_{utilization}$ 4, only $pH_{process}$ had a significant effect, exceptionally on PR2 representing possibly exposed tryptophan residues ($p = 0.009$, $\alpha = 0.95$). No other effects ($T_{process}$ or $t_{process}$) were significant at this $pH_{utilization}$ ($p > 0.05$, $\alpha = 0.95$). Likewise at $pH_{utilization}$ 7, only $pH_{process}$ had a significant impact on PR1 ($p = 0.009$, $\alpha = 0.95$), PR2 ($p = 0.020$, $\alpha = 0.95$) and PR3 ($p = 0.003$, $\alpha = 0.95$). The scores observed at 1% (w/w) protein suspensions showed no significant effects from the modification conditions ($p > 0.050$, $\alpha = 0.95$). Overall, it was clear that the $pH_{process}$ during ingredient modification affected protein folding complexity with a large dependency of the $pH_{utilization}$. It is stressed, though, that caution regarding essential conclusions must be taken, since chemical and physical changes with non-protein components and potential quenchers may affect fluorescent data of protein concentrates. Two approaches to bypass spectral interferences in such complex matrix are recommended, 1) extraction of the proteins to avoid other chemical reactions, thus revealing the true nature of the proteins and 2) testing dilutions during analyses is encouraged to remove physical attenuation of fluorescent signals.

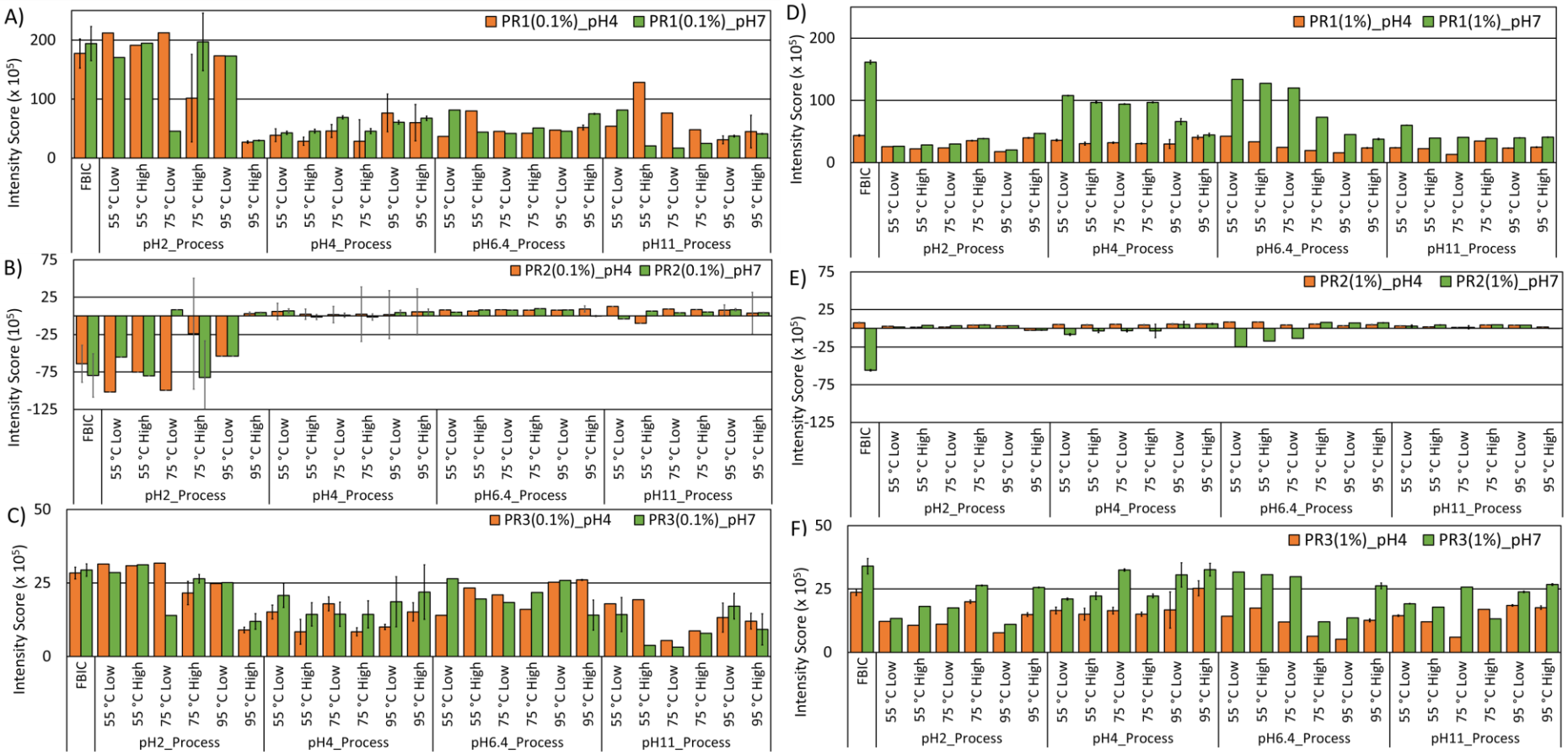

Figure 18 – Fava Bean Protein Folding**.** Intrinsic Protein Fluorescence by PARAFAC shown as score intensities of all *ingredient-buffered-suspensions* measured at $pH_{utilization}$ 4 and 7. A (PR1), B (PR2) and C (PR3) show scores at 0.1% (w/w) protein concentration, and D (PR1), E (PR2) and F (PR3) show scores at 1% (w/w) protein concentration. PR1, 2 and 3 represent 1st, 2nd and 3rd protein-associated components detected by the PARAFAC loadings.

Calorimetric analysis of nine specific ingredients supported the results of protein charge, solubility and protein folding; a predominance of $pH_{utilization}$ was observed (**Table 12**). As seen, FBIC had lower Tp at $pH_{utilization}$ 4 than at $pH_{utilization}$ 7, indicating a relatively higher heat stability of fava proteins at $pH_{utilization}$ 7. This lower denaturation temperature at isoelectric pH is due to lower structural integrity corresponding to the difference in protein folding complexity, the change in surface charge resulting in precipitation in effect decreased solubility. On the contrary, the proteins in FBIC at neutral pH had a net negative surface charge (and perhaps buried hydrophobic areas), which are typical for native folded proteins, thereby exhibiting higher thermal stability.

Table 12 – Thermal properties (DSC) of FBIC and less or extremely modified ingredients[§] at the two $pH_{utilization}$ (4 and 7).

Ingredient	Fava Protein modification	Enthalpy of Denaturation, ΔH (J/ g protein)		Denaturation Peak, Tp (°C)	
		$pH_{utilization}$ 4	$pH_{utilization}$ 7	$pH_{utilization}$ 4	$pH_{utilization}$ 7
FBIC	-	−4.54 ± 0.39 (0%)	−6.15 ± 0.65 (0%)	83.82 ± 0.09	91.07 ± 0.17
pH 2_55 °C_Low	Undetermined	0 (100%)	0 (100%)	-	-
pH 2_95 °C_High	Hydrolysis	0 (100%)	0 (100%)	-	-
pH 4_55 °C_Low	Undetermined	−3.32 ± 0.39 (~27%)	−3.32 ± 0.93 (~46%)	83.63 ± 1.93	94.17 ± 0.22
pH 4_95 °C_High	Hydrolysis	0 (100%)	0 (100%)	-	-
pH 6.4_55 °C_Low	Undetermined	−3.30 ± 0.39 (~27%)	−6.44 ± 1.13 (~0%)	83.37 ± 0.14	91.57 ± 0.20
pH 6.4_95 °C_High	Intense Aggregation	0 (100%)	0 (100%)	-	-
pH 11_55 °C_Low	Undetermined	−1.23 ± 0.04 (~73%)	−1.87 ± 0.13 (~70%)	87.19 ± 0.22	95.30 ± 0.20
pH 11_95 °C_High	Intense Aggregation	0 (100%)	0 (100%)	-	-

Note: % values show the extent of fava protein denaturation i.e. the enthalpy difference between the specific ingredient and fava bean initial concentrate (FBIC) at the respective particular pHutilization

[§] = ingredients corresponding to the lowest i.e. pH2, 4, 6.4 or 11 at 55 °C for 30 min (pHX_55 °C_Low) and highest level of modification i.e. pH2, 4, 6.4 or 11 at 95 °C for 360 min (pHX_95 °C_High).

The enthalpy of FBIC at the isoelectric pH was (numerical) lower than at neutral pH, reflecting greater structural integrity at $pH_{utilization}$ 7. Fava proteins from ingredients that were vigorously modified at high $T_{process}$ and $t_{process}$, i.e. pHX_95 °C_High, were all completely denatured (ΔH = 0), in accordance with results that these ingredients contained either hydrolyzed proteins and intensively aggregated proteins (**Figure 14**, **Figure 15**; **Table 12**). Ingredients modified rather gently at low $T_{process}$ and $t_{process}$, i.e. pHX_55 °C_Low, had very different fractions of denaturation between 27 and 100% at

both $pH_{utilization}$, showing that under these conditions, the fava protein structures were affected differently. The ingredient pH2_55 °C_Low contained extremely modified proteins through an undetermined reaction. The proteins in the ingredient pH6.4_55 °C_Low were least affected, but with increasing ΔH with $pH_{utilization}$, i.e. from isoelectric to neutral pH similar to FBIC. For the other gently modified ingredients, pH4_55 °C_low and pH11_55 °C_Low, the ΔH did not change with $pH_{utilization}$ (**Table 12**). Typically, native proteins unfold and refold with the changes in the medium due to conformational flexibility. The rigidness in ΔH indicated that the proteins in some of these modified ingredients have lost their potential to refold between the two $pH_{utilization}$ as a consequence of the modification conditions. Different extent of protein denaturation (complete and partial) due to the modification conditions were identified, while protein renaturation ($\Delta H_{pH7} - \Delta H_{pH4} > 0$ J/g) or structural rigidity ($\Delta H_{pH7} - \Delta H_{pH4} \sim 0$ J/g) between utilization conditions were observed for the selected ingredients. The results indicate the possibility of other protein-associated modifications of various degrees aside from acid-hydrolysis and intensive aggregation leading to different states of partially or completely denatured proteins, that could impact protein and functional properties.

IV.3.3.3. Effect of modification & utilization conditions on fava protein functionality

Foaming parameters, i.e. foam capacity (FC) and foam stability (FS), were high for FBIC (> 100%) at both $pH_{utilization}$ 4 and 7 (**Figure 19**), though both FC and FS were higher at $pH_{utilization}$ 4 compared to $pH_{utilization}$ 7 (by 15%). Also, as seen in **Figure 19**A and **Figure 19**B, the FC of all modified ingredients at both $pH_{utilization}$ 4 and 7 was very high (> 100%). However, while FC ($p > 0.05$, $\alpha = 0.95$) at $pH_{utilization}$ 4 was independent on the modification conditions, the FC at $pH_{utilization}$7 was significantly dependent on $pH_{process}$ ($p = 0.000$, $\alpha = 0.95$) and $t_{process}$ ($p = 0.019$, $\alpha = 0.95$). Despite some effect of modification conditions, the FC was always high (> 100%) for all ingredients at both $pH_{utilization}$. The FS, however, was affected differently by conditions of ingredient modification and utilization. All modified ingredients maintained high FS (> 100%) at $pH_{utilization}$ 7, though FS was also significantly affected by $pH_{process}$ ($p = 0.000$, $\alpha = 0.95$) and $t_{process}$ ($p = 0.043$, $\alpha = 0.95$). But at $pH_{utilization}$ 4, FS changed severely as a function of $pH_{process}$ ($p = 0.043$, $\alpha = 0.95$). In fact, twelve modified ingredients gave unstable foams (< 50%) which were labelled *foam-breakers* (**Figure 19**A). Apparently, these *foam-breakers* were produced at treatment $pH_{process}$ 2 (pH2_55 °C_Low, pH2_55 °C_High, pH2_75 °C_Low and pH2_95 °C_Low), $pH_{process}$ 4 (all ingredients), and $pH_{process}$ 11 (pH11_55 °C_Low and pH11_55 °C_High). The modification

conditions associated with the *foam-breakers* seemed inconsistent (**Figure 19**), suggesting that there might be more than a unique phenomenon causing foam instability at pH 4. Overall, *foam-breakers* were only formed at $pH_{utilization}$ 4, whereas all ingredients retained their high FS (> 100%) at $pH_{utilization}$ 7.

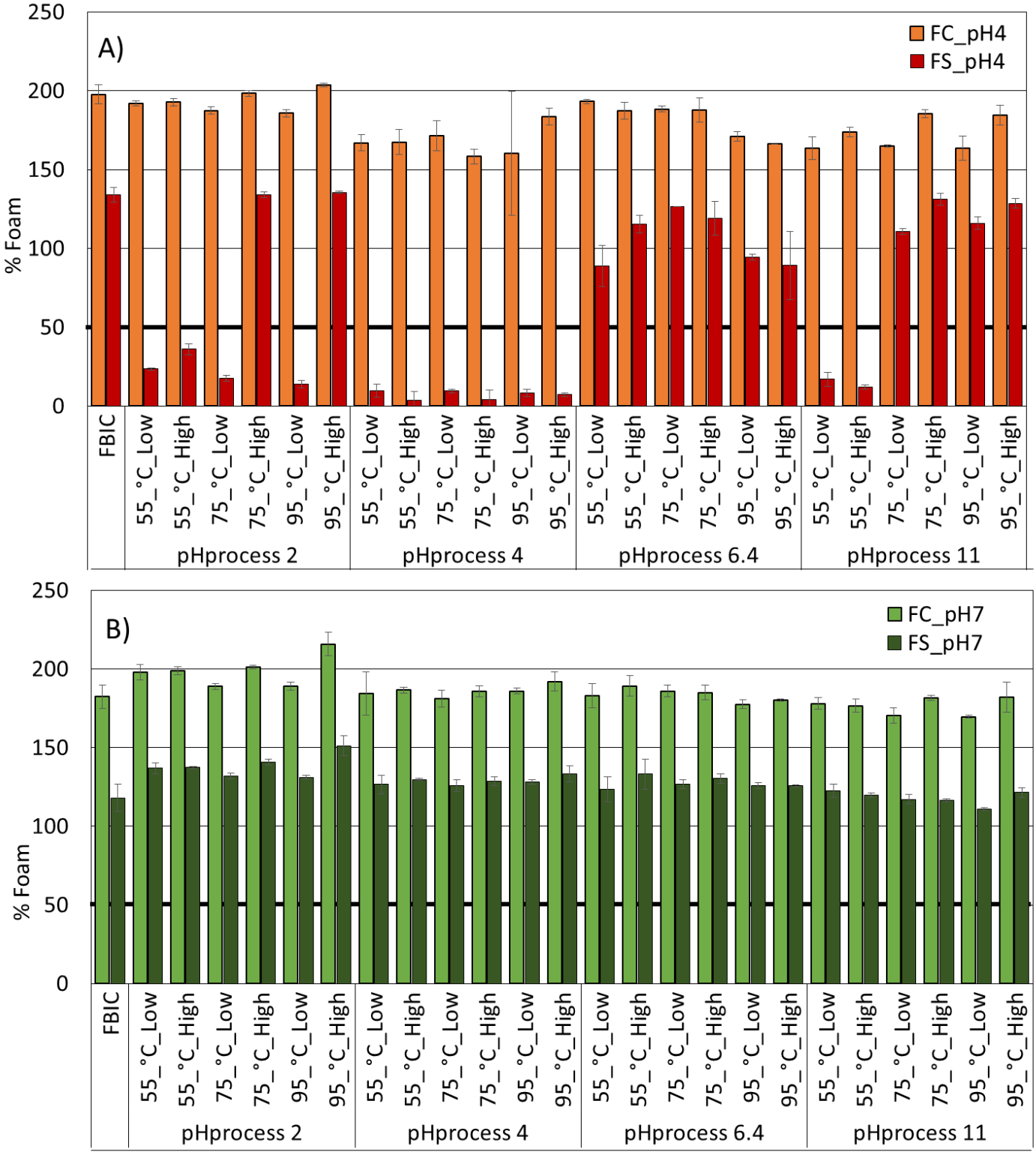

Figure 19 – Fava Bean Ingredient Foamability. Foam capacity (FC) and foam stability (FS) of FBIC and the *ingredient-buffered-suspensions* at A) $pH_{utilization}4$ and B) $pH_{utilization}7$. FC and FS ≥ 50% were considered as 'stable', whereas FS<50% represent *foam-breakers*.

The time dependency of FC was monitored for ingredients containing acid-hydrolyzed and aggregated fava proteins (**Figure 20**). The ingredients pH2_75 °C_High and pH2_95 °C_High containing acid-hydrolyzed proteins (lines with circled markers in **Figure 20**), did not show a remarkable difference in the foaming property compared to FBIC at the two $pH_{utilization}$ (**Figure 19** and **Figure 20**A). However, one of these ingredients was a *foam-breaker* (pH4_95 °C_High) due to FS << 50% (**Figure 19**A), and as seen the foam destabilization occurred fast within the first 10 min at $pH_{utilization}$ 4 (**Figure 20**A). This ingredient contained hydrolyzed proteins and was expected to be a foam stabilizer, hence, the reason for this surprising foam breakage is still unclear. At $pH_{utilization}$ 7, the ingredients with acid-hydrolyzed proteins showed a slight increase in FC (5-18%) and FS (13-28%, 30 min) compared to FBIC (**Figure 20**B). It seems that acid-mediated protein hydrolysis had an improving role in FC and FS but only at $pH_{utilization}$ 7. Previous reports on fava protein hydrolysis (however enzymatic), releasing buried amino acid residues led to a decrease in surface tension and thus, resultant improvement in foam stability at isoelectric and neutral pH [30], [303], [304]. Owing to the differences between acid and enzymatic hydrolyses of proteins, it is not surprising why a difference in the results were noticed here [305], [306]. Effects of acid-hydrolysis needs to be studied in greater detail for fava proteins.

The time dependency of the ingredients containing intensively aggregated proteins was comparable to at both $pH_{utilization}$, with slightly lower FC (7-17% decrease) and FS (4-34% decrease, 30 min) at $pH_{utilization}$ 4 (**Figure 20**A) compared to FC (0-7% decrease) and FS (<6% decrease, 30 min) at $pH_{utilization}$ 7 (**Figure 20**B). Two states of non-thermally aggregated fava proteins, large (>1µm), insoluble and supra-molecular (<1µm), soluble aggregates, have been identified, where the latter was found to have superior foam properties [87]. Interestingly, the sizes of all the aggregates, before or after modification have always been > 1µm (**Figure 14**).

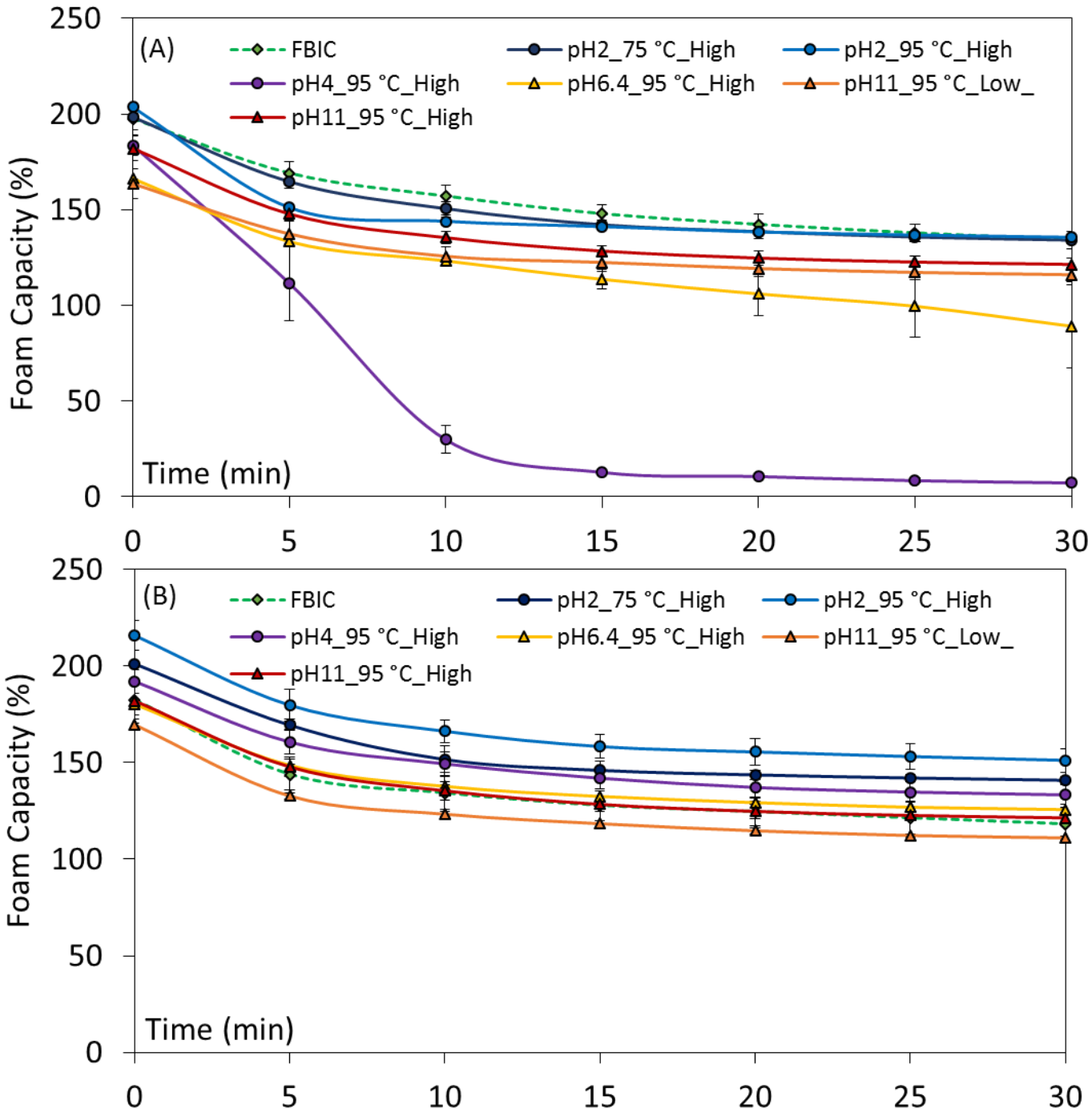

Figure 20 – Foaming Kinetics of Modified Fava Bean Proteins. Foam capacity development during 30 min of the *ingredient-aqueous-suspensions* containing hydrolyzed proteins (pH2_75 °C_High, pH2_95 °C_High and pH4_95 °C_High) and those containing intensively aggregated proteins (pH6.4_95 °C_High, pH11_95 °C_Low and pH11_95 °C_High), compared to FBIC at A) $pH_{utilization}$ 4 and B) $pH_{utilization}$ 7.

Emulsion capacity and stability, denoted by $D[4;3]_{Day0}$ and $D[4;3]_{Day7,}$ were governed majorly by the $pH_{utilization}$ and less by the $pH_{process}$ (**Figure 21**). At $pH_{utilization}$ 4, emulsions of all the ingredients at Day0 immediately creamed after production, indicating the detrimental impact of the isoelectric pH on protein-stabilized emulsions. Obviously, emulsions were still creamed throughout the storage period, and emulsion instability was as expected. The range of particle sizes was between 35 - 130 µm (from green to red) for the emulsions, which remained rather constant for every emulsion throughout Day1 and Day7 (**Figure 21**A). Significance of the effects of $T_{process}$ were observed on $D[4;3]_{Day0}$ ($p = 0.019$, $\alpha = 0.95$) and on $D[4;3]_{Day7}$ ($p = 0.032$, $\alpha = 0.95$). The differences in the values could have been a function of $T_{process}$ dependent aggregation reactions occurring during modification (**Figure 14**). At $pH_{utilization}$ 7 (**Figure 21**B), a considerable difference was seen in emulsion capacity and stability. Unlike $pH_{utilization}$ 4, all emulsions produced at $pH_{utilization}$ 7 were homogeneous immediately after production (from blue to yellow). The $D[4;3]_{Day0}$ ranged between 4-81 µm, with $pH_{process}$ 11 modified ingredients producing emulsions with the lowest $D[4;3]_{Day0}$. Despite some changes in D[4;3] of certain ingredient emulsions with time (**Figure 21**), the values restored back to initial at Day7. $pH_{process}$ had significant effect on the $D[4;3]_{Day0}$ ($p = 0.000$, $\alpha = 0.95$) and $D[4;3]_{Day7}$ ($p = 0.000$, $\alpha = 0.95$) values. This was clear as although most of the emulsions were stable during storage, all the emulsions from $pH_{process}$ 2 modified ingredients creamed and clarified at Day1. Therefore at $pH_{utilization}$ 7, emulsion capacities were equivalent to each other, with differences in D[4;3] values, but during storage, the stability was affected by $pH_{process}$.

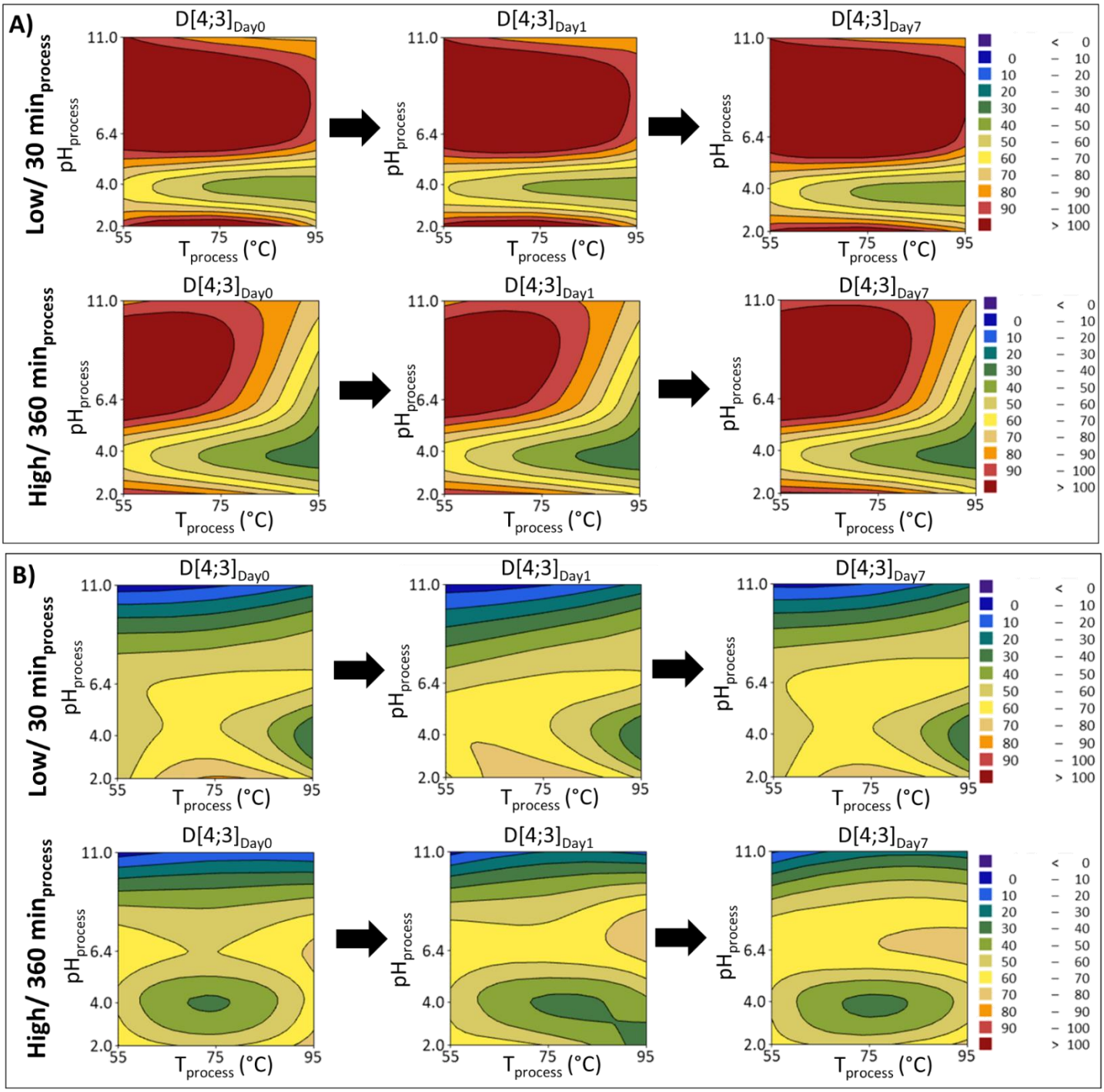

Figure 21 – Faba Bean Ingredient Emulsification. Contour Plot (Interpolation Method) of oil droplet Sauter mean diameter D[4;3] of the emulsions formed from *ingredient-aqueous-suspensions* of different modified ingredients, separated by $t_{process}$ i.e. Low/ 30 min and High/ 360 min, at A) $pH_{utilization}$ 4 and B) $pH_{utilization}$ 7. Color scale represents particle size in µm.

Creaming of emulsions did not correspond to their D[4;3] values. Thus, no specific relationship between emulsion stability and D[4;3] was noticed in these experiments as expected from Stokes law [307], [308]. As Stokes relationship plays well for oil droplet diameter, the distortion observed could be due to the presence of precipitates formed either due to protein precipitation at $pH_{utilization}$ 4, but also due to the aggregation reactions during ingredient modification (as in **Figure 14**). The effect of isoelectric point, as seen as an effect on protein charge, solubility, fluorescence and thermal integrity, can be well related to the formation of protein precipitates, and thus preventing the proteins to form a stable O/W interface to create an emulsion. During ingredient modification, intensive aggregation reaction leads to formation of particles of size > 200 µm as seen in **Figure 14**. These aggregates were produced in the *modified-suspensions*, which were then freeze-dried and then milled. Nevertheless, the aggregates can be still seen in the emulsions by virtue of their high D[4;3] detected, along with the presence of smaller oil droplet sizes in their bimodal PSD (**Figure 22**). Unlike the case of isoelectric precipitation, the emulsions formed from ingredients containing protein aggregates were not destabilized. Rather, these emulsions existed as a stable system of both the oil droplets as well as the protein aggregates together. Importance of retention of emulsion stability by protein aggregates also have been previously reported [5], [267]. Acid-mediated protein hydrolysis did not show any notable differences in the emulsion properties (**Figure 21**). No differences in the failed emulsions at $pH_{utilization}$ 4 were expected, but even at $pH_{utilization}$ 7, the size distribution of the emulsions from ingredients containing hydrolyzed proteins showed less of a bimodal distribution with higher presence of larger aggregates (**Figure 22**). Also, the ingredients modified at $pH_{process}$ 2 all gave unstable emulsions, including those having hydrolyzed proteins. Detrimental effects of $pH_{process}$ 2 in ingredient emulsion stability needs to be investigated. Limited hydrolysis of fava proteins has been favorable, but complete hydrolysis has been detrimental to emulsion properties [30], [303]. Stability of emulsions from pH4_95 °C_High and instability of emulsions from pH2_75 °C_High and pH2_95 °C_High, despite all containing hydrolyzed proteins, calls for an interest to look deeper into the degree of hydrolysis and effects on structural changes and functionalities of fava proteins.

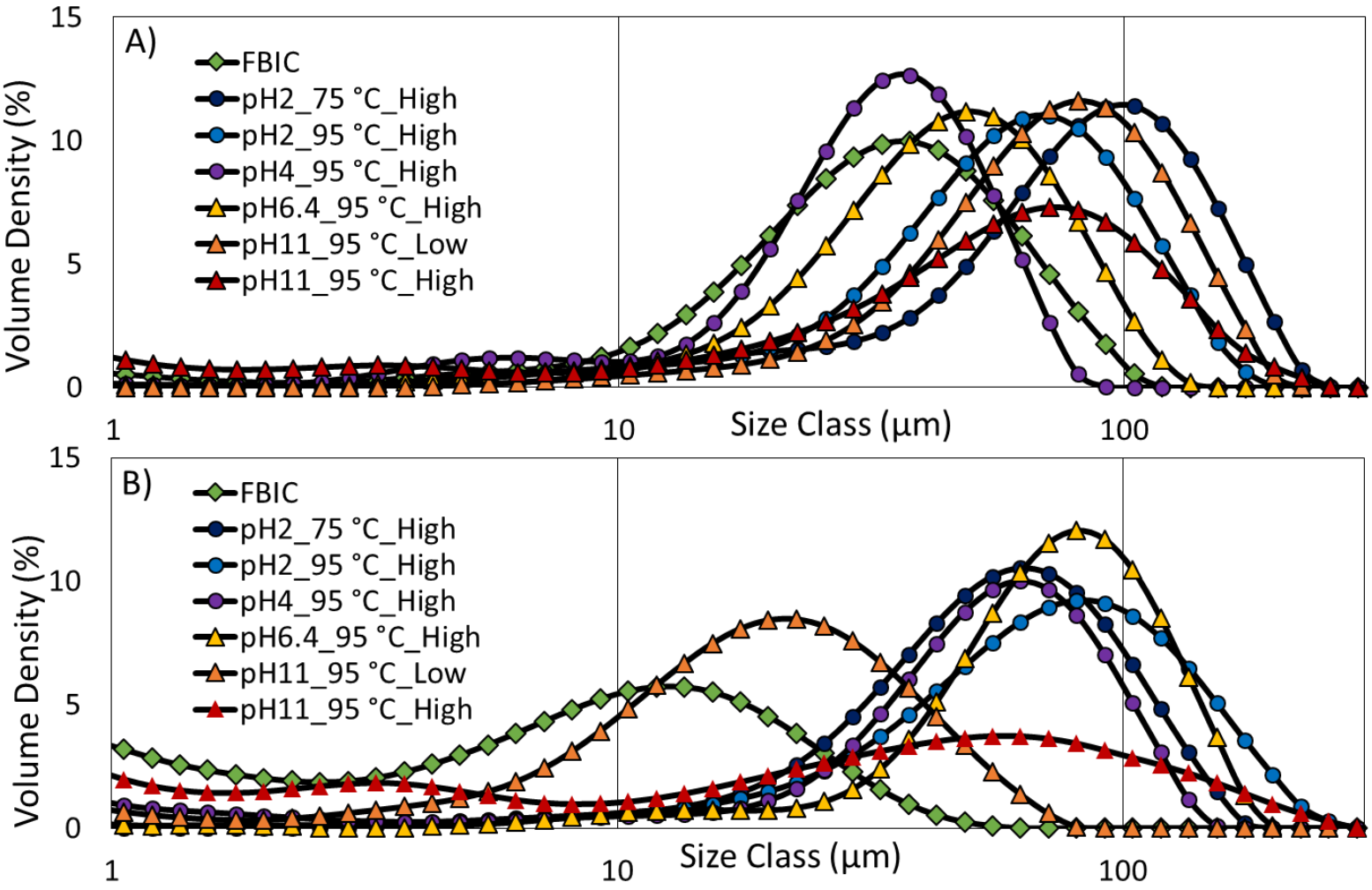

Figure 22 – Emulsions from Modified Fava Bean Proteins. Comparison of particle size distribution of the emulsions produced from *ingredient-aqueous-suspensions* (Day 0) containing hydrolyzed proteins (pH2_75 °C_High, pH2_95 °C_High and pH4_95 °C_High) and those containing intensively aggregated proteins (pH6.4_95 °C_High, pH11_95 °C_Low and pH11_95 °C_High), compared to that of the fava bean initial concentrate (FBIC) at A) $pH_{utilization}$ 4 and B) $pH_{utilization}$ 7.

To sum up, modified fava proteins' physico-chemical and functional properties were influenced by the $pH_{utilization}$ to a great extent. Identified specific protein modifications, aggregation and hydrolysis, had different relationships to the functionalities, foaming and emulsification and, also, with very different dependency on the $pH_{utilization}$ (**Table 13**). However, within each $pH_{utilization}$, associations between charge and solubility was not clear. Interpretation of the protein fluorescence was greatly dependent on the protein dilution

during utilization. Foam capacity and stability measurements were well associated with each other, where the hydrolysis of fava proteins positively influenced the foaming properties. Emulsion oil droplet diameter (D[4;3]) measurements did not correspond well to their visual inspection, i.e. an increase or decrease in the D[4;3] did not correspond necessarily to higher emulsion capacity nor stability. For instance, fava protein aggregation forming larger particles increased the D[4;3], but this did not disturb their emulsifying ability at favorable pH. Additionally, higher foaming property did not correspond to higher emulsification. These aggregated proteins that stabilized emulsions, did not necessarily improve foamability in all ingredients. Therefore, it is difficult to predict functionality from another, and also just by measuring the protein properties. Protein modifications thus need to be monitored during ingredient processing to predict changes in functionalities to a certain degree.

Lastly, the effects of protein modifications were not reflected on the protein properties measured. Thermal stability evaluation by DSC suggested possibility of other reactions occurring at different other conditions. Since fava concentrate is a complex matrix of macro- and micro-constituents, other non-protein associated reactions could influence the inter-dependence between the properties. Therefore, it might be essential to monitor protein as well as non-protein interactions and reactions during modification and utilization of the ingredients [10], [89], [302].

Table 13 – Interplay between fava protein modifications, properties and functionality

		Intensive Aggregation			Acid-Hydrolysis		
		pH6.4_95 °C_High	pH11_95 °C_Low	pH11_95 °C_High	pH2_75 °C_High	pH2_95 °C_High	pH4_95 °C_High
pH*$_{utilization}$ *4	Absolute Zeta Potential	+ + +	+ + + + + +	+ + + + + + + + +	+ + + + + + + +	+ + + +	+ + + + + +
	Nitrogen Solubility	-	-	+	+	+ +	-
	Protein Folding I $^{\alpha}$	- -	- -	- -	-	- -	- -
	Protein Folding II $^{\alpha}$	- - -	- - -	- - -	- -	- - -	- - -
	Protein Folding III $^{\alpha}$	-	- -	- -	-	- -	-
	Foaming capacity (FC)	-	-	-	=	+	-
	Foaming stability (FS)	-	-	-	=	+	- -
	D[4;3]$_{Day0}$ $^{\beta}$	- -	-	-	-	-	- -
	D[4;3]$_{Day7}$ $^{\beta}$	- -	-	-	-	-	- -
pH*$_{utilization}$ *7	Absolute Zeta Potential	+ +	-	-	+	+	+
	Nitrogen Solubility	- -	- - -	-	- -	-	- -
	Protein Folding I $^{\alpha}$	- -	- -	- -	+	- -	- -
	Protein Folding II $^{\alpha}$	- - -	- - -	- - -	+	- - -	- - -
	Protein Folding III $^{\alpha}$	- -	-	- -	-	- -	-
	Foaming capacity (FC)	-	-	=	+	+	+
	Foaming stability (FS)	+	-	+	+	+	+
	D[4;3]$_{Day0}$	+ +	+	+	+ +	+ +	+
	D[4;3]$_{Day7}$	+ +	+	+	+ +	+ +	+

[α] Protein folding I-III represents fluorescence PARAFAC components 1-3 determined for 0.1% (w/w) protein suspensions

[β] Failure of emulsion formation at $pH_{utilization}$4

0% Change in Property: ''='';

0-50% Increase / Decrease in Property: ''+/ -'';

50-100% Increase / Decrease in Property: ''++/ - -'';

100-150% Increase / Decrease in Property: ''+++/ - - -'';

150-200% Increase / Decrease in Property: ''++++/ - - - -'';

200-250% Increase / Decrease in Property: ''+++++/ - - - - -'';

250-300% Increase / Decrease in Property: ''++++++/ - - - - - -'';

> 300% Increase / Decrease in Property: "+++++++/ - - - - - - -";

All % changes are calculated with respect to the fava bean initial concentrate (FBIC)

IV.3.4. Conclusion

Processing of fava bean concentrate at industry simulated conditions resulted in two opposite protein modifications: acid mediated hydrolysis and protein aggregation. Their effects were not mirrored in the physico-chemical properties. Though certain trends were observed in foam and emulsion properties, their effects were to a large extent governed by pH during ingredient utilization. Protein acid-hydrolysis improved foaming only at neutral pH, but had an unclear trend regarding emulsification. Aggregation did not improve foaming, but retained emulsion stability at neutral pH. In general, isoelectric pH during application was not suitable for foam stability, emulsion capacity nor emulsion stability. There may be other unexplored reactions leading to protein modifications, and causing differences in their thermal integrity. Considering physico-chemical and functional properties, their relationship is also mostly dependent on the application pH. The current investigation shows this inter-dependence, but encourages the need to dive further into the different protein-associated interactions that can occur in fava concentrate. Fava bean concentrate exhibits a multi-component character and thus can be of functional value for the food industry.

IV.3.4.1. Acknowledgements

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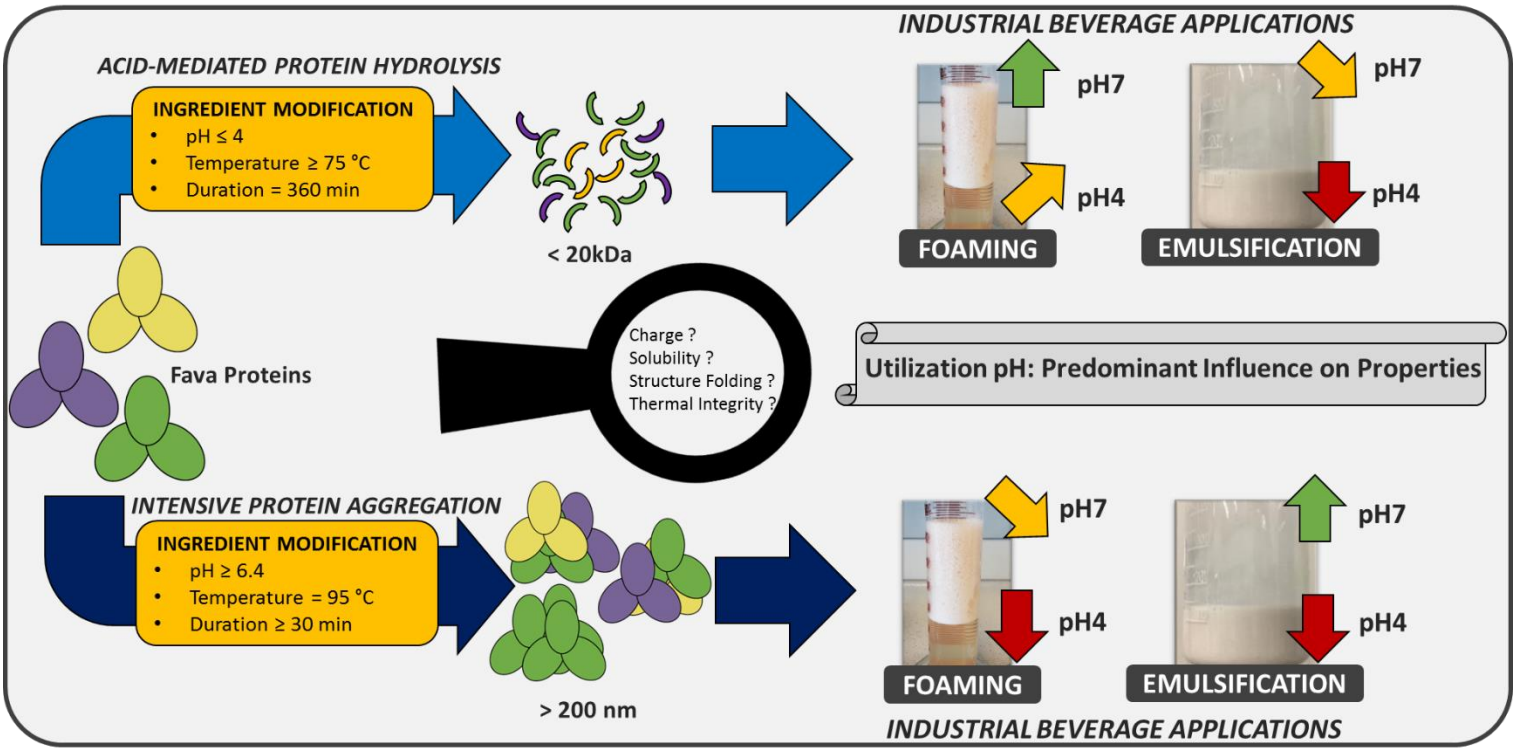
IV.3.4.2. Author Contributions

Conceptualization, S.S., M-N.M., A.S-E., J.Z.; methodology, validation, formal analysis, investigation and data curation, S.S., Å.R., J.S.; resources, J.Z., D.B., J.A.; writing—original draft preparation, S.S.; writing—review and editing, All.; visualization, S.S.; supervision, Å.R., V.O., K.O., J.Z., A.S-E. and M-N.M.; project administration, M-N.M., A.S-E., J.Z.; funding acquisition, M-N.M., D.B., J.A. All authors have read and agreed to the published version of the manuscript.

IV.4. Key Highlights



- Foam and emulsion properties are governed by protein-associated but separate mechanisms owing to the differences in the dispersed phases. Thus these functionalities are not correlated to each other.

- Foam and emulsion capacity and stability are associated with different protein and non-protein characteristics of fava concentrate, suggesting the complexity of using a multi-component matrix as an ingredient.

- The two statistical tools, PCA and Pearson's Correlation are complementary to each other, and they both required in a comprehensive understanding of the impact of process conditions on different properties and the interrelationships between them. Rapid analysis of large data sets can be made through this approach, thus rendering it very useful for industrial research on plant-based ingredients.

- Strong correlations between functional and physico-chemical properties were observed by protein charge, solubility and intrinsic fluorescence. Their behavior along pH during utilization and modification was coherent.

- The process conditions, especially the pH during utilization, drove both foam and emulsion properties. In particular, the utilization pH around the isoelectric point of fava proteins (pH 4) was not suitable for foam stability, emulsion capacity nor emulsion stability. The interplay between all the properties as a function of utilization pH was very clearly seen.

- Ingredient modification of fava bean concentrate by pH, temperature and treatment duration, resulted in two main protein structural modifications: acid-mediated protein hydrolysis and protein aggregation. These reactions had an impact on functionalities, but only to a certain extent as the effect of utilization pH was always predominant. Protein acid-hydrolysis slightly improved foaming only at neutral utilization pH, but had an unclear trend regarding emulsification. Aggregation did not improve foaming, but retained emulsion stability at neutral pH.

- Contrary to the utilization pH, the interplay between modification conditions, functional and protein properties was not so clear. For instance, foam-breakers were a result of modification, but the reason for their foam breakage was not found. Ingredient modification conditions were not particularly mirrored in the physico-chemical properties, and the properties once again depended largely on the pH of utilization.

- There were indications of other reactions that could have caused structural modifications in fava proteins, as observed by the Differential Scanning Calorimetry (DSC).

V

Can Process Drive Odor & its Perception?

V. Can Process Drive Odor & its Perception?

V.1. General Introduction

This chapter goes beyond functional properties of fava bean ingredients and attempts to understand how process conditions drive odor perception, and if olfaction can be correlated to flavor volatile chemistry. The study also dives into the literature to suggest indications of different flavor generating reactions that could possibly be important for fava bean ingredient processing, and looks into the flavor release as a function of the beverage matrix.

As the research approach chosen was a multi-dimensional one, this study is meant to be complementary to the Chapter IV, so as to evaluate the same ingredients, but on a different aspects important for acceptability. With this objective, the same modified ingredients as previously were used in two distinct models of beverage application (pH 4 and 7). Odor perception and headspace volatile chemistry were evaluated during ingredient utilization.

For odor evaluation, a sensory panel was recruited with 21 panelists. They were first trained to memorize 36 different odor attributes with the help of references. Then a qualitative method called Check-All-That-Apply (CATA) test was performed where the panelists selected the attributes characterizing each ingredient suspension. During the discussions, 4 key different odor attributes (green, cooked, "sweet" and rancid) were found to describe many of the ingredients. Therefore, the ingredient suspensions were evaluated on the basis of these 4 odor intensities as well.

Furthermore, headspace volatiles of all the ingredient suspensions were entrapped by SPME fibers and the entrapped volatiles were analyzed by Gas Chromatography coupled with Mass Spectrometry (GC-MS). The detected volatiles were grouped according to different chemical families and were also individually analyzed by Principal Component Analysis (PCA).

At the end, relationships between odor attributes, headspace volatiles and process conditions were established to understand the interplay between them.

V.2. Flavor of Fava Bean (*Vicia faba* L.) Ingredients: The Interplay between Odor Perception & Headspace Volatile Chemistry along with Processing & Application Conditions

Manuscript in Preparation

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Abstract

Application of plant-based sources for human consumption is challenged by consumer acceptance. Fava bean and its ingredients face this similar challenge in the food market. This study attempts to understand how ingredient processing and application conditions drive fava bean flavor. An approach to evaluate odor perception along with the analysis of headspace volatile compounds detected during ingredient utilization was performed. Precisely, a protein-rich ingredient, i.e. air classified fava bean concentrate, selected for its high industrial potential, was modified by pH (2, 4, 6.4 and 11), temperature (55, 75 and 95 °C) and treatment duration (30 and 360 min), in an experimental design that produced 36 different modified ingredients, which were further subjected to two distinct models of beverage application (pH 4 and 7). Results showed that the "green" perception detected in the initial concentrate evolved more into "cooked" perception with ingredient processing. Application conditions drove aroma changes, ranging from a "sweet" to "rancid" perception when changed from neutral to acidic pH. Aldehydes were generated in many ingredients, as well as furanoids at pH 2, terpenoids at pH 4, alcohols at pH 6.4 or ketones at pH 11. Lipid oxidation was suggested as the major contributor of the aroma composition in the ingredient suspensions. Reactions involving proteins, sugars and carotenoids degradations, including Maillard reaction and caramelization, also played a role in the flavor generation. Different suspension matrices at application pH might have influenced the release of pH-dependent volatiles. All of these data also made it possible to understand the molecules at the origin of the different sensory notes, and to highlight the role of process conditions and mainly the pH during ingredient application. Thus, various flavor profiles can be driven by process conditions for fava concentrates – making it promising for several beverage applications.

Keywords: Aroma**,** sensory, plant-based, HS-SPME-GC-MS, CATA

V.2.1. Introduction

The demand for healthy foods is rising and by 2050, a global sustainable transformation in food system is essential to feed nearly 10 billion people [61]. Fava bean (*Vicia faba* L.) is a promising source for human consumption. Fava bean crop has a great agronomic potential owing to higher crop yield per harvest area, and growth at temperatures as low as 12.5 °C. Also, by virtue of its high protein content, i.e. 23-41 % (w/w) d.b., there is a large nutritional and functional potential for food applications [3], [5]. Despite its potential, there are sensory (flavor and color) and anti-nutritional limitations that need attention to increase consumer acceptability [5]. Fava bean is processed to form ingredients (flours, concentrates and isolates) and these ingredients can further be modified using process conditions (ingredient modification) to eventually Increase their utilization in industrial food applications in industrial food applications (ingredient utilization) [5], [6]. These processing steps can drive generation of undesirable flavor compounds, but at the same time, also limit the presence of off-flavors in certain conditions [19].

Flavor perception of foods is a complex process that involves the senses of smell and taste, and chemesthesis, requiring the interactions between non-volatile and volatile molecules with sensory receptors in oral and nasal cavities [19]. Concerning pulse sensory properties, unpleasant bean odor is often linked to i) the perception of green, grassy and beany notes, which are mainly attributed to aldehydes, alcohols and ketones; and ii) a bitter and astringent taste which is associated with sapid-glycosylated compounds such as saponins and phenolic compounds (isoflavones, flavonols, phenolic acids, etc.) [35], [52], [83]. Odor is one of the first key indications of flavor in foods, reflecting its quality and acceptability [51], [84]–[86]. Plant intrinsic aroma depends on its genetic makeup, but also on the availability of precursors, distribution of enzymes and presence of favorable conditions for the reactions to take place [19], [83]. A minor part of the fava volatiles manifest during the bean development, but a majority of them derive from degradation of lipids, amino acids, carbohydrates and carotenoids through enzymatic and/ or non-enzymatic reactions [32]. These reactions are impacted throughout the ingredient supply chain, i.e. from bean harvest until final food application [5], [19].

Lipid oxidation is the primary cause of flavor generation in pulses. For instance, pea flavor has extensively been studied, throwing light on many lipid oxidation products such as aldehydes, ketones, alcohols and pyrazines, giving a combination of green, beany, earthy, and hay-like sensory notes [19], [309]. Fava bean flavor reactions have been investigated

too, but to a lesser extent and not particularly in direct correlation with sensory attributes. In fava, free or esterified unsaturated fatty acids undergo enzymatic reaction by broad bean lipoxygenase (BBL), and can also undergo auto-oxidation due to the presence of initiators (e.g. light, chlorophyll, metal ions) and/ or temperature [5]. Other reactions including amino acids and sugars degradation are possible, along with their rearrangement by Strecker's degradation and Maillard reaction, and lead to additional flavor development [32], [35]. Process conditions largely impact flavor, as they influence the extent and possibility of flavor-associated reactions and thus govern flavor formation. For fava bean, the impact of process conditions on the bean flavor itself has been studied to a certain extent. Fava seeds under microwave treatment (950 W for 1.5 min) or heat treatment (>70 °C, > 2 min) give modified flavor compared to fresh beans due to the inactivation of the BBL [36]–[38]. Fava ingredients, including flours that are dehulled and milled, contain high BBL activity, thus suggesting possibilities of enzymatic lipid oxidation [39]. The effect of pH has also been tested on fava isolates for flavor modification – where dried pea-like flavor predominated at neutral pH, and unpleasant fruity flavor developed at acidic pH [40]. Despite some understanding on fava flavor, there is a need of a comprehensive knowledge of the chemistry of fava bean flavor with process conditions in relation to sensory perception. In this way, the food industry can choose the right kind of processing for a target food application with expected aroma perception. Thus it will be a step further towards acceptability of the use of fava bean as promising food ingredients.

Fava bean concentrate, produced by air-classification, is identified as a gently processed ingredient [90]. In this study, this ingredient was modified by various process conditions before its utilization in model food. The impact of industrially relevant process conditions such as pH, temperature and treatment duration, was established on two main aspects: (1) odor perception: qualitative and quantitative sensory properties, and (2) volatile chemistry: headspace release of volatiles in conditions close to beverage application. Relationships were established to understand the interplay between processing, flavor generation and volatile chemistry in producing different ingredients with diverse flavor profiles. With this, their potential in a variety of food applications could be foreseen.

V.2.2. Materials & Methods

V.2.2.1. Ingredient Preparation

V.2.2.1.a. Fava Bean Initial Concentrate (FBIC)

Fava bean concentrate containing 65% (w/w d.b.) proteins was obtained from Vestkorn Ingredients (Holstebro, Denmark). The concentrate was produced by milling of dried and dehulled beans followed by air classification [214].

V.2.2.1.b. Modified Ingredients

The FBIC was modified as described below: 20% (w/w) suspensions were prepared with Milli-Q water (Millipore, France) and stirred for 30 min at 500 rpm using an overhead dissolver stirrer (IKA Works, Inc., Staufen, Germany); the pH ($pH_{process}$) was then adjusted to 2, 4 or 11 using 6 mol/L hydrochloric acid or sodium hydroxide (Sigma Aldrich, Missouri, United States), and the suspensions were further stirred for 30 min at 500 rpm. Additionally, a series with the natural suspension pH was also considered ($pH_{process}$ 6.4) and further stirred for 30 min at 500 rpm. All the suspensions were then heated (Tprocess) in a temperature-controlled bath (Lochner Labor+Technik GmBH, Germany) maintained at 55, 75 or 95 °C and agitated at 700 rpm for a duration (tprocess) of either 30 min (Low) or 360 min (High). All the treatments at $pH_{process}$ 4 were performed in triplicates in order to assess reproducibility. The different suspensions that were produced were further frozen at −20 °C, followed by freeze-drying (Döhler GmbH, Dahlenburg, Germany) and milling to 0.08 mm mesh size by an ultra-centrifugal mill ZM 200 (Retsch GmbH, Germany). Hence, different modified ingredient powders were obtained, which were named as $pH_{process}$ _Tprocess_tprocess (e.g. pH2_55 °C_Low) based on the conditions used to modify them.

V.2.2.2. Odor Sensory Profiling

Sensory odor profiling of the different ingredients was performed using three stages after panel recruitment: a) attribute generation and selection, b) panel training using references for each attribute and c) evaluation of odor description and intensity profiling of the main odor key notes for all the samples.

V.2.2.2.a. Sample Preparation and Presentation

For the sensory evaluation, all ingredients (FBIC and modified ingredients) were suspended to 5% (w/w) powder concentration in deionized water and stirred for 30 min at 20 °C before analysis. Furthermore, the pH of these suspensions was readjusted to

pHutilization (4 or 7) using 6 mol/L hydrochloric acid or sodium hydroxide respectively. The references and products suspensions were presented to the panelists in 80 mL plastic cups covered with a lid. The samples were labeled with random three-digit numbers.

V.2.2.2.b. Subjects

Twenty-one volunteer panelists (13 women and 8 men, 18-40 years in age) were recruited based on their ability and willingness to participate in this study. The panelists had previously different levels of experiences in sensory study participation. The overall aim of the experiment was communicated to them beforehand, where they gave their free and informed consent and additionally received for their participation. Prior to the sessions, the panelists were asked not to smoke or consume coffee, tea or other flavor-intense foods. All sessions were conducted and monitored by the authors. The experimentation was performed at UMR SayFood (Université Paris-Saclay, INRAE, AgroParisTech, on the sites of Grignon & Massy, France). All communication was done in French.

V.2.2.2.c. Panel Training

The panel training was conducted with three objectives: a) to memorize different odor attributes with the references provided; b) to reach a consensus between all the judges on the choice of the attributes to use and their definition; and c) to train to evaluate the key odor by selection of the main notes characterizing the samples and evaluation of the perceived intensities of a reduced number of attributes. The complete list of attributes was selected after discussion with the panelists to describe all the perception of the ingredients and various reference products were proposed to subjects to help for recognition and learning of various sensations. After the training sessions, a total of 36 attributes was finally selected for the subsequent sessions (**Table 14**) for Check-All-That-Apply test (CATA test). Additionally, four different classes of attributes were identified during the discussions: green (notes vertes), “sweet” (notes sucrées), rancid (notes rances) and cooked notes (notes cuites). The lexicon “sweet” has been used to describe aroma of beverages (e.g. brewed coffee), where the term is associated with caramel/vanilla aroma notes [271]. The intensities of these four odor notes were evaluated for all the samples, following to CATA test.

Table 14 – Final list of attributes used for Check-All-That-Apply test and associated references used for panel training

Attributes in English	Attributes in French	Reference
Cut Grass	*Herbe coupée*	10% w/w *(Z)*-3-hexenol (CAS: 928-96-1) in ethanol
Celery	*Céleri*	Cubes of fresh cut celery
Hay	*Foin*	Horse hay from experimental farm (Grignon, France)
Lentil	*Lentille*	Liquid from canned lentils (Auchan, France)
Potato	*Pomme de terre*	Liquid from canned lentils (Auchan, France)
Mung Bean	*Haricot mungo*	Liquid from canned mung bean sprouts (Auchan, France)
Fresh	*Frais*	10% w/w L-menthol (CAS: 89-78-1) in ethanol
Wood	*Bois*	Bits of tree barks (Grignon, France)
Earthy	*Terre*	Moist soil (Grignon, France)
Spices	*Epice*	Four spices mix (Auchan, France)
Caramel	*Caramel*	Caramel sauce (Vahiné, France)
Rancid	*Rance*	Vegetable oil stored for several years (Grignon, France)
Grilled	*Grillé*	Grilled almonds (Auchan, France)
Burnt	*Brûlé*	Almonds grilled until black (Grignon, France)
Smoky	*Fumé*	Smoky barbecue sauce (Auchan, France)
Coffee	*Café*	Arabica coffee powder (Auchan, France)
Chocolate	*Chocolat*	Cacao powder (Auchan, France)
Hazelnut	*Noisette*	Whole hazelnuts (Auchan, France)
Coconut	*Coco*	Grated coconut (Auchan, France)
Orange Blossom	*Fleur d'oranger*	Orange Blossom Aroma (Fabster, France)
Vanilla	*Vanille*	10% w/w ethyl vanillin (CAS: 121-32-4) in ethanol
Banana	*Banane*	Banana concentrated aroma(Fabster, France)
Almond	*Amande*	Almond oil (Auchan, France)
Citrus	*Citron*	Fresh cut pieces of lemon
Red Wine	*Vin Rouge*	Red wine 'Les Fiefs de Lagrange' (Saint-Julien, France)
Vinegar	*Vinaigre*	Vinegar (Auchan, France)
Milk	*Lait*	Whole milk (Lactel, France)
Butter	*Beurre*	10% w/w 2,3-butanedione (CAS: 431-03-8) in ethanol
Cream	*Crème*	Whole milk fresh cream (Yoplait, France)

Egg	*Oeuf*	Hard boiled eggs, peeled and cut (Auchan, France)
Meat	*Viande*	Beef bouillon (Auchan, France)
Ammoniac	*Ammoniac*	10% w/w ammoniac (CAS: 7664-41-7) in ethanol
Soap	*Savon*	Unscented soap bar (Le Petit Marseillais, France)
Chemical	*Chimique*	10% w/w ethyl acetate (CAS: 141-78-6) in ethanol
Cigarette	*Cigarette*	Nil[§]
Petrol	*Pétrole*	Nil[§]

[§] - This attribute is well known by the panelists, and the reference product for it is avoided due to safety and sensory reasons [272]–[275].

V.2.2.2.d. Odor Attribute Profiling & Intensity Scaling

Sample evaluation session was conducted after the training sessions, using the LimeSurvey platform (LimeSurvey GmbH). Each sample was evaluated by two methods: a) selection of most pertinent attributes for the sample using Check-All-That-Apply (CATA) method (**Table 14**); and b) perceived intensity scaling ranging from 0 (none or negligible perception) to 10 (very intense) of four principal notes identified: green, "sweet", rancid and cooked notes.

The samples were presented to the judges in an order according to a Latin Square experimental design to account for possible carry-over effects. The tests were carried out in single replicate, where analysis of 12 out of 37 ingredients represented true triplicates (from production of ingredients to their odor analyses).

V.2.2.3. Flavor Volatile Chemistry

V.2.2.3.a. Sample Preparation for Volatile Compounds Analysis

A 2 g mixture of 10% (w/w) ingredient suspensions, readjusted to pHutilization 4 or 7 and then introduced with 100 ng of d7-heptanol standard, were prepared using the following method: the ingredients (FBIC and modified ingredients) were suspended in triplicates in deionized water in 20 mL SPME vials which were immediately sealed with aluminum polytetrafluoroethylene coated silicone septum caps in order to avoid loss of volatiles. The vials were agitated by IKA Vortex 2 (IKA Works, Inc., Staufen, Germany) for 30 min at 20 °C, followed by a pH adjustment of either pH 4 or 7 using 0.1 mol/L hydrochloric acid or 0.1 mol/L sodium hydroxide respectively (Sigma Aldrich, Missouri, United States). Additionally, 100 ng of d7-heptanol (Ref: D6920, Cluzeau Infor Labo C.I.L, France) from a 0.1 µg/mg ethanol stock solution was introduced into the vials after the pH adjustment. The required amounts of acid, base and standard stock were added into the vials using a 50 µL eVol™ syringe (Trajan Scientific and Medical, Australia).

V.2.2.3.b. Extraction of Volatile Compounds from the Headspace

The volatile compounds were extracted from the headspace of the samples by automated solid-phase micro-extraction (HS-SPME). Prior to extraction, each vial was incubated at 50 °C for 36 min under agitation (10/1 s on/off) to reach equilibrium between the matrix and the headspace. For the ensuing extraction, a gray-notched divinylbenzene/carboxen/polydimethylsiloxane DVB/CAR/PDMS fiber (2 cm, Supelco) was exposed to the headspace for 42 min at the same temperature. The fiber was then desorbed on a GC injection port, which was held at 250 °C, during 2 min. A 10 min fiber reconditioning procedure was performed at 270 °C between samples. The tri-phase fiber was selected to ensure an efficient extraction of a wide range of volatile compounds [170], [276], [277].

V.2.2.3.c. GC-MS Semi-Quantitative Analysis of Volatiles

The headspace extracts were analyzed by gas chromatography (GC) in a Trace GC Ultra system coupled to an ISQ single quadrupole mass spectrometer (MS, Thermo Scientific, Rodano, Italy). A non-polar ZB-5MSPLUS column (30 m x 0.25 mm x 0.25 µm, Zebron, Phenomenex, United States) was chosen for separation. Helium was used as carrier gas at a constant flow rate of 1.2 mL/min. The parameters were based on the method optimized by Cepeda-Vázquez (2017) in the same equipment, with slight adjustments following pre-tests to achieve a better chromatographic separation of the volatile compounds present in faba beans. Injections were done in splitless mode. The GC oven was programmed as follows: initial temperature 40 °C (held for 5 min), then raised at 1 °C/min until 90 °C and 15 °C/min until a final temperature of 240 °C (held for another 5 min). Mass spectrometry was carried out using electron impact at 70 eV as ionization mode. MS transfer line and ion source temperatures were set to 250 °C and 200 °C, respectively. A standard solution of deionized water with 100 ng d7-heptanol was analyzed for every sequence of 10 runs to assure the steadiness of the system's response over time. Data acquisition was done in full scan mode from m/z 33 to 300. Each compound was identified and confirmed by means of the Wiley 8 and NIST 08 mass spectral libraries, calculation of normal alkane retention index (RI) and comparison to NIST Chemistry Web- Book Standard Reference Data Program 69 indices. The chromatographic peak areas for each compound were calculated by extracting the quantifier ions specific for that compound, and then integrated using the Quan browser of Xcalibur 2.1.0 (Thermo Fisher Scientific Inc., United States). The retention indices were calculated using the isothermal and non-isothermal formulae established by Kovatz (Eq. 1) and Van den Dool and Kratz (Eq. 2), respectfully (National Institute of Standards and Technology (NIST), 2008), based on the retention

times of a series of alkanes (C5-C17, C19-C23) analyzed under the same conditions. Eq. 1 was only applied in the peaks eluted in the first and last 5 min of analysis, for which the oven temperature was held constant, while Eq. 2 was used for all additional peaks.

$$I_x = (100 * n) + 100 * \frac{(logt_x - logt_n)}{(logt_{n+1} - logt_n)} \quad \text{(Eq. 1)}$$

$$I_x = (100 * n) + 100 * \frac{(t_x - t_n)}{(t_{n+1} - t_n)} \quad \text{(Eq. 2)}$$

where Ix = retention index of the volatile, tx = retention time of the volatile, tn and tn+1 = retention times of the smaller and larger alkanes corresponding to the volatile.

The integrated volatile peak areas above the limits of quantification were selected and normalized with d7-heptanol peak areas of their respective chromatograms and the ingredient dry weights measured for each chromatographic analysis.

V.2.2.4. Data Analysis

Statistical data analysis was conducted using XLSTAT 2021.1. (Addinsoft, France).

V.2.2.4.a. Analysis of Odor Profile

A matrix of the selected attributes from CATA data across all samples and judges was obtained in a binary form (0 or 1). First, Coqran's Q test ($p \leq 0.05$) was used to identify the attributes that significantly discriminated the ingredient samples, followed by the Critical difference (Sheskin) multiple pairwise comparison between the attributes. Correspondence analysis (CA) with Chi-square distancing was conducted on these significant attributes, across all judges and samples. For the perceived intensities, analysis of variance (ANOVA) with post-hoc treatment using Newman-Keuls (SNK) method ($p \leq 0.05$) was performed across all the samples and judges. Furthermore, means of the intensities noted across all judges for each ingredient suspension was created and a Principal Component Analysis (PCA) by Pearson's correlation method was conducted on this matrix.

V.2.2.4.b. Analysis of Volatile Profile

A matrix of the normalized peak areas of all quantifiable volatiles was obtained across all ingredient suspensions. ANOVA with post-hoc treatment using Newman-Keuls (SNK) method ($p \leq 0.05$) and PCA Pearson's correlation method was conducted on this matrix.

V.2.2.4.c. Association between Processing, Odor Perception and Volatile Chemistry

The relationships between the composition, the odor characteristics and intensities, as well as the relative amounts of volatile constituents of different ingredient suspensions, were examined using Multiple Factor Analysis (MFA). Four qualitative variables ($pH_{process}$, Tprocess, tprocess, pHutilization), along with three quantitative matrices (CATA data, average odor intensities and normalized volatile peak areas) were used for the MFA. Summation of the binary CATA data for each sample, across all judges was prepared exclusively for this analysis.

V.2.3. Results

V.2.3.1. Odor Perception of Fava Ingredients

V.2.3.1.a. Panel Performance

The panel performances were first evaluated and controlled considering repeatability, discrimination and homogeneous criteria of sensory profiling results. Overall performances were assessed using ANOVAs with three independent variables (product type, subjects, and replicate) and their first-order interactions (**Table 15**). This three-way ANOVA was performed on the odor attribute intensities (green, "sweet", rancid and cooked notes) evaluated on a selected group of fava bean modified ingredients, i.e. the $pH_{process}$ 4 series, which was performed in triplicate. A significant product effect was observed for 3 out of 4 attributes (except for the cooked note), indicating that the panelists distinguished among the different samples ($p < 0.05$). The significance of the first order interactions revealed whether the panelists consistently scored attributes across replicates (subject * replicate), if there was consistency in scoring different ingredient suspensions among the panelists (product type * subject), and whether ingredient suspensions behaved consistently across replicates (product type * replicate). At first, the interaction between the replicate and product type (product type * replicate) was not significant for all attributes (**Table 15**). With a considerable decrease in the F-value, the subject and product type interaction remained significant for all attributes, suggesting possible heterogeneously of evaluation between panelists or effect of different uses of scale between panelists, as described in literature [310], [311]. Detailed analysis of results permitted to confirm this last hypotheses. Therefore, all these performance results suggest that the panelists' scoring was globally consistent (discrimination, repeatable and homogeneous), even if there was some inconsistency in the case of certain attributes, which was taken into account in the analysis of the results.

Table 15 – Panel Performance Results. Three-way ANOVA results illustrating panel performance for odor attribute intensities of green, "sweet", rancid and cooked notes. Three independent variables were evaluated for a particular ingredient series ($pH_{process}$ 4) that was modified as triplicates: a) subject: representing the panelists; b) replicate: representing process as well as analytical replicates; and c) product type: representing each type of ingredient suspended at either $pH_{utilization}$ 4 or 7.

Attribute	Subject		Replicate		Product Type		(Subject * Replicate)		(Subject * Product Type)		(Replicate * Product Type)	
	F	p-Value	F	p-Value	F	p-Value	F	p-Value	F	p-Value	F	p-Value
Green	37.3	**<0.0001**	3.5	**0.032**	2.0	**0.029**	2.8	**<0.0001**	1.9	**<0.0001**	0.7	0.802
"Sweet"	15.9	**<0.0001**	5.2	**0.006**	15.2	**<0.0001**	2.5	**<0.0001**	1.6	**<0.0001**	0.6	0.953
Rancid	26.1	**<0.0001**	9.1	**0.000**	26.6	**<0.0001**	2.8	**<0.0001**	1.9	**<0.0001**	1.3	0.194
Cooked	31.1	**<0.0001**	2.0	0.139	1.5	0.142	1.9	**0.001**	1.7	**<0.0001**	0.8	0.706

V.2.3.1.b. Difference of sensory description between Ingredients from CATA test

All 36/36 attributes were checked at least one time during the test. On an average, 7 attributes per product were checked by the judges to describe a sample. The least checked attributes across all samples were petrol (1%), coffee (4%) and cigarette (4%), and the most checked were hay (46%), mung bean (39%), potato (35%), lentil (30%) and cut grass (30%). Results showed significant differences of perception between the different ingredient suspensions on 28 out of 36 attributes (Cochran's Q test ($p \leq 0.05$)). The significant attributes were presented on **Table 16**, completed by the differences between ingredient suspensions with the post-hoc multiple pairwise comparison test by Critical difference (Sheskin) method. Comparison of the samples showed that the ingredients modified by process conditions, and then suspended at two pH gave a diverse mix of odor complexities (**Table 16**). Specifically, a high variation between the ingredients at the different pHutilization was observed. Taking FBIC into account, attributes like cut grass, celery, fresh and spices were more frequently used at pHutilization 7 compared to pHutilization 4. Perception of rancid note was more frequently selected to describe the FBIC at pHutilization 4. Therefore, it seemed that the pHutilization had an impact in releasing different odor attributes for the same ingredient. The same effect was observed for the other ingredients modified by process conditions. The frequencies of odor attributes differed due to the pHutilization for the same modified concentrate. Also, there were differences between different modified concentrates, and between them and the

FBIC. Taking one attribute as an example i.e. chocolate – it did not describe the FBIC at pHutilization 7 but was selected as a descriptor for all the modified concentrates at this pHutilization. Meanwhile at pHutilization 4, the attribute was again absent for the FBIC and present but for just a few modified concentrates (**Table 16**). The effect of process conditions, particularly the pHutilization in driving different odor perception, was much clearly observed by the CA performed on significant attributes (**Figure 23**). Here, the ingredient suspensions were divided primarily by the first dimension (F1 = 36.56%), where more vinegar, meat, egg, chemical, rancid, burnt, citrus, red wine, spices were pronounced in the pHutilization 4 suspensions compared to chocolate, almond, hazelnut, banana, vanilla, coffee, caramel, fresh, milk, lentil and wood attributes featured in mostly pHutilization 7 suspensions (**Figure 23**). The attributes smoky, cut grass, celery, earthy, mung bean were shared between the two clusters of pHutilization. Separation of the ingredient suspensions by the second dimension (F2 = 12.04%) was unclear and might have been caused by a combination of other process conditions ($pH_{process}$, $T_{process}$ and/ or $t_{process}$). However, this separation was lower compared to the impact of pHutilization.

After this qualitative comparison between different ingredient suspensions, there was a need to describe their differences based on intensities of odor notes. Four major odor attributes were also quantified for all the samples by the panel.

Table 16 – Odor CATA Test Results. Multiple pairwise comparison Critical difference / Sheskin procedure of significant odor attributes for the suspensions of fava bean initial concentrate (FBIC) and the same modified by process conditions ($pH_{process}$, $T_{process}$ and/ or $t_{process}$) along CATA evaluation results.

		$pH_{process}$ 2						**$pH_{process}$ 4**						**$pH_{process}$ 6.4**						**$pH_{process}$ 11**					
		55 °C		**75 °C**		**95 °C**		**55 °C**		**75 °C**		**95 °C**		**55 °C**		**75 °C**		**95 °C**		**55 °C**		**75 °C**		**95 °C**	
	FBIC	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
$pH_{utilization}$ 4																									
Cut Grass	0.190 abc	0.333 abc	0.286 abc	0.333 abc	0.333 abc	0.286 abc	0.238 abc	0.381 abc	0.333 abc	0.286 abc	0.333 abc	0.333 abc	0.333 abc	0.238 abc	0.190 abc	0.333 abc	0.286 abc	0.286 abc	0.238 abc	0.333 abc	0.143 ab	0.286 abc	0.286 abc	0.238 abc	0.238 abc
Celery	0.238 ab	0.143 ab	0.238 ab	0.143 ab	0.238 ab	0.238 ab	0.143 ab	0.095 a	0.095 a	0.190 ab	0.190 ab	0.238 ab	0.143 ab	0.095 a	0.095 a	0.095 a	0.095 a	0.143 ab	0.095 a	0.143 ab	0.286 ab	0.238 ab	0.238 ab	0.143 ab	0.238 ab
Lentil	0.333 a	0.238 a	0.190 a	0.238 a	0.286 a	0.143 a	0.238 a	0.238 a	0.238 a	0.143 a	0.238 a	0.190 a	0.286 a	0.238 a	0.286 a	0.286 a	0.143 a	0.095 a	0.238 a	0.238 a	0.095 a	0.190 a	0.143 a	0.190 a	0.286 a
Mung Bean	0.429 a	0.333 a	0.429 a	0.286 a	0.429 a	0.476 a	0.476 a	0.381 a	0.429 a	0.381 a	0.286 a	0.333 a	0.429 a	0.429 a	0.429 a	0.524 a	0.381 a	0.524 a	0.429 a	0.524 a	0.619 a	0.429 a	0.429 a	0.381 a	0.333 a
Fresh	0.238 ab	0.095 a	0.238 ab	0.095 a	0.143 ab	0.286 ab	0.048 a	0.238 ab	0.238 ab	0.190 ab	0.286 ab	0.238 ab	0.190 ab	0.286 ab	0.143 ab	0.333 ab	0.143 ab	0.190 ab	0.286 ab	0.238 ab	0.190 ab	0.190 ab	0.190 ab	0.286 ab	0.143 ab
Wood	0.143 a	0.286 a	0.190 a	0.238 a	0.143 a	0.381 a	0.286 a	0.190 a	0.238 a	0.286 a	0.333 a	0.095 a	0.095 a	0.143 a	0.143 a	0.190 a	0.238 a	0.286 a	0 a	0.190 a	0.190 a	0.190 a	0.143 a	0.143 a	0.238 a
Earthy	0.238 a	0.381 a	0.286 a	0.190 a	0.286 a	0.238 a	0.143 a	0.095 a	0.238 a	0.143 a	0.095 a	0.143 a	0.095 a	0.190 a	0.095 a	0.143 a	0.238 a	0.095 a	0.143 a	0.286 a	0.143 a	0.190 a	0.333 a	0.143 a	0.238 a
Spices	0.048 a	0.048 a	0.048 a	0.095 a	0.095 a	0.048 a	0.190 a	0.048 a	0.048 a	0.095 a	0.190 a	0.095 a	0.143 a	0.095 a	0.190 a	0.095 a	0.048 a	0.095 a	0.190 a	0.190 a	0.143 a	0.190 a	0.048 a	0.238 a	0.143 a
Caramel	0 a	0.048 a	0.095 a	0.048 a	0.048 a	0.143 a	0.048 a	0.190 a	0.048 a	0.095 a	0.048 a	0.095 a	0 a	0.143 a	0.190 a	0.048 a	0.143 a	0.095 a	0.333 a	0.048 a	0.048 a	0.048 a	0 a	0.143 a	0.048 a
Rancid	0.381 abcd	0.714 d	0.619 bcd	0.667 cd	0.524 abcd	0.429 abcd	0.476 abcd	0.381 abcd	0.571 abcd	0.714 d	0.429 abcd	0.619 bcd	0.524 abcd	0.333 abcd	0.476 abcd	0.667 cd	0.619 bcd	0.714 d	0.429 abcd	0.524 abcd	0.429 abcd	0.429 abcd	0.571 abcd	0.571 abcd	0.381 abcd

Burnt	0.143	0.095	0	0.095	0.095	0	0.095	0	0.095	0.048	0.048	0.095	0.048	0	0	0	0	0.095	0	0.095	0.048	0.048	0.095	0.143	0.286
	ab	ab	a	ab	ab	a	ab	a	ab	ab	ab	ab	ab	a	a	a	a	ab	a	ab	ab	ab	ab	ab	ab
Smoky	0.190	0.095	0.143	0.048	0.190	0.048	0.381	0.048	0.048	0.143	0.095	0.190	0.190	0.048	0.238	0.190	0.381	0.048	0.095	0.333	0.190	0.286	0.190	0.095	0.238
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Coffee	0	0	0	0.048	0.048	0.048	0	0	0	0	0	0.048	0.048	0	0	0	0.095	0.048	0.048	0	0	0	0.095	0	0.048
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Chocolate	0	0	0	0.048	0.048	0.048	0	0	0.048	0.095	0	0	0.095	0	0.143	0	0	0	0	0.048	0.048	0	0	0.048	0.048
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Hazelnut	0.048	0.190	0.143	0.286	0.286	0.143	0.095	0.286	0.238	0.095	0.048	0.238	0.143	0.286	0.143	0.143	0.143	0.048	0.190	0.095	0.143	0.143	0.095	0.190	0.048
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Orange Blossom	0.095	0	0.143	0	0.048	0	0	0.048	0.095	0.048	0.286	0.286	0.095	0.190	0.095	0.048	0	0	0.429	0.048	0.048	0.048	0	0	0.048
	abc	a	abc	a	ab	a	a	ab	abc	ab	abc	abc	abc	abc	abc	ab	a	a	bc	ab	ab	ab	a	a	ab
Vanilla	0.095	0.048	0.143	0.095	0	0.095	0.095	0.143	0.143	0.190	0.095	0.190	0.095	0.190	0.095	0	0.095	0.095	0.190	0.048	0.095	0.048	0.048	0.143	0
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Banana	0.190	0.048	0.095	0.095	0.095	0.143	0.143	0.095	0.190	0.095	0.143	0.143	0.190	0.333	0.143	0.095	0.048	0.048	0.190	0.048	0.095	0.048	0.048	0.048	0.048
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Almond	0.143	0.381	0.048	0.286	0.143	0.143	0.143	0.333	0.238	0.238	0.190	0.095	0.095	0.333	0.190	0.190	0.143	0.143	0.095	0.143	0.143	0.095	0.143	0.238	0.095
	ab	ab	a	ab	ab	ab	ab	ab	ab	ab	ab	a	a	ab	ab	ab	ab	ab	a	ab	ab	a	ab	ab	a
Citrus	0.143	0.143	0.143	0.095	0.143	0.190	0.238	0.095	0.143	0.143	0.238	0.190	0.333	0.095	0.190	0.048	0.143	0.095	0.476	0.095	0.190	0.286	0.095	0.095	0.238
	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	a	ab	ab	b	ab	ab	ab	ab	ab	ab
Red Wine	0.048	0	0.048	0	0.143	0.143	0.143	0.048	0.048	0	0.095	0	0.143	0.095	0.143	0	0.095	0.048	0.238	0.048	0.190	0.190	0.143	0.048	0.143
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Vinegar	0.286	0.190	0.286	0.238	0.143	0.143	0.333	0.190	0.333	0.286	0.429	0.333	0.429	0.095	0.381	0.286	0.381	0.333	0.524	0.524	0.571	0.571	0.571	0.476	0.619
	abcd	abcd	abcd	abcd	abcd	abcd	abcd	abcd	abcd	abcd	abcd	abcd	abcd	abc	abcd	abcd	abcd	abcd	bcd	bcd	cd	cd	cd	abcd	d
Milk	0.143	0.143	0.286	0.238	0	0.143	0.048	0.238	0.095	0	0.143	0.095	0.048	0.429	0.238	0.238	0.238	0.095	0.095	0.143	0.143	0.143	0.190	0.143	0.286
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Egg	0.238	0.095	0.095	0.190	0.048	0.095	0.048	0.190	0.048	0.095	0.238	0.190	0.095	0.190	0.143	0.095	0.190	0.238	0.143	0.143	0.333	0.048	0.190	0.286	0.238
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Meat	0.286	0	0	0.048	0.143	0.048	0.238	0.143	0.048	0	0.143	0.048	0.190	0.143	0.190	0.048	0.095	0.190	0	0.143	0.286	0.190	0.238	0.238	0.381
	ab	a	a	ab	ab	ab	ab	ab	ab	a	ab	ab	ab	ab	ab	ab	ab	ab	a	ab	ab	ab	ab	ab	b
Ammoniac	0.095	0.095	0.095	0.238	0.143	0.143	0.190	0	0.190	0.143	0.143	0.143	0.190	0.095	0.286	0.238	0.143	0.286	0.095	0.190	0.286	0.238	0.333	0.286	0.333
	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a

Chemical	0.095 ab	0.190 ab	0.381 b	0.143 ab	0.143 ab	0.143 ab	0.190 ab	0.190 ab	0.048 ab	0.238 ab	0.143 ab	0.238 ab	0.238 ab	0 a	0.048 ab	0.143 ab	0.143 ab	0.190 ab	0.143 ab	0 a	0.143 ab	0.238 ab	0.286 ab	0.190 ab	0.143 ab
$pH_{utilization}$ 7																									
Cut Grass	0.714 c	0.333 abc	0.381 abc	0.476 abc	0.333 abc	0.238 abc	0.333 abc	0.381 abc	0.476 abc	0.333 abc	0.381 abc	0.476 abc	0.238 abc	0.667 bc	0.476 abc	0.429 abc	0.381 abc	0.286 abc	0.190 abc	0.238 abc	0.429 abc	0.238 abc	0.333 abc	0.190 abc	0.095 a
Celery	0.524 b	0.143 ab	0.238 ab	0.286 ab	0.143 ab	0.095 a	0.286 ab	0.238 ab	0.048 a	0.190 ab	0.143 ab	0.048 a	0.095 a	0.190 ab	0.190 ab	0.238 ab	0.095 a	0.143 ab	0.048 a	0.143 ab	0.190 ab	0.143 ab	0.048 a	0.190 ab	0.143 ab
Lentil	0.286 a	0.381 a	0.333 a	0.381 a	0.429 a	0.286 a	0.190 a	0.333 a	0.571 a	0.429 a	0.381 a	0.429 a	0.429 a	0.286 a	0.333 a	0.381 a	0.524 a	0.238 a	0.524 a	0.286 a	0.238 a	0.476 a	0.286 a	0.333 a	0.286 a
Mung Bean	0.381 a	0.286 a	0.381 a	0.238 a	0.524 a	0.429 a	0.476 a	0.429 a	0.381 a	0.429 a	0.286 a	0.476 a	0.333 a	0.524 a	0.524 a	0.333 a	0.286 a	0.333 a	0.143 a	0.381 a	0.238 a	0.190 a	0.238 a	0.238 a	0.286 a
Fresh	0.524 ab	0.095 a	0.333 ab	0.286 ab	0.286 ab	0.238 ab	0.238 ab	0.381 ab	0.381 ab	0.190 ab	0.286 ab	0.381 ab	0.238 ab	0.667 b	0.238 ab	0.333 ab	0.286 ab	0.143 ab	0.381 ab	0.333 ab	0.143 ab	0.333 ab	0.286 ab	0.238 ab	0.143 ab
Wood	0.143 a	0.333 a	0.333 a	0.143 a	0.190 a	0.238 a	0.238 a	0.238 a	0.238 a	0.333 a	0.238 a	0.190 a	0.238 a	0.190 a	0.333 a	0.238 a	0.429 a	0.190 a	0.190 a	0.190 a	0.143 a	0.286 a	0.286 a	0.333 a	0.476 a
Earthy	0.238 a	0.429 a	0.381 a	0.333 a	0.333 a	0.238 a	0.143 a	0.238 a	0.333 a	0.381 a	0.190 a	0.286 a	0.238 a	0.286 a	0.190 a	0.238 a	0.286 a	0.238 a	0.190 a	0.333 a	0.286 a	0.286 a	0.476 a	0.333 a	0.429 a
Spices	0.143 a	0.048 a	0.095 a	0.095 a	0.048 a	0.143 a	0.190 a	0.143 a	0 a	0.048 a	0.095 a	0.048 a	0.143 a	0 a	0.095 a	0.095 a	0.095 a	0 a	0.048 a	0 a	0.048 a	0.095 a	0 a	0.048 a	0.048 a
Caramel	0 a	0.190 a	0.095 a	0.048 a	0.095 a	0.238 a	0.190 a	0.095 a	0.048 a	0.048 a	0.048 a	0 a	0.286 a	0.095 a	0.190 a	0.190 a	0.238 a	0.143 a	0.286 a	0.048 a	0.333 a	0.095 a	0.238 a	0.143 a	0.238 a
Rancid	0.190 abcd	0.286 abcd	0.143 abcd	0.095 abc	0.286 abcd	0.429 abcd	0.333 abcd	0.238 abcd	0.333 abcd	0.238 abcd	0.190 abcd	0.143 abcd	0.190 abcd	0.095 abc	0.095 abc	0.095 abc	0.143 abcd	0.095 abc	0.048 ab	0.095 abc	0.048 ab	0.095 abc	0.048 ab	0.095 abc	0 a
Burnt	0 a	0.095 ab	0.048 ab	0.048 ab	0 a	0.048 ab	0.095 ab	0 a	0.048 ab	0.095 ab	0 a	0 a	0 a	0 a	0 a	0.048 ab	0.048 ab	0.048 ab	0.095 ab	0.048 ab	0.048 ab	0.095 ab	0 a	0.143 ab	0.333 b
Smoky	0 a	0.143 a	0.095 a	0.190 a	0.095 a	0.143 a	0.429 a	0.190 a	0.143 a	0.095 a	0.190 a	0.190 a	0.143 a	0.048 a	0.190 a	0.048 a	0.190 a	0.190 a	0.238 a	0.190 a	0.286 a	0.190 a	0.190 a	0.286 a	0.286 a
Coffee	0 a	0.143 a	0.095 a	0 a	0.048 a	0.095 a	0.048 a	0.048 a	0 a	0 a	0.095 a	0.048 a	0.095 a	0 a	0 a	0 a	0.095 a	0 a	0.190 a	0.048 a	0.095 a	0.048 a	0.095 a	0 a	0.095 a
Chocolate	0 a	0.190 a	0.095 a	0.143 a	0.095 a	0.048 a	0.095 a	0.048 a	0.048 a	0.048 a	0.095 a	0.095 a	0.190 a	0.095 a	0.095 a	0.143 a	0.143 a	0.143 a	0.238 a	0.143 a	0.143 a	0.143 a	0.095 a	0.143 a	0.190 a

Hazelnut	0.143 a	0.238 a	0.381 a	0.238 a	0.381 a	0.190 a	0.143 a	0.333 a	0.286 a	0.381 a	0.286 a	0.429 a	0.571 a	0.190 a	0.381 a	0.238 a	0.333 a	0.381 a	0.238 a	0.286 a	0.333 a	0.429 a	0.429 a	0.524 a	0.524 a
Orange Blossom	0.286 abc	0.095 abc	0.143 abc	0.095 abc	0.143 abc	0.095 abc	0.095 abc	0.048 ab	0.048 ab	0.143 abc	0.190 abc	0.143 abc	0.238 abc	0.095 abc	0.095 abc	0.095 abc	0.048 ab	0.095 abc	0.190 abc	0.095 abc	0 a	0.143 abc	0 a	0.048 ab	0.095 abc
Vanilla	0.048 a	0.190 a	0.095 a	0.190 a	0.143 a	0.143 a	0.048 a	0.238 a	0.143 a	0.143 a	0.190 a	0.143 a	0.143 a	0.048 a	0.095 a	0.095 a	0.190 a	0.286 a	0.238 a	0.048 a	0.238 a	0.238 a	0.143 a	0.143 a	0.095 a
Banana	0.048 a	0.048 a	0.095 a	0.095 a	0.143 a	0.190 a	0.190 a	0.095 a	0.143 a	0.190 a	0.190 a	0.143 a	0.143 a	0.190 a	0.238 a	0.286 a	0.190 a	0.190 a	0.143 a	0.286 a	0.286 a	0.333 a	0.286 a	0.286 a	0.143 a
Almond	0.190 ab	0.381 ab	0.190 ab	0.333 ab	0.238 ab	0.333 ab	0.286 ab	0.524 ab	0.190 ab	0.286 ab	0.286 ab	0.333 ab	0.286 ab	0.381 ab	0.286 ab	0.381 ab	0.286 ab	0.476 ab	0.381 ab	0.381 ab	0.571 ab	0.333 ab	0.333 ab	0.381 ab	0.286 ab
Citrus	0.238 ab	0.095 ab	0.143 ab	0.190 ab	0.095 ab	0.048 a	0.190 ab	0.048 a	0.095 ab	0 a	0.143 ab	0.095 ab	0.143 ab	0.190 ab	0.095 ab	0.048 a	0 a	0 a	0.095 ab	0 a	0 a	0 a	0 a	0.048 a	0.048 a
Red Wine	0.048 a	0 a	0.095 a	0.048 a	0 a	0.095 a	0.238 a	0 a	0 a	0.048 a	0 a	0.143 a	0.143 a	0.048 a	0.095 a	0.048 a	0.048 a	0.048 a	0 a	0.048 a	0 a	0.095 a	0 a	0.143 a	0.048 a
Vinegar	0.048 ab	0 a	0.095 abc	0 a	0.048 ab	0.143 abcd	0.190 abcd	0 a	0.048 ab	0.048 ab	0 a	0.095 abc	0 a	0.048 ab	0.095 abc	0.048 ab	0.048 ab	0.095 abc	0.143 abcd	0 a	0 a	0.143 abcd	0.095 abc	0.095 abc	0.143 abcd
Milk	0.095 a	0.048 a	0.095 a	0.095 a	0.238 a	0.190 a	0.143 a	0.286 a	0.190 a	0.381 a	0.381 a	0.238 a	0.095 a	0.476 a	0.190 a	0.286 a	0.190 a	0.429 a	0.095 a	0.143 a	0.095 a	0.190 a	0.190 a	0.143 a	0.048 a
Egg	0.095 a	0.143 a	0.095 a	0.048 a	0.095 a	0.143 a	0.143 a	0.095 a	0.095 a	0.048 a	0.095 a	0 a	0.095 a	0 a	0.048 a	0 a	0.095 a	0 a	0 a	0.095 a	0.048 a	0.048 a	0.048 a	0.095 a	0.143 a
Meat	0 a	0.143 ab	0.143 ab	0.048 ab	0 a	0.095 ab	0.190 ab	0 a	0 a	0 a	0 a	0.048 ab	0.095 ab	0 a	0.048 ab	0 a	0 a	0 a	0.048 ab	0 a	0.048 ab	0.048 ab	0.048 ab	0 a	0.143 ab
Ammoniac	0 a	0.143 a	0.095 a	0.190 a	0.143 a	0.095 a	0.095 a	0.143 a	0.095 a	0.048 a	0.095 a	0.095 a	0 a	0 a	0 a	0 a	0.095 a	0.048 a	0.095 a	0.095 a	0.095 a	0.143 a	0.143 a	0.190 a	0.190 a
Chemical	0.095 ab	0.095 ab	0.143 ab	0.095 ab	0.143 ab	0.095 ab	0.190 ab	0.048 ab	0.048 ab	0 a	0.095 ab	0 a	0 a	0 a	0.048 ab	0 a	0.143 ab	0.095 ab	0.048 ab	0.095 ab	0.048 ab	0.048 ab	0.048 ab	0 a	0.095 ab

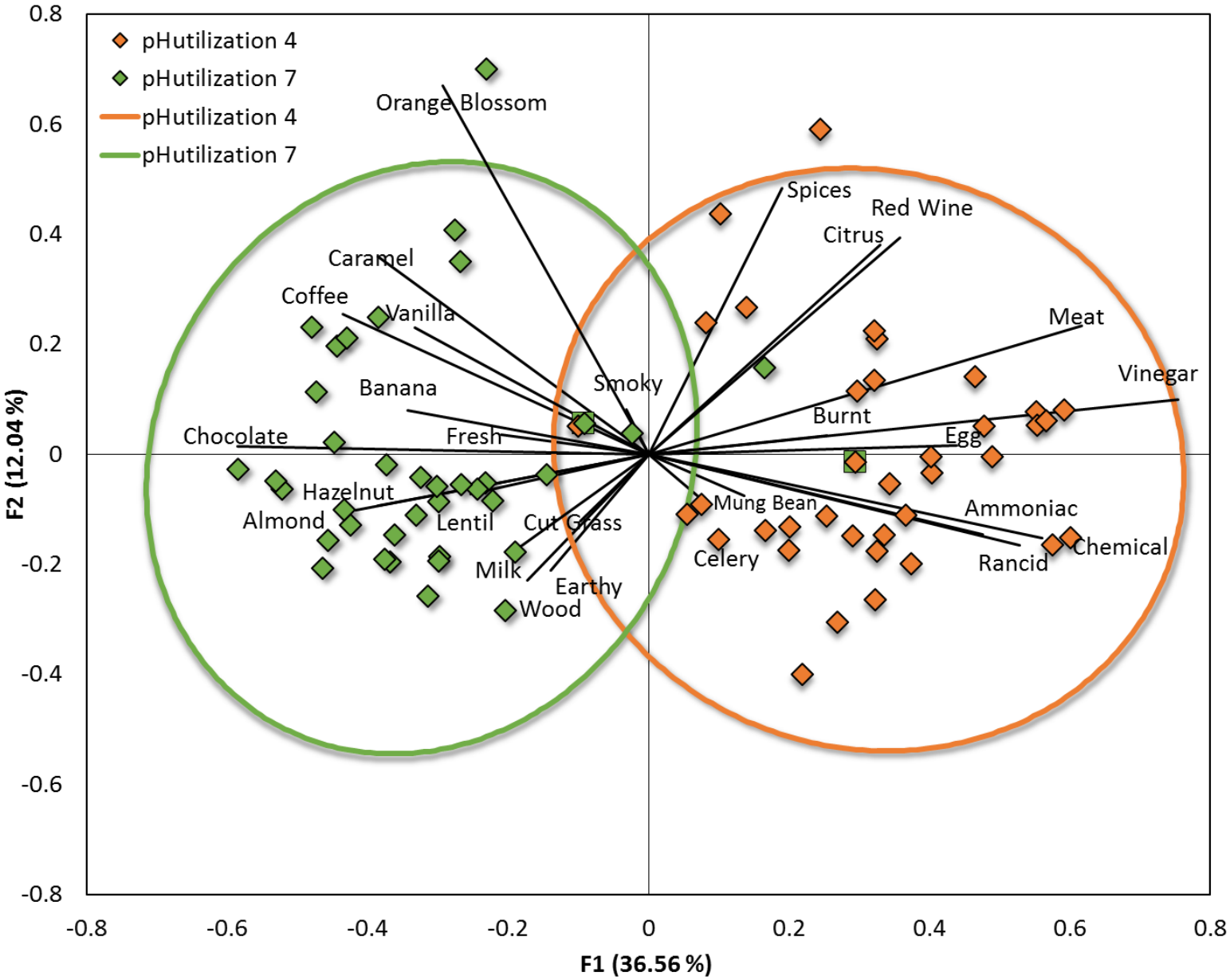

Figure 23 – Odor Attributes of Fava Concentrate Suspensions. Correspondence analysis (CA) of the check-all-that-apply (CATA) data projecting different ingredient aqueous suspensions as points and significant odor attributes as lines on the biplot plane. The ingredients studied are fava bean initial concentrate (FBIC) and the same modified by pH ($pH_{process}$), temperature ($T_{process}$) and treatment duration ($t_{process}$) and

then utilized at two pH ($pH_{utilization}$). Confidence ellipse (α = 0.05) were constructed on the sample coordinates grouped by the $pH_{utilization}$.

V.2.3.1.c. Difference in Note Intensities between Ingredients

For a clearer understanding on the effects of process conditions on different odors, the intensity profiling of the ingredients' suspensions was performed, based on the evaluation of key odors. Four-way ANOVA (**Table 17**) presented significant effects of all process conditions ($pH_{process}$, $T_{process}$, $t_{process}$ and $pH_{utilization}$) on green, "sweet", rancid and cooked perceived intensities. Differences among groups were calculated by the Newman-Keuls post-hoc analysis. The FBIC concentrate had significantly higher green note and lower "sweet" note intensities compared to the modified ingredients grouped by different $pH_{process}$. It was also significantly lower in cooked notes as compared to the ingredients modified by $pH_{process}$ 2 and 11 and in rancid notes compared to the ingredients modified by $pH_{process}$ 2. Ingredients suspended at different $pH_{utilization}$ were significantly different in green, "sweet" and rancid notes ($p \leq 0.05$, **Table 17**). At $pH_{utilization}$ 7, the perceptions of green and "sweet" notes were significantly perceived higher compared to samples at $pH_{utilization}$ 4 ($p \leq 0.05$, **Table 17**). On the other hand, rancid intensity was perceived significantly higher in samples at $pH_{utilization}$ 4 ($p \leq 0.05$, **Table 17**) than $pH_{utilization}$ 7. It must be noted that while ANOVA showed significant differences in all odor intensities, the post-hoc analysis presented singular groups for $T_{process}$ and $t_{process}$ – suggesting the presence of high variations within the groups themselves. A higher order of complexity in the effects on note intensities could thus be hypothesized due to possible physicochemical or sensory interactions [312].

Table 17 – Difference in Odor Note Intensities. Four-way ANOVA (α = 0.05), followed by the Newman-Keuls post-hoc analysis illustrating the means of different note intensities across samples and panelists that varied as a function of different process conditions (effects). The ingredients studied are fava bean initial concentrate (FBIC) and the same modified by pH ($pH_{process}$), temperature ($T_{process}$) and treatment duration ($t_{process}$) and then utilized at two pH ($pH_{utilization}$).

Effects		Green	"Sweet"	Rancid	Cooked
$pH_{process}$	FBIC	6.155 a	2.914 b	3.134 b	3.179 b
	$pH_{process}$ 2	4.895 b	4.202 a	4.257 a	4.326 a
	$pH_{process}$ 4	4.662 b	4.687 a	3.301 b	3.722 ab
	$pH_{process}$ 6.4	4.744 b	4.638 a	3.293 b	3.774 ab

	$pH_{process}$ 11	4.558 b	4.071 a	3.309 b	4.349 a
	p-Value	***<0.0001***	***<0.0001***	***<0.0001***	***<0.0001***
$T_{process}$	FBIC	4.953 a	4.317 a	3.466 a	3.926 a
	55 °C	5.118 a	3.801 a	3.351 a	3.874 a
	75 °C	4.987 a	3.975 a	3.553 a	3.755 a
	95 °C	4.953 a	4.317 a	3.466 a	3.926 a
	p-Value	***<0.0001***	***<0.0001***	***<0.0001***	***0.000***
$t_{process}$	FBIC	5.017 a	4.046 a	3.461 a	3.802 a
	Low	5.017 a	4.046 a	3.461 a	3.802 a
	High	4.976 a	4.215 a	3.454 a	4.007 a
	p-Value	***<0.0001***	***<0.0001***	***<0.0001***	***0.000***
$pH_{utilization}$	$pH_{utilization}$ 4	4.726 b	3.498 b	4.470 a	3.801 a
	$pH_{utilization}$ 7	5.280 a	4.707 a	2.448 b	3.940 a
	p-Value	***<0.0001***	***<0.0001***	***<0.0001***	***0.000***

The PCA biplot highlighted the description of the ingredient suspensions in regards of their respective perceived odor intensities. It explained 76% of variances (**Figure 24**). The biplot presented predominant effect of the $pH_{utilization}$ in influencing the intensities of rancid and "sweet" notes. The "sweet" and rancid notes were associated away from each other, where at $pH_{utilization}$ 4, rancid notes were perceived more intense whereas at $pH_{utilization}$ 7, higher intensity in "sweet" notes was perceived. Cooked and green notes were oppositely associated with each other, and were not in relation with the "sweet" and rancid notes. The exact reasoning between their opposition on the PCA biplot was not so clear for the "sweet" and rancid notes (**Figure 24**), compared to significant differences seen from ANOVA (**Table 17**). But a notable impact of $pH_{utilization}$ 7 can be seen in the perception of cooked and green notes. For instance, the FBIC used at $pH_{utilization}$ 7 gave a significantly higher perception of green notes (**Table 17**) and is on the extreme end of the quadrant of rancid notes (**Figure 24**). Additionally, the FBIC at $pH_{utilization}$ 7 was negatively associated with the cooked notes. It is interesting that green note of the same FBIC, but at $pH_{utilization}$ 4, was not perceivable. The ingredients that gave higher perception of cooked notes were also mostly from the $pH_{utilization}$ 7 suspensions.

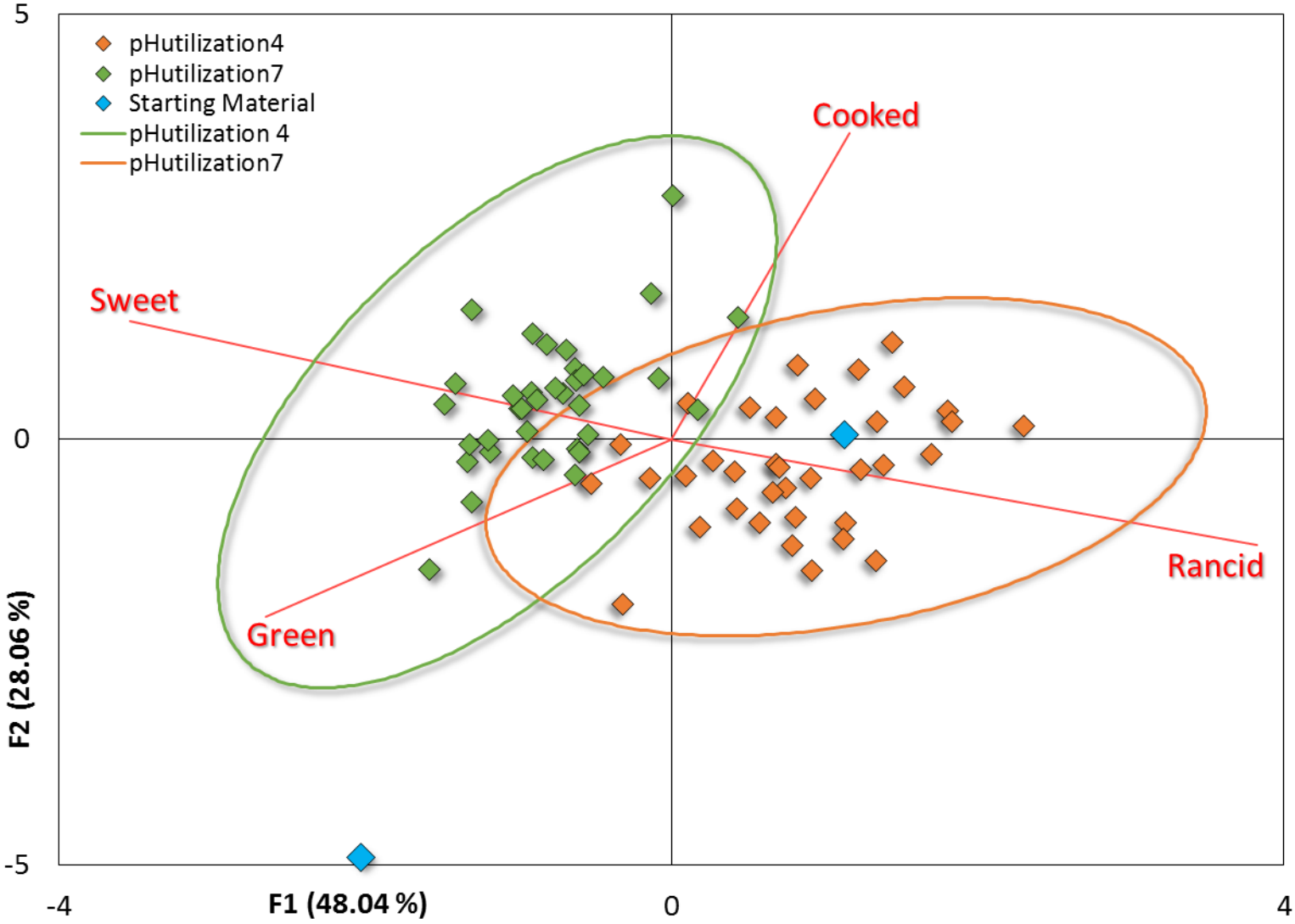

Figure 24 – Odor Intensities of Fava Bean Concentrate Suspensions. PCA projections of the different ingredient aqueous suspensions as points and their odor note intensities as lines on the plane. The ingredients studied are the FBIC (fava bean initial concentrate) and the same modified by pH ($pH_{process}$), temperature ($T_{process}$) and treatment duration ($t_{process}$) and then utilized at two pH ($pH_{utilization}$). Confidence ellipses ($\alpha = 0.05$) were constructed on the sample coordinates grouped by the $pH_{utilization}$.

V.2.3.2. Headspace Volatile Analysis

The volatile compounds present in the headspace of the ingredient suspensions were extracted and analyzed by HS-SPME-GC-MS. Eighty-eight volatile compounds were identified in the variety of ingredients suspended at $pH_{utilization}$ 4 and 7 including 11 alkanes, 4 alkenes, 13 alcohols, 25 aldehydes, 12 ketones, 2 esters, 3 organic acids, 2 aromatic hydrocarbons, 6 furanoids, 5 pyranoids, and 5 other compounds including sulfur and chlorinated hydrocarbons and 1 naphthalene (**Table 18**).

Table 18 – Detected Headspace Volatiles in Fava Bean Concentrate. Volatile compounds analyzed by HS-SPME-GC-MS from fava bean ingredients. The ingredients studied are the FBIC (fava bean initial concentrate) and the same modified by pH ($pH_{process}$ 2, 4, 6.4 and 11), temperature ($T_{process}$ 55, 75 and 95 °C) and treatment duration ($t_{process}$ 30 and 360 min) and then utilized at two pH ($pH_{utilization}$ 4 and 7).

Chemical Group	Compound	CAS Number	Retention Index	Ions used for Identification & Semi-Quantification
Alkanes	Pentane	109-66-0	<766	41, 43, 57, 72
	Heptane	142-82-5	<766	57, 71, 100
	4-Methyloctane	2216-34-4	880	85, 98, 128
	3-Methyl-6-Methyleneoctane	74630-07-2	975	55, 69, 70, 71, 83, 84, 112, 140
	2,7-Dimethyloctane	1072-16-8	994	56, 57, 71, 85, 86, 99, 100
	2,2,4,6,6-Pentamethylheptane	13475-82-6	994	56, 57, 71, 85, 99, 112, 113, 155
	Decane	124-18-5	999	57, 71, 85, 98, 99, 114
	2,2,4,4-Tetramethyloctane	62183-79-3	1076	57, 99, 113
	Undecane	1120-21-4	1099	56, 57, 70, 71, 85, 99, 113, 127, 156
	Dodecane	112-40-3	1200	55, 57, 71, 85, 99, 112, 127, 1170
	Tetradecane	629-59-4	1400	57, 71, 85, 99, 198
Alkenes	1-Heptene	592-76-7	<766	55, 56, 69, 70, 98
	1-Octene	111-66-0	794	55, 56, 70, 83, 84, 112
	2,4-Dimethyl-1-Heptene	19549-87-2	869	55, 57, 69, 70, 83, 126
	4-Cyanocyclohexene	100-45-8	1072	54, 67, 79, 80, 106,107
Alcohols	1-Penten-3-ol	616-25-1	<766	57, 71, 86
	(Z)-3-Methylcyclohexanol	5454-79-5	<766	57, 71, 81, 96
	(E)-3-Methylcyclohexanol	7443-55-2	<766	57, 71, 81, 96, 97, 112
	3-Methyl-1-Butanol	123-51-3	766	45, 55, 57, 70
	1-Pentanol	71-41-0	781	55, 56, 57, 70
	3-Hexen-1-ol	544-12-7	876	55, 67, 82,100
	1-Hexanol	111-27-3	884	45, 55, 56, 57, 69, 84

	1-Heptanol	111-70-6	987	55, 56, 57, 68, 69, 70, 83, 98
	1-Octan-3-ol	589-98-0	991	55, 59, 83, 101, 112
	2-Ethyl-1-Hexanol	104-76-7	1077	57, 70, 83, 84, 98, 112, 130
	1-Octanol	111-87-5	1090	55, 56, 69, 70, 84, 97, 98, 130
	(Z,Z)-4,5-Dimethyl-2-Hepten-3-ol	55956-37-1	1097	55, 71, 72, 100, 109, 124, 142
	Linalool	78-70-6	1099	55, 67, 69, 71, 80, 83, 93, 94, 121, 136, 139
Aldehydes	Acetaldehyde	75-07-0	<766	43, 44
	2-Methylpropanal	78-84-2	<766	41, 43, 72
	Butanal	123-72-8	<766	44, 57, 72
	3-Methylbutanal	590-86-3	<766	44, 57, 58, 71, 86
	2-Methylbutanal	96-17-3	<766	57, 58, 71, 86
	Pentanal	110-62-3	<766	44, 58, 71, 86
	(E)-2-Pentenal	764-39-6	774	55, 70, 91, 106
	3-Methylhexanal	19269-28-4	792	55, 57, 70, 81, 86, 114
	Hexanal	66-25-1	854	44, 55, 56, 57, 67, 72, 82
	2-Hexenal	505-57-7	875	55, 69, 83,98
	Heptanal	111-71-7	961	44, 45, 55, 57, 68, 70, 71, 81, 86, 96
	(*Z*)-2-Heptenal	57266-86-1	981	55, 70, 83, 97, 112
	(E,E)-2,4-Heptanedienal	4313-03-5	997	53, 67, 81, 82, 95, 110
	Octanal	124-13-0	1069	57, 69, 84, 85, 100, 128
	2-Octenal	2363-89-5	1086	55, 57, 69, 70, 83, 97, 98, 111, 126
	4-Nonenal	2277-16-9	1098	54, 55, 67, 83, 84, 96, 98, 122, 140
	Nonanal	124-19-6	1178	44, 45, 54, 55, 56, 57, 67, 70, 82, 98, 114, 124
	2-Dodecenal	4826-62-4	1180	55, 69, 70, 83, 84, 97, 98, 111, 138, 164, 182
	2-Nonenal	18829-56-6	1191	55, 70, 83, 84, 96, 111, 122, 140
	Decanal	112-31-2	1295	55, 57, 70, 71, 82, 95, 112, 128, 138
	2,4-Nonadienal	5910-87-2	1295	53, 67, 81, 82, 95, 109, 138
	2,4-Decadienal	2363-88-4	1398	55, 67, 81, 83, 95, 96, 123, 152

	2-Undecenal	2463-77-6	1399	54, 57, 69, 70, 82, 83, 84, 97, 98, 111, 124, 150, 168
	2-Butyl-2-Octenal	13019-16-4	1399	55, 69, 83, 95, 111, 125, 1182
	9-Octadecanal	5090-41-5	1904	55, 69, 81, 83, 98, 111, 121, 135, 152, 248, 266
Ketones	2-Butanone	78-93-3	<766	57, 72
	2-Pentanone	107-87-9	<766	58, 70, 71, 86
	3-Penten-2-one	3102-33-8	766	69, 70, 84
	2-Heptanone	110-43-0	894	58, 71, 99, 114
	1-Octen-3-one	4312-99-6	989	55, 70, 83, 97, 111, 126
	6-Methyl-5-Hepten-2-one	110-93-0	993	55, 69, 93, 108, 126
	3-Octen-2-one	1669-44-9	1079	55, 69, 83, 97, 111, 126
	3,5-Octadien-2-one	38284-27-4	1089	53, 55, 79, 81, 95, 109, 124
	3-Nonanone	925-78-0	1094	55, 57, 58, 72, 85, 95, 99, 113, 114, 142
	2-Nonanone	821-55-6	1096	57, 58, 59, 71, 85, 99, 127, 142
	3-Undecanone	2216-87-7	1299	57, 72, 73, 85, 96, 123, 1170
	2-Undecanone	112-12-9	1299	58, 71, 85, 96, 112, 110, 155, 170, 171
Esters	3-Methylbutylacetate	123-92-2	888	55, 61, 70, 73, 87, 115, 130
	(*Z*)-3-Octenylacetate	69668-83-3	1091	54, 68, 69, 81, 82, 95, 110, 111
Acids	Acetic Acid	64-19-7	<766	45, 60
	Octanoic Acid	106-32-1	1199	57, 60, 70, 88, 101, 127, 129, 172
	Nonanoic Acid	112-05-0	1298	57, 60, 73, 83, 98, 115, 129, 1158
Aromatic Hydrocarbons	2-Phenylethanol	60-12-8	1178	51, 65, 77, 91, 92, 93, 122
	4-Propylbenzaldehyde	28785-06-0	1299	51, 65, 89, 91, 92, 105, 119, 120, 147, 148
Furanoids	2-Methylfuran	534-22-5	<766	53, 81, 82
	Furfural	98-01-1	866	67,96
	2-Butylfuran	4466-24-4	894	53,67,81,82,95,96,124
	2-Pentylfuran	3777-69-3	995	53, 81, 82, 95, 138
	(*Z*)-2-(2-Pentenyl)furan	70424-13-4	998	53,79,94,107,136

	5-Heptyldihydro-2(3H)-furanone	104-67-6	1399	55, 56, 73, 85, 100, 128, 129, 166
Terpenoids	1-R-α-Pinene	7785-70-8	971	77, 79, 91, 92, 93, 105, 121, 136
	3-Carene	13466-78-9	1070	65,67,79,91,93,105,121,136
	D-Limonene	138-86-3	1076	53, 67, 68, 79, 93, 107, 121, 136
	(+)-α-Terpineol	7785-53-7	1198	55, 59, 67, 81, 93, 95, 121, 136, 115
	(Z)-β-Farnesene	28973-97-9	1454	55, 67, 69, 81, 93, 105, 120, 133, 148, 161, 162, 189, 204
Other compounds	1,2-Dihydro-1,1,6-trimethylnaphthalene	30364-38-6	1399	11, 511, 157, 172, 173
	Dimethyldisulfide	624-92-0	769	47, 79, 94
	Trichloromethane	67-66-3	<766	47, 83, 85, 87
	1-Chloropentane	543-59-9	775	55, 56, 69, 83, 84
	1-Chlorooctane	111-85-3	1087	55, 69, 70, 91, 93, 105, 107, 148
Deuterated Standard	D7-heptanol	1219804-99-5	858	45, 62,77

Table 19 – Average Normalized Volatiles Peak Areas in Fava Bean Concentrate. Average peak areas of volatile compounds from fava bean initial concentrate (FBIC) and modified ingredients, detected by HS-SPME-GC-MS. The areas have been normalized by the respective peak area of the deuterated standard (d7-heptanol and the dry weight quantities of the ingredient used for the analysis ((Peak Area$_{compound}$/ PeakArea $_{d7\text{-}heptanol}$)/ g ingredient d.b.). The table illustrates semi-quantified volatiles at pH$_{utilization}$ 4 and 7.

Compound	FBIC	pH$_{process}$ 2						pH$_{process}$ 4						pH$_{process}$ 6.4						pH$_{process}$ 11					
		55 °C		75 °C		95 °C		55 °C		75 °C		95 °C		55 °C		75 °C		95 °C		55 °C		75 °C		95 °C	
		Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
pH$_{utilization}$ 4																									
Pentane	0.4	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.3	0.0	0.0	0.1	0.0	0.0	0.0
Heptane	3.8	8.1	5.1	6.5	3.8	3.8	4.2	2.7	2.8	3.2	2.8	3.1	3.7	3.0	3.0	2.7	2.5	2.4	3.1	1.9	5.1	1.4	1.5	1.7	1.7
4-Methyloctane	0.0	0.6	0.4	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.3	0.3	0.3	0.4	0.0	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0
3-Methyl-6-Methylene-Octane	0.9	0.0	0.7	0.5	0.5	0.6	0.0	0.0	0.0	0.4	0.0	0.4	0.0	0.4	0.4	0.6	0.5	0.5	0.0	0.8	1.1	0.6	0.6	0.5	0.5
2,7-Dimethyloctane	3.0	1.0	0.0	6.0	4.2	6.0	3.0	2.8	2.4	3.7	2.4	2.9	1.3	0.6	0.6	1.0	0.9	0.9	0.5	1.6	1.5	1.3	1.3	1.5	1.6
2,2,4,6,6-Pentamethylheptane	0.6	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.2	0.1	0.3	0.1	0.2
Decane	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.1
2,2,4,4-Tetramethyloctane	2.8	0.0	0.0	0.0	0.2	0.4	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.4	0.3	0.4	0.4	0.4	0.2	0.3	0.8	1.4	0.6	0.6	0.9
Undecane	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dodecane	0.0	0.3	0.8	1.5	0.4	1.1	1.2	0.9	1.0	1.0	1.0	1.0	1.1	0.7	0.7	0.7	0.2	0.4	0.4	0.7	0.4	0.5	0.4	0.4	0.7
Tetradecane	0.1	0.3	0.2	0.5	0.1	0.3	0.4	0.2	0.3	0.3	0.3	0.2	0.4	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.2	0.3
1-Heptene	0.0	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0
1-Octene	0.0	0.7	0.4	0.6	0.2	0.2	0.3	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
2,4-Dimethyl-1-Heptene	0.0	0.0	0.2	0.0	0.2	0.2	0.2	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.0	0.0	0.2	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0
4-Cyanocyclohexene	0.0	0.0	0.0	0.5	0.5	0.4	0.0	0.0	0.2	0.2	0.2	0.3	0.6	0.0	0.0	0.0	0.3	0.3	1.0	0.0	0.0	0.0	0.0	0.0	0.3
1-Penten-3-ol	0.8	0.3	0.3	0.3	0.1	0.3	0.2	0.4	0.4	0.4	0.4	0.4	0.3	0.7	0.6	0.6	0.5	0.5	0.5	0.3	0.2	0.2	0.3	0.2	0.2
(Z)-3-Methylcyclohexanol	3.4	4.2	3.5	3.4	1.7	2.5	1.3	1.3	1.8	1.4	1.8	1.9	3.0	0.9	0.9	1.0	2.3	1.7	6.8	0.5	0.6	0.4	0.5	0.6	0.6
(E)-3-Methylcyclohexanol	2.2	3.4	2.9	2.7	1.3	2.0	0.8	0.8	1.2	1.0	1.2	1.4	2.2	0.2	0.2	0.4	1.2	0.8	5.3	0.2	0.2	0.1	0.2	0.3	0.3

3-Methyl-1-Butanol	0.9	0.0	0.2	0.2	0.0	0.2	0.0	0.7	1.0	0.5	1.0	0.4	0.3	3.2	2.8	2.7	1.8	2.3	1.4	0.4	0.3	0.3	0.4	0.3	0.3
1-Pentanol	5.3	7.3	3.2	6.3	1.6	2.2	1.8	4.6	3.6	4.3	3.6	2.7	1.4	1.9	1.7	2.1	2.2	2.4	1.6	0.8	0.5	0.6	0.8	0.8	0.6
3-Hexen-1-ol	0.5	0.2	0.4	0.3	0.8	0.4	0.3	0.5	0.4	0.4	0.4	0.4	0.6	0.4	0.4	0.6	0.5	0.4	0.7	0.5	0.0	0.0	0.6	0.0	0.0
1-Hexanol	51.1	7.9	9.3	7.8	8.9	10.6	8.8	13.8	17.2	20.0	17.2	15.7	9.9	23.9	24.7	39.9	36.5	63.6	25.4	13.7	11.6	9.9	12.7	13.4	10.3
1-Heptanol	0.8	3.7	1.7	3.1	1.0	1.0	0.8	1.2	0.9	1.2	0.9	0.6	0.3	0.3	0.4	0.4	0.4	0.6	0.3	0.0	0.0	0.4	0.0	0.0	0.0
1-Octan-3-ol	0.8	6.8	3.8	6.0	2.4	2.5	2.1	1.6	1.2	1.5	1.2	1.1	0.7	0.7	0.6	0.7	0.6	0.6	0.5	0.7	0.6	0.5	0.5	0.5	0.4
2-Ethyl-1-Hexanol	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.7	0.2	0.2
1-Octanol	2.0	10.0	4.3	8.0	2.7	2.8	2.6	3.3	2.2	2.6	2.2	1.6	0.9	0.9	0.9	0.9	1.1	1.1	0.7	0.7	0.5	0.6	0.5	0.5	0.5
(Z,Z)-4,5-Dimethyl-2-Hepten-3-ol	0.7	9.7	2.7	8.3	1.3	1.4	1.5	0.4	0.4	0.6	0.4	0.4	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Linalool	0.0	1.5	3.2	3.2	0.8	2.3	0.0	0.2	1.1	0.2	1.1	1.2	2.4	0.0	0.0	0.0	0.2	0.0	0.7	0.2	0.0	0.2	0.0	0.0	0.2
Acetaldehyde	0.7	0.4	0.4	0.4	0.3	0.5	0.5	0.3	0.3	0.3	0.3	0.2	0.2	0.6	0.5	0.3	0.3	0.3	0.3	0.5	0.8	0.5	0.7	0.8	0.6
2-Methylpropanal	0.1	0.2	0.2	0.2	0.1	0.2	0.2	0.1	0.3	0.1	0.3	0.2	0.3	0.5	0.6	0.2	0.4	0.2	0.6	0.5	0.0	0.4	0.4	0.4	0.3
Butanal	0.0	0.4	0.2	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.3	0.2	0.2
3-Methylbutanal	0.2	0.1	0.1	0.1	0.0	0.1	0.1	0.3	0.3	0.2	0.3	0.2	0.2	1.5	2.3	0.4	0.4	0.4	0.4	0.6	0.3	0.4	0.5	0.5	0.5
2-Methylbutanal	0.0	0.0	0.0	0.0	0.2	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.7	2.2	3.1	0.7	1.0	0.7	1.8	0.0	1.2	0.0	1.3	1.2	0.0
Pentanal	0.7	7.0	4.1	5.4	2.5	2.8	2.9	0.9	1.2	1.3	1.2	1.3	1.4	0.5	0.3	0.4	0.4	0.3	0.5	1.0	0.9	0.7	0.8	0.9	0.7
(E)-2-Pentenal	0.6	3.1	2.1	2.4	1.6	1.8	2.6	0.4	0.5	0.6	0.5	0.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.4	0.4	0.3	0.4	0.1
3-Methylhexanal	0.0	0.4	0.5	0.4	0.3	0.4	0.2	0.4	0.5	0.5	0.5	0.5	0.4	0.4	0.5	0.6	0.5	0.5	0.3	0.9	1.1	0.6	0.7	0.5	0.3
Hexanal	372.8	691.5	467.8	570.6	347.9	356.1	287.4	269.2	212.6	258.3	212.6	212.1	114.0	135.7	88.5	218.9	153.1	91.1	56.1	209.1	189.4	175.4	188.8	190.3	104.9
2-Hexenal	1.0	3.9	2.8	3.4	1.6	2.3	0.7	1.4	0.8	1.4	0.8	1.1	0.2	0.6	0.3	0.5	0.1	0.3	0.0	1.5	0.5	0.8	0.0	0.3	0.0
Heptanal	3.8	32.6	21.1	25.9	19.4	20.3	24.2	8.3	8.4	10.4	8.4	10.5	8.9	2.6	2.1	3.9	4.1	2.8	2.1	8.6	7.3	9.6	6.5	6.6	6.3
(Z)-2-Heptenal	2.4	24.9	17.1	20.6	8.7	12.4	7.3	4.1	3.7	4.9	3.7	3.9	2.6	0.5	0.4	0.5	0.8	0.7	0.6	1.6	1.5	1.2	1.0	1.0	0.8
(E,E)-2,4-Heptanedienal	0.4	1.0	1.5	0.8	0.9	0.4	0.2	0.0	0.3	0.2	0.3	0.3	0.5	0.0	0.0	0.0	0.0	0.3	0.9	0.0	0.0	0.0	0.0	0.0	0.0
Octanal	0.7	11.1	5.6	7.5	3.3	3.4	3.3	1.1	1.1	1.5	1.1	1.3	0.8	0.3	0.3	0.5	0.6	0.4	0.4	1.6	1.3	1.3	1.8	1.5	1.4
2-Octenal	4.4	59.9	30.3	46.7	21.5	22.6	21.2	6.4	5.9	7.4	5.9	7.0	6.2	1.5	1.0	1.2	1.5	1.6	1.2	9.2	6.8	8.6	3.9	4.2	2.0
4-Nonenal	0.0	0.6	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nonanal	4.3	30.9	15.3	23.1	10.1	10.2	8.8	6.9	5.3	6.8	5.3	5.5	4.4	2.6	2.2	4.0	5.0	3.3	2.5	6.8	5.3	5.3	4.1	5.0	5.1
2-Dodecenal	0.4	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-Nonenal	0.4	13.7	9.5	10.1	5.4	4.9	3.7	0.7	0.5	0.6	0.5	0.5	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.4	0.0	0.0	0.0

Decanal	0.1	2.8	1.2	1.8	0.6	0.4	0.4	0.2	0.2	0.2	0.2	0.2	0.1	0.0	0.0	0.1	0.2	0.1	0.0	0.2	0.2	0.2	0.1	0.2	0.1
2,4-Nonadienal	0.8	15.4	9.5	12.6	5.8	5.5	4.2	0.9	0.8	1.0	0.8	0.7	0.5	0.2	0.0	0.0	0.1	0.1	0.0	0.3	0.1	0.2	0.0	0.0	0.0
2,4-Decadienal	0.5	4.8	3.1	3.9	2.4	2.0	2.1	0.3	0.3	0.3	0.3	0.3	0.2	0.0	0.0	0.0	0.0	0.1	0.0	0.3	0.1	0.2	0.0	0.1	0.0
2-Undecenal	0.2	5.0	1.9	3.8	1.0	0.9	0.7	0.3	0.3	0.3	0.3	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1
2-Butyl-2-Octenal	0.0	0.2	0.2	0.2	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
9-Octadecanal	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-Butanone	0.1	0.5	0.3	0.4	0.4	0.2	0.4	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.6	0.5	0.3	0.3	0.3	0.5	0.6	0.6
2-Pentanone	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
3-Penten-2-one	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.1
2-Heptanone	0.8	10.7	3.8	7.7	2.2	1.9	2.1	1.2	1.4	1.3	1.4	1.2	1.5	1.1	1.2	1.9	3.3	3.2	14.5	28.4	38.6	34.1	94.0	72.3	97.6
1-Octen-3-one	0.0	4.4	3.4	3.5	1.9	2.4	1.6	0.5	0.7	0.7	0.7	0.7	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.2	0.2	0.0	0.0	0.0
6-Methyl-5-Hepten-2-one	0.0	1.4	1.0	1.1	0.6	1.2	0.6	0.4	0.4	0.5	0.4	0.4	0.4	0.0	0.0	0.0	0.4	0.0	0.0	0.4	0.6	0.4	0.6	0.5	0.5
3-Octen-2-one	0.5	0.0	60.1	135.3	31.0	26.5	12.7	14.2	10.3	14.0	10.3	6.5	1.9	0.0	0.0	0.0	0.5	0.5	0.0	0.4	0.0	0.3	0.0	0.0	0.0
3,5-Octadien-2-one	0.0	13.6	9.5	15.0	6.1	7.2	3.8	5.0	4.0	4.4	4.0	3.4	1.7	0.0	0.0	0.0	0.7	0.5	0.0	0.5	0.0	0.0	0.0	0.0	0.0
3-Nonanone	0.0	0.3	0.4	0.5	0.3	0.4	0.0	0.0	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.3	0.3	0.3	0.3	0.4	0.0	0.3	0.0	0.3	0.0
2-Nonanone	0.0	0.3	0.2	0.3	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.3	0.9	0.8	0.9	3.7	1.6	2.7
3-Undecanone	0.0	0.4	0.4	0.4	0.3	0.5	0.6	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.3	0.4	0.1	0.1	0.1	0.2	0.2
2-Undecanone	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.1
3-Methylbutylacetate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.2	0.2	0.2	0.0	0.0	0.2	0.0	0.3	0.4	0.0	0.5	0.4	0.0	0.0	0.0
(Z)-3-Octenylacetate	0.0	0.2	0.3	0.3	0.2	0.3	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.3	0.2	0.2
Acetic Acid	0.4	0.9	0.5	0.6	0.3	0.5	0.4	0.3	0.4	0.3	0.4	0.3	0.5	0.8	0.6	0.6	0.8	1.0	1.4	0.1	0.2	0.1	0.1	0.2	0.3
Octanoic Acid	0.2	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.4
Nonanoic Acid	0.0	0.3	0.1	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-Phenylethanol	0.5	1.0	0.4	0.6	0.0	0.3	0.4	0.3	0.2	0.2	0.2	0.2	0.2	0.5	0.5	0.5	0.4	0.4	0.5	0.3	0.0	0.0	0.0	0.0	0.0
4-Propylbenzaldehyde	0.0	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0
2-Methylfuran	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.9	0.6	0.5	0.0	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Furfural	0.0	0.8	1.7	0.7	1.2	1.6	65.4	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0
2-Butylfuran	0.6	17.7	9.5	11.0	5.3	4.5	3.8	0.9	1.0	1.0	1.0	1.0	1.1	0.0	0.0	0.5	0.8	0.8	3.2	5.4	7.1	6.2	17.1	13.8	19.3
2-Pentylfuran	5.4	107.6	79.8	66.9	40.8	33.3	17.5	5.4	6.8	6.1	6.8	7.1	9.1	1.1	0.9	1.2	2.3	2.8	11.2	0.0	2.0	0.0	4.0	0.0	0.0
(Z)-2-(2-Pentenyl)furan	0.3	3.0	2.9	2.3	1.9	1.4	0.9	0.2	0.4	0.3	0.4	0.5	0.9	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0

5-Heptyldihydro-2(3H)-furanone	0.0	0.3	0.1	0.3	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0
1-R-α-Pinene	6.2	0.0	0.4	0.0	0.0	0.3	0.0	0.3	0.4	0.3	0.4	0.3	0.3	0.8	0.9	0.7	0.5	0.7	0.9	0.9	1.4	0.6	0.5	0.5	0.5
3-Carene	17.4	1.2	4.1	1.4	2.6	1.6	1.2	0.8	1.2	1.1	1.2	1.0	1.3	3.2	3.4	2.6	1.9	2.8	4.7	7.1	10.3	3.4	3.9	4.0	5.1
D-Limonene	1.9	24.5	30.1	19.5	14.8	15.1	16.1	20.0	20.2	15.6	20.2	19.1	24.0	11.6	14.9	23.7	13.3	12.0	14.2	20.4	5.2	9.8	3.4	4.0	3.3
(+)-α-Terpineol	0.0	0.3	0.8	0.5	1.1	0.9	0.5	0.0	0.2	0.1	0.2	0.2	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0
(Z)-β-Farnesene	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.3	0.2	0.2	0.2	0.2	0.3
1,2-Dihydro-1,1,6-trimethylnaphthalene	0.0	0.1	0.2	0.1	0.4	0.2	1.0	0.0	0.1	0.0	0.1	0.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dimethyldisulfide	0.0	0.6	0.4	0.4	0.3	0.3	0.4	0.0	0.1	0.1	0.1	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.3	0.7	0.4	0.4	0.3
Trichloromethane	0.8	3.0	7.7	4.5	1.9	16.2	4.2	10.8	12.7	11.0	12.7	15.7	24.6	0.8	1.2	3.0	0.8	1.8	5.9	3.5	2.0	5.2	4.1	4.3	3.6
1-Chloropentane	0.0	1.6	1.2	1.2	1.0	1.0	1.7	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.3	0.1	0.2	0.0
1-Chlorooctane	0.0	9.4	4.1	5.9	2.4	2.1	3.8	0.2	0.2	0.2	0.2	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$pH_{utilization}$ 7																									
Pentane	1.7	0.2	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.0	1.4	0.0	0.1	0.3	0.4	0.3	0.1	0.0	0.0	0.0	0.0	0.0
Heptane	4.0	5.4	3.7	4.5	5.6	3.3	5.3	2.6	2.6	3.1	2.6	3.0	3.0	3.7	2.9	2.0	3.5	3.0	3.9	2.7	2.3	2.2	2.2	2.2	2.6
4-Methyloctane	0.0	0.3	0.5	0.4	0.6	0.4	1.0	0.7	0.8	0.7	0.8	0.6	0.3	0.8	0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.0	0.0	0.0	0.0
3-Methyl-6-Methylene-Octane	1.7	0.3	0.8	0.6	0.6	0.7	0.3	0.0	0.3	0.5	0.3	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	1.2	0.7	0.0	0.0	0.0
2,7-Dimethyloctane	9.3	1.6	2.7	2.8	1.7	2.6	1.6	1.5	1.3	1.9	1.3	1.5	0.6	1.4	0.0	0.7	1.3	1.6	0.0	1.6	2.4	1.5	1.0	0.9	0.9
2,2,4,6,6-Pentamethylheptane	2.3	0.1	0.2	0.2	0.1	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.0	0.3	0.2	0.2	0.3	0.3	0.0	0.3	0.5	0.3	0.2	0.1	0.1
Decane	0.0	0.0	0.1	0.1	0.0	0.1	0.3	0.6	0.6	0.6	0.6	0.6	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0
2,2,4,4-Tetramethyloctane	9.4	0.2	0.8	0.7	0.6	0.9	1.1	0.6	0.4	0.7	0.4	0.3	0.1	1.2	0.8	0.6	1.1	1.4	0.7	1.2	2.5	1.2	0.7	0.6	0.5
Undecane	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dodecane	0.0	0.2	0.7	1.2	0.3	1.2	1.1	1.5	1.5	1.8	1.5	1.3	1.3	1.3	1.1	0.6	0.0	0.8	0.9	1.3	1.5	2.1	0.9	0.7	0.7
Tetradecane	0.0	0.1	0.1	0.2	0.1	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.6	0.4	0.2	0.0	0.2	0.2	0.4	0.5	0.7	0.4	0.5	0.5
1-Heptene	0.0	0.1	0.1	0.1	0.1	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.2	0.1	0.1	0.0	0.0
1-Octene	0.0	0.2	0.1	0.2	0.1	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2,4-Dimethyl-1-Heptene	0.0	0.3	0.4	0.4	0.4	0.3	0.5	0.4	0.3	0.4	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
4-Cyanocyclohexene	0.0	0.0	0.2	0.0	0.5	0.4	0.0	0.0	0.0	0.0	0.0	0.3	0.6	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0

1-Penten-3-ol	1.5	0.3	0.3	0.4	0.4	0.3	0.4	0.5	0.5	0.5	0.5	0.4	0.3	1.2	0.8	0.5	0.7	1.0	0.6	0.4	0.2	0.3	0.4	0.3	0.3
(Z)-3-Methylcyclohexanol	1.5	3.5	3.7	3.2	3.9	2.7	2.1	1.1	1.7	1.3	1.7	2.0	3.0	0.8	0.9	0.7	2.8	2.4	7.0	0.7	0.6	0.6	0.7	0.6	0.8
(E)-3-Methylcyclohexanol	0.4	2.8	2.9	2.5	2.7	2.0	0.9	0.5	1.0	0.6	1.0	1.1	1.8	0.0	0.0	0.2	1.4	1.1	4.8	0.0	0.0	0.0	0.2	0.2	0.2
3-Methyl-1-Butanol	4.7	0.2	0.3	0.3	0.4	0.3	0.2	0.8	1.1	0.6	1.1	0.5	0.4	5.0	4.2	2.6	3.0	4.5	2.1	0.7	0.5	0.9	0.7	0.6	0.6
1-Pentanol	10.6	7.0	3.1	7.0	3.7	2.2	3.0	3.3	2.6	2.7	2.6	2.0	1.1	3.0	2.1	1.7	2.8	3.7	1.8	1.0	0.9	0.9	1.1	1.0	0.9
3-Hexen-1-ol	0.7	0.4	0.5	0.5	0.6	0.8	0.4	0.5	0.5	0.5	0.5	0.5	0.3	0.0	0.5	0.4	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.4
1-Hexanol	277.1	9.4	10.6	10.6	12.7	10.6	6.0	14.6	16.9	20.9	16.9	12.9	7.9	23.4	25.5	28.4	43.5	81.9	24.5	15.4	12.2	14.6	16.2	14.8	12.9
1-Heptanol	1.6	2.6	1.1	2.1	0.8	0.7	0.8	0.8	0.7	0.7	0.7	0.5	0.3	0.0	0.0	0.0	0.0	0.9	0.0	0.3	0.3	0.4	0.0	0.0	0.0
1-Octan-3-ol	0.8	6.4	3.1	5.3	1.8	2.0	2.0	1.4	1.2	1.3	1.2	1.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.6	0.5	0.4	0.4	0.3
2-Ethyl-1-Hexanol	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.4	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1
1-Octanol	3.2	5.8	2.6	4.5	1.7	1.7	2.3	2.3	1.7	1.7	1.7	1.2	0.8	0.0	0.0	0.0	1.1	1.2	0.0	0.5	0.6	0.5	0.0	0.0	0.0
(Z,Z)-4,5-Dimethyl-2-Hepten-3-ol	0.0	2.7	0.6	1.1	0.3	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Linalool	0.0	1.5	3.5	3.2	0.7	2.6	0.0	0.2	1.4	0.2	1.4	1.5	2.9	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
Acetaldehyde	7.0	0.3	0.3	0.3	0.4	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.4	1.7	0.6	0.4	0.6	0.8	0.7	0.8	0.4	0.5	0.9	0.7	0.8
2-Methylpropanal	0.0	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.3	0.2	0.3	0.2	0.0	0.8	1.0	0.2	0.5	0.5	0.6	0.4	0.5	0.4	0.4	0.4	0.0
Butanal	0.0	0.2	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.2	0.2	0.2	0.3	0.3	0.4
3-Methylbutanal	0.0	0.1	0.1	0.2	0.2	0.2	0.2	0.5	0.5	0.3	0.5	0.3	0.3	2.0	2.7	0.4	0.6	0.7	0.4	0.8	0.8	0.6	0.6	0.8	0.8
2-Methylbutanal	0.0	0.0	0.0	0.4	0.6	0.4	0.4	0.7	0.9	0.5	0.9	0.6	0.8	3.9	5.0	0.8	1.7	1.3	2.1	0.0	2.2	1.6	1.8	1.9	1.8
Pentanal	0.5	4.3	2.3	3.2	2.6	1.5	2.3	0.6	0.7	0.8	0.7	0.8	0.5	0.3	0.0	0.2	0.3	0.0	0.0	0.7	0.6	0.6	0.5	0.7	0.6
(E)-2-Pentenal	0.0	1.5	1.1	1.2	1.4	1.1	3.6	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.2	0.0	0.0	0.0
3-Methylhexanal	0.0	0.3	0.5	0.5	0.5	0.4	0.3	0.3	0.4	0.4	0.4	0.5	0.3	0.7	0.5	0.3	0.5	0.7	0.0	0.9	1.1	0.7	0.3	0.2	0.0
Hexanal	30.3	635.8	445.3	515.5	484.6	323.7	251.7	197.2	158.6	209.4	158.6	166.3	61.9	87.8	57.9	90.0	128.9	72.2	46.9	211.4	167.4	207.2	149.7	145.3	74.8
2-Hexenal	0.0	1.8	1.4	1.3	0.8	0.9	0.4	1.0	0.6	1.0	0.6	0.7	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.4	0.0	0.0	0.0
Heptanal	0.0	23.7	13.3	17.8	12.2	10.2	13.7	4.6	4.8	6.1	4.8	5.7	3.9	1.5	1.0	1.0	2.0	1.6	1.1	4.1	3.5	6.0	2.2	2.1	1.4
(Z)-2-Heptenal	0.9	6.5	3.4	3.6	1.8	2.2	1.6	1.3	1.1	1.3	1.1	1.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.4	0.4	0.0	0.0	0.0
(E,E)-2,4-Heptanedienal	0.0	2.6	1.1	1.6	2.6	1.2	0.4	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0
Octanal	0.0	5.4	2.2	3.1	1.0	1.0	1.5	0.7	0.7	0.9	0.7	0.7	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.7	0.7	0.4	0.5	0.4
2-Octenal	1.0	15.7	6.6	8.0	3.8	3.8	4.1	1.9	1.7	2.2	1.7	1.9	1.5	1.2	0.0	0.0	0.0	0.0	0.0	2.1	1.9	2.3	0.9	0.9	0.0
4-Nonenal	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Nonanal	0.0	16.3	6.9	9.8	3.3	4.0	2.9	3.7	3.2	4.0	3.2	3.2	2.5	1.9	1.4	1.5	3.6	2.6	1.8	3.0	2.8	3.2	1.5	1.5	1.3
2-Dodecenal	1.2	0.2	0.4	0.3	0.2	0.3	0.3	0.3	0.2	0.4	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.4	0.3	0.0	0.0	0.0
2-Nonenal	0.0	3.5	2.3	1.8	1.0	1.3	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Decanal	0.0	1.4	0.6	0.7	0.2	0.2	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0
2,4-Nonadienal	0.0	3.5	2.0	2.0	1.0	1.1	1.0	0.3	0.3	0.3	0.3	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2,4-Decadienal	0.0	0.9	0.6	0.6	0.5	0.4	0.6	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0
2-Undecenal	0.0	0.9	0.4	0.8	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1
2-Butyl-2-Octenal	0.0	0.6	0.2	0.5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9-Octadecanal	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-Butanone	0.3	0.4	0.3	0.4	0.3	0.2	0.6	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.5	0.4	0.3	0.4	0.3	0.5	0.5	0.7
2-Pentanone	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3-Penten-2-one	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.2	0.2	0.1	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0
2-Heptanone	0.0	13.7	4.8	11.0	3.3	2.3	2.9	1.1	1.5	1.2	1.5	1.2	1.5	1.4	1.4	1.3	3.7	4.4	14.4	28.9	40.6	44.1	80.6	60.7	85.6
1-Octen-3-one	0.0	0.3	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6-Methyl-5-Hepten-2-one	0.0	1.1	0.8	1.0	0.6	1.0	0.7	0.4	0.4	0.4	0.4	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.4	0.0	0.0	0.0
3-Octen-2-one	0.0	102.0	40.4	79.6	19.8	17.1	9.6	10.7	8.2	10.7	8.2	5.2	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.4	0.0	0.0	0.0
3,5-Octadien-2-one	0.0	12.9	9.3	13.4	4.8	7.4	4.2	5.4	5.0	5.3	5.0	4.5	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.4	0.0	0.0	0.0
3-Nonanone	0.0	0.2	0.3	0.4	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.3	0.0	0.0	0.0
2-Nonanone	0.0	0.2	0.2	0.2	0.1	0.1	0.5	0.5	0.4	0.6	0.4	0.3	0.1	0.0	0.0	0.1	0.2	0.2	0.3	0.9	1.0	1.3	1.1	1.0	1.3
3-Undecanone	0.0	0.2	0.3	0.3	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.1	0.3	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.1
2-Undecanone	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0
3-Methylbutylacetate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0
(Z)-3-Octenylacetate	0.0	0.0	0.2	0.3	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.3	0.2	0.0	0.0	0.0
Acetic Acid	0.2	0.0	0.1	0.0	0.3	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.3	0.4	0.2	0.1	0.2	0.0	0.2	0.1	0.2
Octanoic Acid	0.0	0.0	0.0	0.1	0.0	0.0	0.6	0.7	0.6	0.6	0.6	0.5	0.0	0.3	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nonanoic Acid	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-Phenylethanol	0.0	0.4	0.3	0.3	0.0	0.3	0.3	0.4	0.3	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4-Propylbenzaldehyde	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-Methylfuran	2.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	1.6	0.8	0.5	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Furfural	0.0	0.6	1.4	0.4	1.4	1.3	58.9	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

2-Butylfuran	0.0	11.6	6.4	7.2	3.9	2.3	2.8	0.5	0.7	0.6	0.7	0.6	0.8	0.0	0.0	0.0	0.0	0.0	3.1	5.1	7.1	7.7	14.4	10.8	14.8
2-Pentylfuran	1.3	68.5	61.6	38.6	36.6	21.1	27.3	5.8	7.7	6.2	7.7	8.1	7.3	0.9	0.8	0.6	2.3	3.2	11.7	0.0	0.7	0.0	0.0	0.5	0.5
(Z)-2-(2-Pentenyl)furan	0.0	1.5	1.7	1.1	1.1	0.8	0.6	0.0	0.0	0.0	0.0	0.4	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5-Heptyldihydro-2(3H)-furanone	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1-R-α-Pinene	15.7	0.3	0.5	0.3	0.4	0.4	0.0	0.4	0.4	0.4	0.4	0.4	0.4	1.4	1.6	0.7	0.9	1.3	1.4	1.2	1.6	0.9	0.0	0.3	0.0
3-Carene	27.7	1.1	4.5	1.6	3.6	1.9	1.5	1.0	1.3	1.3	1.3	1.2	1.4	5.0	4.8	2.1	2.7	4.5	6.5	7.6	11.4	4.3	1.8	1.5	1.4
D-Limonene	2.0	20.6	28.7	17.8	17.1	15.7	17.7	21.0	21.4	19.9	21.4	20.4	25.3	13.6	18.6	16.0	16.3	14.6	16.7	24.4	5.7	14.8	1.8	1.4	0.9
(+)-α-Terpineol	0.0	0.2	0.7	0.4	0.9	1.0	0.6	0.0	0.2	0.0	0.2	0.2	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(Z)-β-Farnesene	0.7	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.3	0.1	0.2	0.2	0.3	0.7	0.9	0.7	0.4	0.3	0.3
1,2-Dihydro-1,1,6-trimethylnaphthalene	0.0	0.1	0.1	0.1	0.3	0.3	1.0	0.0	0.1	0.0	0.1	0.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dimethyldisulfide	0.0	0.2	0.2	0.0	0.0	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Trichloromethane	0.9	4.8	12.0	6.2	1.0	31.1	11.7	12.7	16.0	11.8	16.0	18.5	37.4	0.7	0.6	0.5	1.1	1.0	1.3	7.2	9.8	14.8	7.7	9.9	8.5
1-Chloropentane	0.0	0.9	0.6	0.8	0.9	0.7	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1-Chlorooctane	0.0	3.7	1.8	2.5	1.4	1.2	3.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

The volatile compounds detected were further grouped by chemical classes, and their relative cumulative areas presented further clarity in understanding the chemistry of ingredients with respect to process conditions (**Figure 25**).

Firstly, similar to the results from the odor sensory profiling of the FBIC suspension, the headspace volatile chemistry also revealed differences at the two $pH_{utilization}$. Overall, aldehydes and alcohols were primarily detected for the FBIC at both $pH_{utilization}$ (**Figure 25**). For the FBIC at $pH_{utilization}$ 4, the detected signals of aldehydes were the highest, i.e. 76% of the total chromatogram peak area compared to 10% for FBIC at $pH_{utilization}$ 7. At $pH_{utilization}$ 7, an essential difference was seen where alcohol signals (instead of aldehydes) were predominantly detected, contributing to 72% of the total peak area *vs* 13% at $pH_{utilization}$ 4. Amongst these volatile groups, hexanal and 1-hexanol were particularly considered owing to their predominance in peak area signals within their respective chemical families (**Table 19**). Hexanal gave the highest normalized signal (372.8 area/ $area_{d7\text{-}heptanol}$/ g d.b.) amongst all the detected volatiles at $pH_{utilization}$ 4, whereas at $pH_{utilization}$ 7, 1-hexanol gave the highest signal (277.1 area/ $area_{d7\text{-}heptanol}$/ g d.b.). Hexanal drastically decreased when the FBIC was suspended at $pH_{utilization}$ 7 (30.3 area/ $area_{d7\text{-}heptanol}$/ g d.b.) compared to $pH_{utilization}$ 4. On the other hand, 1-hexanol was lower at $pH_{utilization}$ 4 (51.1 area/ $area_{d7\text{-}heptanol}$/ g d.b.) compared to $pH_{utilization}$ 7. Looking further into other individual molecules, there was also a qualitative difference in the volatile profiles observed. To be precise, 32 volatile compounds were detected in the FBIC at $pH_{utilization}$ 7. In addition to these, 26 additional compounds were detected at $pH_{utilization}$ 4, including aldehydes (butanal, heptanal, octanal, nonanal, decanal, 2-methylpropanal, 3-methylbutanal, *(E)*-2-pentenal, 2-hexenal, *(E,E)*-2,4-heptanedienal, 2-nonenal, 2,4-nonadienal, 2,4-decadienal, 2-undecenal, 9-octadecanal), alcohols (2-ethyl-1-hexanol, *(Z,Z)*-4,5-dimethyl-2-hepten-3-ol, 2-phenylethanol), furanoids (2-butylfuran, *(Z)*-2-(2-pentenyl)furan), one alkane (tetradecane), one alkene (1-heptene), ketones (2-heptanone, 3-octen-2-one) and organic acids (octanoic acid, nonanoic acid). The effect of $pH_{utilization}$ was also observed after ingredient modification. Except for furfural, 3-methylbutylacetate, 2-ethyl-1-hexanol and 9-octadecanal, the rest of the volatile compounds was significantly different between $pH_{utilization}$ 4 and 7 ($p \leq 0.05$) for all the ingredients. Remarkably, the effect of $pH_{utilization}$ causing difference in the detection levels of aldehydes and alcohols was not as pronounced in the modified ingredients – suggesting a reduced impact of the change in matrix on the relative release of volatile classes as in the case of native, mildly process ingredient such as the fava air-classified concentrate (**Figure 25**). All this data suggested that the two $pH_{utilization}$ might have led to changes in the ingredient suspension matrices enabling or disabling the release of volatiles in the headspace.

Apart from the effect of $pH_{utilization}$, there were differences between the ingredients by virtue of the process conditions ($pH_{process}$, $T_{process}$ and $t_{process}$) used for their modification (**Figure 25**). Differences in the proportions of volatile class signals also showed effects of process

conditions during ingredient modification, especially for $pH_{process}$. Noticing the different volatile groups detected in different series of ingredients modified by $pH_{process}$, aldehyde signals were the highest in proportion amongst all the ingredients, independent of the $pH_{process}$ series. But interestingly, the volatile groups having secondary relative contribution differed based on $pH_{process}$, i.e. furanoids, terpenoids, alcohols and ketones signals at $pH_{process}$ 2, 4, 6.4 and 11 series respectively (**Figure 25**). Looking closely into the different individual molecules, there were qualitative and quantitative differences found between the different modified ingredients (**Table 19**). To compare different ingredients by their $pH_{process}$, 86, 87, 72 and 78 volatiles were respectively detected in $pH_{process}$ 2, 4, 6.4 and 11 series at $pH_{utilization}$ 4 *versus* respectively 88, 80, 49 and 67 volatiles at $pH_{utilization}$ 7 (**Table 19**). All volatiles, except furfural, 3-methylbutylacetate, 2-ethyl-1-hexanol and 9-octadecanal, were significantly different within the different groups of ingredients processed by $pH_{process}$, $T_{process}$ and $t_{process}$ ($p \leq 0.05$). Comparing volatile generation by different $pH_{process}$ series, the non-pH adjusted process generated the least amount of volatiles compared to the pH-modified processes during the modification step. This suggested that $pH_{process}$ may have driven changes in flavor through possibly different reactions. Furthermore, the impact of $T_{process}$ and $t_{process}$ was significant in determining to what extent these volatile generating reactions could occur

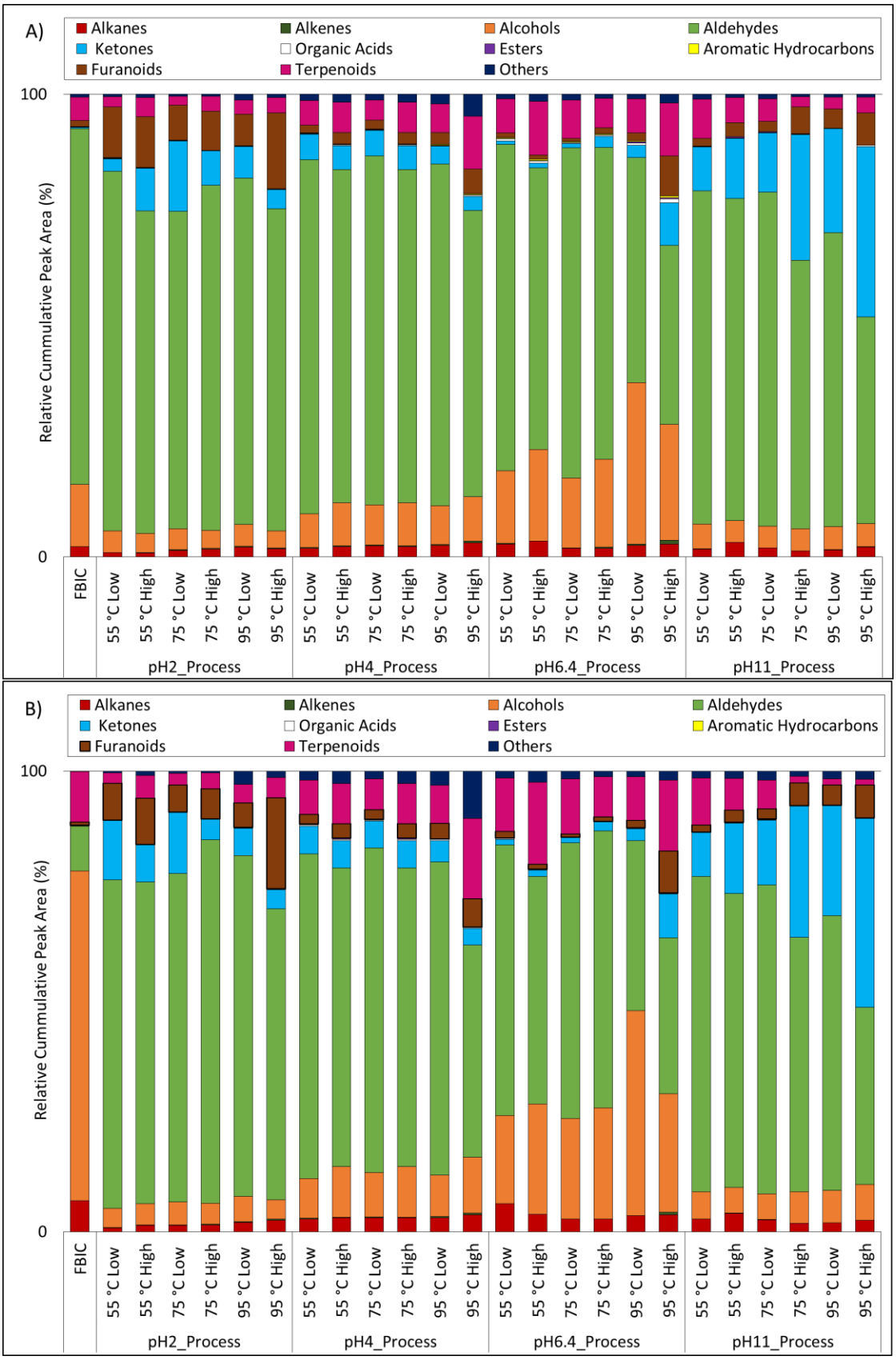

Figure 25 – Volatiles Families Detected for Fava Bean Concentrate Suspensions. in Cumulative relative peak areas of the different chemical classes of volatile compounds detected from different ingredients suspensions by HS-SPME-GC-MS analysis. The

different ingredients included fava bean initial concentrate (FBIC) and ingredients modified by $pH_{process}$, $T_{process}$, and $t_{process}$ and then further suspended at A) $pH_{utilization}$ 4 and B) $pH_{utilization}$ 7.

Thus, in a nutshell, two main effects were clear: 1) effect of $pH_{utilization}$, which was strikingly noticeable for the FBIC and less strong but still significant affecting the release of volatile compounds for all the modified ingredients; and 2) effect of $pH_{process}$, which was evident while looking into the relative proportions of the different volatile groups and causing changes in chemical complexity. Despite these results, it was still unclear as to how $T_{process}$ and $t_{process}$ affect volatile complexity, due to the heavy influence of matrix in the release of volatiles. As these conditions were statistically proven to be significant in influencing the volatile change, PCA was used to have a better overview of the volatile complexity as a function of process effects. Since the $pH_{utilization}$ was seen to cause distinct difference in the volatile release, the individual PCA plots for $pH_{utilization}$ 4 (**Figure 26**A, 56% explained variance) and $pH_{utilization}$ 7 (**Figure 26**B, 53% explained variance) were chosen for a better understanding. In both plots, the different ingredient suspensions (colored points on the plot) were separated based on their $pH_{process}$ during modification. Certain ingredients ($pH_{process}$ 2) were positively associated to the variation of large number of volatiles in the plots (**Figure 26**A and **Figure 26**B). Furthermore, all the clusters were associated to the two different $pH_{utilization}$. For instance, the FBIC at $pH_{utilization}$ 4 was closer to the cluster of $pH_{process}$ 11 ingredients and positively associated with the release of volatiles including acetaldehyde, 3-penten-2-one, 2-ethyl-1-hexanol, 3-carene and octanoic acid (examples from different volatile groups) (**Figure 26**A). It was negatively associated with the release of volatiles such as undecane, 4-propylbenzaldehyde, 1-heptanol, (+)-α-terpineol, 3-nonanone and acetic acid. The same FBIC was further away from the $pH_{process}$ 11 cluster, and distinctly separated from the rest of the modified ingredients at $pH_{utilization}$ 7 (**Figure 26**B). From these PCA plots, it was also now possible to extract information about the effects of $T_{process}$ and $t_{process}$. Firstly, the extent of the effect of $T_{process}$ and $t_{process}$ depended greatly on the type of $pH_{utilization}$ and $pH_{process}$. The ingredients from $pH_{process}$ 2 series especially showed a great separation, along both F1 and F2 axes, in the effects caused due to $T_{process}$ and $t_{process}$ at both $pH_{utilization}$. Variations of $T_{process}$ and $t_{process}$ were less pronounced for $pH_{process}$ 6.4 and 11 series as they lied only along F2 axes that accounted for 12-15% of variances explained in both the PCA plots. Effects of $T_{process}$ and $t_{process}$ were the least impactful for $pH_{process}$ 4 series (**Figure 26**A and **Figure 26**B).

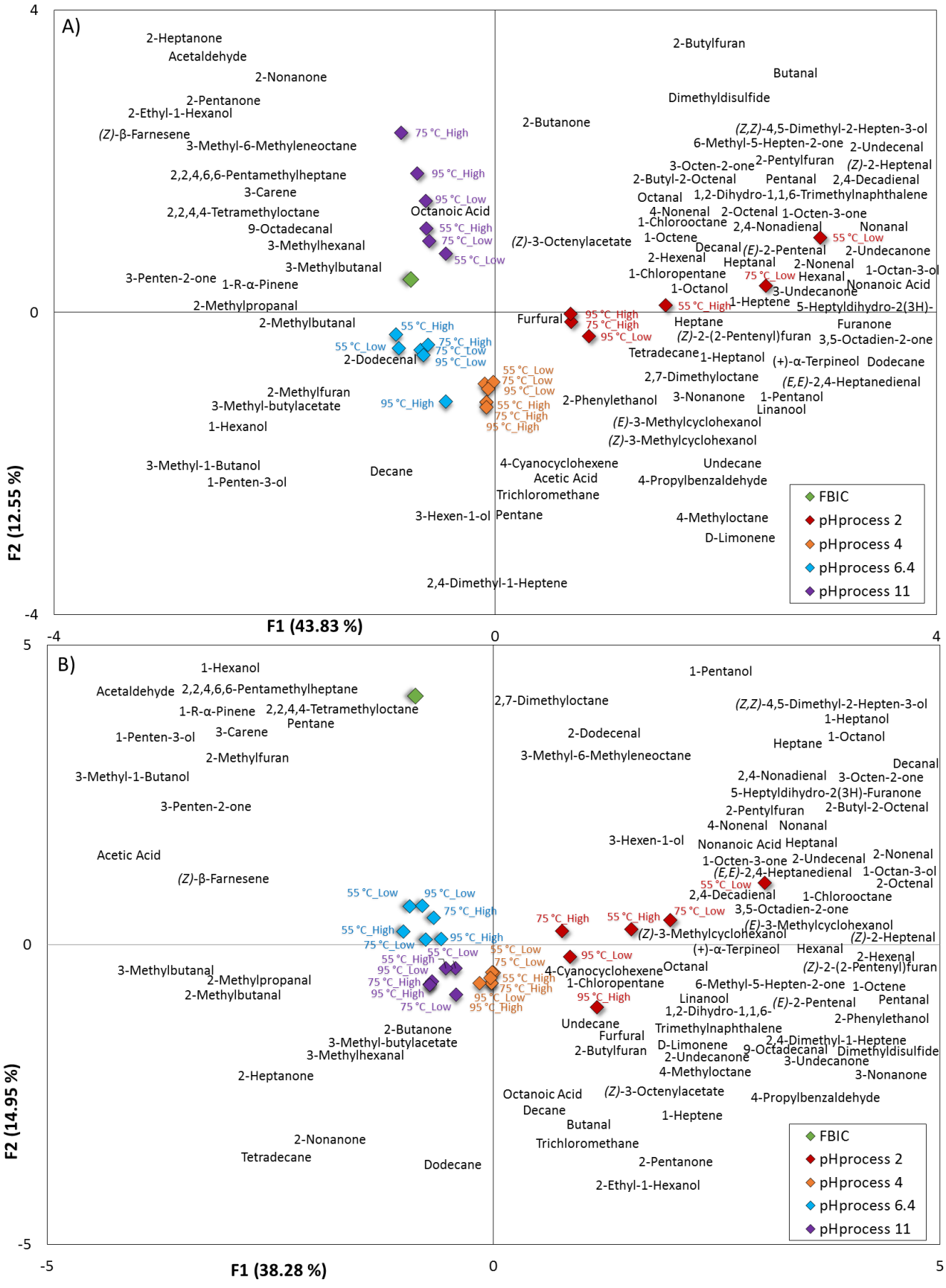

Figure 26 – PCA Projections of Volatiles Detected in Fava Concentrate Suspensions.

PCA Projections of the normalized peak areas of volatile compounds detected by the

HS-SPME-GC-MS. These volatiles were released from suspension of different ingredients including fava bean initial concentrate (FBIC) and ingredients modified by $pH_{process}$, $T_{process}$, and $t_{process}$ and then further suspended at A) $pH_{utilization}$ 4 and B) $pH_{utilization}$ 7. The points on the plot represent ingredient suspensions and the volatile cluster labels are rearranged around their points for better visualization.

V.2.4. Discussion

Odor description and quantification, and HS-SPME-GC-MS analysis of volatile compounds were evaluated together using MFA (**Figure 27**), in order to estimate resemblances and discrepancies between odor perception and chemical composition [313]. Through this, an attempt to simplify this complexity was made, first by looking into the odor intensities that divided the quadrants into four separate perceptive zones. The first was a more "sweet", less rancid perceptive zone, specifically defined by three clusters viz. a) banana, almond, lentil and vanilla notes; b) hazelnut, chocolate, caramel notes and c) coffee, earthy, woody notes. The second was a more cooked, less green zone of perception, specified by a) smoky and burnt, b) red wine as well as c) ammoniac and meat note clusters. The third zone was more green, less cooked perceptive and was characterized by a) milk, orange blossom, b) fresh and c) cut grass note clusters. Finally, the last quadrant was more rancid, less sweet perceptive zone, associated with a) spices, b) egg, vinegar, chemical, c) rancid and d) citrus, mung bean and celery note clusters (**Figure 27**).

MFA analysis contributed to the understanding of odor perception from the composition in flavor compounds and the impact of process conditions. The first dimension was characterized by $pH_{process}$, $T_{process}$ and $t_{process}$, thus presenting the degree of ingredient processing; whereas the second represented more the conditions during food application as they were separated by the $pH_{utilization}$ (**Figure 27**). The process conditions used for fava ingredient modification were either more gentler ($pH_{process}$ = 4 and 6.4, $T_{process}$ = 55°C, $t_{process}$ = Low) or more vigorous ($pH_{process}$ = 2 or 11, $T_{process}$ > 55°C, $t_{process}$ = High) and this division could be seen clearly from the biplot. The FBIC (unmodified air classified concentrate) also lied in the quadrant of gentle processing, thus making this distinction further apparent.

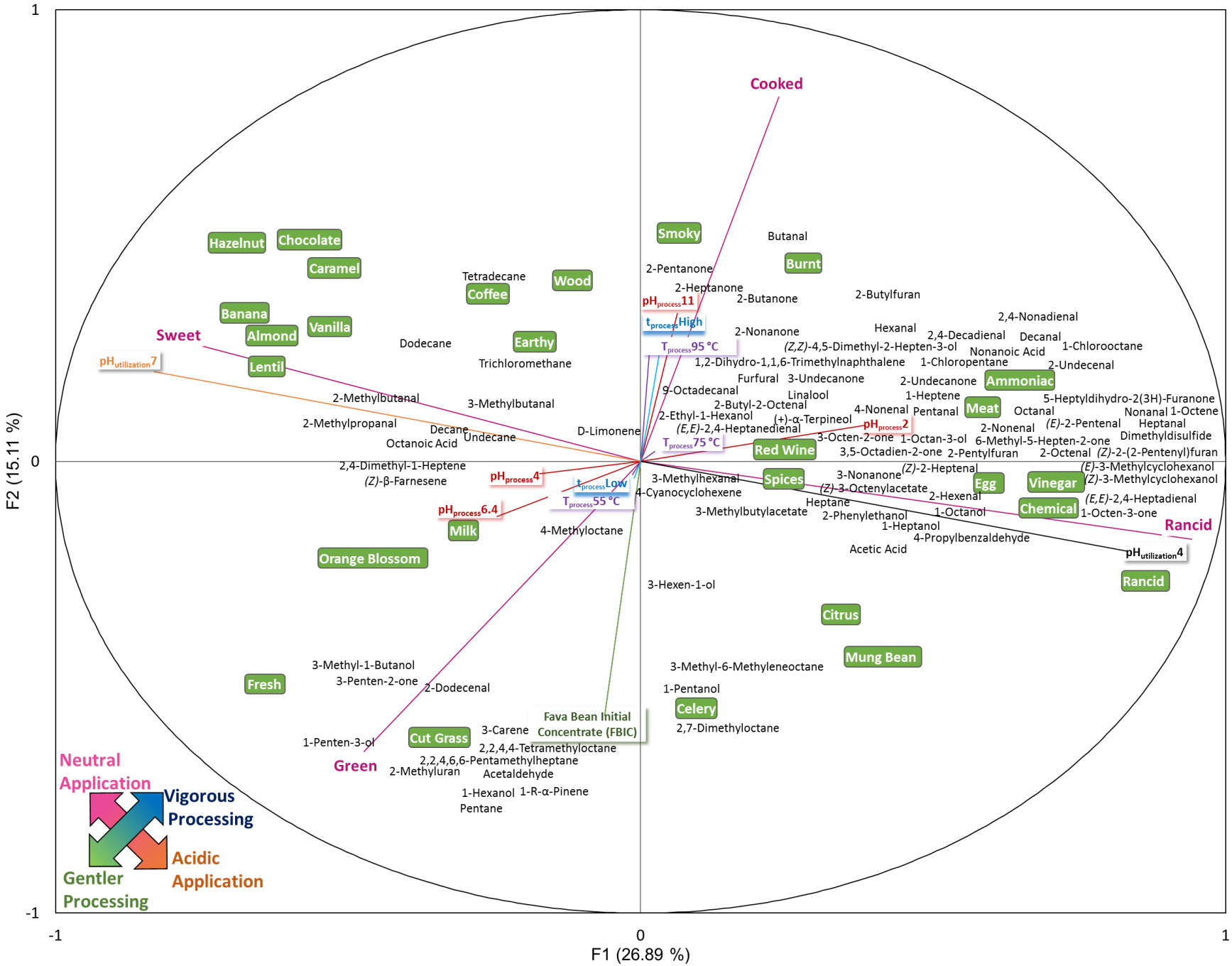
1
0
-1
F2 (15.11 %)
-1
0
1
F1 (26.89 %)
Cooked
Sweet
Green
Rancid
Hazelnut
Chocolate
Caramel
Banana
Almond
Vanilla
Lentil
Coffee
Wood
Earthy
Smoky
Burnt
Meat
Ammoniac
Red Wine
Spices
Egg
Vinegar
Chemical
Rancid
Milk
Orange Blossom
Fresh
Cut Grass
Fava Bean Initial Concentrate (FBIC)
Citrus
Mung Bean
Celery
pH utilization 7
pH utilization 4
pH process 11
pH process 2
pH process 4
pH process 6.4
t process High
t process Low
T process 95 °C
T process 75 °C
T process 55 °C
Butanal
2-Pentanone
2-Heptanone
2-Butanone
2-Butylfuran
2-Nonanone
Hexanal
2,4-Nonadienal
2,4-Decadienal
Decanal
1-Chlorooctane
Nonanoic Acid
(Z,Z)-4,5-Dimethyl-2-Hepten-3-ol
1,2-Dihydro-1,1,6-Trimethylnaphthalene
1-Chloropentane
2-Undecenal
Furfural
3-Undecanone
2-Undecanone
9-Octadecanal
Linalool
1-Heptene
5-Heptyldihydro-2(3H)-Furanone
2-Butyl-2-Octenal
2-Ethyl-1-Hexanol
(+)-α-Terpineol
4-Nonenal
Pentanal
Octanal
Nonanal
1-Octene
(E,E)-2,4-Heptanedienal
2-Nonenal
(E)-2-Pentenal
Heptanal
3-Octen-2-one
1-Octan-3-ol
6-Methyl-5-Hepten-2-one
Dimethyldisulfide
3,5-Octadien-2-one
2-Pentylfuran
2-Octenal
(Z)-2-(2-Pentenyl)furan
(Z)-2-Heptenal
(E)-3-Methylcyclohexanol
3-Methylhexanal
3-Nonanone
(Z)-3-Octenylacetate
(Z)-3-Methylcyclohexanol
4-Cyanocyclohexene
2-Hexenal
(E,E)-2,4-Heptadienal
Heptane
3-Methylbutylacetate
2-Phenylethanol
1-Octanol
1-Octen-3-one
1-Heptanol
4-Propylbenzaldehyde
Acetic Acid
3-Hexen-1-ol
3-Methyl-6-Methyleneoctane
1-Pentanol
2,7-Dimethyloctane
3-Methyl-1-Butanol
3-Penten-2-one
2-Dodecenal
1-Penten-3-ol
3-Carene
2,2,4,4-Tetramethyloctane
2,2,4,6,6-Pentamethylheptane
2-Methyluran
Acetaldehyde
1-Hexanol
1-R-α-Pinene
Pentane
Tetradecane
Dodecane
Trichloromethane
2-Methylbutanal
3-Methylbutanal
2-Methylpropanal
Decane
Undecane
Octanoic Acid
D-Limonene
2,4-Dimethyl-1-Heptene
(Z)-β-Farnesene
4-Methyloctane
Neutral Application
Vigorous Processing
Acidic Application
Gentler Processing

Figure 27 – Interplay Between Process Conditions, Odor Perception & Volatile Chemistry. MFA projections of three quantitative data matrices on a bi-dimensional plane, i.e. that of odor intensity, odor attributes, along with the headspace volatile chemistry (normalized peaks of detected volatiles by HS-SPME-GC-MS) of the different ingredient suspensions. The FBIC was the fava bean initial concentrate which was then modified by $pH_{process}$ (2, 4, 6.4 and 11), $T_{process}$ (55, 75 and 95 °C) and $t_{process}$ (30 and 360 min) and then utilized at two $pH_{utilization}$ (pH 4 and 7). These processes were grouped into gentler ($pH_{process}$ = 4 and 6.4, $T_{process}$ = 55°C, $t_{process}$ = Low) or vigorous ($pH_{process}$ = 2 or 11, $T_{process}$ > 55°C, $t_{process}$ = High) types of ingredient processing; or into acidic ($pH_{utilization}$ 4) or basic ($pH_{utilization}$ 7) types of ingredient application.

V.2.4.1. Effect of Degree of Ingredient Processing

As illustrated by the results, the degree of ingredient processing clearly impacts their odor and volatile composition. Gentler processing, including the presence of the FBIC, was associated to more green perception, where the FBIC was closely attributed to cut grass notes (**Figure 27**). Perception of "green", "beany", "raw" and "fresh" notes have often been associated to raw legumes including beans, pea, lupin and soy [32], [35].The FBIC used in this study was indeed obtained by milling of dried and dehulled beans followed by air classification. Thus, certain volatile compounds can occur naturally, but most of them are formed by processing [5], [314]. The series of reactions that enable formation of volatiles start right from the harvesting to storage and then to bean processing. Bean dehulling and milling results in breakdown of tissues and exposure of flavor precursors (e.g. lipids, proteins and sugars) to active enzymes (e.g. lipases, lipoxygenases, dehydrogenases), thus enabling substrate degradation into flavor molecules [5], [19], [248]. For pulses in general, grassy notes have been noted owing to higher alcohols and aldehydes levels [5], [83], [170]. This is in accordance with the higher levels of aldehydes and alcohols found for the FBIC in this study (**Figure 25**). Higher levels of aldehydes and alcohols, as well as certain furans and ketones, have often been associated with the oxidation of poly-unsaturated fatty acids (PUFA), i.e. linoleic and linolenic acids in pulses, leading to the formation of grassy, beany and green attributes [19], [32], [35], [315]. Lipid oxidation is also reported as the primary contributor to flavor production in pulse ingredients [32], [35], [162]. As indicated in **Figure 27**, the FBIC was along the cluster of gently processed ingredients, being strongly associated with acetaldehyde, 1-hexanol, 2,2,4,6,6-pentamethylheptane, 2,2,4,4-tetramethyloctane and 2-methylfuran, that are possible indicators of lipid oxidation [32], [35]. The FBIC also contained 1-R-α-pinene and 3-carene which are products of carotenoids degradation in plants [35], [248], [316]. This means that the FBIC, however very gently processed, has already undergone some level of volatile generation, in proportions that contributed to more green odor perception.

Moving further to the concentrates that were more but rather gently processed, $pH_{process}$ 4 and 6.4 were associated to more milk, fresh and orange blossom attributes, and lied in the same quadrant as the FBIC (**Figure 27**). For the $pH_{process}$ 6.4 ingredient suspensions, aldehyde and alcohol signals were predominant, with certain variations due to the extent of processing ($T_{process}$ and $t_{process}$). Nevertheless, the volatile proportions were similar to that of the FBIC suspensions at $pH_{utilization}$ 4 (**Figure 25**). For the $pH_{process}$ 4 series, aldehyde signals followed by those of terpenoids were predominant in most of the ingredient suspensions (**Figure 25**). Terpenoids, along with certain alcohols and aldehydes, are popularly studied with regard to the aroma of fruits [317]. Terpenoids such as 1-R-α-pinene, 3-carene and *(Z)*-β-farnesene were associated with these two series, that all have been identified to give a fruity, orange, citrus, sweet and milky aroma [183]. These, along with many other terpenoids derived from

carotenoid degradation, are found mainly in citrus oils [32]. Therefore, in addition to lipid oxidation reactions, carotenoid degradation could also have been favored through these processes – rendering a slightly deviated perception from the FBIC suspensions (**Figure 27**). Now, moving towards an even higher degree of processing, the perception was driven towards a more cooked one, where two distinct zones were defined by $pH_{process}$ 2 and 11 (**Figure 27**). Processing at $pH_{process}$ 2 was associated more towards red wine, meat and ammoniac notes. Whereas $pH_{process}$ 11 was related to smoky and burnt odor attributes and to even $T_{process}$ 95 °C and $t_{process}$ High conditions (**Figure 27**). As explained for raw pulses that give a rather green/ grassy perception, bean processing at higher temperatures leads to a decrease in grassy notes, with the evolution of nutty, roasted and cooked odor – a similar trend as the results obtained in this study [83], [170]. Thermal treatment has been strongly associated to the generation of burnt, cooked, smoked and baked aroma – which was also seen for the concentrates modified at higher temperatures ($T_{process} \geq 75$ °C) [32], [318], [319]. In fact, fava bean flours have been used in the fortification of corn chips where the degree of processing led to the formation of burnt notes within defined acceptable levels [319]. With regard to the cooked flavor-associated reactions that are accelerated by the $T_{process}$, vigorous conditions of extreme pH, such as $pH_{process}$ 2 and 11 have been associated with Maillard reaction, lipid oxidation, protein and sugar degradation reactions [32], [35]. Ingredient modification at $pH_{process}$ 2 was associated with very high volatile complexity (**Figure 26** and **Figure 27**). Earlier reports have shown that acidic processes in systems rich in proteins lead to protein hydrolysis, where lower hydrophobic interactions remove opportunity of flavor binding and thus enabling higher flavor release [32], [258], [320]. Examples of this were seen in the higher generation of volatile compounds, especially for $pH_{process}$ 2 ingredients (**Figure 26** and **Figure 27**). Furfural is formed by either Maillard reaction (Amadori or Heyn's products) or from sugar degradation. Furfural is often associated with fragrant, bread, woody, sweet, baked and almond attributes [183]. Vigorous processes resulted in a higher furfural formation, suggesting the advent of the reactions and generation of associated odor attributes (**Figure 27**). Maillard reaction products also include aldehydes, ketones, sulfur compounds and pyrazines that have been found to impart a "burnt" flavor in soy, along with certain Strecker aldehydes and furanoids [35]. Furanoids (2-alkylfurans) such as 2-butylfuran and 2-pentylfuran are known to be formed from PUFA oxidation products in different pulses [32], [35], [169]. Generation of these compounds has been favored by the vigorous processes (**Figure 27**). In protein-rich matrices, alkaline processes on the other hand, enable more binding of proteins with compounds such as butanal, hexanal, 2,3-butanedione and a higher release of ketones including 2-heptanone [263]. This matrix-effect could have played a role for the ingredients modified by $pH_{process}$ 11, where relatively higher signals of ketones and aldehydes were detected (**Figure 25**). These ketones include products from lipid oxidation such as 2-butanone, 2-pentanone and

2-heptanone (from linoleic and α-linolenic), 3-octen-2-one (from linoleic acid) and 3,5-octadien-2-one (from α-linolenic acid). 2-butanone and 2-pentanone are also produced from thermal decomposition of amino acids (e.g. alanine, valine, isoleucine, cysteine) and sugars (e.g. glucose, sucrose) [35], [169]. Furthermore, acidic and alkaline processing of proteins leads to their deamidation, where at elevated temperatures, ammonia is released due to the conversion of asparagine and glutamine residues into their carboxylic forms – an explanation as to why there was a higher generation of ammoniac odor attributes in some cases [23].

V.2.4.2. Effect of Conditions of Ingredient Application

Apart from the conditions used for ingredient modification, the conditions of utilization also impact largely the odor perception. Precisely, utilization in a more neutral application ($pH_{utilization}$ 7) was linked to a more "sweet" perception, and the same at acidic application was linked more to rancid perception (**Figure 27**). One study concerning gels prepared from fava protein isolates also presented this type of contrast between neutral and acidic pH, where at the acidic pH (pH 4), unpleasant and fruity off-flavors were perceived [40]. Amongst all the ingredients studied, the FBIC showed highest impact in headspace volatile proportions and odor profiles at the two different utilization conditions (**Figure 25**). Changes in volatile protonated states due to the pH, along with the changes in suspension matrix and probable enzyme activation/ inactivation are suggested in this study, that could impact volatile generation and release. Acidic pH retains acidic volatiles to a lesser extent compared to neutral pH. At pH 4 for instance, organic acids are released to a greater extent into the headspace (partial protonation of $–COO^-$ to –COOH groups) whereas the basic odorant molecules such as amines and pyrazines are retained at this pH (protonation of $–NH_2$ to $–NH_3^+$ groups) [32], [321]. In another study, acetic acid was in much higher levels at the $pH_{utilization}$ 4, which is associated with strong, pungent acidic odor [183]. Since lipid oxidation has been seen to be an important flavor generating reaction, enzymatic oxidation of lipids by broad bean lipoxygenase (BBL) can be also anticipated for the FBIC. In fava bean flour, BBL activity can be as high as 0.33 mmol/min/g flour. This could increase the potential for flavor formation [39]. Lack of drastic shifts in volatile group proportions in modified ingredients shows that the BBL activities could have decreased due to ingredient modification's conditions. Thermal treatments above 70 °C for 2 min have been known to inactivate BBL, suggesting a high sensitivity of this enzyme to process conditions [37]. Therefore, it would be interesting to complete this study by looking into possibilities of BBL activity in the FBIC and evolution of this activity with the degree of processing ad well as conditions of utilization. Furthermore, in protein-rich systems such as the ones studied here, protein unfolding and resultant precipitation at pH close to their isoelectric points may impact the release of certain molecules compared to another condition favoring the native

proteins [32]. For fava proteins, the isoelectric pH is between 4 and 5, and thus $pH_{utilization}$ 4 is much distinct when compared to $pH_{utilization}$ 7 owing to protein precipitation [5], [322]. Finally, process conditions affected the complexity of the volatiles through different reactions. The effect of pH was predominant, and governed the changes in matrix along with the types of flavor imparting reactions that could occur, followed by the effects of $T_{process}$ and $t_{process}$ that may have determined the degree to which these reactions can take place – thus altogether driving odor in many possible directions (**Figure 27**). Odor perception and volatile chemistry are both very complex in nature, interdependent and also dependent on process conditions. The **Figure 27** explained 42% of the interplay between odor perception, volatile chemistry and process conditions, suggesting that only a part of the whole phenomena has been explained.

V.2.5. Conclusions

Fava bean is a multi-component system containing all precursors for flavor formation. Process conditions drove changes in odor perception in protein-rich fava bean concentrates. Initially, the unmodified concentrate had stronger green notes, but this evolved into more cooked notes with the extent and type of processing – mainly governed by the pH during concentrate modification. This effect was confirmed with headspace volatile analysis of ingredient suspensions. Aldehyde signals were primarily detected in ingredient suspensions headspace. But furanoids, terpenoids, alcohols and ketones signals had the next higher contribution for modifications at pH2, 4, 6.4 and 11 respectively. From gentler to vigorous process conditions, perception can be modified from more green to more cooked flavors, whereas different conditions of application (e.g. pH) can modulate between "sweet" or rancid perceptions. The utilization pH was predominant in driving odor perception, especially for the unmodified concentrate which also showed drastic changed in volatile groups at different pH. Ingredient modification reduced this radical impact by pH to a certain extent. Lipid oxidation was deemed noteworthy in generating volatiles, along with other reactions including proteins, sugars and carotenoids degradation. Studies looking into the nature of these reactions are encouraged. Role of enzymes needs to be further investigated in imparting flavor during utilization conditions, especially for unmodified concentrates. Just as the odor perception is driven by process conditions, taste and color of such promising ingredients need to be assessed along with process conditions in a similar approach – where perception is explained along with food chemistry.

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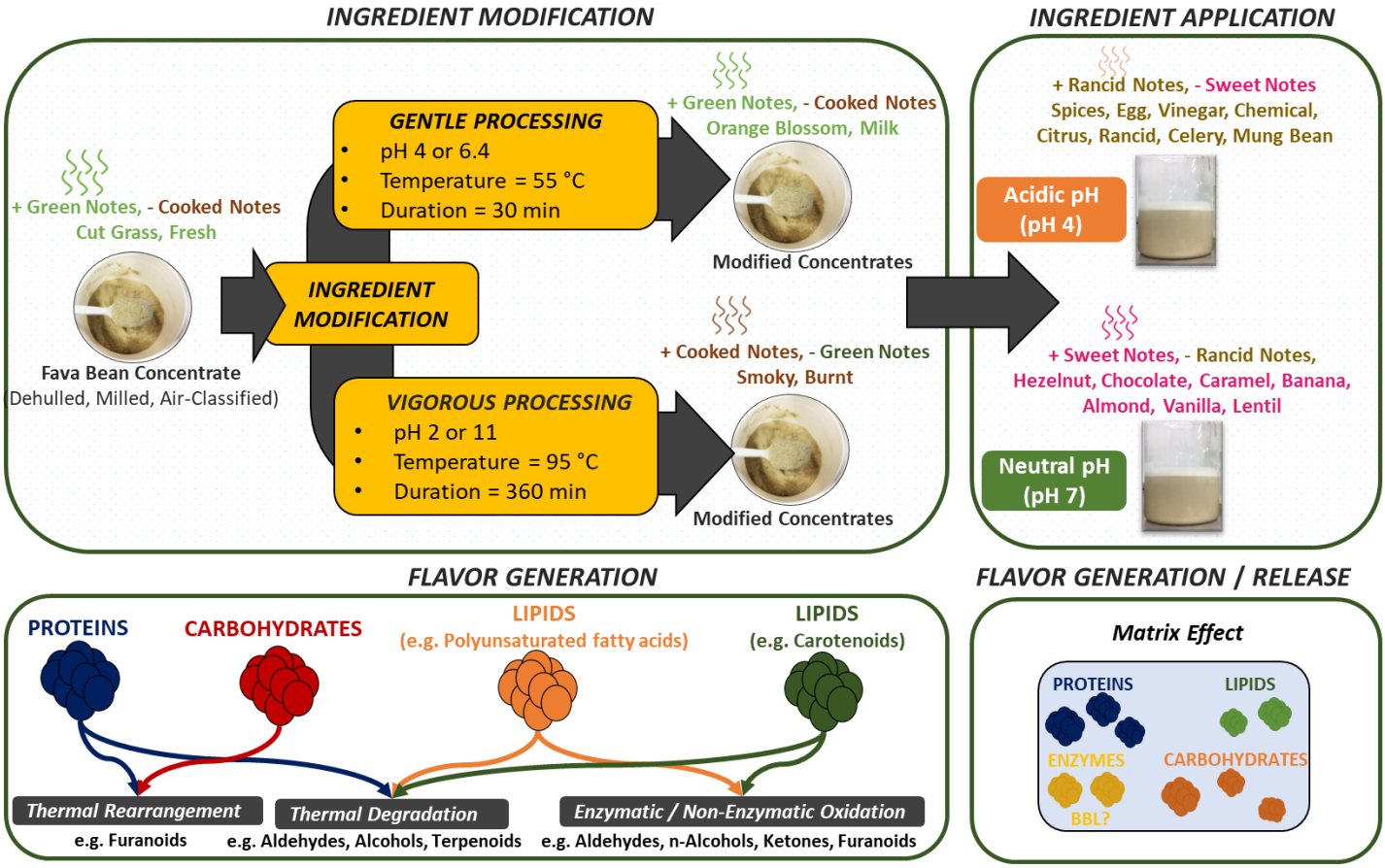
V.2.5.2. Author Contributions

Conceptualization, S.S., M-N.M., A.S-E.; methodology, validation, formal analysis, investigation and data curation, S.S., G.Z., A.P., N.D., D.F., M-N.M., A.S.E.; resources, J.Z., D.B., J.A.; writing—original draft preparation, S.S.; writing—review and editing, All.; visualization, S.S., A.S-E.; supervision, J.Z., A.S-E. and M-N.M.; project administration, M-N.M., A.S-E., J.Z.; funding acquisition, M-N.M., D.B., J.A. All authors have read and agreed to the published version of the manuscript.

V.3. Key Highlights



- The different ingredient suspensions evaluated in this study were significantly distinct in their odor profiles. During the evaluation of odor of different ingredient suspensions, the different ingredients were described by the help of 28 out of 36 different odor attributes. In addition, these ingredient suspensions had different intensities of green, cooked, "sweet" and burnt notes.

- Process conditions, especially pH during utilization, was found to drive odor perception. This effect was prominent for the fava bean initial concentrate. For the native and the modified ingredient suspensions, there was a shift from higher rancid odor intensity to a higher "sweet" intensity from pH 4 to 7. Utilization at pH 4 gave noticeable attributes of vinegar, meat, egg, chemical, rancid, burnt, citrus, red wine, spices notes, whereas utilization at pH 7 gave features of chocolate, almond, hazelnut, banana, vanilla, coffee, caramel, fresh, milk, lentil and wood notes.

- Process conditions during ingredient modification also drove odor perception. Initially, the unmodified concentrate and ingredients gently processed were perceived with stronger green notes. From gentler to vigorous process conditions, perception was modified from more green to more cooked one.

- Headspace volatile analysis detected 88 different volatiles, belonging to the category of aldehydes, alcohols, ketones, furanoids, terpenoids, alkanes, alkenes, organic acids, esters and some chlorinated and sulfur compounds.

- For the fava bean initial concentrate, there was a predominant effect of the utilization pH in the different proportions of headspace volatile peak areas. But then, ingredient modification reduced this drastic impact of utilization pH to a certain extent. The utilization pH may have led to changes in the suspension matrix, resulting in a difference in volatile release.

- Process conditions during ingredient modification generated flavor molecules from lipid oxidation, carotenoids degradation, Maillard reaction, proteins and sugars degradation reactions. Lipid oxidation was quite important in generating volatiles, along with other reactions. Aldehyde signals were primarily in proportion to the other volatile signals from different ingredient suspensions. But proportions of furanoids, terpenoids, alcohols and ketones signals had the next higher contribution across ingredients modified by pH 2, 4, 6.4 and 11 respectively.

VI

Can Process Drive Sensorial & Nutritional Impact of Non-Volatile Compounds?

VI. Can Process Drive Sensorial & Nutritional Impact of Non-Volatile Compounds?

VI.1. General Introduction

Fava bean acceptability is determined by functional, nutritional and flavor aspects, where micro-non-volatile components present play a very important role. The objective of this study was in fact to study these non-volatile components and their evolution as a function of process conditions and to suggest if these modifications can be important for fava bean ingredients for industrial food applications.

With this, the approach was to conform experimental design that as already used for functional and odor studies. Therefore, the same types of process conditions were used for the modification of fava bean ingredients. However, this time, only selected process conditions were studied. For acidic (pH 2 and 4) and alkaline (pH 11) processes, two conditions were studied: mild (55 °C_Low) and vigorous (95 °C_High). Additionally, a series with the natural suspension pH was considered, i.e. without any pH modification (pH 6.4), and ingredients modified at 55, 75 and 95 °C, at 30 and 360 min were investigated. From the fava bean initial and modified concentrates, non-volatile compounds were extracted using a hydro-alcoholic mixture, and detected by UHPLC-PDA-QToF-MS.

With this approach, the first objective was to detect what kind of molecules are present in the extracts of the different ingredients and to semi-quantify them. Secondly, their changes as a function of process conditions were observed statistically. The molecules were then grouped into different families to see their evolution with different types of process conditions. For the ingredients modified at non-adjusted pH, a more detailed analysis on the effects of temperature and process duration were recorded. Finally, literature evidences on the molecules detected were noted. Additionally, hypotheses of process conditions influencing extractability and structural degradation were proposed.

Eventually, with the evolution of the different non-volatile compounds, indications of changes in fava bean ingredient properties were proposed, and the need to complete the study with different nutritional and sensory related studies were discussed.

VI.2. Process Conditions Modify Non-Volatile Components In Fava Bean (*Vicia faba* L.) Ingredients: New Insights into the Interplay Between Process Conditions, Phenolic Compounds and Saponins.

Manuscript in Preparation

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Abstract: Fava bean (*Vicia faba* L.) has a great potential as a protein source for nutritional and functional needs in industrial food applications. Non-volatile micro-constituents including phenolic compounds and saponins play an important role in fava bean's ingredients acceptability and quality. While phenolic compounds are known for their antioxidant properties, bitterness, taste and color, saponins mainly contribute to their bitterness and astringency. Their detection and transformation could help food industry to reach solutions of producing highly acceptable fava ingredients and products for human consumption. With this objective, an industrially relevant, minimally processed, protein-rich ingredient, i.e. fava bean concentrate, was modified by process conditions of pH (2, 4 and 11), temperature (55, 75 and 95 °C) and treatment duration (30 and 360 min). Further simplified modification process, i.e. without any pH adjustment (natural pH 6.4), was studied in greater detail. Hydro-alcoholic extracts were produced from these different ingredients and analyzed by UHPLC-PDA-QToF-MS to yield different molecules belonging to flavonoids (flavan-3-ols, flavones, flavonols), phenolic acids (hydroxycinnamic acids) and saponins. Most of the glycosylated phenolic compounds were present at higher levels in the native concentrate compared to the modified ingredients, but with an interesting interplay between the process conditions used to produce these ingredients, their extractability and their structural modifications. Amongst saponins, soyasaponin β (higher bitterness) was predominant in the native concentrate whereas saponin Bb (lower bitterness) became higher in the modified ingredients, especially in the ones modified without any pH adjustment. Evolution of both phenolic compounds and saponins due to process conditions suggests possible changes in flavor, color, antioxidant and even anti-nutritional profile of the ingredients.

Keywords: Flavonoids, phenolic acids, soyasaponin β, plant-based, UHPLC-PDA-QToF-MS, Principal Component Analysis

VI.2.1. Introduction

With the rising population and the need to have sustainable and healthy foods, consumers along with researchers and food industries are moving their interest towards plant-based products [61], [323]. Fava bean (*Vicia faba* L.) is a plant-based source rich in proteins (25-43% w/w d.b.) and thereby has a great future for human nutrition [3]. Despite its potential, possible sensory (off-flavor and undesirable color) and anti-nutritional limitations need to be addressed so that ingredients and food products from fava bean can be more acceptable to consumers [3], [5].

In the quest towards fava bean acceptability, non-volatile micro constituents including phenolic compounds and saponins can play a key role as they constitute several types of molecules that are linked to different nutritional, functional and sensorial properties [3], [5], [41]–[44]. Phenolic compounds are secondary metabolites that are synthesized through phenylpropanoid metabolism pathways. They do not play a role in fundamental life processes, but are rather involved in protecting plants from infestations and infections in addition to attracting see-dispersing animals [75]. Saponins are triterpene glycosides constituting a non-polar aglycone backbone along with one or more sugar moieties. These two classes of non-volatiles have been associated to a number of different nutritional and sensory properties, thus having a multivalent character. Nutritionally, phenolic compounds have been drawing attention owing to their antioxidant potential, where these can prevent various oxidative stress and fight lifestyle diseases such as cancer [41], [42]. Saponins have been shown to lower plasma cholesterol concentrations and thereby reduce the risk of heart disease [7], [43], [155]. The inhibitory effects of saponins and phenolic compounds on pancreatic lipase and α-glycosidase have also been recorded [324]; It is associated with lower absorption of hydrolyzed lipids and carbohydrates, thus beneficial against diet-related disorders such as obesity and diabetes [75], [184], [324]. A part of the multivalent character of these non-volatiles is also negative, affecting safety of fava bean [3], [5], [50]. Indeed, anti-nutritional factors comprise certain phenolic compounds, saponins, in addition to phytic acid conjugates, lectins and favism-inducing pyrimidine glycosides (vicine and convicine). In sensory aspects, phenolic compounds and saponins also play an important role linked to flavor and color perception and thus to acceptability of fava bean [3], [5], [19], [185]. Flavor in pulses are indeed a result of volatile or non-volatile compounds that bind with their respective receptors inside the oral and/or nasal cavity [19]. In particular, taste perception (e.g. bitterness and astringency) is related

to the dissolution of non-volatile, sapid-glycosylated compounds including phenolic compounds and saponins in the saliva, followed by the stimulation of specific receptors in the oral cavity [19], [32], [51], [52]. Color perception is also an essential part of sensory acceptability, and is associated with non-volatile compounds, including phenolic compounds and products of enzymatic and non-enzymatic browning reactions [5], [53]–[55].

Phenolic compounds and saponins have both been detected in fava beans [324], [325]. Specifically, phenolic compounds are either flavonoids, i.e. flavan-3-ols, flavones, flavonols, flavononols, isoflavones and proanthocyanidins, or phenolic acids, i.e. hydroxybenzoic and hydroxycinnamic acids [45], [46]. Saponins exist in many forms, including soyasapogenol B, soyasaponin β, soyasaponin Bb and ayukisaponin IV [5], [47], [48], [58]. Total phenolics and saponins contents have been measured in fava bean, but their individual molecules and the role of processing in their extractability and structural modifications have been dealt in lesser detail [252], [325]–[328]. Yet, ingredient processing can modify both phenolic compounds and saponins. Bean treatment (dehulling, soaking and heat treatment) prior to ingredient production reduces tannins, phytic acids and saponins to a considerable extent [56], [57]. Precisely, pulse storage above 30 °C, pulse soaking, germination at slightly acidic pH, and soaking/extraction in ethanol or methanol solvents all have resulted in saponin variants with lower bitterness [47], [48], [58]. In fava beans, saponins are lowered by soaking, dehulling, cooking and/or germination before ingredient production [57], [59]. Thus, processing seems to impart changes in the non-volatile aspects that related to the fava limitations, but most of the treatment studied until now have been on bean processing and not primarily on ingredient processing for final food applications. As there are many ways of producing and modifying fava ingredients, transformation of non-volatile by ingredient processing needs to be studied in depth. In this way, food industries could target these limitations and their chemical origins, and monitor their changes due to processing so that more acceptable fava ingredients are generated for the food market.

In this study, a rather mildly processed, protein-rich ingredient, i.e. fava bean concentrate has been examined. Industrially relevant process conditions such as pH, temperature and treatment duration were used to modify this concentrate. Non-volatile molecules were extracted using a hydro-alcoholic mixture and their identification and evolution with respect to processing were investigated with the aim to better understand the changes in fava bean potential and limitations.

VI.2.2. Materials & Methods

VI.2.2.1. Ingredient Preparation

VI.2.2.1.a. Fava Bean Initial Concentrate (FBIC)

Fava bean concentrate containing 65% (w/w d.b.) proteins was obtained from Vestkorn Ingredients (Holstebro, Denmark). The concentrate was produced by milling of dried and dehulled beans followed by air classification [214].

VI.2.2.1.b. Modified Ingredients

The FBIC was modified as described below: 1.5 kg of 20% (w/w) suspensions were prepared with deionized water and stirred for 30 min at 500 rpm using an overhead dissolver stirrer (IKA Works, Inc., Staufen, Germany); the pH ($pH_{process}$) was then adjusted to 2, 4 or 11 using 6 mol/L hydrochloric acid or 3 mol/L sodium hydroxide (Sigma Aldrich, Missouri, United States), and the suspensions were further stirred for 30 min at 500 rpm. All the suspensions were then heated ($T_{process}$) in a temperature-controlled bath (Lochner Labor+Technik GmBH, Germany) maintained at 55 or 95 °C and agitated at 700 rpm for a duration ($t_{process}$) of 30 min (Low) or 360 min (High). Therefore for each $pH_{process}$ (2, 4 and 11), two conditions are followed: mild (55 °C_Low) and vigorous (95 °C_High) to yield in total 6 modified-suspensions.

Additionally, a series with the natural suspension pH was considered, i.e. without any pH modification ($pH_{process}$ 6.4): 1.5 kg of 20% (w/w) suspensions were prepared with deionized water and stirred for 30 min at 500 rpm using an overhead dissolver stirrer (IKA Works, Inc., Staufen, Germany). The suspensions were then maintained at 55, 75 or 95 °C and agitated at either Low (30 min) or High (360 min) durations to yield additionally 6 modified-suspensions.

All the modified-suspensions produced were further frozen at −20 °C, followed by freeze-drying (Döhler GmbH, Dahlenburg, Germany) and milling to 0.08 mm mesh size by an ultra-centrifugal mill ZM 200 (Retsch GmbH, Germany). Hence, different modified ingredient powders were obtained, which were named as $pH_{process}\_T_{process}\_t_{process}$ (e.g. pH2_55 °C_Low), based on the conditions used to modify them.

VI.2.2.2. Analysis of Non-Volatile Compounds

VI.2.2.2.a. Extraction of Non-Volatile Compounds

Non-volatile compounds were extracted according to a protocol adapted from Chaieb et al., 2015 and Love et al., 2020 [278], [279]. A suspension of each ingredient (FBIC or modified ingredients) was prepared in a 30 mL glass vial (VWR, Rosny sous bois, France) by adding to the 0.6g of ingredient, 4mL of a mixture composed of absolute ethanol (Carlo Erba, Val de Reuil, France) and Milli-Q water (Millipore, France) (70/30, v/v) and containing 1mg/L of added leucine enkephaline (CAS 81678-16-2; Waters, Milford, USA) for internal calibration. After sealing the vial by butyl/PTFE septum cap, the mixture was stirred in a multi-post magnetic agitator (2Mag MIXdrive 6 HT, Germany) at 350 rpm for 60 min at room temperature, then centrifuged (Thermo Fisher Scientific Heraeus Multifuge X3R) at 20 °C and 3600 g for 10 min. The supernatant, henceforth called hydro-alcoholic extract, was then filtered through a 0.22 µm nylon filter (25 mm diameter, AIT, France) into a 2 mL HPLC vial closed with silicone/PTFE septum (AIT, France). Each sample was prepared in triplicate from three different ingredient suspensions.

VI.2.2.2.b. Analysis of the Extracts Containing Non-Volatile Compounds

Analysis of the hydro-alcoholic extracts was performed by ultra high performance liquid chromatography coupled with a photodiode array detector and a quadrupole-time-of-flight hybrid mass spectrometer (UHPLC-PDA-QToF-MS). Analyses were performed on a Waters Acquity H-Class apparatus composed of a quaternary solvent manager pump (QSM), a refrigerated sample manager flow-through needle (SM-FTN) thermostated at 10 °C, and a column oven coupled to a photodiode array detector (PDA) and a high resolution quadrupole-time-of-flight (QToF) hybrid mass spectrometer Xevo G2-S QToF, equipped with an electrospray ionization source (ESI).

1µL of the filtered hydro-alcoholic extract was injected onto an Acquity Ethylene Bridged Hybrid (BEH) C18 column (100 x 2.1 mm, 1.7 µm particle diameter, 130 Å) thermostated at 30 °C. The mobile phase was composed of [A] water and [B] acetonitrile, both acidified with formic acid (Carlo Erba, Val de Reuil, France) at 0.1 % (v/v). An elution gradient was performed at a flow rate of 0.49 mL/min according to the following conditions: isocratic 10 % of [B] between 0 and 0.99 min; linear gradient from 10 to 20 % of [B] until 6.70 min; linear gradient from 20 to 100 % of [B] until 26 min; linear gradient between 100 and 10 % of [B] until 28 min; isocratic 10 % of [B] between 28 and 30 min. The MS full scan analysis

was under negative polarity, using the resolution mode for a scan time of 0.5 s and a mass range from m/z 50 to 1500 acquired in centroid; The collision energy was fixed at 6 eV; The internal calibration of the QToF analyzer was performed every 20 s at a continuous flow of 5 µL/ min of leucine enkephalin (1 mg/L) for a total of 3 scans lasting 0.2 s each; The ESI parameters consisted of a capillary voltage of 0.5 kV, a sampling cone of 40 V with a cone nitrogen gas flow of 50 L/h, a source offset of 80 V kept at 120 °C and a desolvation gas (nitrogen) at 550 °C with a flow of 1200 L/h. The MS/MS analyses were performed on ions of interest by data dependent acquisition (DDA), switching from MS (noted MS^1) to MS/MS (noted MS^2) when the intensity of a candidate ion was above a threshold of 20 000 intensity/scan, and then performing a scan of the daughter ions for 0.5 sec of the selected precursor to confirm their identity, under the same MS conditions than the ones described above, at a constant collision energy of 30 eV; The internal calibration and the ESI parameters were also identical to those described above. The MS-analysis was performed by two successive steps in order to focus i) on major pseudo molecular ions (MS^1), and ii) on products ions after fragmentation (MS^2 DDA mode, with fragmentation). Simultaneous acquisition was performed with the PDA detector, at 20Hz from 190 to 500 nm, with a resolution of 1.2 nm.

The UV and mass spectra were acquired and treated by MassLynx software. The data treatment was extracted with open source software for mass spectrometry files mzMine 2 [280]. The peak areas obtained were normalized with the leucine enkephalin signal area and by the exact amount of ingredient used for preparing the sample, in dry weight.

VI.2.2.3. Statistical Analysis

Statistical data analysis was conducted using XLSTAT 2021.1 (Addinsoft, France). A matrix of the normalized peak areas of all detected compounds by UHPLC-PDA-QToF-MS was obtained across all the hydro-alcoholic extracts. Three-way ANOVA ($pH_{process}$, $T_{process}$, $t_{process}$) with post-hoc treatment using Newman-Keuls (SNK) method ($p \leq 0.05$) and PCA using Pearson's correlation method were conducted on this matrix.

VI.2.3. Results and Discussion

VI.2.3.1. Non-Volatiles Detected in Fava Hydro-Alcoholic Extracts

Forty one non-volatile compounds were detected by UHPLC-PDA-QToF-MS from hydro-alcoholic extracts obtained from fava bean concentrates. These consisted majorly of phenolic compounds, particularly flavonoids (3 flavan-3-ols, 8 flavones and 26 flavonols) and phenolic acids (2 hydroxycinnamic acids) (**Table 20**). Looking closely into the flavonoids that were detected in this study, most of them were mono-, di- or tri-

glycosylated by various sugar moieties - hexose (e.g. glucose or galactose) or dehoxyhexose (e.g. rhamnose), in O- or C-glycosylation, thus leading to several identification hypotheses corresponding to different possible isomeric forms (noted I, II, III, etc.) (**Table 20**). Flavonols were the most numerous, in particular glycosylated derivatives of kaempferol and quercetin, but the aglycone form of kaempferol was also detected (**Table 20**).

All the detected molecules have already been found in notable amounts in vegetables and legume seeds in their glycoside forms [180], [329]. Kaempferol and quercetin derivatives, glycosylated with mainly glucose, rhamnose, and galactose moieties, along with hydroxycinnamic acids, have been detected in the leaves of fava bean crops whereas quercetin glycosides were the main flavonol derivatives in pea [330]. Absence of any tannins in the non-volatile fraction detection was not surprising as the fava concentrate selected was already dehulled before its milling. Indeed, 72 to 82% of total phenolics recovered in the seed coat are tannins, 96% corresponding to proanthocyanidins, which are primarily responsible for detrimental impact on fava bean seed color [185]. Despite a direct lack of data on proanthocyanidins in the ingredients analysed in this study, an indirect link could be made with the detected catechins and epicatechins (**Table 20**); These molecules, along with epicatechin gallates, are monomeric flavan-3-ols that condense oxidatively to form proanthocyanidins and condensed tannins [331]. Phenolic compounds have selected solubility and therefore, their extractability may be different according to the solvent mixtures [332]. For instance, aqueous-acetonitrile extracts from faba beans yield isoflavones (diadzein and genistein) [333] whereas acetone extracts help detect anthocyanins (cyaniding and delphidin) in addition to coumaric and sinapic acids [178].

The detected molecules have been noted in literature for nutritional and taste properties. Total phenolics content generally accounts for 0.5-2% (g/kg) in fava bean seeds, associated not only with antioxidant potential but also with taste, color and anti-nutritional properties [97], [182], [278], [334]–[336]. Nutritionally, phenolic compounds have a dual role. Some of them, especially flavonols (e.g. quercetin, kaempferol), flavan-3-ols (e.g. catechins, epigallocatechins), tannins and phenolic acids (e.g. ferulic, caffeic, *p*-coumaric and sinapic acids), have health benefits (antioxidant, antimutagenic and anticarcinogenic properties) owing to their response in decreasing oxidative stress [278], [327], [328], [337], [338]. Superior antioxidant activity of faba seeds and leaves, compared to common vegetables (cabbage, broccoli, spinach, etc.), has also been reported [339].

Despite these nutritional benefits, certain phenolic compounds, e.g. tannins (proanthocyanidins), phenolic acids (ferulic and caffeic acids), flavones (apigenins), flavonols (kaempferol, quercetin) interact covalently or non-covalently with protein residues, decreasing protein bioavailability and thus, rendering them as anti-nutritional factors [3], [156]. With regard to taste, aglycones of quercetin and kaempferol have been known also to trigger bitter taste receptors [19].

Table 20 – Average Normalized Non-Volatiles Peak Area in Fava Bean Concentrates. Non-Volatile compounds detected by UHPLC-PDA-QToF-MS in the hydro-alcoholic extracts from fava bean initial concentrate (FBIC) and ingredients modified by process conditions (pH, temperature and treatment duration). The average detected peak areas have been normalized with internal standard per weight of ingredient used for extraction (Peak Area $_{Compound}$/ Peak Area $_{Leucine\ enkephaline}$/ g ingredient d.b.)

						$pH_{process}$ 2		$pH_{process}$ 4			$pH_{process}$ 6.4					$pH_{process}$ 11			
Retention Time (min)	**UV Spectrum**	**Ion selected by MS^1 for MS^2 analysis (m/z)**	**Ions detected by MS^2 analysis (m/z)**	**Proposed Identification**	**FBIC**	**pH2_55 °C_Low**	**pH2_95 °C_High**	**pH4_55 °C_Low**	**pH4_95 °C_High**	**pH6.4_55 °C_Low**	**pH6.4_55 °C_High**	**pH6.4_75 °C_Low**	**pH6.4_75 °C_High**	**pH6.4_95 °C_Low**	**pH6.4_95 °C_High**	**pH11_55 °C_Low**	**pH11_95 °C_High**	**p-Value**
Internal Standard																		
7.98		554.2621	nd	Leucine enkephaline	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	-
Flavan-3-ols																		
1.84	nd	289.0713	161.0460(40%) 151.0470(73%) 123.0420(100%) 109.0279(90%) 97.0269(40%)	Epicatechin	0.97	0.11	0.06	0.16	1.17	0.61	0.64	0.85	1.57	1.29	3.41	0.00	0.00	**<0.0001**
2.64	nd	289.0712	289.0701(100%) 245.0817(95%) 205.0501(40%) 203.0697(60%) 151.0389(30%)	Monoglycosylated catechin	0.22	0.00	0.00	0.04	0.04	0.07	0.04	0.15	0.09	0.13	0.00	0.00	0.00	**<0.0001**
3.07	nd	289.0713	161.0415(30%) 151.0407(19%) 137.0235(20%) 123.0447(100%) 109.0278(80%)	Catechin	2.42	0.13	0.00	0.33	0.56	0.79	0.67	1.05	1.12	1.29	1.31	0.00	0.00	**<0.0001**

Flavones																		
1.43	nd	563.1424	nd	Diglycosylated Apigenin (I) *(1 hexose + 1 deoxyhexose)*	0.70	0.06	0.09	0.08	0.05	0.00	0.00	0.00	0.00	0.31	0.00	0.18	0.06	**<0.0001**
1.52	nd	739.2094	nd	Triglycosylated Apigenin (I) *(2 hexoses + 1 deoxyhexose)*	0.12	0.00	0.00	0.00	0.01	0.00	0.00	0.03	0.02	0.01	0.03	0.07	0.01	**<0.0001**
4.43	nd	563.1409	nd	Diglycosylated Apigenin (II) *(1 hexose + 1 deoxyhexose)*	0.11	0.02	0.03	0.02	0.01	0.00	0.00	0.03	0.00	0.01	0.00	0.00	0.00	**<0.0001**
4.96	nd	739.2090	nd	Triglycosylated Apigenin (II) *(2 hexoses + 1 deoxyhexose)*	0.06	0.01	0.03	0.02	0.03	0.10	0.11	0.04	0.05	0.03	0.02	0.00	0.06	**0.003**
5.48	nd	593.1516	nd	Diglycosylated Luteolin (I) *(1 hexose + 1 deoxyhexose)*	0.00	0.00	0.02	0.02	0.02	0.20	0.25	0.06	0.05	0.06	0.01	0.00	0.05	**0.020**
5.68	nd	593.1515	nd	Diglycosylated Luteolin (II) *(1 hexose + 1 deoxyhexose)*	0.04	0.02	0.03	0.06	0.00	0.30	0.47	0.19	0.18	0.19	0.00	0.02	0.06	0.084
6.41	nd	593.1516	593.1473(20%) 285.0385(100%) 284.0337(45%) 257.0399(5%)	Diglycosylated Luteolin (III) *(1 hexose + 1 deoxyhexose)*	0.41	0.09	0.19	0.51	0.19	1.91	2.46	0.72	0.67	0.66	0.42	0.15	0.05	**<0.0001**
7.73	nd	593.1513	nd	Diglycosylated Luteolin (IV) *(1 hexose + 1 deoxyhexose)*	0.00	0.00	0.00	0.00	0.00	0.01	0.08	0.00	0.00	0.00	0.00	0.00	0.02	**0.001**
Flavonols																		

6.98	nd	477.1035	nd	Monoglysosylated Isorhamnetin *(1 hexose)*	0.03	0.00	0.03	0.05	0.03	0.26	0.31	0.12	0.12	0.06	0.00	0.00	0.00	**0.003**
2.96	nd	771.1986	nd	Triglycosylated Kaempferol (I) *(3 hexoses)*	0.22	0.02	0.00	0.03	0.06	0.06	0.00	0.10	0.12	0.11	0.14	0.18	0.00	**0.003**
3.27	270(m); 330	739.2091	739.2058(20%) 593.1489(100%) 431.0960(5%) 285.0427(25%) 151.0065(5%)	Triglycosylated Kaempferol (II) *(1 hexose + 2 deoxyhexoses)*	0.88	0.22	0.00	0.25	0.34	0.96	0.69	1.13	1.24	1.24	1.05	0.73	0.10	**0.047**
3.37	nd	593.1517	593.1462(45%) 503.1145(15%) 473.1048(70%) 383.0749(70%) 353.0666(100%)	Diglycosylated Kaempferol (I) *(1 hexose + 1 deoxyhexose)*	0.03	0.47	0.00	0.66	0.00	2.02	1.71	2.18	2.44	2.38	0.00	0.89	0.09	**<0.0001**
3.52	271(m); 280(s); 340	739.2090	739.2065(5%) 593.1477(100%) 430.0866(15%) 284.0306(10%) 151.0025(3%)	Triglycosylated Kaempferol (III) *(1 hexose + 2 deoxyhexoses)*	1.50	0.20	0.01	0.21	0.44	1.02	0.70	0.42	1.43	1.45	0.81	0.59	0.07	0.268
3.56	nd	739.2099	nd	Triglycosylated Kaempferol (IV) *(1 hexose + 2 deoxyhexoses)*	0.11	0.10	0.02	0.11	0.00	0.00	0.00	0.86	0.00	0.00	0.35	0.17	0.04	0.696
4.16	nd	755.2044	nd	Triglycosylated Kaempferol (V) *(1 hexose + 2 deoxyhexoses)*	0.01	0.01	0.01	0.04	0.03	0.13	0.06	0.10	0.10	0.00	0.08	0.05	0.00	**0.038**
4.19	nd	755.2037	nd	Triglycosylated Kaempferol (VI) *(1 hexose + 2 deoxyhexoses)*	0.16	0.01	0.01	0.00	0.02	0.01	0.05	0.04	0.05	0.13	0.03	0.00	0.00	**0.002**

4.69	nd	593.1513	nd	Diglycosylated Kaempferol (II) *(1 hexose + 1 deoxyhexose)*	0.19	0.02	0.00	0.03	0.04	0.15	0.09	0.14	0.14	0.10	0.11	0.06	0.00	**<0.0001**
4.75	nd	739.2089	739.1949(5%) 593.1511(100%) 430.0861(5%) 285.0352(8%) 150.9674(2%)	Triglycosylated Kaempferol (VII) *(1 hexose + 2 deoxyhexoses)*	0.54	0.11	0.02	0.17	0.20	0.52	0.54	0.53	0.59	0.59	0.45	0.30	0.04	**<0.0001**
4.82	271(m); 280(s); 340	771.1989	771.1961(100%) 609.1425(5%) 284.0299(15%) 429.0809(13%)	Triglycosylated Kaempferol (VIII) *(3 hexoses)*	0.60	0.10	0.01	0.13	0.17	0.35	0.12	0.52	0.55	0.56	0.43	0.24	0.06	**0.000**
5.12	271(m); 280(s); 340	739.2092	739.2070(100%) 593.1518(20%) 575.1350(8%) 284.0318(35%) 151.0011(3%)	Triglycosylated Kaempferol (IX) *(1 hexose + 2 deoxyhexoses)*	3.33	0.65	0.18	0.69	1.13	2.84	2.54	3.22	3.61	3.68	2.77	1.37	0.34	**0.000**
5.83	271(m); 350	739.2092	739.1898(15%) 593.1498(35%) 431.0985(100%) 285.0270(30%)	Diglycosylated Kaempferol (III) *(1 hexose + 1 deoxyhexose)*	0.66	0.15	0.00	0.21	0.26	0.64	0.48	0.75	0.87	0.86	0.72	0.40	0.04	**<0.0001**
6.25	nd	447.0930	nd	Monoglycosylated Kaempferol (I) *(1 hexose)*	0.00	0.00	0.01	0.00	0.00	0.02	0.10	0.00	0.00	0.00	0.00	0.00	0.00	**<0.0001**
6.70	nd	447.0927	nd	Monoglycosylated Kaempferol (II) *(1 hexose)*	0.07	0.00	0.09	0.05	0.08	0.36	0.88	0.17	0.00	0.00	0.13	0.04	0.08	**0.005**
6.74	nd	447.0924	nd	Monoglycosylated Kaempferol (III) *(1 hexose)*	0.00	0.00	0.02	0.03	0.00	0.21	0.00	0.08	0.27	0.27	0.08	0.00	0.00	0.253
10.52	nd	285.0400	nd	Kaempferol	0.00	0.00	0.12	0.00	0.00	0.04	0.14	0.00	0.00	0.00	0.00	0.00	0.00	**0.032**

2.97	nd	755.2040	nd	Triglycosylated Quercetin (I) *(1 hexose + 2 deoxyhexoses)*	0.16	0.00	0.01	0.00	0.03	0.00	0.00	0.03	0.02	0.04	0.06	0.10	0.00	**0.040**
3.01	nd	755.2036	nd	Triglycosylated Quercetin (II) *(1 hexose + 2 deoxyhexoses)*	0.17	0.02	0.03	0.02	0.03	0.01	0.00	0.04	0.01	0.00	0.06	0.08	0.01	**0.000**
4.02	271(m); 280(s); 340	771.1991	771.1946(5%) 609.1459(100%) 463.0828(10%) 301.0328(25%) 300.0259(5%)	Triglycosylated Quercetin (III) *(2 hexoses + 1 deoxyhexose)*	0.39	0.07	0.03	0.09	0.11	0.24	0.04	0.30	0.33	0.31	0.28	0.14	0.02	**<0.0001**
4.20	278(m); 330	609.1465	463.0844(20%) 447.1008(45%) 446.0828(100%) 301.0315(55%) 299.0180(35%)	Diglycosylated Quercetin (I) *(1 hexose + 1 deoxyhexose)*	0.08	0.01	0.06	0.02	0.09	0.18	0.14	0.27	0.19	0.11	0.17	0.14	0.06	0.451
4.27	nd	609.1460	nd	Diglycosylated Quercetin (II) *(1 hexose + 1 deoxyhexose)*	0.64	0.09	0.00	0.10	0.07	0.31	0.26	0.12	0.12	0.25	0.17	0.21	0.03	**0.004**
4.37	271(m); 345	755.2039	755.2020(100%) 300.0262(40%)	Triglycosylated Quercetin (IV) *(1 hexose + 2 deoxyhexoses)*	0.31	0.05	0.02	0.04	0.08	0.21	0.16	0.27	0.29	0.29	0.22	0.08	0.00	**<0.0001**
4.80	nd	755.2040	nd	Triglycosylated Quercetin (V) *(1 hexose + 2 deoxyhexoses)*	0.14	0.01	0.00	0.00	0.01	0.02	0.01	0.03	0.02	0.01	0.05	0.06	0.00	**0.000**
5.05	nd	755.2036	nd	Triglycosylated Quercetin (VI) *(1 hexose + 2 deoxyhexoses)*	0.09	0.02	0.00	0.02	0.02	0.03	0.01	0.07	0.08	0.08	0.06	0.04	0.00	**0.001**

5.38	270(m); 350	609.1465	nd	Diglycosylated Quercetin (III) *(1 hexose + 1 deoxyhexose)*	0.16	0.03	0.05	0.09	0.07	0.49	0.63	0.22	0.23	0.22	0.15	0.03	0.00	**<0.0001**
Hydroxycinnamic acids																		
2.92	nd	207.0658	nd	Caffeic acid ethyl ester	0.37	0.08	0.10	0.07	0.08	0.27	0.24	0.29	0.32	0.28	0.27	0.16	0.04	**<0.0001**
4.73	nd	193.0500	nd	Ferulic acid	0.09	0.00	0.00	0.01	0.00	0.36	0.31	0.22	0.14	0.23	0.00	0.26	0.05	**<0.0001**
Saponins																		
13.14	nd	941.5113	941.5112(100%) 923.4975(5%)	Saponin Bb	2.99	1.90	2.01	2.12	5.60	7.27	8.35	11.28	17.83	15.32	19.08	11.95	2.93	**<0.0001**
14.19	292	1067.5410	1067.5121(100%) 1049.5405(3%)	Soyasaponin β	13.78	0.57	0.00	0.93	0.04	6.05	4.00	3.41k	2.17	3.40	0.00	0.00	0.00	**<0.0001**

Note: Significant differences are indicated by * (α=0.05) from three way ANOVA using $pH_{process}$, $T_{process}$ and $t_{process}$ as the factors.
% values for each fragment detected represent relative intensity of the ion signals during MS analysis
Different isomeric forms are denoted by roman numerals I, II, III, etc.
For UV spectra, m: maximum height of peak, s: shoulder
nd – not detected (absent or inferior to the limit of detection)

Saponins were also detected in the different fava concentrates of this study : soyasaponin β and saponin Bb (**Table 20**). Saponins in fava beans can range from 3.5 to 63 g/kg d.b., depending on the type of cultivar and on the type of ingredient and ingredient processing history [43], [58], [325], thus reinstating the importance of detecting and monitoring saponins levels in fava bean ingredients. Just as in this study, different saponin forms including soyasaponin β and saponin Bb have been previously identified in different varieties of fava bean [111], [176], [340].

Saponins also have a multivalent character just like the phenolic acids, but in different aspects. Particularly, higher content of saponins in ingredients is associated to beneficial effect in lowering plasma cholesterol levels [341], [342]. Saponins, thus, also show nutritional effects, but are often associated with high bitterness impacting acceptability of plant-based foods [5], [19].

Differences are found in the phenolic compounds and saponins profiles in the different ingredients (**Table 20**), induced by process conditions during fava concentrate modification. Thus, it was necessary to look closely into special trends in the effects of $pH_{process}$, $T_{process}$ and $t_{process}$ on these non-volatile compounds, and to look into the zoomed-in effects of $T_{process}$ and $t_{process}$ at a simpler processing type which required no pH adjustment ($pH_{process}$ 6.4). At first, the phenolic compounds were grouped by their chemical families, to see their effects due to the process conditions, followed by a more detailed analysis of individual detected molecules by Principal Component Analysis (PCA). A similar approach was used for saponins.

VI.2.3.2. Effect of Process Conditions on Phenolic Compounds from Fava Beans

Three-way ANOVA on the different phenolic compounds across all the ingredients tested, i.e. FBIC *vs* the modified concentrates, presented significant differences in 34 phenolic compounds (**Table 20**), including 3/3 flavan-3-ols, 7/8 flavones, 22/26 flavonols and 2/2 hydroxycinnamic acids ($p \leq 0.05$). Changes in chemical profile of phenolic compounds were observed due to processing at different $pH_{process}$, $T_{process}$ and $t_{process}$ (**Figure 28**). First and foremost, the effect of $pH_{process}$ was prominent, causing significant phenolic modifications ($p \leq 0.05$). Ingredient modification at acidic $pH_{process}$ (2 and 4) resulted in the decrease of all phenolic families compared to the FBIC. Alkaline processing, i.e. modification at $pH_{process}$ 11, also led to a decrease with respect to the FBIC (**Figure 28**). Thus, in brief, both acidic and alkaline conditions of ingredient modifications decreased overall phenolics contents. Following this observation, ingredient modification without pH adjustment ($pH_{process}$ 6.4) decreased the phenolics contents, but to a lower extent compared to the acidic/alkaline processes. This predominant effect of pH in the stability of phenolic compounds has been studied for various plant sources [343]–[345]. The effect of lower (pH < 4) and higher pH

(pH > 7) has been shown to cause changes in phenoxide ions, impacting the resonance stabilization states of certain phenolic compounds including catechin, epigallocatechin, ferulic acid and caffeic acid [343], [345]. Acidic pH has shown to be less aggressive for certain phenolics, e.g. hydroxycinnamic acids [344]. In this study, however, a significant decrease was seen in the hydroxycinnamic acids ($p \leq 0.05$). Alkaline treatment has also shown to form cross-linking between peptides and polyphenols, thereby decreasing their extractability [346], which could explain lower contents in the samples of this study produced at pH 11.

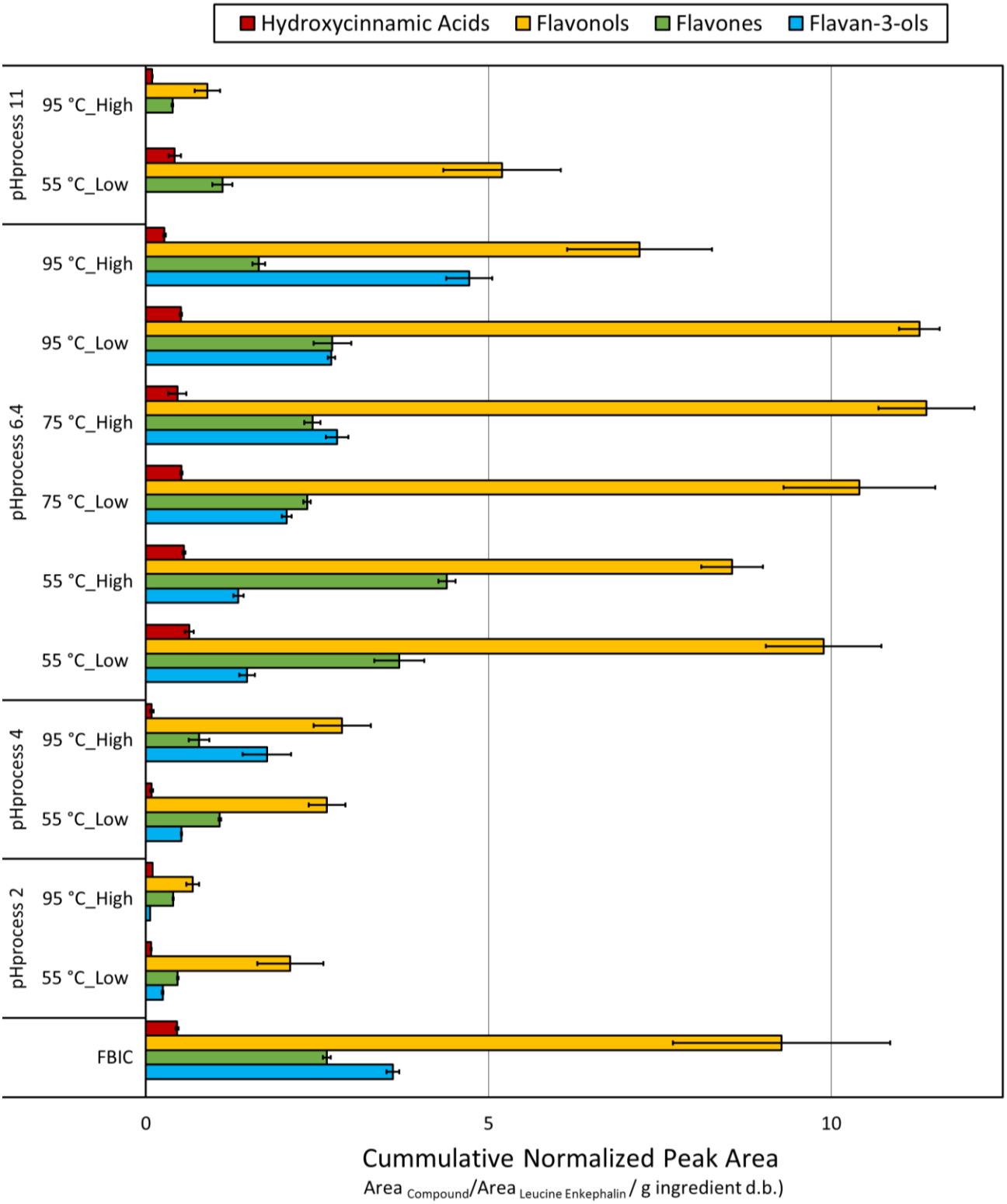

Figure 28 – Processing effect on phenolic compounds from fava beans ingredients. Cumulative normalized peak areas (Peak Area $_{Compound}$/ Peak Area $_{Standard}$/ g ingredient d.b.) of different phenolic families including flavan-3-ols, flavones, flavonols and hydroxycinnamic acids. These compounds were analyzed by UHPLC-PDA-QToF-MS from hydro-alcoholic extracts obtained from fava bean initial concentrate (FBIC) and the same modified by different process conditions, i.e. pH ($pH_{process}$), temperature ($T_{process}$) and treatment duration ($t_{process}$).

Along with the predominant effect on phenolics content due to the $pH_{process}$, it must be noted that the degree of processing within each $pH_{process}$ (from 55 °C_Low to 95 °C_High, with intermediates at 75°C for $pH_{process}$ 6.4), also influenced the level to which these evolutions occurred. Compared to the initial concentrate (FBIC), all phenolic families decreased in the modified ingredients produced at the highest degree of processing (95°C_High), whatever the $pH_{process}$, except flavan-3-ols at $pH_{process}$ 6.4 which slightly increased (**Figure 28**). This decrease effect was particularly pronounced at $pH_{process}$ 2 and 11. Under intermediate conditions, at $pH_{process}$ 6.4, some compounds also slightly increased compared to FBIC: flavones at 55°C_Low and 55°C_High, and flavonols at 75°C_low, 75°C_High and 95°C_Low. Thus, the trends shown within each phenolic family, suggest the complexity of changes in phenolic compounds with process conditions. It could be a combination between a better extraction of the compounds due to an evolution of the matrices from which they are extracted (more or less denatured ingredients) and a degradation of the molecules under the process conditions. Indeed, similar increases in the levels of glycosylated apigenin and hydroxycinnamic acids (ferulic and *p*-coumaric acids) have already been reported when cereals and fruits were processed at 80 °C, owing to a higher release of these compounds from the thermally denatured matrices [22], [347]. But at the same time, flavonols, flavones and flavan-3-ols were shown to be sensitive to treatments above 80 °C in fruits and vegetables [140]. Thus, this study could be completed by investigations on the interplay between the rupture of ingredient matrices and the loss of phenolic compounds with temperature – a scope for future study.

VI.2.3.3. Effect of Process Conditions on Saponins from Fava Bean Ingredients

Two noteworthy saponins, soyasaponin β and saponin Bb, were detected in the FBIC and in certain modified ingredients (**Table 20**), but with different normalized peak area (**Figure 29**). Soyasaponin β differs from saponin Bb by an additional presence of a 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) conjugation at C_{22} in the molecule, that imparts enhanced bitterness [48]. They have been identified previously in fava bean, along with chickpea, soy and common bean [175], [176].

Three-way ANOVA, as in the case of phenolic compounds, showed significant differences of process conditions in the saponin forms detected across different fava bean concentrates ($p \leq 0.05$) (**Figure 29**). Taking the Soyasaponin β which is DDMP-conjugated, there was a considerable decrease after ingredient modification compared to the FBIC, whereas for saponin Bb which is DDMP-lacking, there was either a notable increase or a decrease, depending on the $pH_{process}$ and the extent of processing ($T_{process}$ + $t_{process}$). Soyasaponin β signals were efficiently decreased compared to the FBIC at $pH_{process}$ 2 (96-100%), $pH_{process}$ 4 (93-100%), $pH_{process}$ 6.4 (56-100%) and $pH_{process}$ 11 (100%) as seen in **Figure 29**. This result

was similar to that of phenolic compounds (**Figure 28**) where both modification at acidic and alkaline conditions were more notable than the modifications without pH adjustment. Within each $pH_{process}$, the effect of the extent of processing (from 55 °C_Low to 95 °C_High) was very clearly observed for Soyasaponin β (**Figure 29**). Saponin Bb signals, on the other hand, varied differently depending on the $pH_{process}$ and the extent of processing ($T_{process}$ + $t_{process}$) (**Figure 29**). Taking $pH_{process}$ 6.4 at different levels of $T_{process}$ and $t_{process}$, soyasaponin β markedly decreased, whereas saponin Bb considerably increased with higher degree of processing (**Figure 29**). As compared to the clear changes observed in soyasaponin β with processing, saponin Bb changed in an ambiguous manner and showed higher dependence to the type of process conditions (**Figure 29**). Soyasaponin β has been reported to readily release its DDMP moiety to form saponin Bb and maltol with enzymatic, acid- and alkaline-mediated and thermal processing [47], [48], [58], [348]. In a study comparing neutral and alkaline pH, this conversion was the highest at pH 10.5 compared to pH 8 [348]. Higher pH would help in better matrix disruption and lower saponin-protein interactions, releasing more saponins from soy [348], [349]. The effect of temperature on saponins is somewhat similar when compared to the phenolic compounds seen earlier. This means that with higher degree of processing, especially $T_{process}$, both saponins conversion or decomposition, but also extractability, needs to be accounted for [279], [348], [350]. Processing at higher temperatures changes matrix, reduces viscosity, increases diffusion rate and creates favorable conditions for the transfer and reaction of components [348], [350], [351]. Total saponin levels have been found to increase with temperature until 60 °C, where the highest extractability was noted due to the disruption of plant matrices, followed by an eventual decrease in their levels from 60 to 80 °C to due saponin hydrolysis and oxidation [350]. In addition to this, saponins may also interact with peptides through hydrophobic interaction, hydrogen bonding or ionic bonding, thus altering their levels of extraction [349].

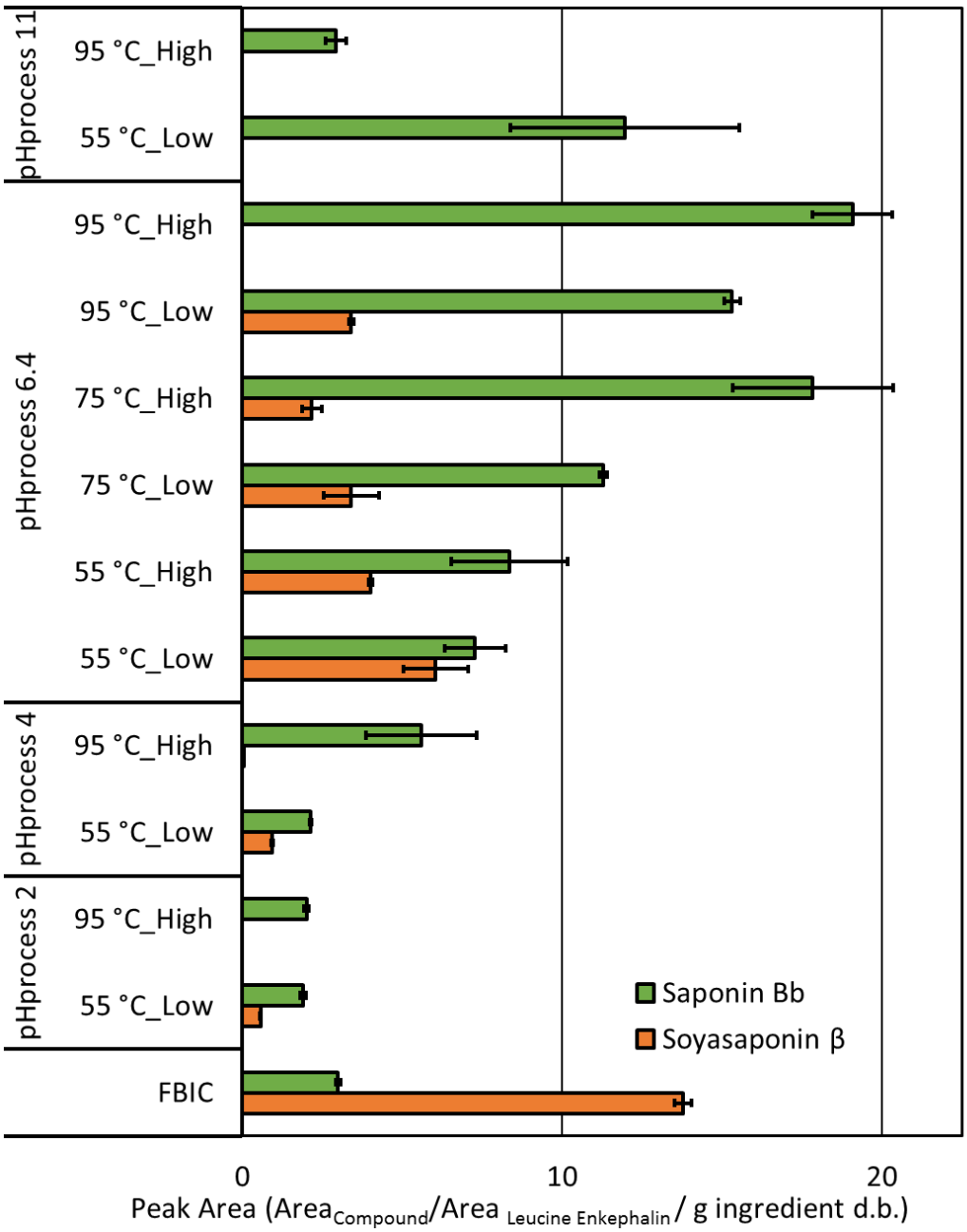

Figure 29 – Processing effect on saponins from fava bean ingredients. Cumulative normalized peak areas (Peak Area $_{Compound}$/ Peak Area $_{Standard}$/ g ingredient d.b.) of the saponins detected in different fava bean ingredients. These compounds were analyzed by UHPLC-PDA-QToF-MS from hydro-alcoholic extracts obtained from fava bean initial concentrate (FBIC) and the same modified by different process conditions, i.e. pH ($pH_{process}$), temperature ($T_{process}$) and treatment duration ($t_{process}$).

VI.2.3.4. Global Effect of Process Conditions on Individual Phenolic Compounds & Saponins from Fava Bean Ingredients

Analysis of different phenolic compounds and saponins described above gave insights on the type of changes that could occur in them due to ingredient modification by the process conditions (**Figure 28** and **Figure 29**). Changes in these non-volatile compounds were substantial for the acidic ($pH_{process}$ 2 and 4) and alkaline ($pH_{process}$ 11) modifications, and less notable for modification without pH-adjustment ($pH_{process}$ 6.4). Despite this clarity, it was difficult to interpret, especially which of the molecules were majorly affected within different groups of phenolic compounds and what kind of process conditions drove these changes.

PCA projections of the different detected non-volatiles, thus, gave a better picture on the variability in the changes of individual molecules as well as the association with trends in the type and degrees of ingredient modifications (**Figure 30** and **Figure 31**). PCA plot of different $pH_{process}$ considered along with the FBIC explained 78% of the variations occurring in the phenolic compounds and saponins (**Figure 30**). The X-axis explains nearly 50% of variance, separating the ingredients modification primarily by the $pH_{process}$. While $pH_{process}$ 2, 4 and 11 were placed towards the left-hand side, the FBIC as well as the $pH_{process}$ 6.4 modified ingredients were placed on the other side of the plot. This meant that the alkaline and acid processes were the most away from the FBIC and the non-pH adjusted modifications were closer to the FBIC by virtue of the variations in these detected molecules. Most of the molecules (especially the glycosylated ones) were positively correlated to either the FBIC or the $pH_{process}$ 6.4 (**Figure 30**). It is important to note that only two molecules including diglycosylated luteolin (IV) and kaempferol were the most linearly and positively correlated to the acid and alkaline mediated modifications. Negative association of these processes are with all the rest of the molecules – which are nearly all glycosylated. This gives a suggestion of possible (partial) deglycosylation of phenolic compounds due to processing at acidic and alkaline conditions. Variations in solubility in extracts due to structural changes in phenolic compounds could be a reason why most of the deglycosylated products after acidic and alkaline processes were not retrieved [21], [42], [352]. Other flavonoids occur in glycosylated forms in nature, where the associated sugar moieties include glucose, rhamnose, galactose, arabinose, xylose [42], [353]. Processing generally results in the hydrolysis of flavonoids, leading to their aglycone forms. Higher association of acidic and alkaline processes with kaempferol supports this hypothesis (**Figure 30**). Aglycone forms are bioavailable for their intestinal absorption, and thus deglycosylation of flavonoids is associated with nutrition and health [42], [353]. In nature, hydroxycinnamic acids including caffeic and ferulic acids exist rarely in free form, as they are mostly found in raw foods as bound forms. Caffeic acid can exist in both free or esterified forms in fruits [42]. Some molecules like catechins might be more stable to acidic treatments [354]. Another observation was that all the vigorously

modifications (at 95 °C_High) lied more or less on the X-axis and thus were the least explained by the PCA plot, whereas the milder modifications (at 55 °C_Low) were relatively well separated by the Y-axis explaining nearly 28% of the variance (**Figure 30**). As previously indicated, vigorous modifications, i.e. at 95 °C_High in all $pH_{process}$ conditions, showed a considerable change in the level of phenolic compounds and saponins (**Figure 28** and **Figure 29**). Therefore, there was a clear indication that at milder conditions of processing (here 55 °C_Low), more differences in the phenolics and saponins profiles could be anticipated. As the fava concentrate is a protein-rich ingredient (65% w/w d.b.), protein - phenolic compounds interactions are hypothesized to have taken place during modifications of the initial concentrate [322]. Acidic conditions have been earlier reported to cause protein hydrolysis in pulse ingredients [258], [306], [355]. Formation of smaller peptides, and higher accessibility of free amino acid residues, would favor reversible and/ or non-reversible interactions with the phenolic compounds, thus lowering their extractability [346], [356]. Alkaline conditions (> pH 8) are also known to cause protein denaturation and conformational changes [28], [262], [322]. Apart from the effects of pH, fava proteins denature at temperatures above 75 °C, and completely at 95 °C, where processes beyond 10 minutes of treatment can further cause protein denaturation [24]. Protein denaturation eventually causes unfolding of the structure and exposure of previously buried amino acid residues into the system. Interactions between proteins and gallic, ferulic and caffeoylquinic acids, as well as with glycosylated quercetins, apigenins and catechin derivatives, are popularly known to be complex and depend on many conditions including pH, temperature, protein concentration and ionic strength [346]. Anti-nutritional behavior by protein indigestibility is also associated with protein interactions with these molecules – thus impacting bioavailability and extractability of the molecules in the systems [75], [291], [346]. Saponin-protein binding, along with inhibition of digestive enzymes, have been reported to cause anti-nutritional effects [3], [5], [357]. From the different changes observed in individual molecules, and certain links between these molecules and their multivalent properties, a hypothesis could be drawn that the antioxidant, taste (bitterness and astringency), color and certain anti-nutritional properties could have all considerably changed due to alkaline and acid mediated ingredient modifications. Thus, investigation of these type of interactions are highly encouraged for further understanding of the fate of such phenolic compounds and saponins.

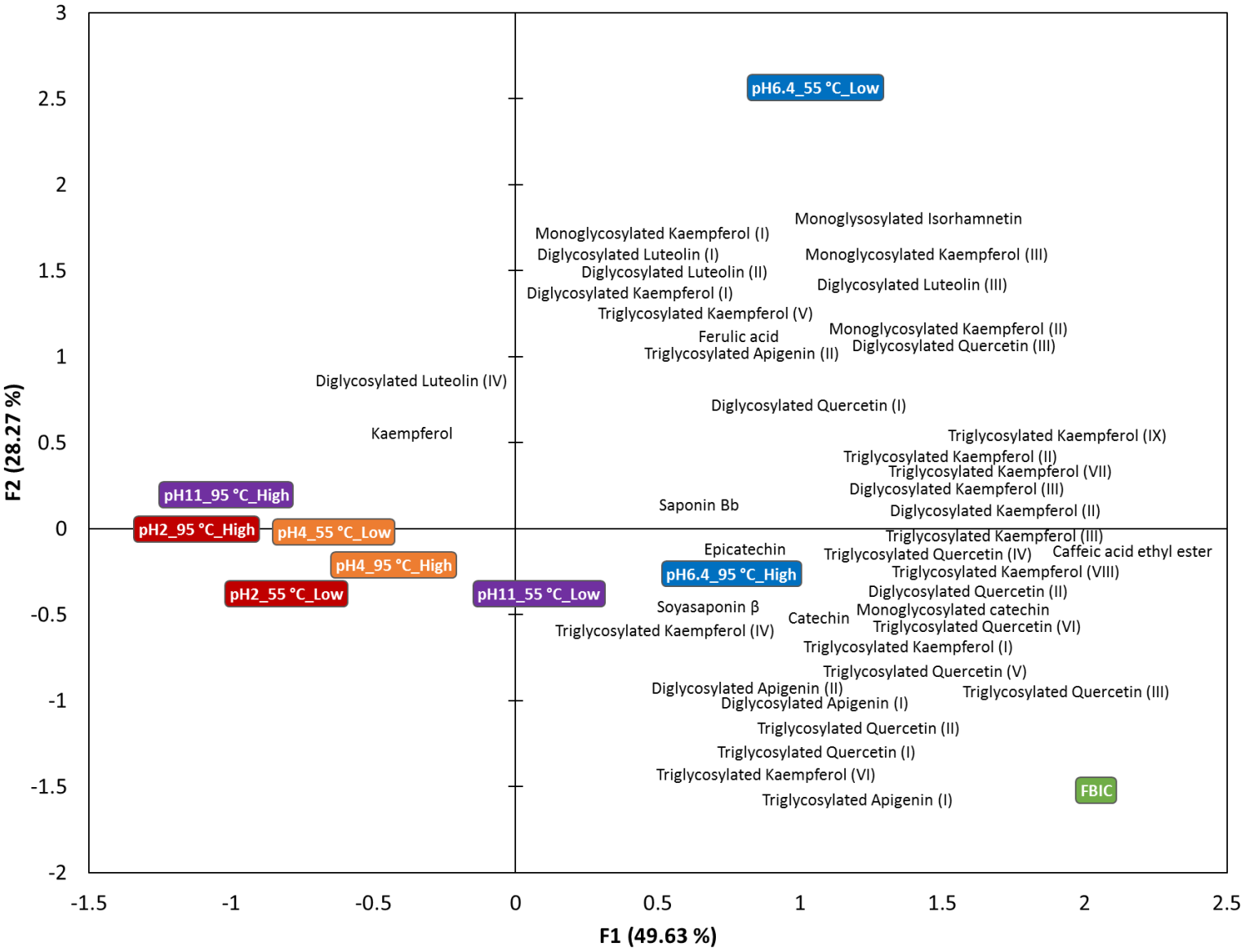

Figure 30 – Phenolic compounds and saponins evolution at different process conditions in fava bean ingredients. PCA projections of peak signal variations between the non-volatiles detected by UHPLC-PDA-QToF-MS. The points on the biplot represent ingredient hydro-alcoholic extracts and the non-volatile cluster labels are rearranged around their points for better visualization.

For fava ingredient processing at $pH_{process}$ 6.4, corresponding to the natural pH of the initial concentrate in suspension, changes at different $T_{process}$ and $t_{process}$ were analyzed through PCA (**Figure 31**). About 75% of the variances were recorded by this PCA plot within the different processes. In the case of **Figure 30**, the $pH_{process}$ 6.4 seemed much closer to the FBIC compared to other $pH_{process}$ modifications. Analysis of different $T_{process}$ and $t_{process}$ levels within $pH_{process}$ 6.4 gave a rather magnified view of the changes in phenolics and saponins profiles from the FBIC (**Figure 31**).

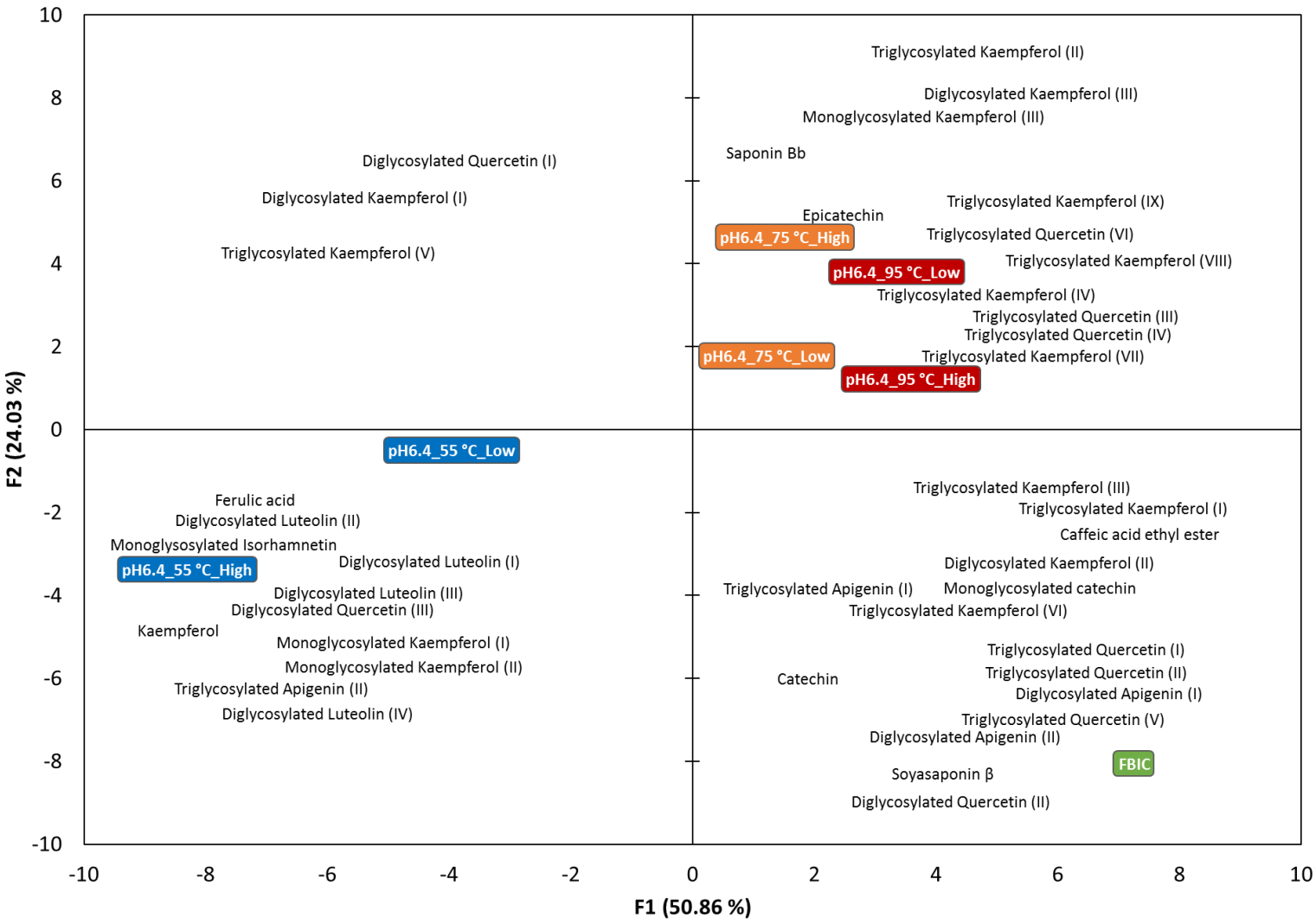

Figure 31 – Phenolic compounds and saponins evolution at $pH_{process}$ 6.4 in fava bean ingredients. PCA projections of peak signal variations between the non-volatiles detected by UHPLC-PDA-QToF-MS. The points on the biplot represent ingredient hydro-alcoholic extracts and the non-volatile cluster labels are rearranged around their points for better visualization.

A much more comprehensive outlook of the phenolic compounds and saponins changes was noted, where the ingredients were separated according to the degree of processing. While the FBIC lied on the lower right quadrant, modifications at 55°C lied on the lower left quadrant and the higher degrees of processing, i.e modifications at 75 and 95 °C, were on the upper right quadrant. The FBIC was positively correlated to caffeic acid ethyl ester, soyasaponin β, along with several di- and tri-glycosylated kaempferols, quercetins and apigenins (**Figure 31**). Interestingly, ferulic acid and saponin Bb, as well as many isomers of luteolin, kaempferol, apigenin, quercetin, epicatechin and catechin, were not associated (positively/ negatively) to the FBIC, but to the other rather milder and vigorous ingredient

modifications. Once again, the question of extractability, dissociation of matrix and degradation of molecules comes into play, as discussed before. The positive association of 55 °C modification with aglycone kaempferol or monoglycosylated luteolin, isorhamnetin, quercetin, kaempferol and apigenin, followed by a negative association with many triglycosylated kaempferols and quercetins, suggests indication of lower extractability of their higher glycosylated derivatives at these conditions. Whereas for the vigorous processes, i.e. at 75 and 95 °C modifications, there might have been possible dissociation of the above-mentioned monoglycosylated forms, but also improved extractability of the several other mono-, di- and tri- glycosylated molecules by rupturing their matrices (**Figure 31**). Different molecules in their various glycosylated forms have variable extractabilities to a process condition – therefore indicating why there is a heterogeneous trend observed in the association between different molecules and the degrees of process conditions [41], [42], [228].

Amongst the saponins, higher degree of $T_{process}$ and $t_{process}$ clearly increased saponin Bb peak signals as seen in the raw data (**Figure 29**). Once again, the interplay between conversion of soyasaponin β to saponin Bb and their extractability play a role in different ingredients, as seen in the two figures (**Figure 28** and **Figure 29**). The varied proportions of saponins, either with or without the DDMP moiety, could suggest likelihood of taste modifications (bitterness and astringency) of these ingredients during food applications [5], [19], [57], [75].

As clear trends between many different kinds of phenolics and saponins were noted, differences in the taste, color and anti-nutritional profiles at $pH_{process}$ 6.4 as a function of $T_{process}$ and $t_{process}$ could also very much be anticipated and therefore interesting to look into in further studies.

VI.2.4. Conclusion

Fava bean concentrate is a complex ingredient composed of macro- and micro-components. Important non-volatile micro-components, including flavan-3-ols, flavones, flavonols, hydroxycinnamic acids as well as saponins, were studied in different modified fava bean concentrates. They are diverse and multivalent in nature, and thus are important determinants of antioxidant potential, taste (bitterness and astringency), color and even antinutritional effects. Many isomeric forms of these molecules, either aglycone of glycosylated (mono-, di- or tri-) by different sugar moieties, were detected from hydro-alcoholic extracts of different fava concentrates modified by pH, temperature and treatment duration. Process conditions, predominantly the pH during ingredient modification ($pH_{process}$), had significant impact on most of the detected non-volatile molecules. Acidic and alkaline processing (pH 2, 4 and 11) were highly distinct compared to the non-pH adjusted

process (pH 6.4) in changing the phenolics and saponins profiles of the ingredients. When looked closely at non-pH adjusted processes, variability in the detected molecules due to increasing degree of processing seemed to be either a function of their variable extractability and their degradation reactions. Changes in phenolic compounds and saponins are complex in nature. Therefore, just by looking into the chemical profiles, possibilities of their multivalent properties, i.e. better bioavailability, higher antioxidant activity, changes in taste, color or even anti-nutritional limitations, cannot be directly extrapolated. Detection and quantification of molecules by UHPLC-PDA-QToF-MS is certainly promising in this regard. But then, this study urges investigations on the changes in taste, color and anti-nutritional factors of fava ingredients, as well as studies using different other types of extraction methods, to have a more comprehensive outlook of the non-volatile profiles of fava ingredients. Studies of this type can help get insights on the impact of processing on ingredient acceptability with an understanding of the associated chemical changes – therefore get closer to knowledge base of suitable fava ingredient processing for industrial food applications.

VI.2.4.1. Acknowledgements

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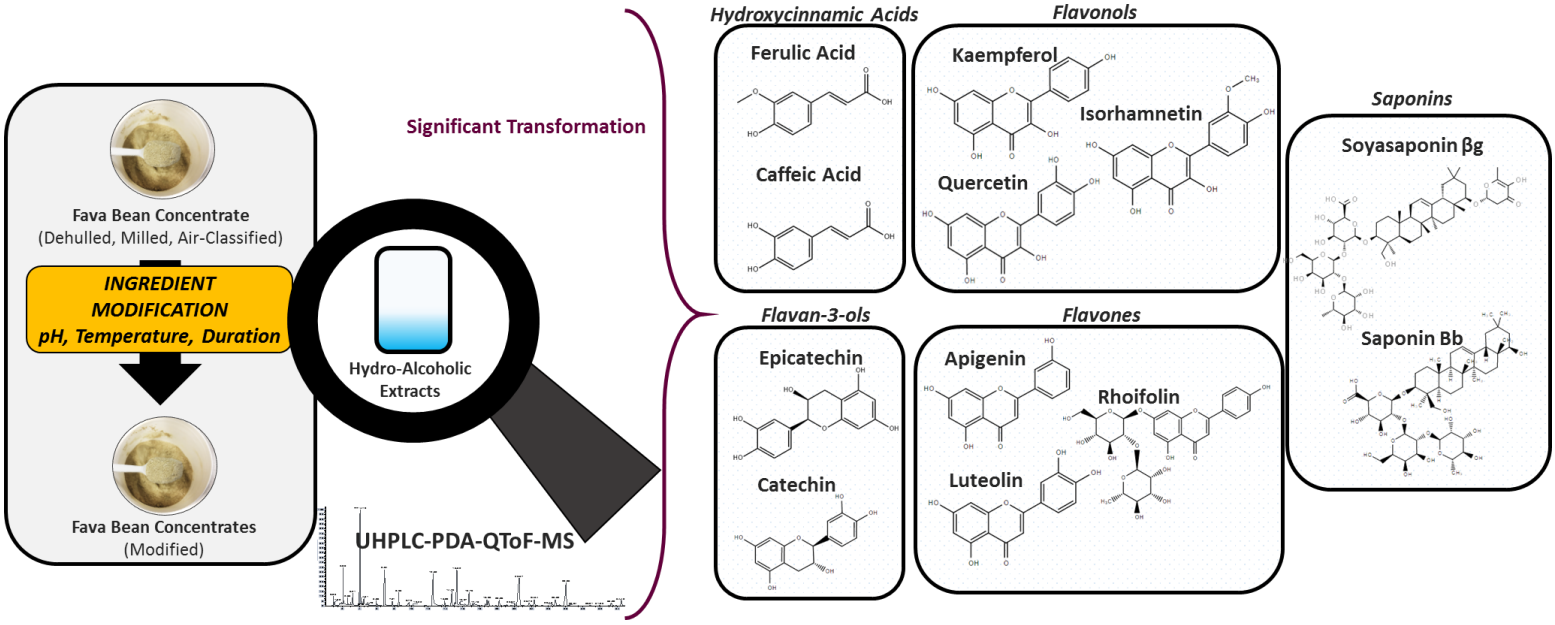
VI.2.4.2. Author Contributions

Conceptualization, S.S., M-N.M., A.S-E.; methodology, validation, formal analysis, investigation and data curation, S.S., E.L-R., G.Z., M-N.M.; resources, J.Z., D.B., J.A.; writing—original draft preparation, S.S.; writing—review and editing, All.; visualization, S.S., E.L-R.; supervision, J.Z., A.S-E. and M-N.M.; project administration, M-N.M., A.S-E., J.Z.; funding acquisition, M-N.M., D.B., J.A. All authors have read and agreed to the published version of the manuscript.

VI.3. Key Highlights



- Non-volatile micro-constituents of fava bean are multivalent in nature, i.e. they comprise different molecules which in turn have different properties influencing flavor, color and nutritional properties.

- 39 phenolic compounds and 2 saponins are detected by UHPLC-PDA-QToF-MS in the hydro-alcoholic extracts obtained from fava bean initial concentrate and the same modified by pH, temperature and treatment duration. These consisted majorly of phenolic compounds, particularly flavonoids (3 flavan-3-ols, 8 flavones and 26 flavonols) and phenolic acids (2 hydroxycinnamic acids). Additionally, two different forms of saponins - soyasaponin β and saponin Bb - were detected in the different ingredient extracts. Ingredient processing in these conditions significantly transformed phenolic compounds and saponins profiles in fava bean extracts.

- pH effect was predominant during ingredient processing with large impact on most of the detected molecules. Both acidic (pH 2 and 4) and alkaline (pH 11) conditions of ingredient modifications decreased overall phenolics contents, whereas ingredient modification without pH adjustment (pH 6.4) decreased the phenolics contents, but to a lower extent compared to the acidic/alkaline processes.

- Most of the glycosylated phenolic compounds were present in higher concentrations in the native concentrate compared to the modified ingredients. There was a complexity of changes in phenolic compounds with the degree of processing by temperature and process duration. Changes could be a combination between a better extraction of the compounds due to an evolution of the matrices from which they are extracted (more or

less denatured ingredients) and a degradation of the molecules under the process conditions.

- Amongst saponins, soyasaponin β (known for its bitterness) was predominant in the native concentrate whereas saponin Bb (lower bitterness) became higher in the modified ingredients, especially in the ones modified without any pH adjustment. Both modification at acidic and alkaline conditions were more notable than the modifications without pH adjustment. The effect of temperature on saponins was again complex, meaning that with higher degree of processing, especially $T_{process}$, both saponin conversion or decomposition, but also extractability, needs to be accounted for.

- Transformation of both phenolic compounds and saponins due to process conditions indicated possible changes in flavor, color, antioxidant and even anti-nutritional profile of the ingredients.

VII

Conclusions & Perspectives

VII. Conclusions & Perspectives

VII.1. Overall Conclusions

Fava bean is a sustainable plant-based source of proteins which presents a high potential in nutritional and functional properties for industrial food applications. There are various ways for producing and modifying industrially relevant ingredients with large consequences on functional and flavor properties. For this PhD work, a minimally processed, i.e. air-classified protein-rich (65% w/w, d.b.) concentrate obtained from dehulled and milled fava beans, was used as a starting material. This material was rich in proteins, but also contained other components, i.e. 2% w/w carbohydrates, 3% lipids, more than 17% dietary fibers and nearly 8% ash (w/w, d.b.). Thus it was deemed as an ingredient with a **multi-component composition** with various properties. In this study, this initial concentrate was modified by various industrially relevant process conditions, to produce different ingredients utilized at two different conditions of application. The originality of the PhD work was to have a multi-component approach to better understand the mechanisms at the origin of functional and flavor properties. For that, a **cross-dimensional approach** was undertaken with the final objective to find the right kind of compromise between different properties of ingredients. First, their functional properties (foamability and emulsification) were investigated. These properties were associated with the study of protein reactions during ingredient modification (acid-mediated protein hydrolysis and protein-protein aggregation) and also with protein characteristics during ingredient utilization (solubility, charge, structural folding and thermodynamic integrity) to better understand mechanisms of functional modifications. Furthermore, odor perception of the ingredients was investigated and correlated to the headspace release of volatiles during ingredient utilization in order to investigate the molecules at the origin of key fava odor. To complete the approach with much deeper understanding, a **multi-process approach** was also undertaken, where not only the process conditions during ingredient modification were studied, but also the conditions of application were considered to highlight the major effects and their molecular origin. Additionally, changes in non-volatile compounds were examined for selected processing combinations to anticipate changes in taste, color, but also certain potential antioxidant and anti-nutritional properties.

To summarize and conclude the study, process conditions were able to drive functional and flavor properties of the fava bean concentrate. Modification of fava bean concentrate resulted in acid mediated protein hydrolysis and protein aggregation. Although certain trends were observed in foam and emulsion properties, their effects were predominantly governed by the pH during ingredient utilization. In general, utilization pH around the isoelectric point of fava proteins (pH 4) was not suitable for foam stability, emulsion capacity nor emulsion stability. Protein acid-hydrolysis improved foaming only at pH 7, but had an

unclear trend regarding emulsification. Aggregation did not improve foaming, but retained emulsion stability at neutral pH. Ingredient modification conditions were not particularly mirrored in the physico-chemical properties, and the properties once again depended largely on the pH of utilization. Results on functional and physico-chemical properties were strengthened by statistical models which facilitated a rapid comprehension of the large data set. Strong correlations between functional and physico-chemical properties were identified and explained by protein properties. There might have been non-protein associated reactions that could have impacted functional and physico-chemical properties, as seen from correlations with fluorescence signals detected in the non-protein region.

In addition to functional properties, flavor was interestingly driven heavily by the modification and utilization conditions, especially by the pH. Initially, the unmodified concentrate was perceived with strong green notes, but this evolved into more cooked notes with the extent and type of processing – mainly governed by the pH during concentrate's modification. This odor evolution will prove interesting depending on the targeted food applications. The utilization pH was also important in driving odor perception, especially for the unmodified concentrate which also showed drastic changes in volatile groups. Ingredient modification reduced this high impact of utilization pH to a certain extent. From gentler to vigorous process conditions, perception can be modified from more green to more cooked flavors, whereas different conditions of application (e.g. pH) can modulate between "sweet" or rancid perceptions. Considering headspace volatiles, numerous aldehydes were primarily detected in ingredient suspensions headspace. But furanoids, terpenoids, alcohols and ketones had the next higher contribution for modifications at pH 2, 4, 6.4 and 11 respectively. Lipid oxidation was deemed primary in generating volatiles, along with other reactions including proteins, sugars and carotenoids degradation. Going deeper into understanding of other elements of flavor, important determinants of antioxidant potential, taste (bitterness and astringency), color and even anti-nutritional effects were investigated. Phenolic compounds (flavan-3-ols, flavones, flavonols, hydroxycinnamic acids) and saponins were significantly impacted by process conditions (especially pH) during ingredient modification. For phenolic compounds, acidic and alkaline conditions (pH 2, 4 and 11) were highly distinct compared to the non-pH adjusted process (pH 6.4) in changing the phenolics and saponins profiles of the ingredients. When looked closely at non-pH adjusted processes, their variability due to increasing degree of processing seemed to be either a function of their variable extractability and/ or reactions involving their structural rearrangement. The study of non-volatiles compounds was the beginning of a quest to understand changes in their different properties further in detail, such as taste perception.

The context of this research work can be used and extended to many kinds of studies related to plant-based ingredients and their applications in foods. The most interesting perspectives

have been outlined into a rather application-oriented and scientific oriented perspectives dealing with questions and concerns related to food industries and food science respectively.

VII.2. Applicative Perspective – An Example of Vegan Cappuccino

An example of a plant-based cappuccino can be now taken to understand how far this PhD work could be applied for industrial beverage applications. This plant-based or vegan cappuccino ideally is a stable, colloidal dispersion formed from pulse or cereal sources – preferable an emulsion which can give a foam. Fava bean concentrate could be added to a cereal- or nut-extracted dispersion to functionalize the drink, or could be used to make a drink all by itself too. In both the cases, understanding of functional and flavor properties would be essential to know how process conditions can deliver the right ingredient to be used for this type of drink. To study what type of fava bean ingredient can be used for cappuccino drink, it must be noted that the analyses made here are just perspectives and a deeper analysis into sensory and functional parameters is needed to really establish and concretize the following information. First, from the present studies, it could be noted that the impact of the process conditions on the odor properties of the ingredients was much more diverse than the impact on functional properties. Therefore, a compromise here would be made by the functional property in order to accommodate flavor profile, in agreement with consumer expectations and the target of population. For this, flavor needs to be analyzed first. In the literature, cappuccino follows the similar lexicon as coffee drinks, with an additional incorporation of milk [358]. Popular flavor (aroma and taste) attributes chosen by the World Coffee Research for coffee beverages are woody, roasted, sour (taste), sweet (taste), sweet aromatics, green, bitter, nutty, fruity and floral; along with many secondary and tertiary levels of coffee attributes [271], [359]. Comparing this with the sensory odor analysis done for different fava bean ingredients in **Chapter V**, one could notice that woody (wood?), roasted (smoky?, burnt?, cooked?), sweet aromatics (sweet?), green (green?), nutty (hazelnut?, almond?), fruity (banana?) and floral (orange blossom?) notes were similar to the odor attributes significantly, characteristics of the studied ingredients (**Figure 32**). Modeling study could be performed to combine odor and processing condition. Qualitative approach could also be used, by placing the target odor notes on the odor map constructed for different fava bean concentrates. Looking at the odor map, various odor perception were distributed in all quadrants except for the quadrant containing rancid perception. This means that no matter the ingredient modification type, it would be the best to avoid acidic application conditions ($pH_{utilization}$ 4) as it would drive a more rancid flavor. The application at neutral pH ($pH_{utilization}$ 7) would help drive a sweeter aroma perception with many similar odor notes such as hazelnut, almond, banana, coffee and wood (**Figure 32**). Of course, these are just indications and one would have to utilize fava ingredients at exact realistic conditions

and perform sensory analysis to be able to determine which ingredient type will be the most appropriate.

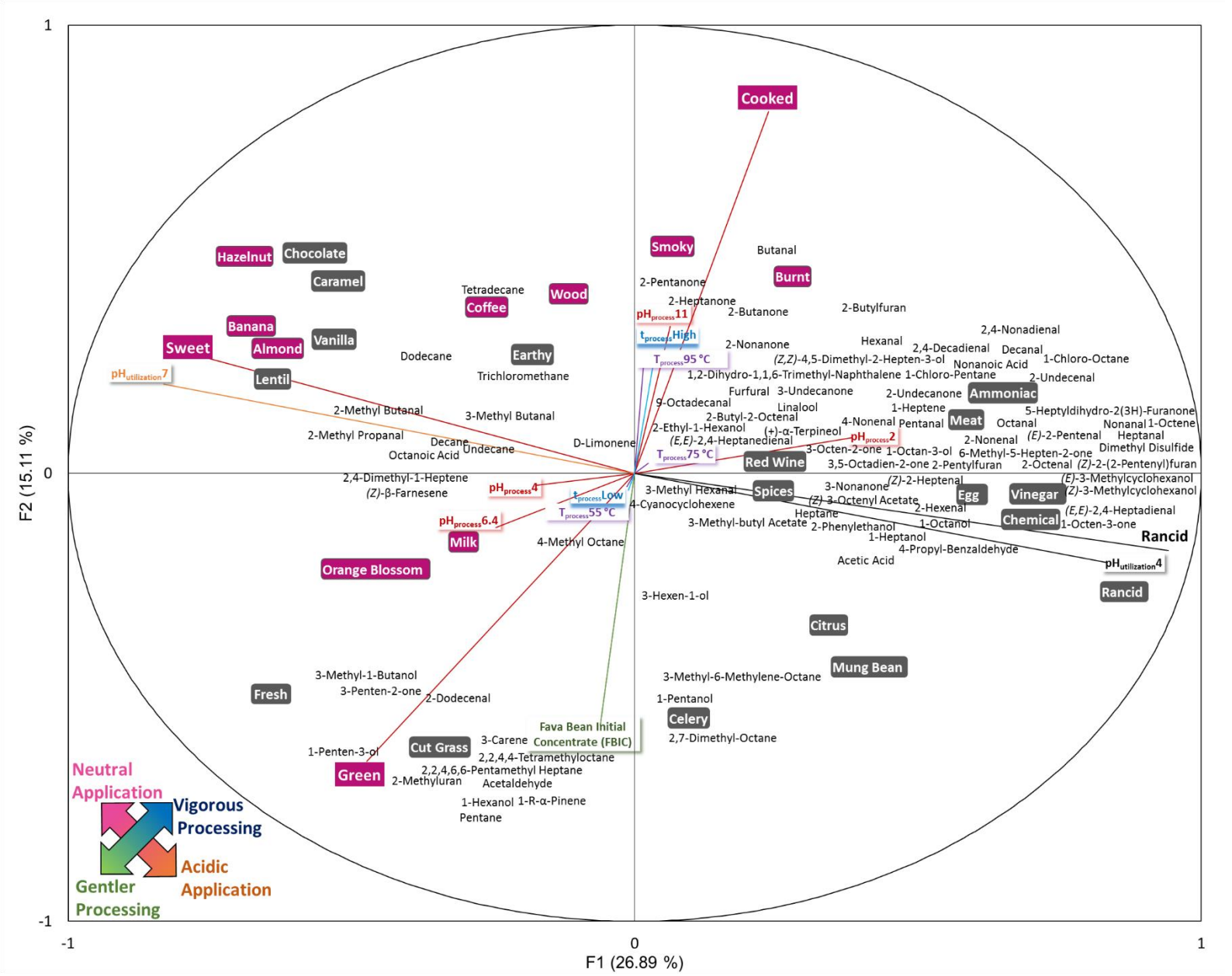


Figure 32. Perspectives for Aroma in Plant-Based Cappuccino. Overview of process conditions driving odor attributes of different fava bean ingredients modified by process conditions. Plant-based cappuccino perspectives are visualized here based on theoretical attributes (in pink) of coffee beverages (**Chapter V**).

Now, moving on to the taste profile, sweetness and bitterness were also important perception defining coffee/ cappuccino beverage. Depending on the information on phenolic compounds and saponins, one could anticipate differences in bitterness and sweetness in the ingredient and subsequent applications. Just as the model used for odor profile, a similar approach with sensory taste profiling and its chemistry could be done as a future perspective.

Apart from the flavor properties, functional properties are also essential for cappuccino owing to colloidal stability (e.g. foam and emulsion capacity and stability). From the information obtained from **Chapter IV**, one could concretely say that utilization pH had a predominant impact on the different properties, and that at neutral pH ($pH_{utilization}$ 7), most of the properties were favored (**Figure 33**). Foam capacity and stability remained superior for all ingredients. Emulsions were successfully formed for all ingredients, but emulsions produced from ingredients processed at $pH_{process}$ 2 were unstable. Applications at neutral pH therefore seem the most promising from both flavor (odor) and functional (foam and emulsion) indications. Amongst modification conditions, $pH_{process}$ 2 may not be ideal if emulsion stability is important for the application, whereas other process combinations may be tested for their applicability. Looking back at the odor profiles, $pH_{process}$ 2 trended more towards meat, ammoniac and chemical attributes – not necessary for cappuccino anyway (**Figure 32**).

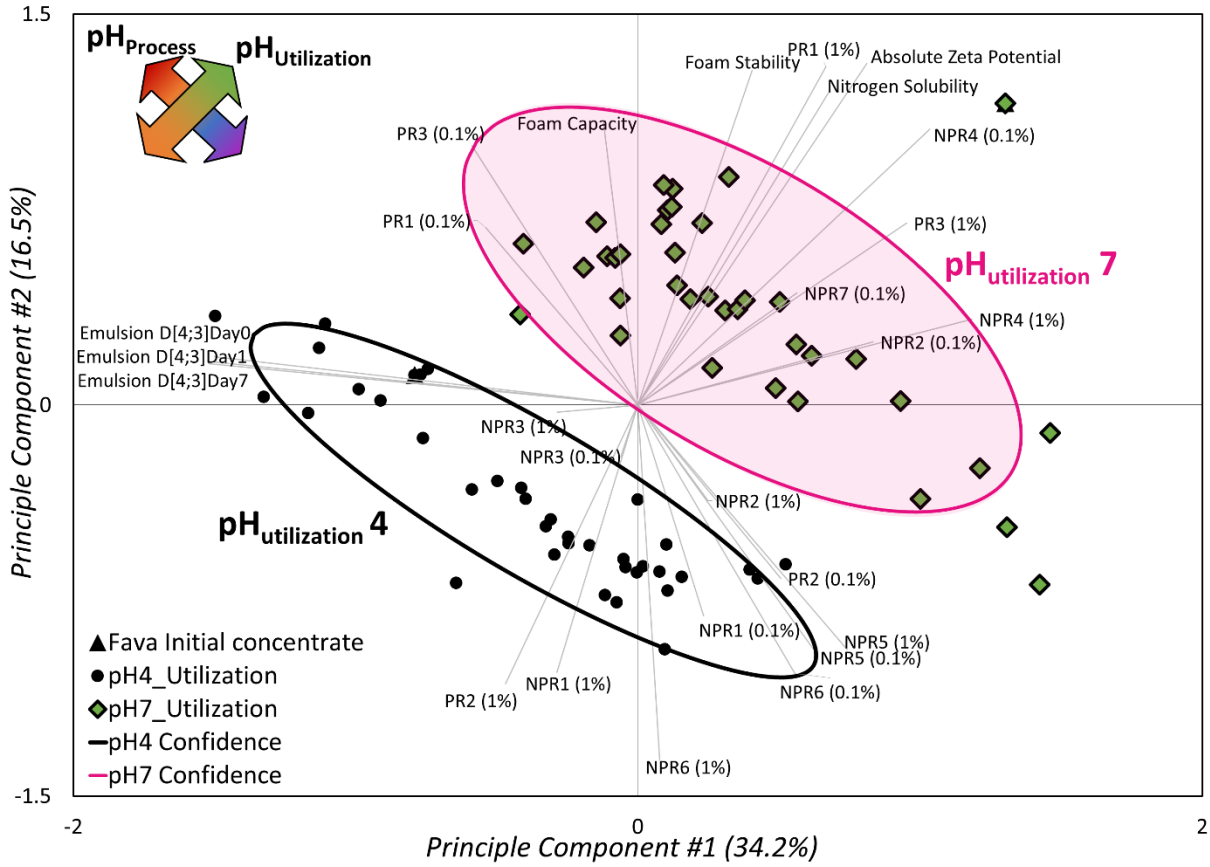


Figure 33. Perspectives for Functionality in Plant-Based Cappuccino. Overview of process conditions driving functional properties of different fava bean ingredients modified by process conditions. Plant-based cappuccino foam and emulsion perspectives are visualized here for successful coffee beverages (**Chapter IV**).

Going beyond the knowledge of functional and odor properties, it is also essential to evaluate consumer acceptance and preference of the ingredient suspensions. Acceptance

and preference are important factors determining food choice, and understanding the drivers of liking is key to developing a "good" food product [360]–[362]. There are many methods to evaluate consumer acceptance and preference and to determine the sensory properties which can explain the liking, such as preference-mapping approaches [361]. As the approach of the PhD work was more process conditions driving functional and flavor properties, it could be interesting to study what functional and flavor properties are expected by the consumers. Thus this would allow a more targeted approach to study what functional and flavor properties does one need to drive to, and for what kind of products – so that the consumers actually like those products.

A cappuccino application is a rather simpler matrix that has been imagined in this scenario. Different other applications of fava bean concentrates using different liquid or solid matrices can be imagined, and would be interesting to study. Amongst liquid matrices are plant-based mayonnaise, spreads, mousses, drinks or yoghurts, that would have to be tested, and also certain solid matrices, including cheese, pasta, cakes, where fava bean ingredients can act as both nutritional and functional agents.

The use of a cross-dimensional (functional and flavor properties) could be extended to other important properties governing ingredient acceptability – functional properties (e.g. gelation), sensory properties (e.g. color, taste, texture), nutritional and health characteristics (essential amino acid profile, antioxidant profile, anti-nutritional factors) – to have a much bigger and comprehensive knowledge on each ingredient, and therefore a more realistic compromise for industrial food applications. Further on, the multi process approach (ingredient modification and utilization) could be extended by other processing steps, including bean variety sourcing, bean pre-treatment, dehulling necessity, protein extraction alternatives (flours/ concentrates/ isolates), etc. Thus in terms of application perspectives, there lies many different possibilities to explore.

VII.3. Scientific Perspectives

VII.3.1.1. Starting Material

To analyze the materials that were used in this PhD approach, other alternatives and reasoning could be developed. To start with, the initial concentrate was studied in this work, which is quite relevant in the industry and also which has been shown to have superior foam, emulsion and gelation properties compared to other types of ingredients like flours and isolates [214]. However, one need not be limited to a specific kind of ingredient and other alternatives such as flours could be interesting as they are much more close to the raw material in the process flow chain. But just as the concentrate, flours may enact more of a multi-component character in the different functional and flavor properties [119]. In addition, flours are also minimally processed and thus an interesting matrix to study. Isolates,

on the other hand, are away from the raw material in the supply chain, thus demand more energy for production, but may show less of a multi-component character as they contain > 90% w/w proteins d.b. [126]. It is important to note that perhaps, the multi-component nature of the fava concentrate was able to produce different ingredients with such a diversity of odor attributes, and this may be different in the case of other food component proportions in flours and isolates.

VII.3.1.2. Process Conditions

Process conditions of pH, temperature and process duration were used to modify the fava bean initial concentrate. New perspectives could include either variations within each type of process conditions or a completely novel process conditions that has not yet been tested in this study. For example, pH changes were done using hydrochloric acid and/ or sodium hydroxide. It could be interesting to check if using alternative acids (acetic acid, citric acid, etc.) or bases (potassium hydroxide, magnesium hydroxide, etc.) could impact functionalities in a similar way. Alternate ways of heating could also be imagined, where conventional water bath heat treatment would be replaced by either sub-critical water treatment, microwave-induced heat treatment or pasteurization at high or ultra-high temperatures. Drying method could also be imagined to include heating before/ during drying e.g. in the case of spray drying. The treatment durations at time points between 30 and 360 min could be tested, where further kinetics of different proteins- and flavor-associated reactions could be monitored in detail. Apart from these conditions and their levels or modes, novel conditions employing for instance high pressure treatment or ultrasound intensity treatment could be studied as well. In addition to the process conditions, more complex aqueous systems involving salts, polysaccharides, lecithin, etc., would need to be checked to have a more realistic idea of how functional properties would be influenced in matrices closer to applications.

VII.3.1.3. Nutrition, Functionality & Flavor

In terms of the different studies performed for evaluation of functional and flavor properties, a few more analytical elements could be used to have a more complete understanding. One could look deeper into foam and emulsion microstructures to characterize not only their macroscopic properties but also their microscopic textures. One could look even deeper into the size of the air droplets in foams to further investigate and characterize the forms. Analyzing surface tension properties of the foams and emulsions also give a good indication of how their stability could be. For the analyses of proteins, gel permeation chromatography could be employed for indications of tertiary structures of the proteins in their soluble states, and perhaps their soluble, supra-molecular aggregated states as well. Further on, for flavor properties, odor perception correlating volatile chemistry could be completed by using gas chromatography coupled with olfactometry analysis to determine the active components in

the ingredient suspension headspace. On top of this, the key aroma volatiles could then be quantified using their deuterated standards, and their relative peak signals compared to other compounds to be seen to evaluate which volatiles are generated and released in predominant quantities. As flavor is completed with odor as well as taste, it would be crucial to evaluate sensory taste parameters of the ingredient suspensions to know how bitterness, sweetness and astringency are driven by the process conditions. This would be complementary to the non-volatile analysis performed on the different ingredients. Last but most importantly, it is of utmost priority of the industry and academia to provide safe and healthy food solutions. Safety of the ingredients thus would need to be evaluated imperatively – especially regarding the anti-nutritional factors which are optimistically reduced during processing (according to literature). Using different types and combination of ingredients in such multicomponent matrices also leads to formation of novel ingredients, and thus their potential safety and health impacts on consumers would need to be investigated and confirmed with the safety standards. Then, the introduction of such plant-based food in the diet involves to further study the plant-based diets in association with health benefits (e.g. lower blood pressure, cholesterol, blood glycaemia and reduced body weight).

VII.3.1.4. Sustainability

Concerning the first point made on the rise in population and the need to have a sustainable global transition it is vital for the future food systems to guarantee food security and health without compromising on economic, social and environmental aspects around these food systems [1], [61], [363]. The concerns of climate change are growing and its effects are appalling [63], [364]. Life cycle analysis of different types of foods have shown to be important in evaluating their entire supply chain history concerning greenhouse gas emissions [1], [363]. In addition to this type of analysis, energy efficiency calculations and global partition of energy resources would help establish energy inputs utilized by patterns of consumption of a particular population and see where modifications can be made. For instance, consumption pattern changes towards a more sustainable diet can cause upto 4-fold decrease in the energy input [63]. Thus, for ingredients derived from fava bean, the entire supply chain investigation would need to be done to assess how sustainable it is to produce fava beans and to use its ingredients – in comparison with both animal or plant-based sources. Such a study has not been done yet for fava bean ingredients. However, indications are reported of this crop being an ergonomically sustainable – and therefore one could be optimistic about the outcome from these type of analyses for fava bean ingredients [3], [5].

Thus, this work opens a wide horizon of different possibilities of approaches and investigations that are essential for plant-based food applications. This study has gained its

originality by initiating different types of approaches and methods to give industrially relevant, scientific solutions for plant-based ingredients. Future studies of this manner would help the food industry face the global food transformation to feed all the 820 million people with sustainable, healthy and good quality foods.

VIII

References

VIII. References

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Titre : Compréhension des mécanismes à l'origine des propriétés fonctionnelles et de la flaveur d'ingrédients riches en protéines issus de féveroles

Mots Clés : Végétale, fonctionnalisation, procédés, mousse, émulsion, hydrolyse des protéines, agrégation des protéines, arome, composés volatiles, composés phénoliques, saponines

Résumé : Afin de rendre les régimes alimentaires occidentaux plus durables, un changement d'alimentation s'impose. Parmi les sources végétales prometteuses, la fèverole (*Vicia faba* L.) peut être transformées en ingrédients, qui peuvent être modifiées, fonctionnalisées, afin d'être plus adaptées à des applications alimentaires. Ce travail de thèse avait pour objectif de comprendre le rôle des conditions de transformation des ingrédients riches en protéines de fèveroles sur leurs propriétés fonctionnelles et leur flaveur. L'impact des conditions de transformation, choisies pour être réalistes sur le plan industriel, a été étudié en utilisant une approche multi-dimensionnelle et trouver ainsi un compromis favorable à l'expression des propriétés des différents ingrédients. Plus précisément, un concentrât de fèveroles riche en protéines, traité selon un procédé de transformation doux à l'échelle industrielle, a été sélectionné puis modifié par différentes conditions de transformation, i.e. le pH (2, 4, 6,4 et 11), la température (55, 75 et 95 °C) et la durée du traitement (30 et 360 min). Trente-six ingrédients différents ont ainsi été produits. Ceux-ci ont ensuite été utilisés à deux pH différents, 4 et 7, dans des systèmes modèles proches d'applications de type boissons. Au cours de l'utilisation de ces ingrédients, la fonctionnalité des boissons (propriétés moussantes et émulsifiantes), la perception olfactive des produits et la composition en composés volatils et non volatils ont été étudiées. Les résultats montrent que les conditions de transformation sont capables de moduler les propriétés fonctionnelles et olfactives du concentrât de fèveroles, l'analyse étant renforcée par le biais de différents modèles statistiques. Les propriétés des mousses et des émulsions sont principalement gouvernées par le pH d'utilisation des ingrédients. Un pH proche du point isoélectrique des protéines de fèverole (pH 4) n'est pas favorable ni à la stabilité de la mousse, ni à la capacité d'émulsification ou à la stabilité de l'émulsion.

Par ailleurs, la flaveur est fortement influencée par les conditions de modification et d'utilisation des ingrédients, en particulier par le pH. Selon les modifications du concentrât initial, en conditions douces ou sévères, la perception peut être modifiée, pour évoluer d'odeurs vertes à des odeurs cuites, tandis que les conditions d'utilisation des ingrédients (pH) peuvent conduire à des perceptions « douces » ou rances. L'oxydation des lipides apparaît importante dans la génération de composés volatiles, de même que des réactions de dégradation des protéines, des sucres et des caroténoïdes. Les propriétés physico-chimiques et sensorielles des composés à l'origine du potentiel antioxydant, du goût (amertume et astringence), de la couleur et des effets antinutritionnels ont également été étudiés. Les composés phénoliques (flavan-3-ols, flavones, flavonols, acides hydroxycinnamiques) et les saponines se sont avérés significativement impactés par les conditions de transformation mises en œuvre lors de la modification des ingrédients, en particulier par le pH.
Ainsi, ce travail a montré que l'itinéraire technologique mis en œuvre jouait un rôle important dans la construction des propriétés des ingrédients issus de fèverole. Il ouvre un nouveau champ d'investigation interdisciplinaire, basé sur la nutrition (aspects antioxydants et antinutritionnels), la durabilité (évaluation du cycle de vie), la fonctionnalité (gélification) et les propriétés sensorielles (texture, arôme, amertume) des fèveroles en tant qu'ingrédients potentiels pour des applications alimentaires.

Title : Understanding of the Mechanisms at the Origin of Functional & Flavor Properties in Protein-Rich Fava Bean Ingredients

Keywords: Plant-based, ingredient functionalization, processing, foam, emulsion, protein hydrolysis, protein aggregation, odor perception, volatile compounds, phenolic compounds, saponins

Abstract: The growing population is demanding new healthy, sustainable solutions for foods and beverages. Fava bean (*Vicia faba* L.) is a promising plant source that is processed to form ingredients and they can be further modified to render them fit for food applications. This PhD work aimed to understand the role of processing conditions on functional and flavor properties, and apply this understanding to produce and use fava bean protein-rich ingredients. It investigated the effects of certain industrially relevant process conditions using a cross-dimensional approach to find the right kind of compromise between different ingredients properties. To be precise, a very gently processed fava bean protein rich concentrate was modified by process conditions such as pH (2, 4, 6.4 and 11), temperature (55, 75 and 95 °C) and treatment duration (30 and 360 min) to produce 36 different ingredients. These were further utilized at two pH (4 and 7) in systems close to beverage applications. During ingredient utilization, beverage functionalities (foam and emulsion) along with odor perception and non-volatile compounds were investigated for all ingredients as a function of process conditions.
Results showed that process conditions were able to drive functional and flavor properties of the fava bean concentrate, strengthened by different statistical models. Foam and emulsion properties were predominantly governed by the pH during ingredient utilization.

In general, utilization pH around the isoelectric point of fava proteins (pH 4) was not suitable for foam stability, emulsion capacity nor emulsion stability. In addition, flavor was heavily driven by the modification and utilization conditions, especially the pH. From gentler to vigorous process conditions, perception can be modified from more green to more cooked flavors, whereas different conditions of application (e.g. pH) can modulate between "sweet" and rancid perceptions. Lipid oxidation was deemed important in generating volatiles, along with other reactions including proteins, sugars and carotenoids degradation. Going deeper into understanding of physico-chemical and sensory properties, determinants of antioxidant potential, taste (bitterness and astringency), color and even anti-nutritional effects were also investigated. Phenolic compounds (flavan-3-ols, flavones, flavonols, hydroxycinnamic acids) and saponins were significantly impacted by process conditions during ingredient modification, especially by pH.
This work opens up new arena for inter-disciplinary study based on nutritional (anti-oxidant and anti-nutritional aspects), sustainability (life cycle assessment), functionality (gelation) and sensory (texture, sweetness, bitterness) considerations of fava bean as potential ingredients for industrial food applications.