HYBRIDMEAT - PRODUCTS FROM ANIMAL AND PLANT SOURCES

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I have no special talents. I am only passionately curious.

Albert Einstein

Co-Authors

The scientific work presented was partially conducted in cooperation with other scientists. Prof. Dr. Jochen Weiss supervised the complete doctoral thesis as project leader and contributed substantially to the conception and interpretation of this work. Dr. Monika Gibis was involved as project coordinator in experimental planning, writing, and proof-reading of all manuscripts. Dr. Nino Terjung supported in conception and was involved in project administration and funding acquisition.

- **Chapter I:** Sandra Ebert designed the studies, conducted the experiments, interpreted results, and wrote the manuscript.
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List of Publications

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Oral presentations

Ebert, S. (2020). Developing hybrid meat products based on technological and technofunctional boundary conditions. Scientific presentation at the *SHIFT20 Virtual Experience,* Online

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Symbols and Abbreviations

Symbol	Definition	Unit
a^*	Green-red color channel	-
A_{KAC}	Peak area of identified volatile compound	min^{-1}
A_{Peak}	Peak area of integrated peak in sel. interval	min^{-1}
AUC	Area under the curve	pH*(mmol H+/kg)
AWL	Average weight loss	%
a_w	Water activity	-
b^*	Blue-yellow color channel	-
BC	Buffering capacity	mmol H^+/(kg*pH)
F	Nitrogen to protein conversion factor	-
L^*	Lightness value	-
pH	Potential of hydrogen	-
pI	Isoelectric point	-
R^2	Coefficient of determination	-
RH	Relative humidity	%
RI	Retention index	-
RWL	Relative weight loss	%
S_0	Surface hydrophobicity	-
v/v	Volume per volume	ml/ml
w/v	Weight per volume	g/ml
w/w	Weight per weight	g/g
Z-Average	Particle size from dynamic light scattering	μm
ΔE	Color differemce	-
ζ-potential	Zeta-potential	mV

Abbreviation	**Definition**
AiF	Arbeitsgemeinschaft industrieller Forschungsvereinigungen
ANOVA	Analysis of variance
BMWi	Bundesministeriums für Wirtschaft und Energie
CIE	Commission Internationale de l'Éclairage
CORNET	Collective research networking
DI-SBSE	Direct immersion stir bar sorptive extraction
e.g.	For example (Latin: exempli gratia)
FEI	Forschungskreis der Ernährungsindustrie e.V.
FTIR	Fourier transform infrared
GDL	Glucono-delta-lactone/Glucono-δ-lactone
GC	Gas chromatography
ICP-OES	Inductively coupled plasma optical emission spectrometry
i.e.	That is to say (Latin: id est)
IGF	Industrielle gemeinschaftsforschung
LC-MS	Liquid chromatography-mass spectrometry
MUFA	Monounsaturated fatty acids
MS	Mass spectroscopy
n. a.	Not available
n. d.	Not determined
n. i.	Not integrable
NPN	Non-protein nitrogen
p	Partial
PUFA	Polyunsaturated fatty acids
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFA	Saturated fatty acids
THAM/TRIS	Tris(hydroxymethyl)aminomethane
TVP	Texturized vegetable protein/Dry texturized (plant) protein
WTP	Wet texturized (plant) protein

Summary

Consumer diversification and concerns about insufficient protein supply and global malnutrition demand for an exploitation of alternative protein sources such as plant proteins. While manufacturers have made substantial progress in industrially-scaled extraction processes and structuring of plant proteins *e.g.* by extrusion, there is still a lack of information on their fundamental functional and organoleptic properties and interactions with other ingredients in traditional formulations. As a result, food product developers are facing a lot of challenges and are often forced to base their work on trial-and-error rather than mechanistically-guided approaches. This is in particular the case for foods where complex raw material requirements and production processes make the manufacture of products with high acceptance and shelf-stability not trivial. This includes the design of hybrid meat products that are composed of mixtures of meat and plant proteins. There, traditional meat products are often set as a benchmark, making the performance of such mixed products mostly unsatisfactory. Establishing composition-material property-functionality relationships may be a first step to overcome these obstacles. Therefore, a variety of plant proteins was assessed for their composition, physicochemical properties, and techno-functionalities to gain an understanding of their suitability for the formulation of hybrid meat products. This included their dispersibility, the miscibility of select plant protein fractions with solubilized meat proteins at varying pH and mixing ratios, and the characterization of their odor-active compounds. The latter included powdered as well as extruded plant proteins due to their increasing relevance in the manufacture of hybrid meat and analogue products. Following this, plant proteins were screened in terms of their performance in hybrid meat formulations and during traditional manufacture with a special focus on dry-cured products in order to define feasible protein sources and application thresholds.

The first part of this thesis showed that aqueous solubility, native pH, and appearance of a variety of 26 plant protein powders from carbohydrate and vegetable oil production correlated with purity and the extraction process. Solubility ranged from as low as 4 % to as high as 100 % based on the protein concentration and prevalence of select protein fractions. For example, large amounts of prolamins (wheat) or glutelins (rice, pumpkin) resulted in low values, while high shares of albumins and globulins promoted moderate to high solubility in sunflower, pea, and potato proteins. A highly soluble (100 %) small molecular weight fraction (< 24 kDa) of the latter was subsequently screened for its particle size and electrostatic and hydrophobic properties as compared to solubilized water- and salt-soluble meat proteins and the miscibility

of both proteins was assessed at pH 3.0 to 7.0 and at select mixing ratios. Phase behavior of mixtures started to change below the isoelectric point (p*I*) of salt-soluble meat proteins (pH ~ 5.5), which was identified as a defining boundary value. Here, one-phase/co-soluble systems (pH > p*I*) transitioned to two-phased/aggregated ones mediated by interactions (pH ≤ p*I*) in between individual meat and meat and potato proteins. This resulted in dense, irregularly shaped meat-potato heteroprotein particles, that deviated from the characteristic assembly of pure meat proteins into regular, anisotropic aggregates. A perturbing effect of potato proteins on the structural, organized association of meat proteins below their p*I* was found. Protein-protein interactions were based on both electrostatics and hydrophobics as shown by variations in surface charge, hydrophobicity, and particle size if sole potato/meat and mixtures were compared. For example, particle size of solubilized meat proteins increased from 18.0 ± 2.9 μm (pH 3.0) to 26.8 ± 9.0 μm (pH 3.0) in 50:50 mixtures. FTIR results confirmed alterations as a function of mixing ratio and pH. Image analysis of microstructures revealed a shift from elongated regular networks towards more disorder and irregularity along with a lower degree of branching. Besides solubility, organoleptic properties influence the suitability of plant proteins as food ingredients. Therefore, odor-active compounds of two pea isolates were analyzed by gas chromatography-mass spectrometry-olfactometry (GC-MS-O) after direct immersion stir bar sorptive extraction (DI-SBSE), and results were compared to those of their respective extrudates to define changes during dry and wet extrusion. Twenty-four odor-active compounds were found, whereof nine represented major (off-) flavor contributors in peas: hexanal, nonanal, 2-undecanone, (*E*)-2-octenal, (*E, Z*)-3,5-octadiene-2-one, (*E, E*)-2,4-decadienal, 2-pentyl-furan, 2-pentyl-pyridine, and γ-nonalactone. The quantity of these nine volatiles was affected distinctively by extrusion. Hexanal was reduced from 3.29 ± 1.05 % (Isolate I) to 0.52 ± 0.02 % (Wet Extrudate I) and (*E,Z*)-3,5-Octadiene-2-one and (*E,E*)-2,4-decadienal decreased by 1.5- and 1.8-fold when powdered and dry texturized pea proteins were compared. As a result of the perturbing effect of soluble potato proteins and the higher amount of off-flavors in pea isolates compared to their extrudates, use of plant powders as additives was rejected in favor of extruded ones for all subsequent studies. As the focus of this work was the development of dry-cured hybrid meat products, the effect of various amounts of extrudates on the traditional formulation and manufacture of this product class was assessed. This included the susceptibility of extrudates towards acid-induced pH-changes as compared to pork meat, as well as their behavior in a traditional acidification and drying processes. To that purpose, pork meat and six wet extrudates from peas, pumpkin, or sunflower seeds were analyzed in their proximate composition and subjected to titration starting from the same

pH-value and using the same acid concentrations. It was shown that wet texturized pumpkin and sunflower proteins had the highest buffering capacity (BC), especially between pH 7.0 and pH 4.5, while pea protein extrudates and pork meat were more prone to acidification and similar in buffering capacity with an average of 881 ± 5 mmol H^+/(kg*ΔpH). The obtained data was then used to relate BC with the compositional elements of extrudates such as minerals, proteins, select amino acid, and non–protein nitrogen. These findings on varying susceptibility towards acids were extended by studies on a minced meat model systems containing pork meat, curing salt, and various amounts (0 to 100 wt%) of wet extrudates and the chemical acidifier Glucono-delta-lactone (GDL). It was shown, that increasing concentrations of plant extrudates resulted in a linear increase of the initial (pH_{0h}), intermediate (pH_{6h}), and final (pH_{48h}) pH of minced meat model systems. A sufficient acidification to common target pH-values in dry-cured meat products (pH ~ 5.0) could be achieved with acidifier amounts of 1.0 wt% up at no more than 15 wt% of extrudates. A mathematical model was proposed to correlate pH, time, acidifier, extrudate concentration, and plant protein origin to aid in the adjustments of formulations at higher extrudate contents, and to describe thresholds of feasible extrudate and acidifier concentrations. The calculated concentrations were then implemented to manufacture dry-cured hybrid sausages where meat was partially replaced by 12.5, 25, 37.5, and 50 % of pumpkin seed extrudates. All recipes reached the target pH-value with an accuracy of pH 5.0 ± 0.06 thereby validating the proposed mathematical correlations. Hybrid recipes with up to 25 % of extrudates were comparable to the traditional all-meat formulation in both the drying behavior and the distribution of moisture and free water. However, higher meat replacement levels promoted distinct changes in drying behavior and product texture where chewiness, hardness, and cohesiveness decreased by up to 70 %.

In conclusion, plant protein functionality differs profoundly from the one of meat proteins, and this functionality also depends on the respective protein source as well as the applied extraction process. Their structuring by extrusion provides beneficial organoleptic changes and eases their incorporation in hybrid formulations. The fundamental characterization of plant proteins in terms of their proximate composition and (physico)chemical properties may be used to establish mathematical correlations to estimate the effect of these novel ingredients in hybrid meat products. Thus, the obtained results offer a valuable basis that manufacturers can draw upon not only to create new foods within this product class but also to broaden and facilitate the application of plant proteins on a large scale.

Zusammenfassung

Die Verfügbarkeit von Pflanzenproteinen muss weiter ausgebaut werden, um sich einerseits der zunehmenden Verbraucherdiversifizierung im Lebensmittelbereich anzupassen und andererseits den Problemen der Rohstoffverknappung und des Welthungers entgegenzutreten. Obwohl viele Industrieprozesse zur Extraktion und Strukturierung von Pflanzenproteinen in den letzten Jahren entwickelt wurden, fehlt es immer noch an Kenntnissen über die grundlegenden funktionellen und sensorischen Eigenschaften pflanzlicher Proteine und deren Wechselwirkungen mit anderen Inhaltsstoffen. Dies erschwert eine mechanistisch getriebene Produktentwicklung, vor allem bei Produkten mit komplexen Rohstoffanforderungen und Produktionsprozessen. Infolgedessen werden nach wie vor viele Produktentwicklungen als kostspielige und zeitraubende Trial-and-Error Versuche konzipiert. Dies schließt die Kategorie der Hybridfleischprodukte mit ein, die aus Mischungen tierischer und pflanzlicher Rohstoffe bestehen. Derzeit ist die Akzeptanz derartiger Produkte noch ungenügend, da die qualitativen Attribute von traditionellen Fleisch- und Wurstwaren oft zum Vergleich herangezogen werden. Um zu einem systematischeren Ansatz beim Design von Hybridprodukten zu kommen und qualitativ hochwertigere Hybridlebensmittel herzustellen, ist die Etablierung eines mechanistischen Zusammenhangs zwischen Rohstoffzusammensetzung, physikochemischen Eigenschaften der darin enthaltenen Pflanzenproteine und deren Technofunktionalität notwendig. Im Zuge dieser Dissertation wurden aus diesem Grund eine große Anzahl an Pflanzenproteinen auf ihre funktionellen Eigenschaften und Eignung in Hybridfleischprodukten untersucht. Dies umfasste ihre Löslichkeit und Mischbarkeit mit gelösten Fleischproteinen in Abhängigkeit von pH und Mischungsverhältnis, die Charakterisierung geruchsaktiver Komponenten ausgewählter Pflanzenproteine, deren Veränderung durch Nass- und Trockentexturierung und schließlich die Bestimmung der Produkteigenschaften hybrider Modellformulierungen mit Rohwurstcharakter.

Im ersten Teil dieser Arbeit wurde gezeigt, dass Löslichkeit, der native pH-Wert, die Farbe und das Erscheinungsbild von Pflanzenproteinen mit ihrer Aufreinigung und dem zugehörigen Extraktionsprozess korrelieren. Hierzu wurden 26 Pflanzenproteinpräparate, die aus der Herstellung von Kohlenhydraten oder Pflanzenölen gewonnen wurden, verwendet. Die Löslichkeit lag zwischen 4 % und 100 % basierend auf der Proteinkonzentration und dem Vorkommen bestimmter Proteinklassen in den Präparaten. So wurde bei einem hohen Anteil an Prolaminen (Weizen) oder Glutelinen (Reis, Kürbis) eine schlechte Löslichkeit festgestellt, wohingegen Sonnenblumen-, Erbsen- und Kartoffelproteine durch die darin enthaltenen

Albumine und Globuline gut bis sehr gut löslich waren. Eine Kartoffelproteinfraktion mit niedrigem Molekulargewicht (< 24 kDa) wurde aufgrund ihrer Löslichkeit von 100 % näher untersucht. Die Partikelgröße und die elektrostatischen und hydrophoben Oberflächeneigenschaften wurden bestimmt und mit Mischungen aus wasser- und salzlöslichen Fleischproteinen zwischen pH 3.0 und 7.0 und bei verschiedenen Mischungsverhältnissen verglichen. Der isoelektrische Punkt (p*I*) der salzlöslichen Fleischproteine (pH ~ 5.5) stellte einen wichtigen Grenzwert für das Phasenverhalten der Mischungen dar, da an diesem Punkt (pH > p*I*) die isotropen Lösungen in einen zwei-phasigen, aggregierten Zustand (pH ≤ p*I*) übergingen. Es entstanden dichte, unregelmäßig geformte „Heteroprotein" Partikel, die sich in ihrer Morphologie wesentlich von der für Fleischproteine typischen anisotropen fasrigen Struktur unterschieden. Dies deutete auf einen Störeffekt der Kartoffelproteine auf die typische Selbstassoziation der Fleischproteinen unterhalb ihres p*I* hin, welcher auf Veränderungen der elektrostatischen (Oberflächenladung) und hydrophoben (Oberflächenhydrophobizität) Protein-Protein Interaktionen zurückgeführt werden konnte. So nahm die Partikelgröße der Fleischproteine im Vergleich zu 50:50 Mischungen von 18.0 ± 2.9 μm (pH 3.0) auf 26.8 ± 9.0 μm (pH 3.0) zu. Eine FTIR Analyse validierte den Zusammenhang der makro- und mikroskopischen Beobachtungen mit Mischungsverhältnis und pH-Wert. Eine Bildanalyse verdeutlichte zudem eine Veränderung der länglichen, vernetzten Fleischprotein-Aggregate hin zu ungeordneten und unregelmäßigen Heteroproteinstrukturen mit geringer Anisotropie.

Da organoleptische Eigenschaften von großer Bedeutung sind, um die Eignung von Pflanzenproteinen in Lebensmitteln abzuschätzen, wurden zwei Erbsenproteinisolate mit Hilfe von Gaschromatographie kombiniert mit Massenspektroskopie und Olfaktometrie (GC-MS-O) untersucht, nachdem sie aus der Lösung an ein Adsorbens gebunden worden waren (DI-SBSE). Die Ergebnisse wurden mit denen der daraus produzierten Trocken- und Nassextrudate verglichen, um den Einfluss des Extrusionsprozesses zu bestimmen. Die Analyse identifizierte 24 geruchsaktive Stoffe von denen neun als wesentliche (Off-) Flavor Komponenten in Erbsen bekannt sind: Hexanal, Nonanal, 2-Undecanon, (*E*)-2-Octenal, (*E, Z*)-3,5-Octadiene-2-on, (*E, E*)-2,4-Decadienal, 2-Pentyl-furan, 2-Pentyl-pyridin, und γ-Nonalacton. Diese neun Substanzen wurden durch die Extrusion merklich beeinflusst. Hexanal verringerte sich von 3.29 ± 1.05 % (Isolat I) bis auf 0.52 ± 0.02 % (Nassextrudat I) und (*E,Z*)-3,5-Octadiene-2-on and (*E,E*)-2,4-Decadienal nahmen um das 1.5- und 1.8-fache ab, wenn die Isolate mit den jeweiligen Trockenextrudaten verglichen wurden.

Die darauffolgenden Studien fokussierten sich – aufgrund des Störeffektes der Kartoffelproteine und dem höheren Gehalt an Off-Flavor Komponenten in Isolaten – auf Extrudate und deren Effekt auf die traditionelle Rezeptur und Herstellung von Produkten mit Rohwurstcharakter. Dies beinhaltete ihre pH-Abhängigkeit und ihr Verhalten während Säuerung- und Trocknung im Vergleich zu tierischen Proteinquellen. Dazu wurden die Zusammensetzung und die Säure-Base Eigenschaften (Pufferkapazität) sechs verschiedener Extrudate aus Erbsen, Kürbis- oder Sonnenblumenkernen bestimmt. Kürbis- und Sonnenblumenextrudate zeigten die höchste Pufferkapazität, vor allem im Bereich zwischen pH 7.0 und 4.5. Erbsenextrudate und Schweinefleisch zeigten geringere, aber ähnliche Werte von 881 ± 5 mmol H^+/(kg*ΔpH). Eine statistische Analyse der Ergebnisse ergab eine Korrelation der Pufferkapazität mit der Extrudat-Zusammensetzung z.B. dem Gehalt an Asche, Proteinen, ausgewählten Aminosäuren und Nicht-Protein Stickstoff. Die gewonnenen Erkenntnisse flossen dann in die nachfolgenden Untersuchungen an Hybridfleischmodellsystemen ein, um so einen Zusammenhang zur Technofunktionlität der Rohstoffe herzustellen. Die Modellsysteme bestanden aus gewolftem Fleisch und Nitritpökelsalzmischungen, denen verschiedene Extrudatkonzentrationen (0 bis 100 wt%) und das chemischen Säuerungsmittel Glucono-delta-lactone (GDL) zugegeben wurden. Steigende Extrudatkonzentrationen führten dabei zu einem linearen Anstieg des pH-Wertes zu Beginn (pH_{0h}), und nach 6 h (pH_{6h}) und 48 h (pH_{48h}) aufgrund der puffernden Wirkung der Proteinextrudate. Typische GDL-Konzentrationen (1.0 wt%) ermöglichten eine pH-Wert Senkung auf den für Rohwürste üblichen Wert von pH ~ 5.0, allerdings nur bis zu einer Extrudatkonzentration von nicht mehr als 15 wt%. Für höhere Extrudatgehalte wurde ein mathematisches Modell entwickelt, welches pH, Zeit, GDL- und Extrudatkonzentration, sowie den Ursprung des Pflanzenproteins korrelierte. Das Modell wurde bei der Herstellung einer Hybridwurst mit Rohwurstcharaketer validiert, in der 12.5, 25, 37.5 und 50 % Fleisch durch Kürbiskern-Extrudate ersetzt wurde. Alle Formulierungen erreichten den gewünschten pH-Wert mit einer Genauigkeit von pH 5.00 ± 0.06. Bis zu einer Fleischreduktion von 25 % waren die Hybride in ihrem Trocknungsverhalten und der Feuchteverteilung mit der rein tierischen Formulierung vergleichbar. Höhere Extrudatkonzentrationen verursachten jedoch merkliche Ungleichverteilungen der Feuchte in den Matrizen und heterogene Produkttexturen.

Zusammenfassend zeigen die Studien dieser Dissertation, dass sich Pflanzenproteine hinsichtlich ihrer Funktionalität nicht nur von tierischen Proteinen, sondern auch untereinander aufgrund der verwendeten Proteinquelle und der darin enthaltenen Proteintypen und dem verwendeten Extraktionsprozess unterscheiden können. Ihre Sensorik kann mit Hilfe von

Extrusion positiv verändert werden, so dass eine Nutzung als Lebensmittelinhaltsstoff erleichtern wird. Eine grundsätzliche Charakterisierung der Zusammensetzung und der physikochemischen Eigenschaften von Pflanzenproteinen ermöglicht es mathematische Korrelationen zu erstellen, die eine Selektion geeigneter Rohstoffe und Konzentrationen für die Herstellung von Hybridprodukten ermöglicht. Die gewonnenen Erkenntnisse könnten so einen wichtigen Beitrag zur Kommerzialisierung von Hybridfleischprodukten leisten, und damit einen breiteren Einsatz von Pflanzenproteinen ermöglichen.

General Introduction and Aim of the Study

The availability for alternative proteins is steadily increasing due to technological progress and the exploitation of new plant sources. Their market value is estimated to reach more than $27 Billion by 2027, thereby supporting the growth of meat alternatives and analogues whose value may be worth around 36 billion US $ by 2027 (Meticulous Research®, 2021; Wunsch, 2020). However, these products possess unique organoleptic properties and key quality attributes such as color, texture, and taste may differ substantially from purely meat-based ones. This limits their acceptance particularly if conscientious meat eaters and flexitarians are envisioned as a target group due to their preference for meat and meat product attributes (Profeta et al., 2021a). The product class of hybrid meats might overcome these challenges since it combines meat proteins with plant-based ones, providing sustainability and health benefits compared to purely meat-based products. The following sections summarize the key drivers for the transition towards plant protein fortified foods, the relevance and properties of hybrid meats, and outlines differences in the organizational, functional, and organoleptic properties of animal- and plant-based proteins. A focus is put on process-related changes and requirements of raw materials and insights into the modulation of plant-protein functionality are provided.

Meat product trends and hybrid meats

The transition from traditional to modern meat consumption

The history of meat consumption is estimated to date back more than 2.5 million years, where societies obtained up to 50 % of their energy intake from animal-based food (Mann, 2018). Besides being a high value protein source, meat delivers important vitamins, minerals, micronutrients, fatty acids, and bioactives, which has enabled the evolution of the human race (Urrego, 2014). Since these ancient times, the emergence of agricultural and crop science as well as increasing knowledge on cooking and processing of food has led to the modern society that we know today. However, the attitude towards meat and meat products has changed considerably within the last decades. This is related to health concerns associated with a high meat consumption *e.g.* higher risks of coronary heart and cardiovascular diseases, as well as concerns around animal welfare and greenhouse gas emissions (González et al., 2020; Mann, 2018; Urrego, 2014). Meanwhile, global consumption levels are still rising, which is also related to the growth of world population that is estimated to reach ten billion people by 2050

(Godfray et al., 2018). This development has implications for the availability and distribution of food. While first world countries have the possibility to choose from an abundance of food resources, less developed ones suffer more and more from malnutrition and hunger. More than 800 million people are considered to be 'food insecure' today (Searchinger et al., 2014). Taken together, it becomes increasingly clear that human diets have to change and alternative proteins sources need to be developed and exploited. There is an opportunity to improve the situation especially if the comparably low feed-to-food conversion ratio of livestock and the high amount of unused food side-streams are considered (Morone et al., 2019; Shepon et al., 2016). For example, the same amount of land used for livestock could be used to produce up to 20-times the amount of legumes and meals, yielding key nutrients such as oil, carbohydrates, and proteins (Chéreau et al., 2016; Nadathur et al., 2016; Tang, 2011).

Driven by these challenges and opportunities, the food and in particular the meat industry is currently investing in the development of innovative, alternative, and more sustainable solutions that appear in supermarkets and complement traditional ones such as seitan, tofu, and tempeh (He et al., 2020). At the same time, a high emotional attachment to meat prevents conscientious consumers to purchase purely plant-based alternatives (Grasso & Jaworska, 2020; He et al., 2020; Profeta et al., 2021b). Because of that, meat hybrids have recently gained interest since they might be a low-threshold option to decrease meat consumption while increasing the intake of plant proteins (Profeta et al., 2021b). As a consequence of this shift, substantial sustainability benefits can be reaped. The following section provides insights into the scientific findings and pinpoints knowledge gaps in the field of hybrid meats.

Establishment and scientific findings on hybrid meats

Hybrid meats are defined as "… *foods in which a portion of meat has been replaced by other plant-based ingredients and more sustainable protein sources*" (Grasso, 2020). This makes them a promising option to overcome the gap in between plain meat- and plant-based products. Besides their current relevance, researchers actually began to investigate the properties of these mixed meat matrices more than 40 years ago (Randall et al., 1976). Initially, soy and wheat proteins were screened for their suitability in hybrid meat products (Keeton et al., 1984; Patana-Anake & Foegeding, 1985; Randall et al., 1976) due to their commercial availability and legal permission to be used in meat products (Klopfer, 1907; Louis & Morton, 1957; Mussman, 1974; Rao & Gerrish, 1974). However, most of these and other research groups have described distinct changes in texture, flavor, and/or reductions in shelf life especially at higher plant protein amounts. These aspects and consumer skepticism towards 'diluted' meat products has

slowed down the commercialization of hybrid meats. However, technological progress in plant protein extraction and modulation *e.g.* in (off-) flavor, purity, and functionality coupled with an increased availability of alternative sources have led to a growing number of studies and developments in this area over the past two decades. **Table 0.1** summarizes these findings related to the plant protein source used, the level of incorporation, and the implications on some functional and organoleptic properties of end products. It becomes apparent that the incorporation of protein-rich flours, concentrates, or isolates improves the cooking yield of heat-stabilized meat products (emulsified, convenience), which was often related to their supporting action in gelation, fat, and/or water binding. Authors also reported that lower contents increased the firmness of end products, while texture started to soften at around ≥ 11 %. Moreover, the red meat color became fainter and changed to a more light and yellow one. Plant proteins powders lack texture, which distinguishes them from the viscoelastic properties of meat fibers. This limitation has been overcome with the emergence of extrusion technologies that can transform unstructured protein doughs to fibers (Sha & Xiong, 2020). The resulting texturized vegetable proteins may then be rehydrated and incorporated as meat replacers at much higher levels and achieve acceptable end products in particular in convenience products (**Table 0.1**). The product class of dry-cured or raw fermented sausages has been mostly disregarded in the field of hybrid meats and the effect of alternative proteins in these matrices remains mostly unknown. This might be related to the high quality requirements on raw materials, more complex processing procedures that need experienced craftsmanship, and their lower popularity overseas.

Until now, most research approaches on hybrid meats have focused mainly on product development. The studies iteratively evaluated the effect of plant proteins at increasing contents in formulations on a trial-and-error basis. As a result, many formulations were supplemented with functional ingredients such as hydrocolloids, starches, or designed as a combination of different plant- and animal-based proteins (**Table 0.1**). This makes it hard to describe the influence of individual meat replacers and their interaction systematically and to compare literature data. At the end a holistic understanding and systematic evaluation of the functional and organoleptic changes of plant protein addition in meat products is needed for a rational development of hybrid meats. This includes a fundamental assessment of the functional properties of plant proteins in comparison to meat proteins and their modulation by external factors such as pH and temperature. In the following, animal and plant-based proteins are described in their structural, compositional, functional, and organoleptic properties to provide a better understanding in the challenges faced when combining them in hybrid meats.

Table 0.1 Overview on plant protein sources and effects in hybrid meat products

Meat Product	Plant protein	Condition	Amount	Other Additives	Effect on product properties	References
Emulsified sausages	Soy	Isolate/ Concentrate	1.2 – 11 %	Konjac flour, carrageenan and/or starch	Cooking yield ↑ Firmness ↑ Lightness & Yellowness ↑ Redness ↓	Cengiz and Gokoglu (2007); Gao et al. (2015a); Herrero et al. (2008); Lin and Mei (2000); Su et al. (2000); Youssef and Barbut (2011)
	Pea	Isolate	11 %	--	Cooking yield ↑ Firmness ↓ Lightness ↑↓, Yellowness & Redness ↓	Su et al. (2000)
	Legume (Pea, bean)	Flour	4 – 16.5 %	Corn flour, sesame and walnut paste or sunflower oil	Cooking yield ↑ Firmness ↑↓ Lightness & Yellowness ↑, Redness ↓	Dzudie et al. (2002); Pietrasik and Janz (2010); Tahmasebi et al. (2016)
	Rice	Flour	2 – 10 %	Corn sirup	Cooking yield ↑ Firmness ↑↓ Lightness & Yellowness ↓, Redness ↓	Ali et al. (2011); Jailson et al. (2016)
	Soy	Extrudate	10 – 40 %	Soy protein isolate, tapioca starch	Cooking yield ↑ Firmness ↓ Lightness & Yellowness ↑, Redness ↑↓	Hidayat et al. (2018)
Convenience	Soy	Isolate/Flour	2 – 10 %	Ice/water, egg, wheat roll, starch, and/or peas	Cooking yield ↑ Firmness ↑ Lightness ↓, Yellowness ↑↓, Redness ↑↓	Danowska-Oziewicz (2012); Gao et al. (2015b); Kenawi et al. (2009); Shahiri Tabarestani and Mazaheri Tehrani (2014)
	Legume (Lupin, pea)	Concentrate/ Flour	3 – 11 %	Water, wheat roll/bread, flour, and/or egg	Cooking yield ↑ Firmness ↑↓ Appearance →↓	Danowska-Oziewicz and Kurp (2017); El-Sayed (2013); Shoaib et al. (2018)
	Rice	Isolate	3 – 11 %	Flour, egg, bread crumbs	Cooking yield ↑ Firmness → Appearance →↓	Shoaib et al. (2018)
	Oat	Flour	3 %	Water	Cooking yield ↑ Firmness ↓ Lightness →, Yellowness ↓, Redness ↑	Bastos et al. (2014)
	Soy	Extrudate	2 – 30 %	Starch	Cooking yield ↑ Firmness ↑ Lightness ↑→, Yellowness & Redness ↑→	Carvalho et al. (2017); Deliza et al. (2002); Gujral et al. (2002); Kilic et al. (2010); Yadav et al. (2013)
Dry-cured/ Raw fermented sausages	Soy	Isolate	2.5 %	Ice	Drip loss ↓	Porcella et al. (2001)
	Chickpea	Concentrate	1.5 – 5 %	Water	Drip loss ↓↑ Firmness ↓ Lightness ↑↓, Yellowness & Redness ↓	Mokni Ghribi et al. (2018)
	Legume (Soy, pea)	Extrudate	10 – 40 %	--	Drip loss ↓ Firmness ↓ Lightness, Yellowness & Redness →	Colomer Sellas et al. (2021); Zepeda Bastida et al. (2018)

Food Protein Properties

An introduction to the functionality of major food ingredients

Carbohydrates, water, oils/fats, and proteins constitute the major functional ingredients in food products. Carbohydrates can be water-soluble such as sugars and starch or water-insoluble such as cellulose depending on whether polymer-polymer molecular interactions or polymer-water interactions are energetically favorable or unfavorable (Guo et al., 2017). This interplay influences water and fat-holding capacities of food, as well as its tendency to undergo glass transitions (Guo et al., 2017; Ubbink et al., 2008). The latter is influenced by the amount of solids which correlates with the content of water and/or oils. Water and oils act as solvents for hydrophilic and hydrophobic substances and solubility is often a key requirements for structure formation and matrix integrity, thereby preventing degradation of other compounds such as *e.g.* vitamins and flavors (Sikorski, 2006). Surfaces and interfaces play a special role in food systems, and especially the interface between water and oils has an effect on the overall bulk properties of food systems and thus the maintenance of their structural and organoleptic properties and microbial safety. When it comes to proteins, all of the aforementioned points are of relevance due to their complex and diverse nature. In addition, susceptibility towards environmental changes plays a role: *i)* the presence of carbohydrates may result in the formation of heteroconjugates or micro- and macro- separated phases (Weiss et al., 2019); *ii)* the content of water or oils may decide on their location within the food *i.e.* in the water-, oil-, or interface (McClements, 2015); *iii)* pH, temperature, ionic strength, and certain chemicals may influence molecular interactions (Zayas, 1997a). This also gives proteins a key role in the development and manufacturing of food products, and thus a holistic understanding of their functional properties is often required for a rational food design. This is particularly important when it comes to mimicking and replacing of animal-based proteins with plant-based ones due to differences in their molecular structure and general function in meat and plant materials. The following sections briefly compare both protein classes with an emphasis on functionality and its relevance in the formation of (hybrid) meat products.

Protein functionality and its relation to solubility

Protein functionality is a result of the various physical and chemical (physicochemical) properties of food proteins, and is related to their amino acid composition and higher structural organizations, *i.e.* secondary, tertiary, and if applicable quaternary structures based on covalent and non-covalent interactions (Zayas, 1997a). For example, the number and presence of

hydrophobic amino acids on the protein surface caused by a specific folding of the protein in water may determine fat solubility and binding, while a high number of hydrophilic ones may promote solubility (in water), swelling, and water binding. The amphiphilicity of proteins due to the presence of both amino acid classes will give rise to surface and interfacial activity and thus to emulsification, foaming, and gelling properties. As a result, the solubility behavior of food proteins consequently plays a crucial role in their functionality since it is a prerequisite for many of the aforementioned and other properties, since the molecules need to be mobile and dispersed throughout the aqueous and/or oil phase. Food protein solubility can be modulated by adding co-soluble biopolymers, ions, and other solutes such as sugar or alcohols. Thus, predicting the behavior of proteins in a complex food matrix is a difficult undertaking since other proteins, carbohydrates, and lipids, as well as the solvents composition needs to be taken into account (Zayas, 1997a). Many proteins exist in the animal, microbial, or plant world and this not only makes every food protein source unique, but also challenging to work with, especially if traditional formulations and process operations are performed with protein alternatives. This raises a question as to whether different sources such as animal- and plant-based ones may be easily combined at all.

Organoleptic properties

Protein composition and functional properties are closely related and determine the complex behavior of food proteins and ultimately the organoleptic properties of food products, *e.g.* color, aroma, taste, and texture. Color is one of the most important aspects in food acceptance and triggers expectations when it comes to other organoleptic properties (Garber et al., 2003; Williams, 1992). It is mostly related to experiences with food and food products meaning that dairy and meat-products products are generally estimated to be white and red, respectively, while an ‘orange vegetable’ reminds us of carrots. The external appearance of food products is further linked to expectancies on aroma and flavor, and thus the smell and taste of food products. Both color and aroma/flavor arise from the interaction of specific food ingredients with our digestion system, in particular the olfactory system in nose and mouth. For example, rancid or other aroma-compounds arise from lipid oxidation, that may have been generated by the presence of triplet oxygen (photooxidation) or enzymes such as peroxidase and lipoxygenase. Protein oxidation can result in off-flavors such as H_2S but may be also intentionally induced to create the unique flavor of dry-aged products (Aalhus & Dugan, 2014; Hellwig, 2019). Enzymatic browning involves cross-reactions of phenols, oxygen, and the enzyme polyphenol oxidase (Martinez & Whitaker, 1995), while non-enzymatic Maillard

browning is the conjugation of amino acid side chains with reducing sugars, which plays an important part in the development of desired and undesired color, aroma, and flavor changes (van Boekel, 2006). Finally, nitrate is used to convert the meat protein myoglobin to the stable red-meat pigment nitrosomyoglobin, which is characteristic for cured meat products (Brombach et al., 2003; Suman & Joseph, 2014).

Texture or mouthfeel is another important organoleptic property. It is related to the mechanical behavior of food products when deformed and thus the manifestation of fluid- and solid-like properties, which are used to describe and differentiate food products via complex mathematical models (Lu et al., 2015). It can be altered by processes such as heat- or acid-induced gelation (proteins, some hydrocolloids), gelatinization (starchy compounds), water- or fat-binding (proteins, hydrocolloids, starchy compounds), and emulsification (surface-active compound). This also underlines the importance of ingredient functionality and interactions, which are highly depending on the external physical (temperature, pressure) and chemical (acid, base, detergents) parameters as shown by a number of authors (Hartel and Hasenhuettl (2013); Higa and Nickerson (2021); Hu et al. (2020); Lavoisier and Aguilera (2019); Wüstenberg (2015); Zayas (1997a)). Texture is the sensual perception of the food when it is orally processed, namely when it is chewed, bitten, or swallowed (Wilkinson et al., 2000). Due to the complexity of the mechanical action in combination with the mixing of saliva and enzymes therein, texture is widely varying organoleptic property that is often however quite specific for a certain product. Because of this it is often closely linked to consumer acceptance or rejection of foods. Today, it is one of the major reasons for the difficulty in producing acceptable meat analogues and hybrids whose structure and mechanical properties deviate from their meat-based counterparts.

Meat structure and protein functionality

Lean meat contains around 74 % of moisture, 5 % of lipids, and around 1 % of minerals and carbohydrates (Keeton et al., 2014). Proteins represent around 20 % of the total share in lean meat and can be divided into three classes: *i)* Sarcoplasmic proteins; *i)* Myofibrillar proteins; and *iii)* Stromal proteins. Their molecular assembly and spatial distribution in the serum phase results in the characteristic fibrous structure of meat (**Figure 0.1**). Sarcoplasmic proteins are a group of water-soluble proteins that represent around one third of the total meat protein and range from as low as 16 kDa to as high as 99 kDa in molecular weight (Keeton et al., 2014; Warner et al., 1997). They are mostly glycolytic enzymes, nucleoproteins, as well as the characteristic red meat pigments myoglobin and hemoglobin. Their name comes from their

location, *i.e.* the sarcoplasm which is the cytoplasm surrounding myofibrils in the myofiber. Myofibrillar proteins are salt-soluble, contractile biopolymers such as myosin (43 %), actin (22 %), titin (8 %), tropomyosin (5 %), and troponin (Keeton et al., 2014). They are responsible for muscle contraction and the structural organization of muscle fibrils and fibers (**Figure 0.1**). Thin filaments are composed of actin, troponin, and tropomyosin molecules, and thin filaments of myosins, whose ATP-mediated contractions or relaxations trigger muscle motion (Astruc, 2014). Their individual assembly leads to the formation of individual sarcomeres and supramolecular structures therby yielding muscle myofibrils, myofibers, muscle fibers, and finally the muscle itself (**Figure 0.1**). The diameters of muscle bundles range from 50 to 300 muscle fibers depending on the purpose and location in the body. Individual structural elements of muscle are spatially separated by layers of stromal proteins (Velleman & McFarland, 2015). These stromal proteins are a mixture of highly viscous, soluble glycoproteins that are pervaded by collagen and elastin fibers thereby forming the connective tissue in skin, tendons, cartilage, ligaments, and bone.

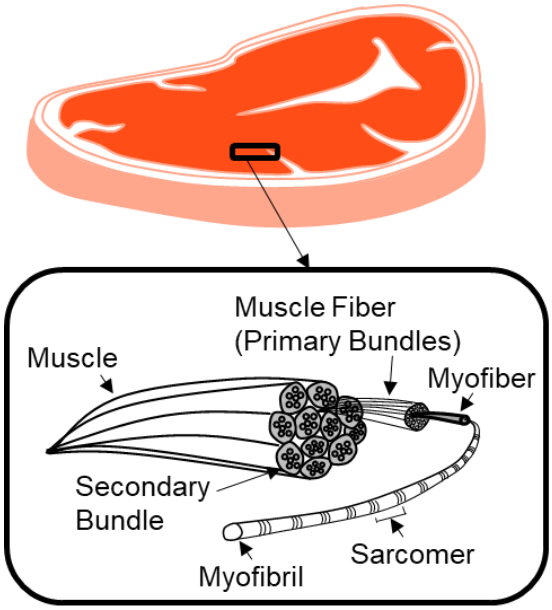

Figure 0.1 Schematic of muscle structure (adapted from Velleman and McFarland (2015))

From a functionality and organoleptic point of view, sarcoplasmic, myofibrillar, and stromal proteins give rise to the characteristic properties of meat and meat products. First, meat color depends on the content of water-soluble myoglobin, hemoglobin, and cytochromes, whose content is depending on the oxidative pattern of the muscle (Yu et al., 2017). Myofibrillar proteins are insoluble at physiological ionic strength (I ~ 0.06 N), but can be solubilized by increasing it to > 0.5 N (Xiong, 2014). This is commonly done during the manufacture of dried or heated meat products in order to obtain the synergistic functionality of both water- and

salt-soluble proteins (**Figure 0.2**), which then results in a characteristic texture and taste. For example, dried sausages base on the partial solubilization of myofibrillar proteins, followed by a coagulation below their isoelectric point p*I* (pH ~ 5.5). The acidified gel can then be solidified by drying until a sliceable, chewy matrix with high microbial stability and visible (back) fat particles is obtained (**Figure 0.2**). Animal backfat contains fatty acids that are embedded in a network of connective tissue, that provides structure and elasticity. It is thus a crucial contributor to the textural characteristics of meat and meat products; a fact that is often overlooked when designing meat analogues (Dreher et al., 2020).

Heated products differ from dried ones in terms of their properties. Here, a higher degree of comminution and an earlier addition of salt (and polyphosphates) promotes a high degree of protein solubilization (**Figure 0.2**). The resulting matrix is relatively homogeneous and consists of fat globules that are surrounded by a protein matrix that is crosslinked due to the heating. This class of product has been named emulsified sausages, even though the system is not truly a classical emulsion - which by definition is a liquid-liquid dispersion with one phase being present in the other in the form of droplets. Rather, emulsified sausages are particle-filled gels. This is because the comminution leading to a dispersion of fat in a concentrated protein suspension is followed by a heating step of > 55 °C to induce a heat-induced gel formation that works best at pH ≥ 6.0 due to a higher electrostatic repulsion of meat proteins above their p*I* (Xiong, 2014).

To conclude, meat technology makes use of the highly specific functional properties of meat proteins that result in structural transitions when mechanical or thermal processes are used thereby giving rise to the characteristic organoleptic properties of dried or heated meat products. The use of multiple, sequentially-arranged process steps and tailored ingredients coupled with raw materials having specific quality characteristics are a prerequisite to obtain acceptable meat products. In essence this is why a mimicking this product class with alternative materials is so difficult and challenging.

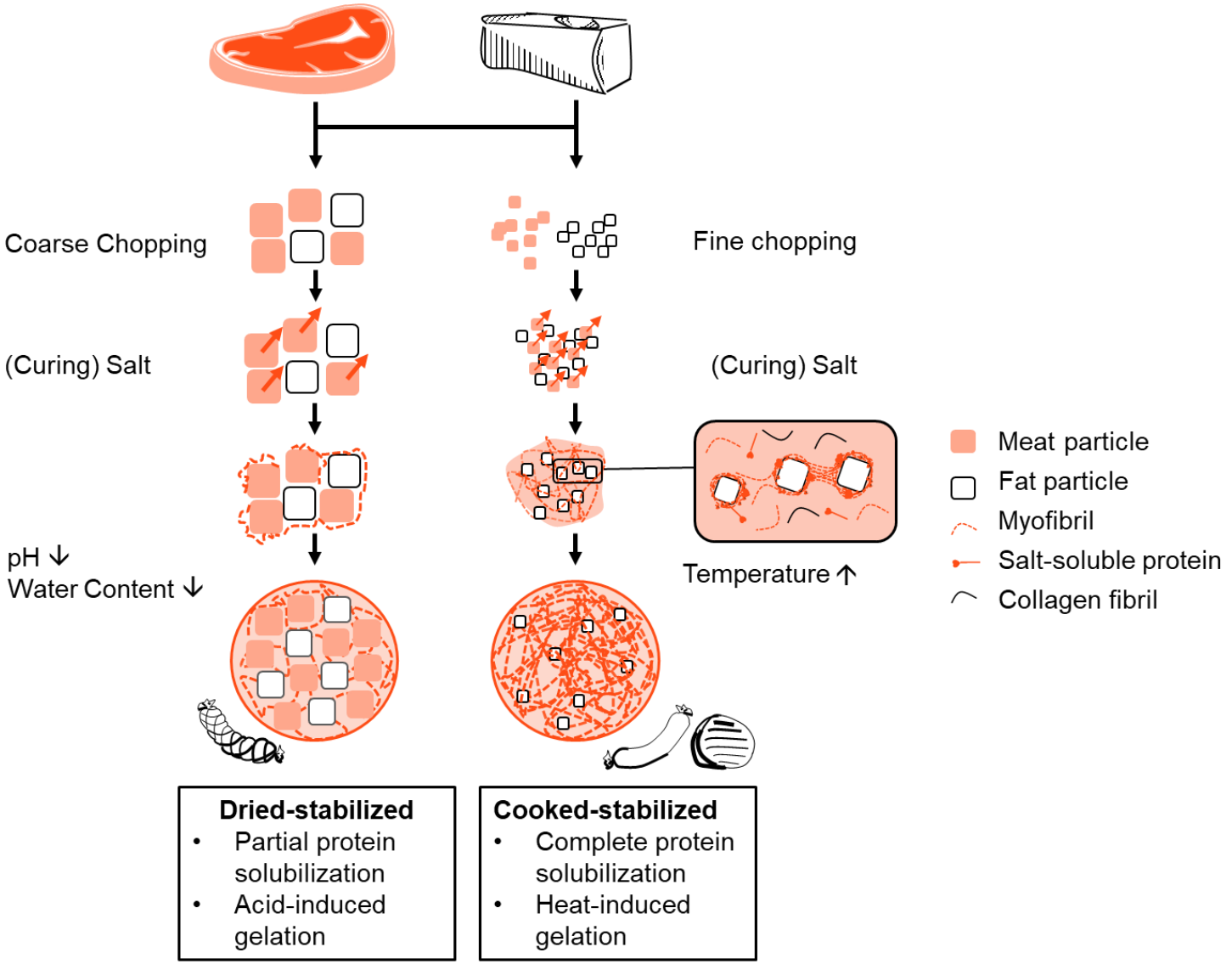

Figure 0.2 Structure formation of dried- and cooked-stabilized meat products (adapted from Kotter and Prändl (1957); Xiong (2014))

Structure of plant materials and protein functionality

Plants materials differ not only from meat, but also among each other according to their biological classification. This includes their capability to flourish, form fruits or seeds, and to grow in specific habitats. However, all plant cell walls show the same fundamental structural organization (**Figure 0.3**). Their cell walls consist of bundles of macro-fibrils (around 10 μm), that consist of bundled micro fibrils (around 1 μm) with poly-glucose as their smallest building block (Miyashiro et al., 2020). The latter is mostly cellulose, while the matrix and fibrous, branched structures around represent hemicellulose and lignin (Horvat, 2016). Upon comparison, the structural organization of plants (**Figure 0.3**) appears to resemble the one of meat (**Figure 0.1**) but relies on carbohydrate polymers instead of proteins as fundamental structural building blocks. But there are more differences than just structural building principles. Animal tissue metabolizes carbohydrates for their energy supply, while plants store proteins in their seeds for growth, maintenance, and protection. This means that polymers serve a completely different purpose in plants and animals. In plants, proteins are located in so-called protein bodies that are finely dispersed in the seed tissue next to larger-sized starch bodies (**Figure 0.3**). Protein bodies in peas were shown to be around 3 μm, while starch bodies were up to 30 μm (Kornet et al., 2020). Both have a high structural integrity meaning that the mechanical disruption of plant materials may liberate but not disrupt or separate the individual structural elements (Kornet et al., 2020).

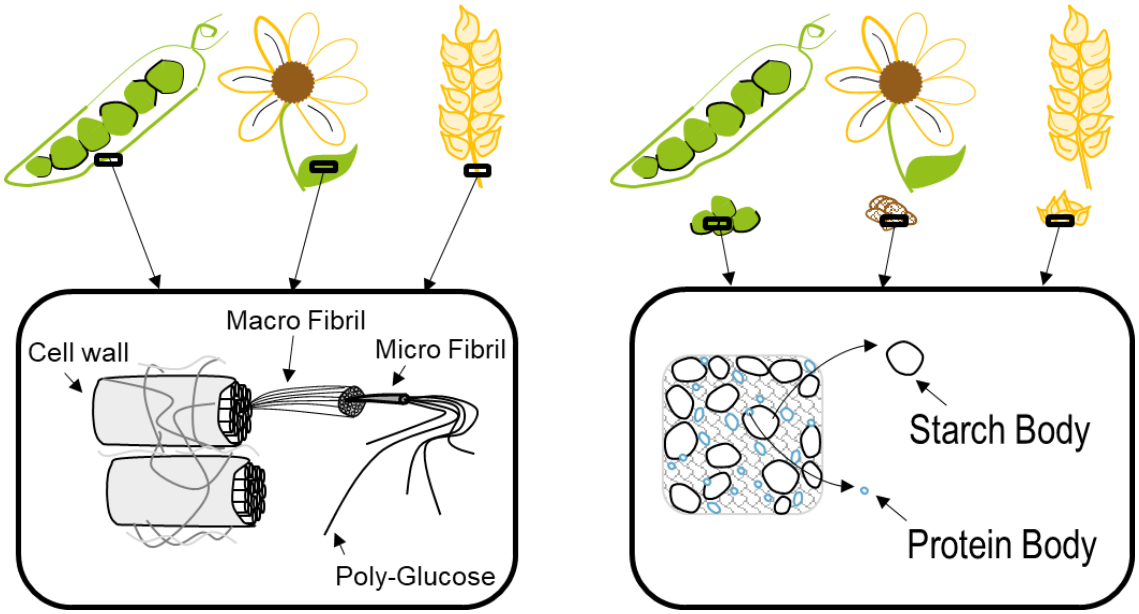

Figure 0.3 Schematic of cell wall and seed structure (adapted from Horvat (2016); Kornet et al. (2020); Miyashiro et al. (2020))

As a result, plant protein extraction processes generally involve multi-step fractionation procedures that may involve the use of salts, chemicals, as well as separation, filtration and/or drying steps that make use of specific (in)solubility properties of carbohydrates, lipids, but also different plant proteins classes. One of the most common classification scheme that these processes rely on is the one created by Osborne, who first evaluated and introduced it for cereal proteins (Osborne, 1907; Osborne, 1924). Since then, the Osborne classification has been revised several times but still relies on the description of protein groups based on their solubility differences, namely albumins, globulins, prolamins, and glutelins. Nowadays, it is additionally connected to the Svedberg classification (2S, 11/12S, or 7S), which is indicative of the size and shape of proteins (Svedberg, 1939) and the subsumption into superfamilies (Shewry & Casey, 1999) (**Figure 0.4**). Albumins are 2S mono- or dimeric proteins with relatively low molecular weight (10 to 20 kDa) and a high hydrophilicity. This makes them readily soluble in water and dilute salt-solutions. They include enzymes and many of the commonly known food allergens *e.g.* amylase and trypsin inhibitors (Mills & Shewry, 2008; Nadathur et al., 2017; Shewry & Casey, 1999). In contrast, 7S and 11/12S globulins are only saline soluble. They may be monomers, tri-, tetra- or hexamers with up to around 500 kDa, whose subunits might be linked covalently (disulfide bridges) or non-covalently. Both 7S and 11/12S globulins are high in leguminous plants where they represent up to 89 % of the total seed protein (Thrane et al., 2017). In contrast, oilseeds contain no 7S, but only 11/12S globulins (Shewry & Casey, 1999). The third class of prolamins received its name from a high content of proline and amide nitrogen. It is characteristic for its solubility in ethanol-water mixtures (60 – 80 %), varying molecular weight (10 to 90 kDa), and abundance in cereals *e.g.* in maize (zeins), oat (avenins), rye (secalins), and wheat (gliadins) (Shewry & Casey, 1999). Although having the highest structural diversity, this class is the most clearly defined by literature (Mills & Shewry, 2008). The remaining Osborne fraction contains the glutelins. Glutelins are insoluble in water, but are extractable with alkali or acids (Shewry & Casey, 1999). They are polymers that consist of a combination of different chain length polypeptides that have intermolecular connections via disulfide bonds and are further divided into high (HMW) and low (LMW) glutelins, whereof the first are the major contributors to gluten elasticity in bread making (Asgar et al., 2010).

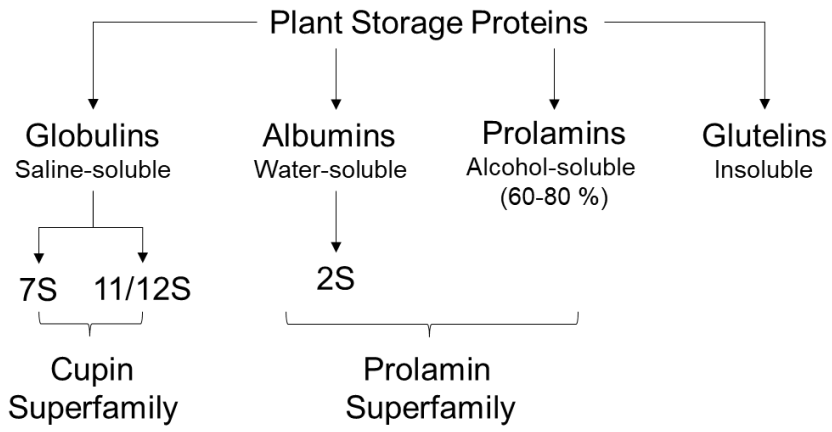

Figure 0.4 Summary of the classification of plant proteins according to Osborne, Svedberg, and their superfamilies

Figure 0.4 shows that albumins and prolamins form the so called "prolamin superfamily", which is marked by the presence of three conserved, homologous regions that contain cysteine residues and undergo a similar posttranslational modification. The 7S and 11/12S globulins represent the cupin superfamily which are defined by a common prokaryotic ancestor protein. It has been suggested that 7S proteins are dissociation products of 11S globulins (González-Pérez & Vereijken, 2007; Mills & Shewry, 2008; Shewry & Casey, 1999).

To sum up, plant proteins are highly diverse macromolecules that differ in their abundance in individual plants and have a wide range of properties especially when it comes to solubility, which is a key physicochemical property giving rise to many functionalities. Yet, it can be said that proteins from the same Osborne fraction and superfamily possess similar techno-functional characteristics. For example, sunflower and canola albumins were shown to have a good solubility irrespective of the ionic strength and most pH-values. The 11/12S globulins in pumpkin (pumpkin globulins), canola (cruciferin), sunflower (helianthinin), and pea (legumin) are all large hexamers with molecular weights up to 470 kDa and at least one disulfide bridge, which is important for gelation (Asgar et al., 2010; González-Pérez & Vereijken, 2007; Nadathur et al., 2016; Rezig et al., 2013; Shewry & Casey, 1999). Further theoretical consideration on the behavior of specific Osborne fractions in plant proteins may be useful to estimate their suitability for certain food products *e.g.* to promote solubility, emulsification, foaming, and/or to build-up texture. However, it becomes increasingly apparently that plant proteins and their functionality differ from meat-based ones in many fundamental aspects due to their different purpose and structural organization.

Structuring plant proteins by extrusion

The previous chapters have discussed protein functionality and its effect on product properties as well as the differences of the structural organization and functional properties of meat- and plant-based proteins. However, there are further aspects to consider when using them as food ingredients. One of these aspects includes their physical state, *i.e.* whether they are used as dispersions, powders or pre-structured fibers. The extraction of plant proteins generally yields protein-rich flours, concentrates, or isolates. All these commercial preparations lack anisotropic structures that emerge through self-assembly of meat proteins. A texturization by extrusion can overcome this challenge since it transforms powders into anisotropic materials with viscoelastic properties thereby providing a structural integrity that enables these systems to withstand a certain level of mechanical and thermal stresses. This is crucial for their application as meat extenders or analogues since their manufacturing involves a sequence of processing steps where stresses are superimposed (Asgar et al., 2010). Extruders are composed of one or two screws and consist of four zones, where a premix of powder and water is fed (zone 1), mixed (zone 2), melted (zone 3), and ejected through an outlet die (zone 4) (Zhang et al., 2019). High pressures and temperatures inside the main barrel (zone 2-3) suddenly change after the material exits the die thereby resulting in a sudden evaporation of water and the formation of viscoelastic, anisotropic texturates with varying aspect ratios and porosities. The latter is depending on the composition of the dough formulation (*e.g.* the water content) that is fed into the extruder, and the extrusion conditions such as screw design and speed, die design and operating temperature. For example, higher temperature may improve porosity but decrease expansion. A high amounts of fiber and fat in the dough may hinder expansion due to a lower capability to plastify and a lubricating effect (Bisharat et al., 2013). Generally, protein contents of around 50 to 70 % are needed to obtain a fibrous texturate (Zhang et al., 2019). During the extrusion process, proteins undergo a sequential structural change, namely an unfolding, association, aggregation, and finally a cross-linking (**Figure 0.5**). Initially, the mixing zone (zone 2) promotes protein unfolding along the direction of the flow thereby exposing hydrophobic amino acids. Following this, high temperature and internal friction forces in the melting zone (zone 3) trigger protein-protein and protein-water interactions which finally results in association, aggregation, and cross-linking and an increase in viscosity.

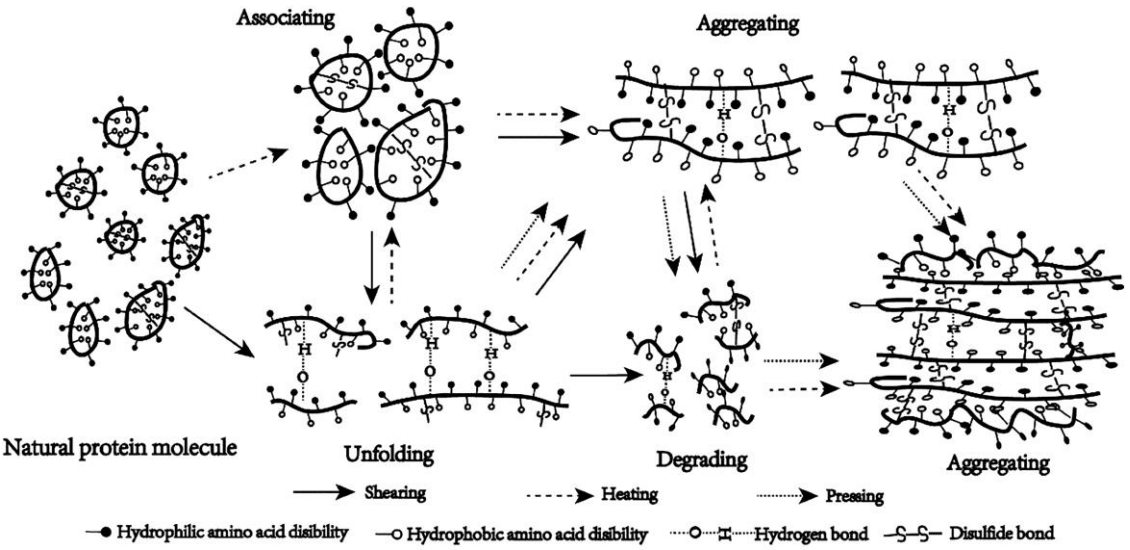

Figure 0.5 Conformational changes of protein during the extrusion process (retrieved from Zhang et al. (2019))

This also means that the behavior of proteins during extrusion depends on their composition such as the number and presence of hydrophobic and hydrophilic amino acids on the proteins surface and their capability to form hydrogen and disulfide bonds. Texturized vegetable proteins from this "low moisture" extrusion (20-40 % of water in the process) are generally rehydrated before their application due to their dry, sponge-like characteristic (Asgar et al., 2010; Zhang et al., 2019). During the last decade, "high moisture" or wet extrusion (40-80 % of water in the process) has gained more and more importance since hydrated, fibrous extrudates can be obtained that do not need (extensive) rehydration. Here, zone 4 is additionally equipped with a cooling die to limit the evaporation of water and to enable a plastification of proteins (Osen & Schweiggert-Weisz, 2016). While the application of texturates is already quite common in meat analogues, their application in hybrid meats has not yet been commercially exploited. This is also related to a lack of understanding of their function and behavior during processing in common unit operations such as heating and drying and their susceptibility towards changes in pH and salt. Moreover, it is still unclear if these alternative proteins may behave synergistic or antagonistic with meat proteins, or if they act as inert particles.

Aims of the Study

This dissertation focusses on the characterization of the properties and behavior of various functional plant-based proteins and their extrudates to gain an insight into their ability to replace meat to generate hybrid meat products. This includes functional and organoleptic analysis as well as a comparison to products made by traditional formulations and unit operations. A special emphasis was put on the product class of dry-cured sausages since their manufacture involves a complex set of raw materials and process steps. Moreover, this product class represents one that is popular with consumers and has a high added value. The overall aim was to establish a better understanding of the properties and interactions in matrices containing both meat and plant proteins in order to provide guidance on product development through a mechanistic rather than a trial-and-error approach. This work was divided into three sections: *i)* the functionality of plant proteins and meat-plant protein interactions *ii)* the functional and organoleptic properties of plant-based extrudates *iii*) the effect of plant proteins on product manufacture and properties of dry-cured hybrid meats. The aim was to develop systematic relationships between protein ingredient properties, their functionality, and performance in processes and hybrid products.

The selection of proteins was first narrowed down by screening a variety of 26 commercially available plant-based protein powders with moderate to high protein content. To that purpose, solubility, color, and composition were correlated with the respective extraction process and the theoretical amount of functional Osborne fractions in order to exclude plant proteins with a low functionality. Following this, model mixing studies with solubilized water-salt soluble meat proteins fractions and soluble plant proteins were carried out in dilute systems to assess protein-protein interactions and to gain insights into influencing factors such as molecular interactions, hydrophobicity, dispersibility, microstructure. A range of techniques was used including FTIR and systems were assessed over a broad pH range (pH 3.0 to 7.0). Next, two protein isolates and their extrudates were analyzed with regards to their volatile profile by GC-MS-O to better understand extrusion-related modulations on organoleptic properties after dry and wet extrusion. After these model studies, the behavior in mixed matrices with varying formulations subjected to unit operations such as acidification and drying to manufacture dry cured hybrids was studied. To that purpose, wet extrudates from six different proteins were analyzed with respect to their susceptibility towards acid induced pH-changes (buffering capacity) and compared to the behavior of pork meat. Two mathematical models were established to describe the influence of different compositional elements on the acid-induced

response and to suggest formulations for hybrid meat matrices that may ensure product safety and yield a consumer-acceptable texture. Finally, four hybrid dry-cured sausages were manufactures in order to validate the obtained knowledge and to analyze their drying behavior, texture, and color in comparison to a traditional meat-only control formulation.

Each chapter of this study was designed based on a specific set of research questions that were developed and aimed to be tested.

i) How does solubility correlate with the applied plant protein extraction method (*e.g.* wet or physical extraction) and the resulting purity (*e.g.* flour, concentrate, or isolate)? Do traditional plant protein classifications such as the Osborne scheme work for commercially produced protein preparations and can such schemes be successfully used to estimate functionality (**Chapter I**)?

ii) What are the major odor-active compounds in plant proteins and how are they modulated by a dry or wet extrusion? Which are the main chemical reactions that induce their formation (**Chapter IV**)?

iii) Which molecular interactions are key to the structural organization and behavior of plant proteins and do these differ from meat proteins? How does the presence of plant proteins affect the functionality (*e.g.* dispersibility, microstructure, folding) of water- and salt-soluble meat proteins (**Chapter II – III**)?

iv) How does pH and its modulation change the functionality of plant- and meat-based proteins? Is there a difference in the susceptibility towards acids and how does this change dispersibility, microstructure, buffering capacity, and final pH (**Chapter II – VI**)?

v) Which adjustments in the formulation and manufacturing process are necessary to produce hybrid meat products with an acceptable texture and a high product safety, especially when considering final product pH and water activity values? What are the application thresholds of plant proteins in hybrid meat matrices (**Chapter VI – VII**)?

Each of these chapters represents a study that was published (**Chapter I – V**) in a peer-reviewed journal or has been accepted for publication (**Chapter VI – VII**).

Functional Plant Proteins and Meat-Plant Protein Interactions

I. Chapter

Survey of Aqueous Solubility, Appearance, and pH of Plant Protein Powders from Carbohydrate and Vegetable Oil Production

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Abstract

There is a need to have functional protein-rich ingredients to formulate a range of vegan and vegetarian products. However, available proteins vary in functionality and in particular in solubility, even if derived from the same plant source. In this survey, appearance, native pH, and aqueous solubility of 26 plant proteins were assessed. Solubility correlated with purity and extraction process applied and differences in between plant sources were related to protein types present in the raw material. For example, proteinswith a prevalence of prolamins (wheat) or glutelins (rice) had lower solubilities than those from pea and sunflower, which consist almost exclusively of globulins and albumins. Moreover, solubility of pea protein isolates was between as little as 8 % to as much as 50 %, while less pure sunflower meals varied only between 19 % to 22 %. Extraction procedures further affected appearance e.g. due to pigment degradation and/or non enzymatic browning. This study serves as reference to researchers currently working with various protein powders and highlights the need to establish relationships between base raw material properties such as solubility, pH, appearance, and applicability in the design of food matrices.

HIGHLIGHTS

- Biological origin and extraction approach cause variations in crude protein solubility.
- Crude protein solubility correlates with the amount of albumins.
- The choice of extraction techniques impacts powder appearance and native pH.

KEYWORDS

Alternative proteins; Plant proteins; Protein solubility; Protein classification; Protein extraction

Introduction

Prevalence of global malnutrition as well as concerns about food security and the sustainability of food production have resulted in an increase in research related to "alternative" proteins (Godfray et al., 2010). These proteins may be derived from plants, microorganisms such as fungi, bacteria, or microalgae, insects, or tissue cultures and are intended to supplement or replace conventional animal-based protein sources (Asgar et al., 2010). There has been a noticeable rise in consumer demand for such ingredients to facilitate a shift towards alternative protein-based diets and novel products are continuously entering the market (Bashi et al., 2019). Alternative proteins from plants may be obtained from side-streams from the carbohydrate and oil processing sector, thereby facilitating a more holistic use of raw materials. Aside from the proteins, such side-streams may contain other valuable compounds such as dietary fibers, vitamins, or secondary plant metabolites with antioxidant or antimicrobial activities (González-Pérez & Vereijken, 2007; Rezig et al., 2013; Tulbek et al., 2017). However, there are still open questions as to how to design criteria of fractionation and extraction cascades. At present, the protein-type classification scheme suggested first by Osborne (1907) is still most commonly used to set up these processes, since it categorizes proteins based on their solubility in polar or non-polar solvents. However, this does not necessarily correspond to other functional properties such as emulsification, foaming, and gelation, especially in novel and emerging protein sources. Moreover, when it comes to aqueous solubility of protein powders, it may not only be the protein class, but also the used extraction procedure that causes changes in functionality. A first step towards establishing relationships between physicochemical properties and functionality in food applications is therefore an assessment of available alternative proteins. In this study, we focused especially on the aqueous solubility since this if often a prerequisite for their suitability in food applications. To that purpose, a variety of plant protein powders from major food crops and oilseeds were evaluated. Plant protein solubility was then correlated to the theoretical amount of water-soluble proteins in the respective genus using Osborne (1907) classification. Further, color and appearance of powders, as well as native pH of their suspensions were assessed.

Materials and methods

Materials

A total of 26 plant protein powders from seven different plant protein genera (pea, wheat, rice, potato, sunflower, pumpkin, canola) were obtained from a range of suppliers (**Table SI.4**).

Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany). Tris (hydroxymethyl) aminomethane (THAM) was purchased from Carl Roth (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Double deionized water was used for all experiments.

Powder analysis

Nitrogen content

The nitrogen content of plant protein powders was analyzed according to Dumas (BVL, 2005b) with a flash combustion method (Dumaterm® DT N Pro, C. Gerhardt GmbH & Co. KG, Königswinter, Germany). In short, plant protein powders were weight accurately into tin foils (Dumafoil®, C.Gerhardt GmbH & Co. KG, Königswinter, Germany), combusted with oxygen at 1030 °C, and reduced with copper at 750 °C. The respective release of nitrogen was reported by an integral thermal conductivity detector through the conductivity change of a standardized helium flow and calculated as total nitrogen (%) by the inherent software (Dumatherm® Manager, C. Gerhardt GmbH & Co. KG, Königswinter, Germany). EDTA was used as a standard for calibration.

Color and visual appearance

Plant protein powders were analyzed with a chroma meter (CR-400 with data processor DP-400, Konica Minolta, Inc., Marunouchi, Japan) against a white calibration standard to obtain lightness L^*, as well as green-red a^* and blue-yellow b^* color values. Analysis of parameters were done with D_{65} as a standard illuminant and an observer angel of 2 °. Additionally, images were taken with an iPhoneX (Apple Inc., Cupertino, USA) under controlled illumination to check the visual appearance of samples.

Analysis of plant protein suspensions

Preparation of plant protein suspensions

Plant protein powders were suspended in double deionized water at a crude protein concentration of 3 wt% based on the specified protein content (**Table SI.4**). Samples were stirred overnight at room temperature to ensure complete hydration and stored at room temperature until further analysis.

Native pH

The native pH of plant protein suspensions was analyzed after overnight stirring (*Preparation of plant protein suspensions*) with a pH-meter (inoLab pH Level 1, Weilheim, Germany). A minimum of five pH measurements was taken per sample.

Nitrogen content

An aliquot of each protein suspension was transferred into an Eppendorf® 3810X microtube (Eppendorf AG, Hamburg, Germany) and centrifuged (20,000 g, 30 min, 25 °C) to separate the soluble from the insoluble fraction. Supernatants (soluble fraction) were carefully decanted and stored at 4 °C until further analysis. The pellet was discarded. Plant protein suspensions and respective supernatants were analyzed in a DUMATHERM® N Pro analyzer (C. Gerhardt GmbH & Co. KG, Königswinter, Germany) as mentioned before (see 2.2.1). Here, liquid samples were weight into tin foils (DumaFoil®, C.Gerhardt GmbH & Co. KG, Königswinter, Germany) equipped with an absorbent for polar samples (DumaSorb®, C.Gerhardt GmbH & Co. KG, Königswinter, Germany) at an adsorber to sample ratio of 1:4 and THAM (0.5% N) was used as a liquid standard. The crude protein solubility CPS (%) was then calculated according to **Eq. I.1**

$$Crude\ Protein\ Solubility\ (\%) = \frac{N_{Supernatant}}{N_{Suspension}} * 100 \qquad \textbf{I.1}$$

where $N_{\text{Suspension}}$ is the nitrogen content (%) of the suspension and $N_{\text{Supernatant}}$ is the nitrogen content (%) of the supernatant.

Average crude protein solubility and variance

The effect of plant protein origin on solubility was observed by calculating the average crude protein solubility (%), as well as the standard deviation and variance among samples of the same genera according to **Eq**. **I.2**, **I.3**, and **I.4**, respectively.

$$Average\ Crude\ Protein\ Solubility\ (\%) = \frac{\sum_{i=1}^{n} CPS_{Genus}\ (\%)}{n_{Genus}} \qquad \textbf{I.2}$$

$$Standard\ Deviation = \sqrt{\frac{\sum(CPS\ (\%) - Average{-}CPS\ (\%))^2}{(n-1)}} \qquad \textbf{I.3}$$

$$Variance = \frac{\sum(CPS\ (\%) - Average\text{-}CPS\ (\%))^2}{(n-1)} \qquad \textbf{I.4}$$

where *CPS* is the crude protein solubility (%) and n is the amount of tested plant protein powders from one genus.

Theoretical albumin content

The theoretical albumin content in plant protein powders was calculated based on the crude protein and mean albumin content (%) of one genus according to literature values **Eq. I.5**

$$Theoretical\ Albumins\ (\%) = Mean\ Albumins\ (\%) * \frac{Crude\ Protein\ (\%)}{100} \quad \textbf{I.5}$$

Statistical Analysis

After checking the assumption of normality (Shapiro-Wilk-test, *p*-value to reject ≤ 0.05) and equal variance ($p \leq 0.05$), a one-way analysis of variance with a Duncan posthoc-test was carried out and an α-level of 0.05 was used to test statistically significant differences among samples using SPSS statistics V23 (IBM Corp., Armonk, USA).

Results and Discussion

To date, most plant proteins typically represent side streams after a separation from their carbohydrates or fats and oils (Amagliani, 2017; Düring et al., 2015; Flambeau et al., 2017; González-Pérez & Vereijken, 2007; Peksa et al., 2009; Rezig et al., 2013; Tulbek et al., 2017). Related processes include wet fractionation techniques, where proteins are separated from other components based on solubility differences, but also simple mechanical pressing of ground raw materials to obtain a protein-enriched pomace. As such, there are differences both in this primary extraction, as well as in the following steps to gain proteins. This can be expected to influence purity, properties, and thus physicochemical properties and functionality of the obtained protein-enriched fractions. In this study, 26 commercially available plant proteins powders from seven plant protein genera including pea (*Pisum*), wheat (*Triticum*), rice (*Oryza*), potato (*Solanum*), sunflower (*Helianthus*), pumpkin (*Cucurbita*), and canola (*Brassica*) were chosen to assess fundamental powder properties such as color, appearance, and protein content, as well as their native pH, and aqueous solubility after dispersion.

Powder properties

Elementary nitrogen was measured according to Dumas (BVL, 2005b) and converted to crude protein using a factor of 6.25, which is generally used to qualitatively compare ingredients for their average protein content (Mariotti et al., 2008). A color analysis was done to obtain

Lightness *L** as well as red-green *a** and yellow-blue *b** balance values. Both properties have implications on the suitability of plant powders for certain food applications since residuals such as carbohydrates, minerals, and especially pigments may lead to consumer rejection. For example, off-colors (in particular green color notes) trigger low acceptance scores in meat analogues, since comparisons are drawn to corresponding meat products (Zamuz et al., 2019).

Based on the obtained results, analyzed powders may be divided into two groups having low and high purity and thus low and high crude protein contents of 48 .4 - 62.1% and 76 .8 - 90.2%, as well as distinct and low inherent color, respectively (**Table I.1**). The first group was comprised of proteins from sunflower or pumpkin seeds received by a physical extraction process (mechanical pressing) of oilseeds after milling (Panic et al., 2013; Pickardt et al., 2017). This is sometimes combined with roasting or salt addition (Pumpkin seed flour, Pumpkin60, R) to improve product safety and to melt solid fat fractions to increase lipid yield (González-Pérez & Vereijken, 2007; Juranovic et al., 2003). Residuals were mostly dietary fiber (carbohydrates) and some fat (**Table SI.4**). Irrespective of some pigment degradation and enzymatic browning through roasting – *i.e.* Pumpkin60,R compared to Pumpkin, 60 UR – high color intensities of oilseed proteins were linked to the presence of lipophilic pigments such as chlorophylls and carotenoids. Organic solvents extraction can be used for their removal, but has become less and less common due to adverse effects on the sustainability and economic value of the end-product as well as protein functionality (Day, 2013). However, they can be further defatted with supercritical CO_2 (*e.g.* Heliaflor 55) or are flaked (*e.g.* Extrufix W/D) to ease handling for subsequent applications such as extrusion (Pickardt et al., 2017).

This simple mechanical separation of vegetable oils and protein-enriched flours is especially contrasting with wet fractionation techniques, where plant proteins with high purity may be obtained after a complex set of process steps. Here, water or other polar solvents are used to separate soluble from insoluble compounds. The general principle relies upon cell tissue disruption followed by a number of sequential mechanical, chemical, and/or enzymatic steps that involve protein solubilization, precipitation, purification, or concentration (Amagliani, 2017; Bourgeois et al., 2015a; Düring et al., 2015; Flambeau et al., 2017; Giuseppin et al., 2008; Johansson & Samuelsson, 2018). Individual process parameters and steps and suitable solvents are chosen according to the physicochemical properties of raw materials, as well as preceding primary extraction steps. In example, wheat may be separated by a simple addition of water by forming a viscoelastic insoluble mass upon hydration (Flambeau et al., 2017).

Table I.1 Nitrogen, calculated protein contents, appearance, and color values (CIELAB-system) of tested plant protein powders (n ≥ 3)

Genus	Trade name	Nitrogen (%)	Crude Protein[1] (%)	Specific Protein[2] (%)	Appea-rance	*L** (-)	*a** (-)	*b** (-)
Pea (*P. sativum*)	PisaneF9	13.3	82.9	71.1		85.9	3.1	20.2
	PisaneC9	12.7	79.6	68.3		86.9	2.8	20.1
	PisaneM9	12.9	80.7	69.2		85.3	2.2	19.2
	PisaneB9	12.8	79.9	68.6		85.4	3.8	19.3
	Pea protein	12.8	80.3	68.9		83.3	3.2	25.9
Wheat (*T.* L.)	VW Gluten	12.6	78.9	69.7		88.3	-0.3	14.9
	EMCEvitC	12.5	77.9	68.8		87.1	0.8	14.4
	VWG75 Food	12.8	79.8	70.5		85.7	0.2	18.8
	Wheat gluten	13.0	81.3	71.8		87.1	0.7	14.9
Rice (*O. sativa*.)	Plantforce Synergy	13.8	86.0	73.5		89.5	0.0	13.6
	Rice protein (H)	13.0	81.4	69.6		81.3	2.1	21.8
	Remypro N80+	12.4	77.4	66.1		84.8	0.5	21.2
	Rice protein (C)	12.3	76.8	65.6		79.2	3.6	19.5
Potato (*S. tuberosum*)	Solanic100T	12.5	78.3	64.9		68.6	3.9	23.4
	Solanic200	14.0	87.2	72.3		78.5	1.8	16.9
	Solanic300	14.4	90.2	74.8		70.6	5.0	22.3
	Potato protein	12.5	77.9	64.6		86.4	0.9	15.4
Canola (*B. napus*)	Teutexx Isolexx	14.1	87.9	75.2		57.0	6.0	25.3
Sunflower (*H. annus*)	Heliaflor45	7.7	48.4	41.0		75.9	1.5	14.0
	Heliaflor55	8.5	53.3	45.1		83.3	0.4	8.7
	Extrufix W	7.5	46.6	39.5		60.3	2.9	15.5
	Extrufix D	8.3	51.6	43.7		68.1	1.3	11.2
Pumpkin (*C. pepo*)	Pumpkin seed flour	9.9	62.1	54.6		72.8	-3.8	26.3
	Pumpkin60, R	9.8	61.2	53.9		72.3	0.8	27.4
	Pumpkin60, UR	9.8	61.0	53.6		78.9	-4.1	23.8
	Pumpkin protein	9.7	60.7	53.4		61.9	0.7	30.9

[1] N x 6.25;
[2] N x Genus-specific conversion factor according to Mariotti et al. (2008); Panic et al. (2013); Sosulski and Imafidon (1990)

In contrast, proteins from pea, rice, and canola were found to have good extraction efficiency in saline media, in particular at elevated pH (Amagliani, 2017; Reinkensmeier et al., 2015; Tan et al., 2011). This makes the addition of alkali a common method to solubilize and re-solubilize proteins before and after their pH-induced precipitation. Similarly, acid coagulation of potato proteins is generally followed by a neutralization step to improve powder dispersibility for their application in food matrices (Johansson & Samuelsson, 2018). Exceptions to this principle of wet fractionation are the two used potato proteins Solanic200 and 300, which are chromatographically separated based on their size and charge (van Koningsveld et al., 2001) to obtain isolates with high purity (**Table I.1**). Aqueous ingredient fractionation further promoted pigment removal due to their hydrophobic nature related to lower color intensities as seen by high L^*-values (70.6 – 89.5) in pea, wheat, rice, and potato proteins and a yellow to beige appearance related to b^*-values in between 13.6 and 25.9. Finally, if Maillard pigments were formed, or carotenoid and chlorophyll degradation due to heat treatments during extraction occurred (Britton et al., 2004; Skibsted et al., 2010), powders were more orange (PisaneC9, Pea protein) or brown (Rice protein (C), Solanic100T, Solanic200, Isolexx).

Despite using 6.25 as a nitrogen-to-protein conversion factor to receive the crude protein content, specific values for each foodstuff exist to assess the nutritional quality of a protein source (Mariotti et al., 2008; Milovanović et al., 2014; Sosulski & Imafidon, 1990) based on the specific protein content. As for the raw materials tested in this study the following literature-based factors were used: wheat (5.52), pumpkin (5.50), pea (5.36), canola (5.35), rice (5.34), sunflower (5.29), potato (5.18) (Mariotti et al., 2008; Milovanović et al., 2014; Sosulski & Imafidon, 1990). Lower magnitudes compared to the standard value of 6.25 are related to differences in amino acid composition and amounts of non protein nitrogen present. As a result, specific protein contents calculated in this study were 12–17% lower than their crude protein contents and ranged from as low as 41.0% (Heliaflor45) to as high as 75.2% (Isolexx) (**Table I.1**). Considering the nutritional value based on the specific protein content canola thus performed best followed by wheat, potato, rice, pumpkin, and sunflower. Irrespective of the specific conversion factors used in this study, it should be stated that published literature values may vary substantially due to differences in the applied analytical methods and/or used raw material. For example, conversion factors for rice were suggested to be 5.17, 5.37, or 5.47 (Mariotti et al., 2008). Therefore, care has to be taken while using these specific factors. Taken together, results from the protein powder characterization showed that high purity of plant protein powders was related to multi-step wet fractionation processes while mechanical pressing as used for oilseed proteins from sunflower and pumpkin resulted in lower protein

contents related to residual carbohydrates, fats, and minerals. A calculation of the specific protein content of plant protein powders may be used to gain information on the nutritional value of plant proteins powders and presence of non-nitrogen compounds leading to a more detailed understanding of the nature of the protein powders obtained from different plant materials and processes. Nevertheless, the use of the average factor of 6.25 to calculate crude protein content is sufficient to facilitate a first qualitative comparison of the purity of a protein powder based on the applied extraction process.

Suspension properties

Native pH

Besides their appearance, the native pH of plant proteins powders plays a crucial role in determining their suitability as food ingredients in low (*e.g.* soft drinks), low to slightly acidic (*e.g.* bread, dairy and meat products), or neutral pH products (*e.g.* vegetable juices, tofu-based products). In this study, the majority of tested plant proteins revealed values in the slightly acidic (pH 4.51) to neutral range (pH 6.89) (**Table I.2**), thus fitting to the second group mentioned. The native pH of plant proteins that originated from wet fractionation was related to the respective last step of their extraction such as neutralization (PisaneF9, Plantforce Synergy) or alkaline re-solubilization (PisaneC9, M9, Pea protein, Isolexx, Solanic100T). Proteins with a lower pH lacked these steps, such as for example Rice protein (H), RemyproN80+, or Potato protein, or were subjected to an alternative chromatographic extraction technique (Solanic 300) (Giuseppin et al., 2008). This makes their application in food quite challenging especially in matrices where pH plays a crucial role during process as it is the case for meat and dairy products. In contrast, protein powders from side streams of the oil production such as pumpkin and sunflower varied little in terms of pH, both within and in between genera (pH 6.34 – 6.89). Results also indicate a low effect of conditioning steps such as flaking (Extrufix D,W) or CO_2- extraction (Pumpkin60, UR, Heliaflor 45, 55) on the native pH, while raw materials that had been subjected to a roasting process (Pumpkin seed flour, Pumpkin60, R, and Pumpkin protein) had slightly lower native-pH. This is likely due to a conjugation of basic amino acids with reducing sugars during Maillard reactions (Skibsted et al., 2010).

Solubility

Solubility of protein powders was analyzed as an important criterion for the applicability of plant-based proteins in food matrices. It was calculated as the ratio of the nitrogen content of

the supernatant and the original plant protein suspension I**.1**. Variations in between and among plant genera were assessed through the average crude protein solubility (**Eq. I.**2) and the solubility variance among proteins from the same biological origin (**Eq. I.**4).

Table I.2 Native pH, aqueous solubility and variance of tested plant protein powders ($n \geq 2$).

Genus	Trade name	Native pH (-)	Crude Protein Solubility[1] (%)	Average Protein Solubility (%)	Solubility variance (-)
Pea	PisaneF9	6.72	28.6 ± 0.3^{aE}	31.7 ± 15.7	246.7
	PisaneC9	7.72	42.4 ± 1.9^{bF}		
	PisaneM9	7.62	49.6 ± 0.4^{cG}		
	PisaneB9	5.80	8.5 ± 0.3^{aC}		
	Pea protein	7.61	29.5 ± 0.7^{aE}		
Wheat	VW Gluten	5.69	20.1 ± 0.9^{aE}	17.0 ± 9.5	89.5
	EMCEvitC	6.08	7.5 ± 0.2^{bBC}		
	VWG75 Food	5.87	11.5 ± 0.1^{cD}		
	Wheat gluten	5.40	28.8 ± 0.1^{dE}		
Rice	Plantforce Synergy	6.84	4.2 ± 0.2^{aA}	4.3 ± 0.3	0.1
	Rice protein (H)	4.51	4.7 ± 0.4^{aAB}		
	Remypro N80+	4.91	4.3 ± 0.1^{aA}		
	Rice protein (C)	6.02	3.9 ± 0.4^{aA}		
Potato	Solanic100T	8.11	5.1 ± 0.1^{aAB}	52.0 ± 53.1	2819.2
	Solanic200	3.82	95.9 ± 0.2^{bH}		
	Solanic300	6.76	100.0 ± 0.4^{bI}		
	Potato protein	3.27	7.0 ± 3.0^{aABC}		
Canola	Teutexx Isolexx	8.05	85.4 ± 1.5^{E}	--	--
Sunflower	Heliaflor45	6.61	21.7 ± 0.2^{bCD}	21.0 ± 1.4	1.9
	Heliaflor55	6.59	22.1 ± 0.3^{bCD}		
	Extrufix W	6.62	21.1 ± 0.6^{bCD}		
	Extrufix D	6.71	19.0 ± 0.1^{aC}		
Pumpkin	Pumpkin seed flour	6.34	11.7 ± 0.2^{aB}	10.7 ± 2.2	4.9
	Pumpkin60, R	6.38	12.5 ± 0.2^{aB}		
	Pumpkin60, UR	6.89	7.5 ± 3.3^{aA}		
	Pumpkin protein	6.39	11.3 ± 0.2^{aB}		

[1] Mean ± standard deviation; Different small (among genus) and capital (in between genera) raised letters indicate significant difference ($p < 0.05$)

Results showed that the majority of analyzed plant proteins had quite low solubilities (≤ 20%), which poses a substantial problem for their use in foods (**Table I.2**), since solubility is also a prerequisite for other functional properties (Day, 2013). Emulsification, foaming, and gelation typically require polymers to be mobile to facilitate molecular rearrangements at interfaces or in networks. The presence of high amounts of insoluble protein aggregates may interfere with those functions (Buchmann et al., 2019; Dai et al., 2019a). Consequences for food products involve lower dispersibility, changes in viscosity as well as altered consistency such as recently shown for infant formula and cakes upon rice, potato, or pea protein addition (Lin et al., 2017;

Roux et al., 2020). Similarly, we determined low solubilities for all rice proteins and the potato proteins (Solanic100T, Potato Protein) obtained by heat- and acid-induced coagulation (**Table I.2**). All in all, notable variations among all tested proteins were observed with values from as little as 3.9 % (Rice protein (C)) to 100% (Solanic300) meaning that some powders were almost completely insoluble while others were completely soluble. A comparison among and in between genera showed that solubilities varied substantially for some plant sources, but much less for others irrespective of supplier and composition. For example, solubilities of pea and wheat powder preparations varied from as little as 8.5% and 7.5% to as much as 42.4% and 28.8% representing a variance of 246.7 and 89.5, respectively. In contrast crude protein solubilities for oilseed proteins were very similar with values of 19.0 – 22.1 % (Sunflower; Variance 1.9) and 7.5 – 12.5 % (Pumpkin; Variance 4.9).

Various authors have reported variations in the functionality of alternative proteins extracts, depending on their respective extraction process and purity (Du et al., 2020; Feyzi et al., 2018; Karefyllakis et al., 2019; Wang et al., 2006). The authors evaluated this in the context of the *i*) applied extraction approach, *ii*) crude protein content, and *iii*) plant protein classes present of plant protein powders. For the latter, the classical classification scheme according to Osborne (1907) has been suggested to be a suitable mean to explain differences between tested genera. It groups proteins according to their solubility in water, saline solutions, aqueous alcohol solutions, and acid/alkali into albumins, globulins, prolamins, and glutelins, respectively.

Table I.3 summarizes Osbourne classification for the tested plant protein genera based on literature values. Due to their water-solubility, albumins are especially interesting when it comes to the aqueous solubility of plant protein powders. **Figure I.1** summarizes the determined crude protein solubility of each plant protein (**Table I.2)** plotted as a function of its theoretical content of water-soluble albumins from literature values and their individual crude protein content (**Figure I.1**) according to **Eq. I.5**. It was shown, that crude protein solubility increased from wheat < rice < pumpkin < sunflower < pea << canola < potato (Solanic200, 300). Exceptions were the two potato proteins Solanic100T, and Potato protein due to their denatured state. As a result, genera comprised mostly of albumins such as potato and canola were those with the best solubility and thus promising functionality for their application in foodstuff. In contrast, plant protein sources with a high amount of scarcely soluble glutelins showed an overall low solubility such as rice (5% albumins, 80% glutelins), wheat (4% albumins, 35% glutelins), and pumpkin (14% albumins, 49% glutelins).

Table I.3 Protein classification of the tested plant protein genera based on literature values and **Osborne (1907)**

Osborne fraction	Albumin	Globulin	Prolamin	Glutelins	Reference
Suitable solvents	Water/ Dilute saline solutions	Dilute saline solutions	Aqueous alcohol solutions	Acid/ Alkali	Osborne (1907)
Pea	20 %	65 %	--	15 %	Chéreau et al. (2016)
Wheat	4 %	7.5 %	45 %	35 %	Schormüller (1965)
Rice	5 %	13 %	3 %	80 %	Ju et al. (2001)
Potato[1]	50-60 %	25-26 %	2-4 %	9 %	Peksa et al. (2009).
Canola	50 %	25 %	5 %	10 %	Chéreau et al. (2016)
Sunflower	20 %	60 %	5 %	15 %	Chéreau et al. (2016)
Pumpkin	14 %	20 %	4 %	49 %	Pham et al. (2017)

[1] Alternative classification available into acid-soluble and -coagulable proteins

Further, better crude protein solubility was related to lower native pH and higher purity – meaning higher protein contents (**Table I.1**) and thus more albumins (**Figure I.1**). This was in accordance with previous results (Adebiyi et al., 2007; Rezig et al., 2013; Wang et al., 2006) where authors also proposed a beneficial effect of higher net electrical charge and repulsion in between individual protein molecules. For example, the wheat protein powder EMCEvitC had a protein solubility of 7.5 ± 0.2% at pH 6.08 at a calculated albumin content of 3.1 %, while wheat gluten had a solubility of 28.8 ± 0.1 % at a pH of 5.40 and an albumin content of 3.3 %. While roasting had no significant ($p > 0.05$) effect on the crude protein solubility (Pumpkin60, UR vs. R), the presence of residual salts or minerals (**Table SI.4**), resulted in an improved solubility as seen for PisaneC9, M9; and Pumpkin seed flour Pumpkin60, R as compared to PisaneF9, B9, and Pumpkin60, UR. Besides the notable differences among coagulated (Solanic100T, Potato Protein) and functional (Solanic200, 300) potato proteins (**Table I.2**), crude protein solubility among pea proteins was significantly different ($p \leq 0.05$) along with a high solubility variance of 246.7 and values ranged from 8.5% to as high as 49.5 %. This was irrespective of their theoretically calculated albumin content of 16.1 ± 0.3 % and thus especially contrasting from the aforementioned relations. Considering the long history of their availability on the market and the related technological progress, suppliers have invented elaborated and complex processes that combine fermentation, precipitation, ultrafiltration, and/ or heating to create tailored end-products for specific applications (Bourgeois et al., 2015a).

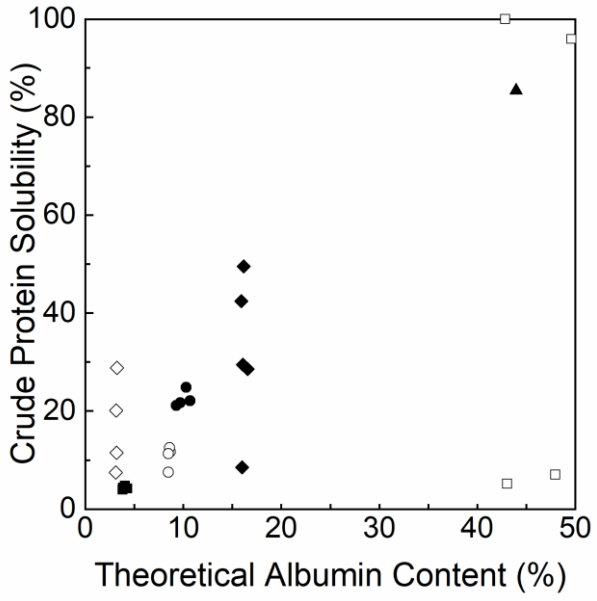

Figure I.1 Correlation of analyzed crude protein solubility (%) and theoretical content of albumins based on **Osborne (1907)** classification from literature for pea (◆), wheat (◇), rice (■), potato (□), canola (▲), sunflower (●), pumpkin (○).

This may not only cause changes of powder composition, but also promote re- or unfolding of proteins, their aggregation or co-precipitation with carbohydrates rendering end products more or less soluble and/or functional (Dai et al., 2020; Weiss et al., 2019). Based on this and our findings it is increasingly becoming clear that plant proteins exist in a wide range of conformational states, that can be impacted and modulated by superimposed processes, contributing to differences in properties within a single genus (Dai et al., 2019a; Grossmann et al., 2019a). Due to these process-related changes, estimations on the functionality of proteins that were in particular subjected to a multistep wet fractionation based on Osborne (1907) classification may not be valid, since it only considers the nature and behavior of individual protein molecules. Moreover, the presence of glycosylated proteins such as for example in potato (Peksa et al., 2009) – but also in other alternative proteins sources such as microalgae (Grossmann et al., 2019b) are also not accounted for in the classical Osborne scheme. Lastly, the distribution of the individual protein classes may also differ depending on the timepoint of harvest and external factors such as the availability of water, nutrients, or the climate. This was for example shown for wheat proteins, that also exhibited large differences in this study (Flambeau et al., 2017). An extension of the classical Osbornes scheme by these means may thus allow to establish a better link between solubility and technofunctionality eventually facilitating predictions on the suitability and behavior of plant proteins for their application in food matrices.

Conclusion

Currently available plant protein powders diverge in terms of solubility, native pH, and color. The degree of divergence varies with plant genera due to the prevalent protein classes, protein content in plants, as well as differences in extraction procedures. While complex multi-stage fractionations allow high purity, they may challenge plant protein applicability due to alterations in functional proteins or native pH. Moreover, emerging sources such as oilseeds still lack extraction processes to remove residual carbohydrates and pigments. Overall, this variability, and in general low solubility of many protein powders makes a prediction of their functionality and use for a particular food application difficult in particular in aqueous food systems such as beverages and matrices with high pH-dependency as it is the case for meat and dairy products. At present, manufacturers may have to use a range of additives such as colorants and buffering agents in order to achieve consumer acceptability in fortified foods. However, this study showed that this is not only due to non-optimal processes in which for example unwanted protein aggregations lead to decreased solubility but is a combination of both applied processes *and* intrinsic composition of proteins in the plant material. Many of these are storage proteins that do not exhibit functionalities that lend themselves to form functional structures, especially contrasting with meat or dairy proteins. More technological progress will be needed to obtain end product with high functionality in order to replace these traditional proteins in foods. An increase in solubilities *e.g.* through mechanical or chemical disruption of aggregates may offer a way forward to improve functionality of these "insoluble" fraction in the future (Dai et al., 2020). Finally, besides the respective solubility behavior, the use of each plant protein should be critically evaluated based on the presence of minor compounds such as antinutritional factors i.e. in leguminous plants (peas) and Solanaceae (potato), that negatively interact with digestive enzymes after ingestion (Savage & Morrison, 2003).

Acknowledgments

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Supporting Information

Table SI.4 Trade name, supplier, price range, and crude composition of analyzed plant protein powders based on manufacturers specifications.

Botanical origin	Trade name	Supplier	Price range [1]	Protein (%)	Fat (%)	Total Carbohydrates [2] (%)	Salt (%)
Pea	PisaneF9	Cosucra	Low	83.6	3.0	2.7	1.9
	PisaneC9	Cosucra	Low	81.7	4.0	3.2	3.7
	PisaneM9	Cosucra	Low	81.7	4.0	3.2	3.7
	PisaneB9	Cosucra	Low	81.7	4.0	3.2	1.0
	Pea protein	Ceresal	Mid	≥ 80.0	5.0-12.0	≤ 4.0-7.0	0.0
Wheat	VW Gluten	Kröner Stärke	Low	80.0	6.0	8.6	0.08
	EMCEvitC	Hydrosol	Low	76.0	6.0	11.0	0.25
	VWG75 Food	Beneo	Low	79.5	3.8	11.7	0.04
	Wheat gluten	Ceresal	Low	82.2	≤ 2.0	≤ 10.5	≤ 1.5
Rice	Plantforce Synergy	Third Wave Nutrition	High	90.0	4.7	4.3	0.0
	Rice protein	Hydrosol	n. a.	75.0-80.0	≤ 5.0	5.0-10.0	n. a.
	Remypro N80+	Beneo	Low	79.0	5.0	9.2	0.25
	Rice protein	Ceresal	Mid	80.0	8.0	≤ 5.0-8.0	0.0
Potato	Solanic100T	Avebe	Mid	78.0	4.1	< 5.5	> 0.1
	Solanic200	Avebe	High	90.5	0.2	< 5.5	0.04
	Solanic300	Avebe	High	93.2	0.2	< 0.3	0.04
	Potato protein	Ceresal	Mid	81.0	2.5	7.5	0.18
Canola	Teutexx Isolex	Teutoburger Ölmühle	High	91.0	4.0	3.0	2.0
Sunflower	Heliaflor45 [4]	All Organic	Mid	47.6	10.0	25.0	0.002
	Heliaflor55 [4]	All Organic	High	53.4	≤ 2.0	29.0	0.002
	Extrufix W [4]	All Organic	Low	47.6	10.0	25.0	0.002
	Extrufix D [4]	All Organic	High	53.0	≤ 2.0	29.0	0.002
	Sunflower flour [4]	Hydrosol	n.a.	40.0-45.0	10.0-15.0	5.0-10.0	n. a.
Pumpkin	Pumpkin seed flour	Fandler	Low	55.0	12.3	19.8	5.1
	Pumpkin60, R [4]	All Organic	Mid	59.3	12.8	15.5	1.99
	Pumpkin60, UR [4]	All Organic	High	60.0	9.0	17.0	0.01
	Pumpkin protein [4]	Ceresal	Low	59.2	14.7	12.6	< 0.25

[1] = Unit price (€/kg) < 5 (low), 5-10 (mid), >10 (high) [2] = Sum of carbohydrates and fiber; [3] = Calculated as sodium x 2.5; [4] =Organic

II. Chapter

Establishing the Mixing and Solubilization Behavior of Pork Meat and Potato Proteins at Acidic to Neutral pH

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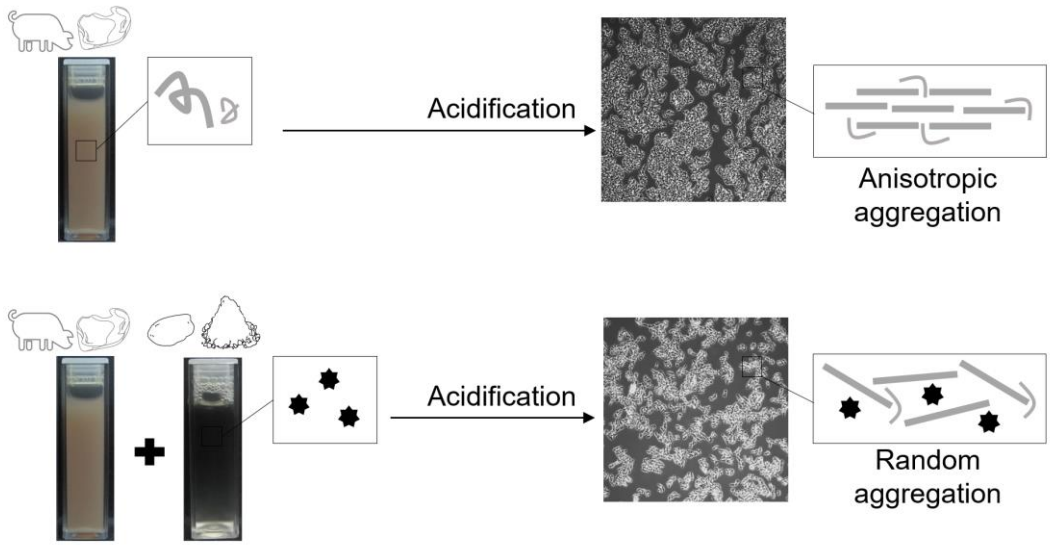
Abstract

Technofunctional properties of plant proteins deviate from those of animal-derived proteins and make their combination in foods challenging. In this work, proteins from a soluble, low-molecular weight potato protein fraction (<24 kDa) and pork meat were salt-solubilized (1.8 wt % NaCl) and mixed in a 50:50 (v/v) ratio to assess their miscibility at three different pH values. The mixture maintained high solubility at pH 7.0 irrespective of the proximity to the p*I* of potato proteins (pH 6.7 ± 0.1) and exhibited lower surface hydrophobicity (174) than individual potato (608) and meat (278) protein fractions. In contrast, mixtures were visible as large heteroprotein particles of dense, irregular shape at pH 5.0 and 3.0. These particles deviated from highly regular, anisotropic aggregates in pure meat dispersions or solutions of potato proteins that were aggregate-free and was related to modulations of electrostatic and hydrophobic meat–potato protein interactions. This study presents the first insights into and a basic understanding of the combinational effects of meat and plant proteins and is useful for further compatibility studies and the development of hybrid products.

KEYWORDS

Plant Protein; Meat Proteins; Protein Interactions; pH-dependency; Anisotropy

GRAPHICAL ABSTRACT



Introduction

Meat and meat products represent an essential part of human diets all over the world. With an increasing global population as well as ecological strains of the food system as a whole, there is a need to decrease overall meat consumption to improve sustainability (Willett et al., 2019). Aside from the approach to develop vegetarian or vegan products, there has also been interest to formulate so-called "hybrid foods" (Alves & Tavares, 2019) representing a category of products in which animal-based raw materials such as meat, milk, or eggs are partially replaced by alternative ones *e.g.* from microalgae, fungi, or plants.

The combination of these traditional and emerging raw materials to formulate appealing foods, however, is nontrivial because changes in functional or organoleptic properties of individual ingredients might occur upon mixing (El-Sayed, 2013; Jailson et al., 2016; Shoaib et al., 2018; Tahmasebi et al., 2016). This is in particular the case for mixtures of proteins with mostly unknown solubility, emulsification, foam stabilization, or gelation abilities (Asgar et al., 2010) or low functionality *e.g.* due to large fractions of insoluble proteins (Dai et al., 2019b; Ebert et al., 2020). Moreover, depending on the extraction procedure used, residual pigments and off-flavor notes may limit application quantities to formulate hybrids (Amagliani, 2017; Ebert et al., 2020; González-Pérez & Vereijken, 2007; Roland et al., 2017). While researchers have begun to investigate mixing effects in select product matrices (Gao et al., 2015d; Jailson et al., 2016; Porcella et al., 2001), knowledge on the molecular and colloidal interactions of meat and plant proteins is still limited. This involves not only fundamental mixing studies but also characterizations that allow a qualification of prevalent noncovalent interactions in such mixtures, *i.e.* electrostatic versus hydrophobic effects (Lund & Jönsson, 2003).

A first key process in the production of many cooked and dried stabilized meat products is a (partial) solubilization of meat proteins to enable a subsequent emulsification, foam formation, or gelation. In this study, we therefore focused on assessing the solubility behavior in mixtures of solubilized proteins from pork meat and a plant protein isolate. For the latter, a low-molecular weight fraction from potato protein was chosen due to its high functionality (*i.e.* solubility, emulsifying ability, and foaming ability), as well as detailed structural and compositional descriptions found in other studies (Ebert et al., 2020; Pouvreau et al., 2004; Pouvreau et al., 2005; Ralet & Guéguen, 2000; Schmidt et al., 2017; van Koningsveld et al., 2002; Zeeb et al., 2018). This makes it a promising ingredient in hybrid meat products to compensate for meat protein functionality upon their partial replacement. Furthermore, using soluble or solubilized

proteins as a starting point prevents insoluble aggregates from affecting the initial interaction behavior, thereby making it a good model system to have a first look at and for gaining basic information for future studies of meat−plant protein compatibility. Due to their different isoelectric points suggested in previous research(Pouvreau et al., 2001; Xiong, 2017), we hypothesized that pH may play a substantial role in the mixing behavior of these two protein sources. Therefore, mixing studies were carried out at three different pH values to evaluate the influence of potato upon meat protein functionality based on electrostatics, hydrophobics, and dispersibility behavior.

Materials and Methods

Lean pork meat SII (GEHA standard) was obtained from MEGA (Stuttgart, Germany). Potato protein isolate Solanic 300 (93.2 % crude protein, 0.2 % fibers, 0.1 % total fat, 0.1 % carbohydrates, and 0.04 % salt) was provided by Avebe (GK Veendam, The Netherlands). *N*,*N*-Dimethyl-6-propionyl-2-naphthyl-amine (PRODAN) and sodium azide (≥ 99.0% pure) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol (≥ 99.9% pure), tris(hydroxymethyl)aminomethane (THAM, ≥ 99.9% pure), sodium chloride (≥ 99.5% pure), sodium hydroxide (≥ 98.0% pure), hydrochloric acid (10.2 % molarity), sodium phosphate dibasic (molecular weight of 142 g/mol), and citric acid monohydrate (molecular weight of 210 g/mol) were acquired from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Doubly deionized (DI) water was used for all experiments.

Protein solubilization and sample preparation

Meat protein solubilization

Meat protein solubilization was performed according to the method of Hermanianto (1995) with slight modifications (**Figure II.1**). Lean pork meat SII (4 °C) was minced with a grinder (type WD114, Seydelmann, Aalen, Germany) using a 3 mm punch disk and chopped with a bowl chopper (Stephan UMC 5, Stephan Machinery GmbH, Hameln, Germany) with 2 parts of ice and 1.8 wt % sodium chloride at 3000 rpm for 2 × 6 min. The meat batter suspension was subsequently centrifuged (J6-MI, Beckman Coulter, Brea, CA) at 20000 g for 30 min at 4 °C. The supernatant was decanted and vacuum filtered (Sartorius Filter Discs 3hw, pore size of 8−12 μm, Sarorius AG, Göttingen, Germany), and 0.03 wt % sodium azide were added to prevent microbial spoilage. The pellet was discarded. Solubilized meat proteins were packed airtight and stored at - 18 °C until further use.

Potato protein solubilization

Solanic 300 potato protein was dispersed in a saline solution (DI water containing 1.8 wt % sodium chloride) at a crude protein concentration of 10.0 wt % (**Figure II.1**). Samples were stirred overnight to ensure hydration. Following this, the native pH of 3.82 was adjusted to a value of 5.8 by using solutions of sodium hydroxide or hydrochloric acid, related to the native pH of pork meat (Keeton et al., 2014) and solubilized meat proteins in this study. Samples were stirred for at least 4 h, and the pH was continuously adjusted to compensate for the buffering capacity of proteins. Resulting potato protein suspensions were centrifuged at 20000 g for 30 min at 4 °C. The supernatant was decanted; 0.03 wt % sodium azide was added, and the mixture stored at 4 °C until further use. The pellet was discarded.

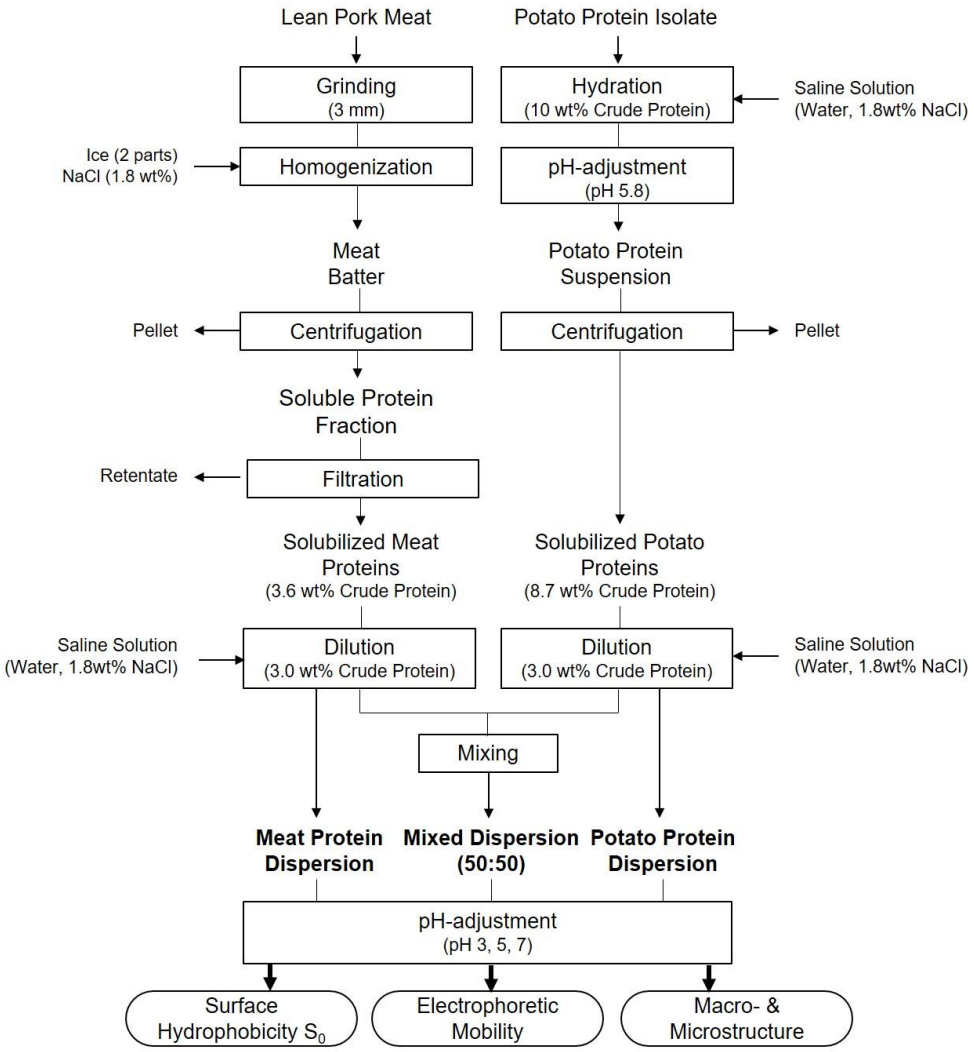


Figure II.1 Schematic overview of the solubilization of water- and salt-soluble pork and potato proteins.

Total nitrogen content

The total nitrogen content of solubilized meat and potato proteins was determined with a flash combustion method with a Dumatherm DT N Pro Analyzer (C. Gerhardt GmbH & Co KG, Königswinter, Germany) according to the Dumas method (BVL, 2005b). Results were used as a prerequisite for subsequent sample preparation and analysis. In short, samples were weighed accurately into tin foils (Dumafoil, C. Gerhardt GmbH & Co. KG) equipped with an absorbent for polar samples (DumaSorb, C. Gerhardt GmbH & Co. KG) at a sample:absorbent ratio of 4:1, combusted with oxygen at 1030 °C, and reduced with copper at 750 °C. The release of nitrogen was then detected by the conductivity change of a standardized helium flow, and the crude protein content was calculated by using a common nitrogen-to-protein conversion factor of 6.25 (Mariotti et al., 2008).

Preparation of sample dispersions

Solubilized meat and potato proteins were diluted in a saline solution (DI water containing 1.8 wt % sodium chloride) to a nitrogen content of 0.48 % (crude protein content of 3.0 wt %). The pH was re-adjusted to 5.8 if necessary. The sodium azide concentration was fixed to 0.03 wt % to prevent microbial spoilage. A mixture of individual meat and potato proteins was prepared at a mixing ratio of 50:50 (v/v). Samples were stirred for at least 2 h at room temperature.

pH-adjustment

Individual meat and potato protein sample dispersions were adjusted to pH 3.0, 5.0, and 7.0 by using hydrochloric acid and sodium hydroxide. Samples were stirred and stored overnight at room temperature. The pH was monitored and adjusted if necessary.

Sample analysis

Protein characterization by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS−PAGE).

SDS−PAGE under reducing conditions was performed with a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Hercules, CA). Solubilized proteins were diluted to 4 mg/mL in DI water and then mixed in a 1:1 ratio with a reducing sample buffer prepared according to the method of Laemmli.28 An aliquot of 10 µL was loaded onto 4 - 20 % Mini-PROTEAN TGX precast gels (Bio-Rad Laboratories), and electrophoresis was performed at 200 V in a 25 mM Tris-HCl buffer solution (pH 8.3, 0.192 M glycine and 0.1 wt% SDS) for 35 min. A standard marker

(Roti-Mark PRESTAINED, 17 - 245 kDa, Carl Roth GmbH & Co. KG) was used to estimate macromolecules in protein dispersions according to their molecular weight. Following this, gels were stained with Coomassie Brilliant Blue for 45 min and destained overnight with a mixture of 10 % (v/v) acetic acid and 15 % (v/v) methanol.

Surface hydrophobicity S_0

Surface hydrophobicity S_0 of individual proteins and their mixture (**Figure II.1**) was analyzed according to a procedure from (Reichert et al., 2015) with slight modifications. Samples were diluted to 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 wt % crude protein in McIlvaine buffer solutions at pH 3.0, 5.0, and 7.0. Buffers were prepared by blending 0.2 M sodium phosphate dibasic and 0.1 M citric acid monohydrate solutions at ratios of 79.45:20.55 (v/v), 48.5:51.5 (v/v), and 17.65:82.35 (v/v), respectively and 1.8 wt % sodium chloride was added. Samples were mixed thoroughly. PRODAN was dispersed in methanol at a concentration of 1.41 mM. For analysis, two aliquots of 180 μL per dilution were transferred into the wells of a 96-well microtiter plate (Nunclon, Delta 96-Well MicroWell, Thermo Scientific) and the fluorescence was measured with and without the addition of 20 μL of a PRODAN solution after shaking and a waiting time of 15 min in the dark. Excitation and emission wavelengths of 365 and 465 nm, respectively, were used, and the surface hydrophobicity was calculated as a slope from the net relative fluorescence intensity versus concentration (v/v) by linear regression. At least two dilution sets were prepared and measured at least in duplicate to maximize the linear fit through coefficient of determination R^2.

Electrophoretic mobility and isoelectric point (pI)

The electrophoretic mobilities of individual proteins and their mixtures at pH 3.0, 5.0, and 7.0 were determined with a particle electrophoresis instrument (Nano ZS, Malvern Instruments, Malvern, U.K.) in disposable folded capillary cells (DTS1070, Malvern Instruments) without further dilution. The ζ-potential and the average particle size (z-average) were calculated via the Smoluchoswski model and the Stokes−Einstein equation, respectively, based on the electrophoretic mobility of protein particles in the applied electric field. The isoelectric point (p*I*) of proteins was interpolated from plots of the ζ- potential versus pH as the point of zero net charge between pH 3.0 and 8.0. For that purpose, sample dispersions were diluted to 0.1 wt % crude protein content, adjusted to a pH value of 8.0, and transferred to disposable folded capillary cells (DTS1070, Malvern Instruments). An integrated titrator device (MPT-2 Titrator, Malvern Instruments) was used to perform sequential titrations by the addition of hydrochloric

acid (0.1 and 0.01 N). The sample charge was measured every 0.2 pH unit. All measurements were taken at 25 °C.

Visual appearance and formation of sediment layer due to phase separation

Pictures of samples were taken after overnight storage with an iPhoneX (Apple Inc., Cupertino, CA) under controlled illumination. Additionally, phase separation was assessed by measuring the height of the sedimented phase in relation to the total height according to **Eq. II.1.**

$$Separation\ Index\ (\%) = \frac{h_{Sediment}}{h_{Total}} * 100 \qquad \textbf{II.1}$$

Optical microscopy and image analysis

The microstructure of samples was investigated with a light microscope (Axio Scope A1) equipped with a digital camera (AxioCam ICc3) (Carl Zeiss Microscopy GmbH, Jena, Germany) at a 100-fold magnification (Objective A-Plan 10×/0.25 M27). Microscopic images were evaluated with ImageJ (ImageJ 1.52a, National Institutes of Health, Bethesda, MD) after a conversion and adjustment to binary and a standardized threshold that was used for all samples. The perimeter (micrometers) and aggregate area (square micrometers) were calculated by an ImageJ shape-descriptor macro.

Statistical analysis

A one-way analysis of variance with a Duncan post hoc test was carried out, and an α-level of 0.05 was used to test statistically significant differences among samples using SPSS statistics V23 (IBM Corp., Armonk, NY). All analyses were performed at least in duplicate, and measurements were repeated at least twice from freshly prepared samples. Image analysis was performed from at least four images, and obtained data were averaged.

Results and Discussion

The functionality of proteins is known to depend on various parameters such as their inherent composition, size, and folding, as well as external factors, *e.g.* pH, ionic strength, and temperature (Zayas, 1997a). This dependency is crucial for their application in food systems due to their complex composition and in particular if different protein sources are mixed (Polyakov et al., 1997). In this study, solubilized protein fractions from pork meat and a functional potato protein fraction (**Figure II.1**) were analyzed in their individual macromolecular composition by SDS−PAGE and mixing behavior at a 50:50 (v/v) ratio with

an emphasis on surface charge, particle size, and surface hydrophobicity. Three different pH values of 3.0, 5.0, and 7.0 representative for points below, around, and above the isoelectric point (pI) of many food proteins (Flambeau et al., 2017; Hoogenkamp et al., 2017; Pelegrine & Gasparetto, 2005; Thrane et al., 2017; Tulbek et al., 2017; Xiong, 2014). Visual observations and microscopic appearance were done additionally and quantitatively interpreted by image analysis.

Protein characterization by SDS-PAGE

SDS-PAGE under reducing conditions was performed to prove successful isolation of obtained meat protein fractions, as well as to characterize their individual protein macromolecules based on their molecular weight (**Figure II.2**). Gels showed that solubilized pork meat proteins were a heterologous mixture with molecular weights ranging from 15 to > 100 kDa with at least 16 different macromolecules with intensified bands from 23 to ~55 kDa. It is known from literature that meat is composed of approximately 16 - 22 % protein, with a majority of it being water-soluble (sarcoplasmic) and salt-soluble (myofibrillar) (Keeton et al., 2014). Water-soluble glycolytic enzymes are visible in the low-molecular weight area, and respective bands include GAPDH (38 kDa), creatine kinase (40 kDa), and/or enolase (54 kDa), as well as 16 kDa myoglobin as the characteristic red colorant in meat (Keeton et al., 2014; Radoslav & Savanović, 2018). Salt-soluble meat proteins mostly represent the actin and myosin family with bands at 24−37 and 95 kDa (actinins) and 42 kDa (actin) and myosin light chains between 15 and 22 kDa (Bhagavan, 2002; Radoslav & Savanović, 2018). High-molecular weight proteins such as myosin (440 kDa), titin, and nebulin (>1000 kDa) were not clearly visible on the SDS gels in this study, possibly caused by a partial proteolysis to smaller fragments of the myosin heavy chains (~100 kDa) as observed by other authors (Bandman & Zdanis, 1988; Ikeuchi et al., 2001; Yates et al., 1983) and their overall low degree of solubilization from meat (Boland et al., 2019).

In contrast to the broad molecular weight distribution and mixture of macromolecules in solubilized meat proteins, that of the potato protein fraction was narrow and limited to < 24 kDa. Bands were visible at 20 - 23, 16, and < 10 kDa consistent with a recent study by Zeeb et al. (2018). Solubilized potato proteins represent a family of acid-soluble macromolecules with protease inhibitor activity (Peksa et al., 2009; Pouvreau et al., 2001) and are obtained by separation from the major glycoprotein fraction (patatins) in potato juice through size- and charge-based exclusion chromatography methods (Schmidt et al., 2017; van Koningsveld et al.,

2001). This mild and highly elaborated extraction results in a high-purity isolate and the absence of aggregated or other proteins that are not protease inhibitors (**Figure II.2**).

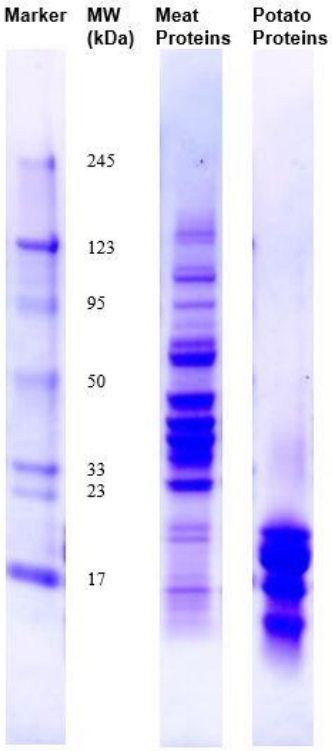


Figure II.2 Molecular weight distribution of solubilized meat and potato proteins determined by SDS−PAGE

Results of SDS−PAGE thus confirmed a successful extraction of water- and salt-soluble proteins from meat. They also illustrated profound differences between the two protein types, with mainly narrowly distributed, low-molecular weight potato proteins and broadly distributed meat proteins having low and higher molecular weights, respectively. Likely, these differences may also lead to varying electrostatic and hydrophobic properties, which may affect their mixing behavior.

pH-dependent hydrophobicity and electrophoretic mobility

Besides their inherent macromolecular composition, protein functionality may also be modulated by external factors such as pH (Zayas, 1997a). This not only involves effects on the effective protein charge but also other noncovalent interactions such as hydrophobic forces. Therefore, both electrostatic interactions (as characterized here by the ζ-potential and related Z-average) and hydrophobicities (S_0) of individual meat and potato protein dispersions and their 50:50 (v/v) mixture were assessed at pH 3.0, 5.0, and 7.0 (**Figure II.1**), and results were then related to their solubility behavior.

Solubilized meat proteins and the 50:50 (v/v) meat/potato protein mixture had the highest surface hydrophobicity S_0 at pH 3.0 with values of 571 ±12 and 405 ±112, respectively (**Table II.1**), but significantly ($p \leq 0.05$) lower values were found at pH 5.0 and 7.0 (174 ± 33 to 278 ± 41, respectively). Large values generally mean a high accessibility of hydrophobic patches to the probe and thus a localization of hydrophobic amino acid residues on the outside of the tertiary structure of macromolecules. This is especially true for proteins that re- or unfold as it is the case for meat proteins below their isoelectric point p*I* (Xiong, 2017) and validated by results obtained in this study (**Table II.1**).

Table II.1 Isoelectric points p*I* (-) of solubilized proteins and their mixture (50:50), surface hydrophobicities (S_0) with coefficients of determination R^2 (-) from linear regression of the measured fluorescence intensity and protein concentration, Particle size as Z-average (μm), and ζ-potentials (mV) at pH 3.0, 5.0, and 7.0[a]

	S_0 (-) and (R^2)	Particle size (μm)	ζ-potential (mV)	p*I* (-)
Meat Proteins				
pH 3	571 ± 12 ^{bE} (0.98)	18.0 ± 2.9 ^{cB}	12.1 ± 0.2 ^{cE}	5.7 ± 0.1
pH 5	215 ± 52 ^{aABC} (0.82)	26.5 ± 0.4 ^{bC}	-0.7 ± 0.9 ^{bBC}	
pH 7	278 ± 41 ^{aBCD} (0.87)	0.9 ± 0.2 ^{aA}	-5.9 ± 0.1 ^{aA}	
Mixture (50:50)				
pH 3	405 ± 112 ^{cD} (0.95)	26.8 ± 9.0 ^{bC}	11.3 ± 1.6 ^{cE}	5.9 ± 0.1
pH 5	187 ± 81 ^{bAB} (0.89)	28.8 ± 3.6 ^{bC}	-0.8 ± 0.1 ^{bBC}	
pH 7	174 ± 33 ^{aAB} (0.87)	1.6 ± 0.1 ^{aA}	-3.7 ± 0.6 ^{aAB}	
Potato Proteins				
pH 3	108 ± 20 ^{aA} (0.72)	0.1 ± 0.1 ^{aA}	3.5 ± 0.1 ^{bD}	6.7 ± 0.1
pH 5	325 ± 28 ^{bCD}(0.89)	0.5 ± 0.1 ^{aA}	0.7 ± 0.2 ^{aCD}	
pH 7	608 ± 6 ^{bE} (0.87)	0.3 ± 0.2 ^{aA}	-0.4 ± 0.1 ^{aBC}	

[a]Superscript lowercase letters indicate significant ($p \leq 0.05$) differences among samples from the same protein fraction. Superscript uppercase letters indicate significant ($p \leq 0.05$) differences among all samples in one column.

In contrast, lower values may not only be related to an overall lower hydrophobicity, and thus a higher hydrophilicity, but also a cross-association of hydrophobic patches prior to analysis as recently proposed for pea proteins that were analyzed according to the same protocol (Reichert et al., 2015). This may be especially true at pH 5.0, where both the meat proteins and the mixed fraction revealed an overall low ζ-potential (- 0.7 ± 0.9 and - 0.8 ± 0.1 mV, respectively), indicating a weak electrostatic repulsion and proximity to the p*I*. As a result, protein-protein interactions through hydrophobic bonds are highly favored (Gehring et al., 2009). Consequential association of hydrophobic patches in homo- or heteroprotein aggregate structures resulted in lowered surface hydrophobicity, due to their inaccessibility to the probe

as, *i.e.* for meat proteins with a value of 215 ± 52 (pH 5.0) compared to values of 571 ± 12 (pH 3.0) and 278 ± 41 (pH 7.0). This was further supported by the large particle sizes (Z-average) of meat proteins and their mixture with >20 µm at pH 5.0 (**Table II.1**). Conversely, low S_0 values at pH 7.0 along with small particle sizes (< 2 μm) and negative, large-magnitude charges are indicative of a strong electrostatic repulsion between proteins of the meat protein fraction and the meat/potato mixture and the absence of (hydrophobic) protein-protein association. In contrast to this, the surface hydrophobicity of the potato protein fraction gradually increased from pH 3.0 (108 ± 20) to pH 5.0 (325 ± 28) and pH 7.0 (608 ± 6), while particle sizes stayed well below 1 μm with no significant changes ($p > 0.05$). Moreover, the absolute value of the ζ-potential was generally lower than those of meat proteins and their mixture but in accordance with previous studies, where low diffuse surface charges of the protease inhibitor fraction were observed, especially around pH 5.0 (Schmidt et al., 2018; Zeeb et al., 2018).

As already noted, protein-protein association and precipitation around the p*I* are promoted due to a preference of protein-protein hydrophobic interactions over protein-water electrostatic interactions due to a zero net charge (Gehring et al., 2009). This also means that the p*I* represents an inflection point where changes in the respective hydrophobic and electrostatic interactions of macromolecules with each other and their surrounding might lead to changes in protein functionality but also trigger complex formation and/or precipitation. In this study, p*I*s of solubilized meat and potato proteins and their mixture were defined at pH 5.7 ± 0.1, 6.7 ± 0.1, and 5.9 ± 0.1, respectively (**Table II.1**). All values represent means, considering the heterologous mixture of proteins, which may not only vary in molecular weight (**Figure II.2**). but also p*I*. If only solubilized meat proteins were considered, individual p*I*s would be found to range from pH 5.0 (salt-soluble myosin) to pH 9.0 (water-soluble enolase), coinciding with a p*I* range between pH 5.0 and 6.0 (Farrar & Deal, 1995; Xiong, 2014). Moreover, previous analysis on the electrophoretic mobility revealed low charge magnitudes at pH 5.8 (Gibis et al., 2017) fitting to the p*I* determined in this study (**Table II.1**). Similarly, acid-soluble protease inhibitors from potato varied in their p*I*s from pH 5.1 to > 9.0 (Pouvreau et al., 2001), which is consistent with increased surface hydrophobicities and overall low charge magnitudes at pH 5.0 and 7.0 in this study (**Table II.1**) and suggests the presence of a heterologous mixture of positively and negatively charged proteins. Interestingly, the p*I* of the 50:50 (v/v) meat/potato protein mixture was shifted more toward that of the meat proteins, revealing its dominance over potato proteins. This was further supported when the ξ-potential of sample dispersions at pH 3.0, 5.0, and 7.0 was considered, because no significant ($p > 0.05$) differences of solubilized meat proteins and the meat/potato mixture were found. Despite the proximity of particle sizes,

mixtures always contained larger particles than the sole meat fractions , *i.e.* 26.5 ± 0.4 μm (meat proteins) and 28.8 ± 3.6 μm (mixture), at pH 5.0 making an interaction of meat and potato proteins and thus the formation of larger heteroprotein particles highly likely. Analysis of hydrophobicities and charges of mixtures of solubilized proteins indicated that these properties appear to be dominated by meat rather than potato proteins. Likely, the emerging properties of heteroprotein aggregates were related to a combination of hydrophobic and electrostatic forces, because the surface hydrophobicity was low compared to those of the individual protein fractions, and electrostatic attraction may occur due to the divergence of the p*I* values of the two types of proteins.

Appearance and image analysis

Surface hydrophobicity and electrophoretic mobility results were further supplemented by visual observations and light microscopy of individual protein dispersions and their 50:50 (v/v) mixture (**Figure II.3, Figure II.4**) Separation index and perimeter and aggregate area were calculated from macroscopic and microscopic images, respectively, to allow for a quantitative and not just qualitative comparison (**Table II.2**).

Table II.2 Separation indices, perimeters, and aggregate area of solubilized proteins and their mixture (50:50) from visual observation and optical microscopy[a]

	Separation index (%)	Perimeter (μm)	Aggregate area (μm²)
Meat Proteins			
pH 3	75.6 ± 5.0 ^{bD}	229.4 ± 86.1 ^{aB}	4233 ± 2043 ^{aB}
pH 5	80.6 ± 1.2 ^{bD}	123.2 ± 18.7 ^{aAB}	944 ± 428 ^{aA}
pH 7	3.0 ± 0.1 ^{aA}	42.7 ± 5.5 ^{aA}	153 ± 64 ^{aA}
Mixture (50:50)			
pH 3	58.3 ± 5.3 ^{bC}	378.4 ± 47.6 ^{bC}	4834 ± 1256 ^{bB}
pH 5	44.7 ± 8.3 ^{bB}	99.4 ± 15.1 ^{aA}	580 ± 166 ^{aA}
pH 7	0.0 ^{aA}	29.5 ± 7.0 ^{aA}	64 ± 20 ^{aA}
Potato Proteins			
pH 3	0.0 ^{aA}	32.1 ± 8.6 ^{aA}	103 ± 65 ^{aA}
pH 5	0.0 ^{aA}	38.9 ± 15.1 ^{aA}	97 ± 26 ^{aA}
pH 7	0.0 ^{aA}	35.1 ± 2.3 ^{aA}	106 ± 20 ^{aA}

[a] Superscript lowercase letters indicate significant ($p \leq 0.05$) differences among samples from the same protein fraction. Superscript uppercase letters indicate significant ($p \leq 0.05$) differences among all samples.

Photographs and microscopic images show that potato proteins were soluble at all tested pH values (**Figure II.3**) and no phase separation occurred (**Table II.2**).This supported previous particle size measurements (**Table II.1**) and is in agreement with findings from other authors

on the good dispersibility of potato proteins (Ebert et al., 2020; Schmidt et al., 2019). Reasons for this may be the broad range of p*I* values in this potato protein fractions (Pouvreau et al., 2001) enabling sufficient electrostatic repulsion between macromolecules, or an overall high hydrophilicity, as shown for other alternative proteins such as those from microalgae. (Grossmann et al., 2019b). Moreover, potato proteins were recently shown to maintain their secondary structure irrespective of changes in pH (Pouvreau et al., 2004; Pouvreau et al., 2005). However, light microscopy revealed the presence of small, round-shaped particulates at pH 5.0 and 7.0 hinting at some protein association, which confirmed results obtained from surface hydrophobicity and electrophoretic mobility measurements (**Table II.1**) Calculations of perimeter and aggregate area based on the microscopic images showed the same trends, but no significant ($p > 0.05$) difference, *e.g.,* perimeters of 32.1 ± 8.6 μm (pH 3.0), 38.9 ± 15.1 μm (pH 5.0), and 35.1 ± 2.3 μm (pH 7.0) (**Table II.2**).

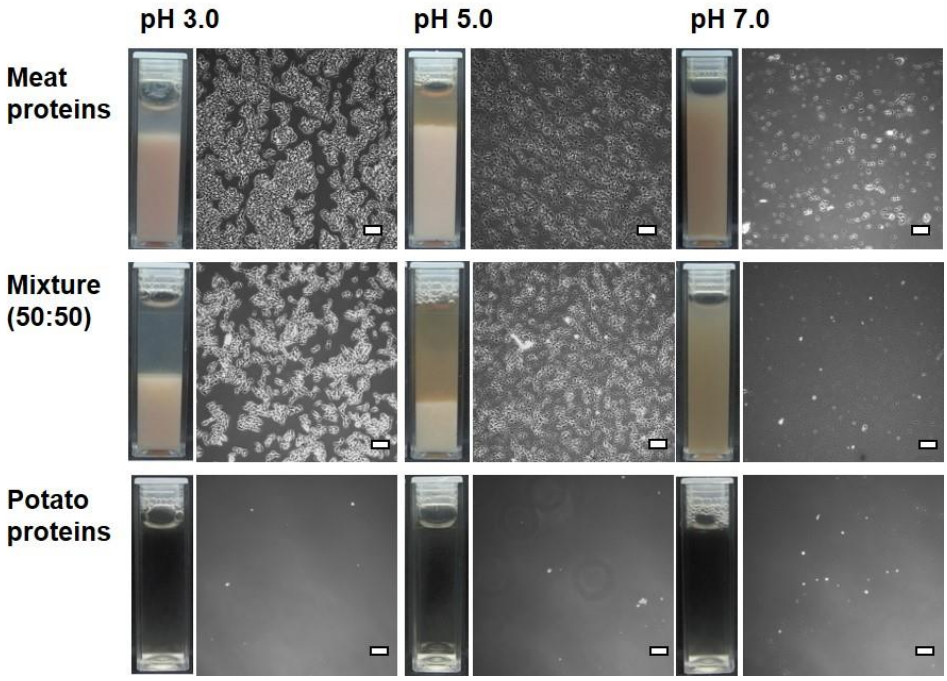


Figure II.3 Macroscopic and microscopic images of solubilized pork and potato proteins and their mixture (50:50) at pH 3.0, 5.0, and 7.0. Magnification of 100-fold. Scale bar of 100 μm

In contrast, meat proteins were monophasic at pH 7.0 and microscopically appeared as small, dispersed, ellipsoid particles with an area of ~ 200 μm^2. At pH 5.0, which was below their overall p*I* (**Table II.1**) and around the p*I* of the salt-soluble myosin (Xiong, 2014), loose aggregation occurred and proteins precipitated into small subunits, that were well-organized and had anisotropic, elongated microstructures (**Figure II.4**). These aggregates further evolved into a three-dimensional network at pH 3.0 which could be linked to the phenomenon of acid-

included gelation based on an unfolding and coagulation of salt-soluble meat proteins below their p*I* (Brewer, 2014; Raghavan & Kristinsson, 2007; Sun & Holley, 2011). Results indicated significantly ($p \leq 0.05$) larger values of perimeter (229.4 ± 86.1 μm) and aggregate area (4233 ± 2043 μm^2), while phase separation was decreasing due to the formation of a dense network. The solubility behavior of the meat/potato protein mixture was again dominated by the behavior of the meat protein fraction with no phase separation at pH 7.0, but precipitation at pH 5.0 and 3.0. Microscopic images further revealed the presence of more irregular, less coherent, and random structures with significantly ($p \leq 0.05$) increased values of perimeter and area. Macro- and microstructural observations thus supported results from previous physicochemical property characterizations, in particular the prevalent behavior of solubilized meat proteins that appeared to dominate structure formation in mixtures with potato proteins. In comparison with the highly ordered anisotropic aggregation of meat proteins in the absence of potato proteins, potato proteins apparently interfered with this structural organization with a decrease in pH, resulting in random precipitation and an altered microstructure. Trends seen in image analysis agreed with results from particle size determinations using dynamic light scattering, albeit values were much higher especially when intensively phase-separated samples or nonspherical irregularly shaped aggregate structures were considered.

Proposed mechanism for the solubility and mixing behavior and meaning for product developers

The solubility behavior of meat proteins depended strongly on pH, while potato proteins possessed a high solubility irrespective of the tested pH value. The behavior of the mixtures was markedly different from that of individual fractions, especially in their microstructural arrangements. The proposed mechanisms at pH 3.0 *(i)*, 5.0 *(ii)*, and 7.0 *(iii)* are illustrated in **Figure II.4** and discussed below.

(i) pH 3.0. The high solubility of potato proteins related to repulsive, positive electrostatic charge and low surface hydrophobicity disturbs the formation of a coherent, anisotropic three-dimensional network of unfolded and coagulated meat proteins in a mixture. This promotes irregular and random precipitation with the formation of smaller, spiked protein aggregates and a dense phase separation.

(ii) pH 5.0. Low surface charge and intermediate surface hydrophobicity trigger some potato protein association. These associates and individual potato proteins interact with salt- and water-soluble meat proteins, thereby interfering with their loosely aligned structural association between their individual pI values and decreasing phase separation.

(iii)pH 7.0. Potato proteins possess high surface hydrophobicity due to the close proximity to their p*I*. Microscopic aggregation occurs but, dispersions remain soluble, and no interaction with solubilized meat proteins occurs due to their electrostatic repulsion.

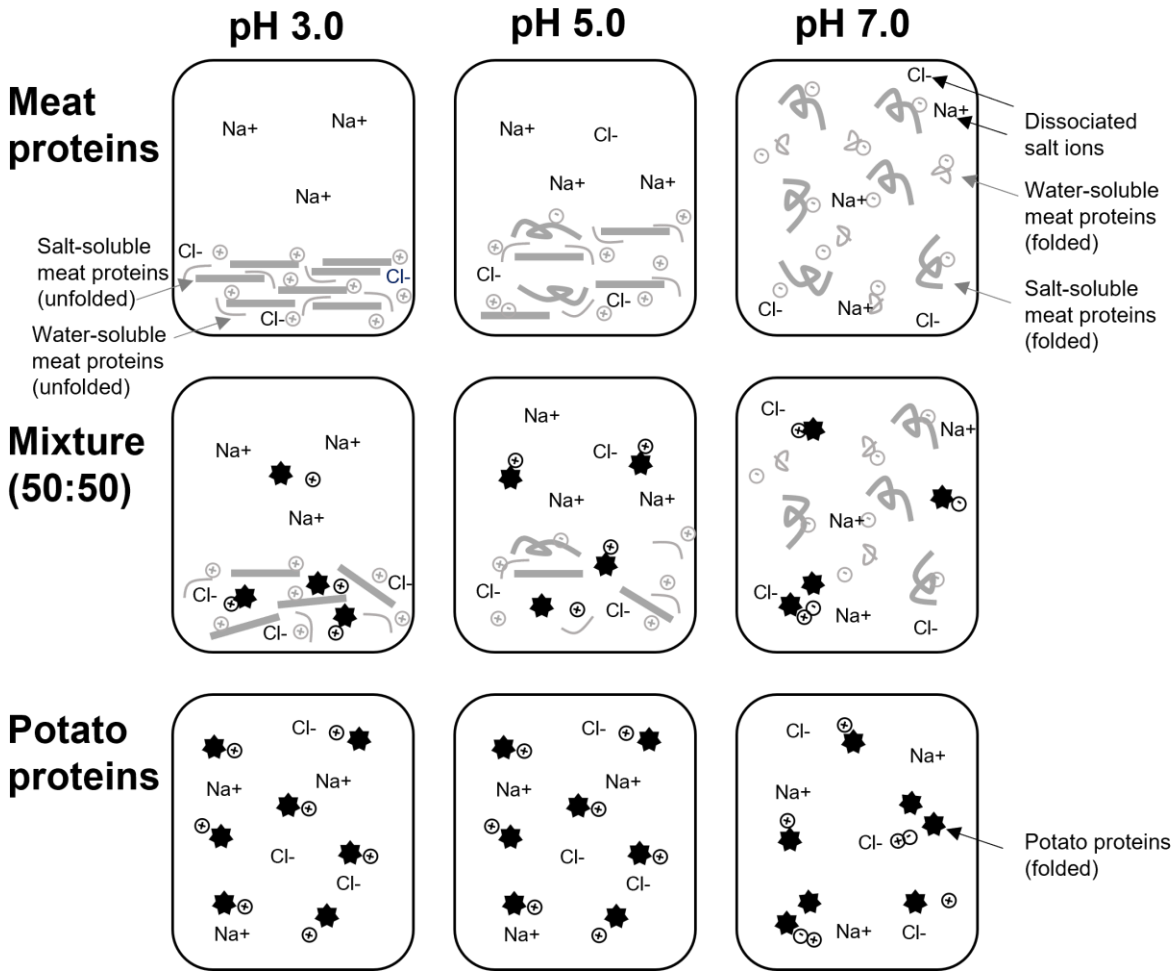


Figure II.4 Proposed mechanistic model describing the solubility and mixing behavior of solubilized meat and potato proteins.

Thus, individual protein dispersions and their mixture are macroscopically soluble at neutral pH irrespective of the proximity to their combined and individual isoelectric points, while precipitation occurs at pH 5.0 and 3.0. This is based on a complex mixture of electrostatic and hydrophobic effects simultaneously happening upon mixing and acidification of solubilized meat and potato proteins, which is especially contrasting with, *i.e.* carbohydrate-protein complexation that is mostly charge-driven. Furthermore, alterations in the microscopic aggregation behavior in a mixture can be related to competitive effects of potato proteins and the structural self-organization of meat-based ones.

These findings may prove to be a connector to link molecular scale to bulk and thus facilitate a more rational product development. This is especially true for meat hybrids such as sausages, in particular if the replacement of meat with a high content of plant matter is considered since the technofunctionality of meat proteins plays a crucial role in the development of their textural and organoleptic properties. On the basis of the obtained results, there may be limits to the ability of meat proteins to maintain the desired anisotropic structure formation that gives rise to bite and mouthfeel and thus critical mixing ratios beyond which an addition of plant proteins is detrimental to the properties of the obtained end product. However, the combination of meat and potato proteins also gives rise to the development of novel products with modulated textural and organoleptic properties. Clearly, further investigation of behavior in subsequent product applications and more complex mixtures is needed to verify this, and effects of intrinsic or extrinsic factors such as, e.g., mixing ratio, salt, and protein concentration or pH and temperature need to be assessed.

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III. Chapter

Aggregation Behavior of Solubilized Meat - Potato Protein Mixtures

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Abstract

Knowledge on the mixing behavior of meat and plant proteins is of importance for the manufacture of so-called hybrid products, a product category that is becoming of interest to food manufacturers. The aim of this study was to assess the pH-dependent miscibility and aggregation behavior in combinations of solubilized pork meat and potato tuber proteins. Analysis on their individual pH-dependent surface charge and size were followed by a visual and microscopic evaluation of their mixtures. Image analysis was used to obtain separation indices, aggregate areas, and aggregate aspect ratios and FTIR spectra were recorded to obtain insights into secondary structural elements. Net zero charge transition points differed, with a higher value for potato (pH 6.8) compared to meat proteins (pH 5.7). In mixtures, homogeneous solutions (pH 5.8 and 7.0) abruptly changed to phase-separated ones at low (pH 3) to medium (pH 5.5) pH. Here, anisotropic, clustered aggregates at high meat protein contents were exchanged for smaller, irregularly-shaped and sized microstructures at low ones and image analysis suggested a perturbing effect of potato on meat proteins to associate and form fibrous aggregates. The isoelectric point of salt-soluble meat proteins (pH ~ 5.5) was described as defining boundary value for co-solubility or interaction in between individual meat and meat and potato proteins. FTIR results confirmed alterations as a function of mixing ratio and pH. Results indicate that significant textural changes can be expected to occur in hybrid matrices, with plant proteins impeding the structural-self-association of meat proteins

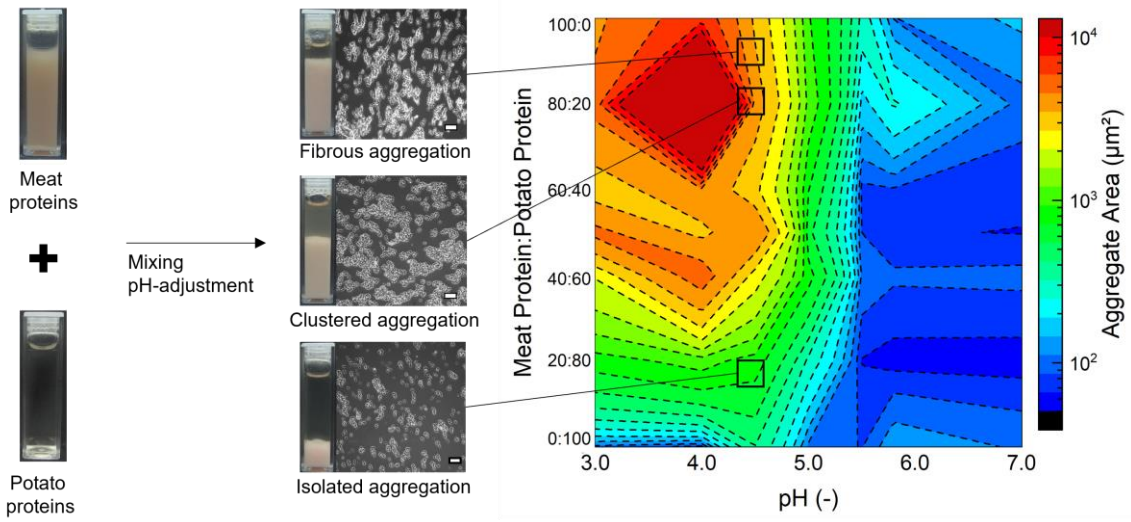
HIGHLIGHTS

- Potato proteins do not act co-solutes but show a complex pH-dependent interaction.
- Interactions of meat with potato proteins change the aggregation below their pI.
- Coherent, stranded meat protein networks evolve to irregular, isolated aggregates.

KEYWORDS

Potato Protein; Pork Meat Protein; Hybrids; Aggregation; Interaction; pH-dependency

GRAPHICAL ABSTRACT



Introduction

Solubility represents one of the most important physicochemical properties of proteins since it determines their ability to act as functional agents such as emulsion and foam stabilizers, or network formers (Zayas, 1997b). Solubility is impacted by a protein's structural characteristic. This includes the amount and location of hydrophilic amino acids affecting surface hydrophobicity and charge at the protein's sur-face, as well as non-covalent interactions of individual amino acid residues or peptide side-chains. It can be modulated by pH, temperature, or the properties of the solvent in which proteins are dispersed, *i.e.* by adding co-solutes or co-solvents (Damodaran, 1997; Yada, 2017). Depending on this, proteins may stay in solution or aggregate and precipitate thereby forming a variety of structures that facilitate (or prevent) their use in a broad variety of food products.

A prominent example of the deliberate modulation of protein solu-bility are many meat products (Gibis et al., 2014). Here, meat proteins are solubilized to a smaller or greater extent to facilitate the formation of comminuted cooked, boiled, or raw fermented sau-sages, as well as cooked or raw fermented hams. This may be done by the addition of salts, ice, and mechanical energy (bowl chopper) during their manufacture to obtain a sausage batter that can be converted into a gelled network (Xiong, 2014) by subsequent heating (cooked) or ripening and drying (raw fermented sausages). In turn, inappropriate pH-values of meat can lead to proteins losing their solubility as is for example the case in pale, soft, exudative (PSE) meat, where pH is low due to stress upon animals prior to slaughtering. This impeded solubility also

leads to a reduced ability to form sausages with acceptable sensory attributes. Moreover, meat proteins have been shown to readily self-assemble into anisotropic, fibrous structures thereby providing products with a characteristic texture and bite (Feiner, 2006; Gibis et al., 2014; Kim et al., 2005; Wilson Iii & van Laack, 1999). This is an ability that many plant proteins lack, since they act as storage rather than structural proteins. The latter are responsible for muscle contraction and mobility while the former act mostly as a nitrogen source for developing plants (Grossmann & Weiss, 2021). Moreover, solubility of plant proteins in aqueous solutions is also related to their type and concentration in the source material as described by Osborne Osborne (1924), including water- (albumins), salt- (globulins), acid- and alkali- (glutelins), or alcohol-soluble (prolamins) ones and might be further affected by the applied extraction method (Ebert et al., 2020). For example, raw materials may have been subjected to temperature or solvent treatments to extract other functional compounds such as lipids prior to protein extraction (González-Pérez & Vereijken, 2007; Johansson & Samuelsson, 2018).

As a result, the inclusion of plant-based proteins into traditional meat products can pose a challenge and unforeseen structural alterations may occur (Asgar et al., 2010) and organoleptic properties of these hybrid products may differ substantially from that of for example traditional meat products (El-Sayed, 2013; Jailson et al., 2016; Porcella et al., 2001). These alterations may not only be related to differences of meat and plant protein functionality, but also on modulations of non covalent interactions that may result in complex formation or repulsion among proteins especially if important process parameters such as the pH value are considered. In this study, we investigated the mixing behavior of a soluble potato protein isolate with solubilized pork meat proteins in an aqueous model system. Pork meat-potato-protein combinations were analyzed in terms of their dispersibility or phase separation, and aggregation behavior by using optical, microscopy analysis, and light scattering. Furthermore, FTIR spectroscopy was done to describe effects of plant protein addition on key secondary structural elements of meat proteins.

Materials and methods

Materials

Lean pork meat SII (GEHA standard) and Solanic®300 potato protein isolate (93.2% crude protein, 0.2% fibers, 0.1% total fat, 0.1% carbohydrates, 0.04% salt) were obtained from MEGA (Stuttgart, Germany) and Avebe (GK Veendam, The Netherlands), respectively. Sodium chloride (purity ≥ 99,5%), sodium hydroxide (purity ≥ 98.0%), hydrochloric acid (molarity 10.2%), and Tris (hydroxymethyl) aminomethane (THAM, purity ≥ 99.9%) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

Protein solubilization and characterization

Meat protein solubilization was adapted from Hermanianto (1995). An overview of individual process steps is shown in the Supplemental Materials (**Figure SIII.6**). In short, lean pork meat was minced to 3 mm with a meat grinder (Type WD114, Seydelmann, Aalen, Deutschland) and chopped in a bowl chopper (Stephan UMC 5, Stephan Machinery GmbH, Hameln; Germany) under the addition of 1.8% sodium chloride and two parts of ice at 3000 rpm for 2 × 6 min. The resulting meat batter was subsequently centrifuged (20,000 g, 30 min, 4 °C) and the supernatant was vacuum-filtered with a pore size of 8 - 12 μm to remove remaining insolubles and fat. The pellet was discarded. Potato protein isolate was dispersed in double deionized water at a nitrogen (crude protein) and sodium chloride concentration of 1.6 wt% (10 wt%) and 1.8 wt%, respectively (**Figure SIII.6**). The suspension was stirred overnight to ensure protein hydration. The pH was adjusted to 5.8 representative of the native pH of solubilized pork meat proteins in this study by adding sodium hydroxide solutions (NaOH, 0.1 - 6 N). It was monitored for at least 4 h and re-adjusted if necessary to compensate for the buffering capacity of proteins. Centrifugation (20,000 g, 30 min, 4 °C) was carried out to obtain the soluble protein fraction. The pellet was discarded.

Total nitrogen content

The total nitrogen content of solubilized meat and potato protein fractions was determined according to Dumas (BVL, 2005b) with a flash combustion method in a Dumatherm DT N Pro Analyzer (C. Gerhardt GmbH & Co KG, Königswinter, Germany). It was used as the calculation basis for the preparation and dilution of sample solutions. Samples were weight accurately into tin foils (Dumafoil®, C. Gerhardt GmbH & Co. KG, Königswinter, Germany), that included an absorbent for polar samples (DumaSorb®, C. Gerhardt GmbH & Co. KG) at

an adsorbent to sample ratio of 1:4 (w/w). A combustion with pure oxygen (1030 °C) and reduction by copper (750 °C) was done to release nitrogen, that was then determined by an integrated thermal conductivity detector through a conductivity change of a standardized helium gas flow. THAM was dispersed in double deionized water at a nitrogen concentration of 0.5 wt% and used as a standard for calibration. The respective crude protein content was calculated using a nitrogen-to-protein conversion factor of 6.25 (Mariotti et al., 2008).

ξ-potential and particle size determination

Solubilized meat and potato proteins were diluted to 0.08 wt% nitrogen (0.5 wt% crude protein) in saline solution (double deionized water, 1.8 wt% NaCl) and pH was elevated to 8.2. Solutions were stirred for at least 4 h and pH was continuously adjusted to compensate for the buffering capacity of proteins. Following this, meat and potato protein solutions were diluted to 0.1 wt% crude protein in double deionized water, pH was checked again, and transferred into disposable folded capillary cells (DTS1070, Malvern Instruments, Malvern, UK) of a light scattering and electrophoresis instrument (Nano ZS, Malvern Instruments, Malvern, UK) via the pump of an integrated titrator device (MPT-2 Titrator, Malvern Instruments, Malvern, UK). Solutions were circulated at least 4-times to ensure homogeneity throughout the measurement circuit. A sequential, automated titration sequence with addition of hydrochloric acid (0.1 and 0.01 HCl) was performed. Threshold values of pH 8.0 and 3.0 were set as starting and end values for the titration, respectively. The accuracy was set to pH ± 0.1 and ξ-potential and particle size (Z-average) were recorded at every 0.2 pH-step. Analysis was carried out at 25 °C.

Preparation of protein mixtures

The nitrogen content of solubilized meat and potato proteins was standardized to 0.48 wt% nitrogen (3.0 wt% crude protein) by dilution in saline solution (double deionized water, 1.8 wt% NaCl). Individual solutions of meat and potato proteins were blended at mixing ratios of 100:0, 80:20, 60:40, 50:50, 40:60, 20:80, and 0:100 (v/v) and adjusted to pH-values of 3.0, 4.0, 4.5, 5.0, 5.5, 5.8, and 7.0 by the addition of hydrochloric acid or sodium hydroxide (0.05 – 3 N). Samples were stirred for at least 4 h and pH was monitored and re-adjusted if necessary, to a pH-accuracy of ± 0.01. Following this, aliquots of 3.5 ml were transferred into disposable cuvettes (BrandTech™ Macro, Polystyrene, Fisher Scientific GmbH, Schwerte, Germany), covered with a cap, and stored at 4 °C overnight, to be subsequently analyzed (see below).

Visual appearance and separation index

Photographic images of sample solutions after overnight storage were taken with a digital camera (Canon Power Shot G10, Canon Deutschland GmbH, Krefeld, Germany). Pictures were taken under controlled illumination and a set sample-to-camera distance. The separation index (%) was calculated according to **Eq. III.1.** There h_{Sediment} was the height of the sedimented layer, while h_{Total} represented the height of the whole sample liquid in the cuvette.

$$Separation\ Index\ (\%) = \frac{h_{Sediment}}{h_{Total}} * 100 \qquad \textbf{III.1}$$

Light microscopy and image analysis

The microstructure of sample solutions was recorded with a light microscope (Axio Scope A1, Carl Zeiss Microscopy GmbH, Jena, Germany), equipped with a digital camera AxioCam, ICc3 (Carl Zeiss Microscopy GmbH, Jena, Germany) at a 100-fold magnification. Aliquots of 10 μl were withdrawn from individual and mixed meat and potato proteins with a BRAND™ Transferpette™ S (Brand GmbH, Wertheim, Germany) after slight mixing, transferred to the microscopic glass slides (VWR International GmbH, Darmstadt, Germany) and carefully fixed with a cover glass (VWR International GmbH, Darmstadt, Germany). Microscopic images were then assessed by image analysis in Image J (ImageJ 1.52a, National Institutes of Health, USA). A shape-descriptor macro was used to determine aggregate area (size of visible protein particles in calibrated units as μm^2) and aggregate aspect ratio (length ratio of the major to the minor axis of protein aggregates; a value of 1.0 describes a perfect circle) of microstructures in order to gain quantitative insight into morphological changes.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of solubilized meat and potato proteins and their mixtures at pH 3.0 to 7.0 were recorded at room temperature. A sample aliquot of 20 µl was transferred to the universal attenuated total reflectance accessory (UATR) of a Spectrum 100 FT-IR (PerkinElmer, Beaconsfield, UK) and slightly air dried on the crystal prior to analysis to avoid water interference. Transmission mode was chosen and spectra were recorded at 4000-650 cm^{-1} at a resolution of 4 cm^{-1} over 64 scans. Obtained data was converted to the second derivative by using the 2nd Savitzky-Golay-derivative with 15 smoothing point. The range in between 1550 and 1700 cm^{-1} was used to specifically assess secondary structures elements in the amide I region (Yang et al., 2015).

Statistical analysis

Significant differences among results were analyzed by a one-way analysis of variances using SPSS statistics V23 (IBM Corp, Armonk, USA) after checking the assumption of normality (Shapiro-Wilk-test, p-value to reject ≤ 0.05) and equal variance ($p \leq 0.05$). A Tukey post-hoc test was carried out at an α-level of 0.05. Variance analysis of FTIR data was done by Excel 365 V2004 (Microsoft Cooperation, New Mexico, USA). Principle component analysis was performed with OriginPro (OriginLab Corp., Northampton, MA; USA). All analyses were done at least in duplicate and measurements were repeated at least twice from freshly prepared samples. Image analysis was carried out with at least four images and calculated data was averaged.

Results and discussion

Deliberate solubility changes are an essential tool to create a variety of foods including sausages, cheeses, or breads and respective production processes involve changes in the complex interplay of water- and salt-soluble macromolecules (Desmond, 2007). Protein-protein interactions are well understood for established products (Acton & Dick, 1984; Gerrard, 2002), but less so for emerging ones such as the class of meat-plant hybrids, which represent mixtures of animal- and plant-based ingredients. It is likely that due to differences in the biological function of proteins in the respective raw material, this can have unforeseen consequences, especially due to interactions between the involved protein classes. Below, we take a look at the mixing behavior of an aqeuous model system composed of salt-solubilized pork meat and potato proteins. The first were obtained through a salt- and ice-supported chopping process to extract the entirety of water- and salt-soluble meat proteins (Hermanianto, 1995; Zeeb et al., 2018), while the latter was dispersed in saline media at the same ionic strength and pH 5.8 (**Figure SIII.6**) representative for the native pH of meat proteins. Both protein sources were selected due to pork being one of the primary functional constituents in traditional sausages (Gibis et al., 2014), and the potato isolate as a readily available and well-characterized side-stream from potato starch production with high solubility (Ebert et al., 2020; Giuseppin et al., 2008; Pouvreau et al., 2005).

Behavior of individual protein solutions

The pH-value influences a protein's functionality by altering its charge, which in turn leads to changes in electrostatic interaction, *i.e.* repulsion or attraction, and therefore strongly affects its solubility and aggregation behavior (Zayas, 1997a). The ξ-potential was therefore assessed as

an indicator of surface charge over a select pH-range (**Figure III.1**). A dynamic light scattering technique was used, where samples were subjected to an externally applied electric field after appropriate dilution and an adjustment to alkaline pH to minimalize salt screening and obtain individually dispersed proteins. Moreover, particle size (z-average) was determined through their diffusion coefficients (Jachimska et al., 2008). Surface charge of solubilized meat proteins changed from negative (- 14.8 ± 0.3 mV) to positive (21.7 ± 0.4 mV) upon decreases in pH from 8.1 to 3.2 (**Figure III.1**) with a zero net charge transition point (isoelectric point p*I*) (Jachimska et al., 2008; Novák & Havlíček, 2016) at pH 5.7. Based on this, high charge magnitudes in the alkaline and acidic regime promoted high electrostatic repulsion resulting in small particle sizes with mean diameters of around 6 μm. In contrast, particle sizes increased in between pH 4.0 to pH 6.2 due to low net charges and reached a maximum of 20.2 ± 1.7 μm at pH 5.4. The determined p*I* of solubilized pork meat proteins represented a mean value coinciding from individual water- and salt-soluble meat proteins that were simultaneously obtained after their salt-supported solubilization and extraction (**Figure SIII.6**) from pork meat at elevated ionic strength (Hermanianto, 1995). For example, glycolytic enzymes and the red meat colorant myoglobin have individual p*I*s in between pH 6.2–6.8 and around pH 9.0, respectively (Farrar & Deal, 1995; Lopez Buesa et al., 1995; McBride et al., 1990; Michels et al., 1986; Satterlee & Zachariah, 1972; Takasawa & Shiokawa, 1983) and a combined p*I* around neutral pH (Xiong, 2017).

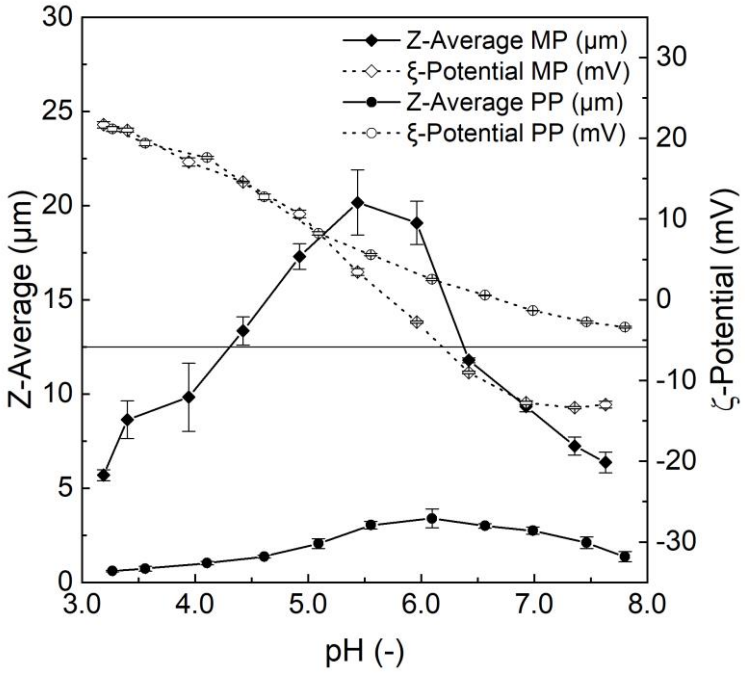


Figure III.1 Particle diameter (Z-average) and surface charge (ξ-potential) of solubilized pork meat and potato proteins as a function of environmental pH.

In contrast, the salt-soluble muscle fiber proteins actin and myosin have a p*I* in between pH 5.4 - 5.6 (Cercel et al., 2015; Fretheim et al., 1985; Sun & Holley, 2011). Considering that the p*I* of the solubilized pork meat was at pH 5.7, a dominance of the salt-soluble meat proteins on the pH-dependent charge transition behavior can be suggested, likely because they were in excess. This was further supported by a maximum particle size at pH 5.4 rather than pH 5.7, as well as high net charges and small particle sizes around neutral pH and thus close to the combined p*I* of water-soluble meat proteins (Xiong, 2017). Moreover. our results are in good agreement with the pH-dependent ξ-potential determined previously for salt-soluble meat proteins (Sun et al., 2013).

Similarly, solubilized potato proteins used in this study are composed of a variety of proteins that are extracted from potato juice by a pH-modulated size exclusion chromatography (van Koningsveld et al., 2001). Resultant acid-soluble proteins are known to possess an inhibiting activity towards some digestive enzymes, and have thus been classified as protease inhibitor fraction (Pouvreau et al., 2001). Whilst small deviations in molecular weights (4 - 23 kDa), p*I*s were shown to vary from pH 5.0 to more than pH 9.0 (Pouvreau et al., 2001; Pouvreau et al., 2004; Pouvreau et al., 2005) revealing an even broader range than water- and salt-soluble meat proteins. This resulted in an overall p*I* at pH 6.7 found in our study and low charge magnitudes in between pH 5.6 to 8.0 (**Figure III.1**) as also described by other authors (Schmidt et al., 2018; Stounbjerg et al., 2019; Zeeb et al., 2018). This charge diversity combined with their low molecular weight are likely the reason for the small degree of protein association observed related to particle sizes of less than 1 to around 3 μm. Taken together, meat proteins had a distinct pH-dependent particle size that was dominated by their salt-soluble protein fractions, while potato proteins showed nearly pH-independent solution behavior. It should be noted that because of the different charge dependencies of the two constituents, there is an area in between their various p*I*s where electrostatic attractions may occur. This in combination with Van der Waals interactions and thermodynamic effects through temperature modulations may promote protein-protein association, dissociation, and re- or unfolding of macromolecules.

Behavior of individual protein mixtures

Visual appearance and separation index

Analysis on the pH-dependent behavior of mixtures of solubilized meat and potato proteins was done by assessing their visual appearance (**Figure III.2**) and determining separation indices (**Figure III.4A**) at different mixing ratios.

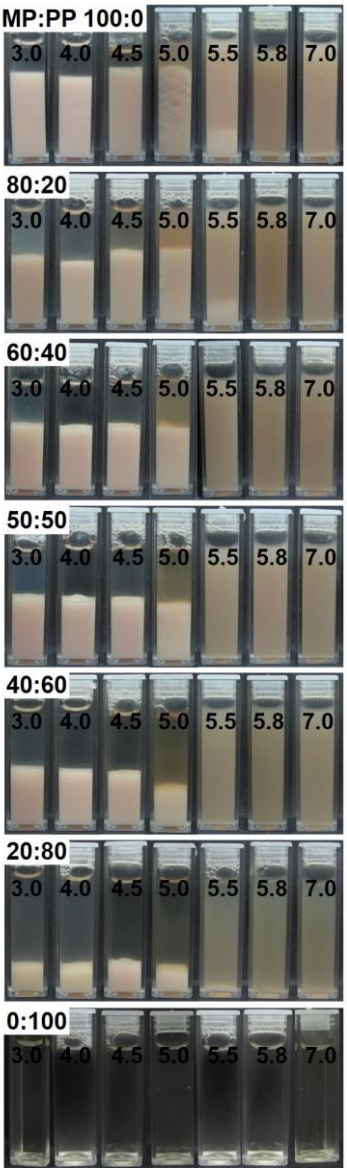


Figure III.2 Visual appearance of test tubes containing protein solutions at various mixing ratios of meat to potato proteins (MP:PP) and pH (3.0 - 7.0).

The tested pH-range of 3.0 - 7.0 was chosen because it encompassed the previously determined points of zero net charge from electrophoretic mobility measurements (**Figure III.1**). Photographic images of test tubes containing solubilized meat proteins and protein mixtures showed distinct changes in solution appearance (**Figure III.2**), indicating regions were they appeared homogenously dispersed, *i.e.* at pH 5.8 and 7.0 while phase separation occurred at and below pH 5.5 seen by increasing sedimentation. This sediment phase either appeared as loos flocks (pH 5.0) or as a dense, pink (pH 4.5) to whitish (pH 3.0 and 4.0) precipitation. This was in line with expectations from results of their electrophoretic mobility measurements (**Figure III.1**) indicating that attractive interactions took place below the determined p*I* of 5.7. In contrast, turbid, but monophasic appearance at pH 5.8 to pH 7.0 was related to weak

interactions among meat proteins and in between meat and potato proteins. Moreover, it was recently shown that both water-soluble pork meat and potato proteins possess a high degree of hydrophilicity related to their amount of charged and polar amino acids, globular structure, and small molecular weight (Xiong, 2014; Xiong, 2017) and the salt-soluble protein fraction was at its solubility peak (Brewer, 2014).

A pH of 5.8 and higher is also one that is typically utilized in the production of emulsified sausages, and care is taken to avoid that pH declines below this critical value, which would lead to an inability to form stable gels upon heating in subsequent process steps (Desmond, 2007; Keeton et al., 2014; Li et al., 2014). The formation of a sediment phase in mixtures at pH values below 5.5 was similar to the formation of a sediment layer in the system composed of solubilized meat proteins only, which indicated their aggregation irrespective of the presence of potato proteins (Cercel et al., 2015; Fretheim et al., 1985; Sun & Holley, 2011). It is interesting to note that at pH 5.0 and 5.5, the solution phase above the sediment phase was turbid as well as in the higher pH region, suggesting that two species of particulates with different size and/or density were present in the mixtures. In contrast, potato proteins were soluble throughout the entire tested pH-range with no difference in appearance between samples. This agreed with the small particle size determined previously in solutions (**Figure III.1**) and was in accordance with earlier studies on such potato fractions and its pH-dependent solubility (Ralet & Guéguen, 2000). To better understand the solution behavior of mixtures, the phase separation index was determined as a function of pH and mixing ratio, and a contour plot thereof is shown in **Figure III.4A**. The contour plot revealed two distinct areas of low (pH 5.8 and 7.0) and high separation indices (pH 3.0 to 5.0) divided by a narrow regime with intermediate values at pH 5.5. The boundary where a transition from soluble (solubilized potato proteins) or well dispersed (mixtures, solubilized meat proteins) to phase separated proteins occurred was thus not located at the measured mean pI of the meat protein fraction (pH 5.7), but the theoretical pI of the salt-soluble fraction (pH ~ 5.5) coinciding with a maximum in particle size obtained by electrophoretic mobility measurements (**Figure III.1**). Moreover, values at a given mixing ratio showed no significant difference ($p > 0.05$) in between pH 3.0 and 5.0 i.e. for mixing ratios of meat to potato proteins from 100:0 to 50:50, but declined significantly ($p \leq 0.05$) from pH 5.0 to 5.5 in all mixtures. A regression analysis on the dependency of mixing ratio and separation index further indicated linearly decreasing phase separation with increasing potato protein share in mixtures related to coefficients of determination of $R^2 \geq 0.89$. This resulted in lowered values in particular at pH 5.5 and higher

as the potato protein share of the mixture increases (**Figure III.4A**) and is indicative of the potato proteins beginning to dominate the phase behavior in that pH-region in particular at mixing ratios of meat to potato protein of 50:50 to 0:100.

Light microscopy and image analysis

Solubilized meat and potato proteins and their mixtures were subsequently analyzed by light microscopy to gain better insights into morphologies of particulates and the extent of aggregation (**Figure III.3**). Microscopic images were further evaluated by image analysis and calculation of the aggregate area (μm^2) and aggregate aspect ratio (-) as an indicator for anisotropy of the observed aggregates. Analysis of solubilized meat proteins revealed the presence of small, rod-shaped, dispersed particles at pH 5.8 and 7.0 (**Figure III.3**), which corresponded to a low turbidity (**Figure III.2**), and a repulsive, negative net charge (**Figure III.1**). Lower pH-values resulted in protein association and consequently larger particulates were formed at pH 5.5. This was followed by the formation of elongated, aligned aggregates at pH 5.0 that evolved into anisotropic, branched microstructures at pH 4.5 and a coherent, three-dimensionally stranded network at pH 3.0 and 4.0 (**Figure III.3**).

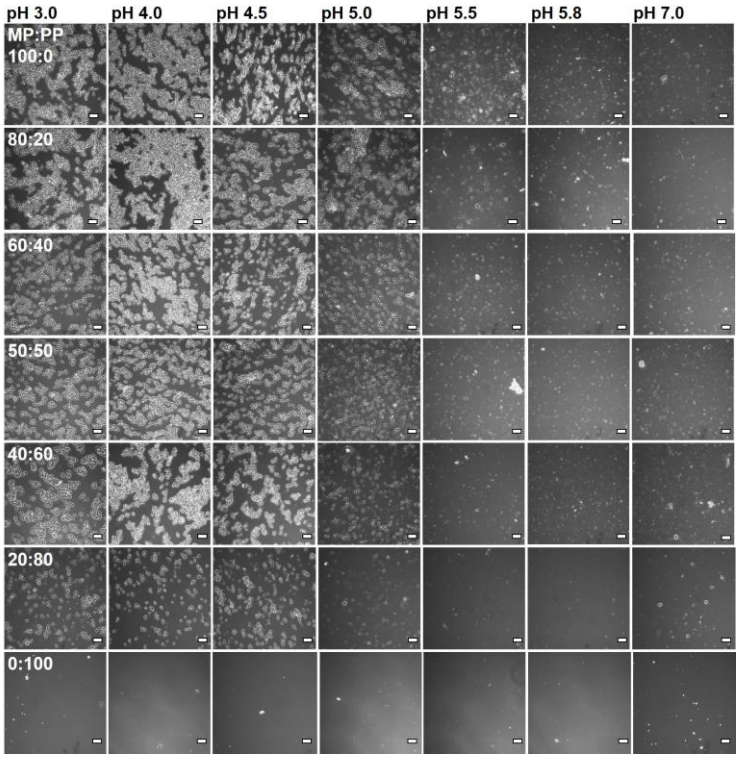


Figure III.3 Microscopic images of protein solutions depending on mixing ratio of meat to potato proteins (MP:PP) and pH (3.0 - 7.0); magnification 100-fold; scale bar equivalent to 100 μm.

Here, proteins may undergo associative phase separation, which involves their complexation close to the p*I* and aggregation and sedimentation (segregative phase separation) upon further pH-decrease (Stounbjerg et al., 2019). Moreover, salt-soluble meat proteins such as myosin are generally known to re- and unfold below their p*I* (pH ~ 5.5), followed by an association and acid-induced coagulation into an insoluble, stranded network that excludes water (Feiner, 2006; Fretheim et al., 1985; Hermansson et al., 1986). These multi-stage processes involved in protein association and aggregation and their high dependency on *e.g.* time, protein concentration and the presence or absence of mechanical forces (Saluja & Kalonia, 2008) underlines the complexity of protein interactions and might explain deviations from dynamic, short-term analysis such as electrophoretic mobility (**Figure III.1**). As shown for the separation index, aggregate area from image analysis of meat protein microstructures (**Figure III.4B, Figure III.4C**) revealed a linear increase in aggregate area (R^2 = 0.99) from pH 5.5 (~ 300 μm^2) to pH 4.0 (~6000 μm^2), while aggregate aspect ratio reached a maximum at all pH-values in this range. In contrast, potato proteins contained only small and barely visible particles at the used magnification (**Figure III.3**) that were rather round and well below 500 μm_2 (**Figure III.4B, Figure III.4C**) which prevented their sedimentation (**Figure III.2**). In mixtures, both microscopic images and quantitative analysis of aggregate area and aspect ratio indicated that increasing potato protein shares impeded the structured highly anisotropic association (pH 5.5) and aggregation (pH 5.0 to pH 3.0) of meat proteins (**Figure III.3, Figure III.4B, Figure III.4C**). Supporting this, a concave shaped transition line between areas of high and low aggregate area and aspect ratio can be discerned from contour plots of mixing ratio versus pH, that was also visible for separation indices visualized in **Figure III.4A**. Replacement of meat in favor of potato proteins *e.g.* at pH 4.0 resulted in a transition from elongated (MP:PP 100:0) or dense (80:20) aggregates networks (**Figure III.3**) to irregularly sized (60:40, 40:60) and finally individual, small particulates (20:80). Furthermore, fibrous (100:0) was exchanged for clustered and increasingly distanced aggregation from 80:20 to 40:60 at pH 4.5 and aggregated protein lumps at pH 5.0 decreased in size and abundance (100:0 and 80:20 compared to 60:40) finally visual as dispersed, small (< 300 μm^2) particulates. All in all, higher potato protein shares induced alterations in the nature of the aggregates formed, and there was a general trend towards more disorder and irregularity, along with a lower degree branching. Aggregates became more spherical instead of stranded and were composed of subunits instead of connected networks meaning a lower capability of meat protein to undergo organized association followed aggregation below pH 5.8. A similar "perturbation" effect has been observed for mixtures of meat and soy proteins, where authors reported weaker gelation

through a dilutive or interfering effect of plant proteins upon meat protein functionality (Foegeding & Lanier, 1987; Lanier, 1991; Sofos et al., 1977). Authors further proposed, a lack of interaction between meat and non-meat proteins, which may impede the formation of coherent networks. Clearly, our results also showed that structure formation is altered depending on pH and/or mixing ratio. Thus, an exchange of meat with potato proteins is likely not a trivial undertaking, as the observed perturbation implies that potato proteins do not act as simple fillers or co-solutes but will rather display a complex interaction behavior.

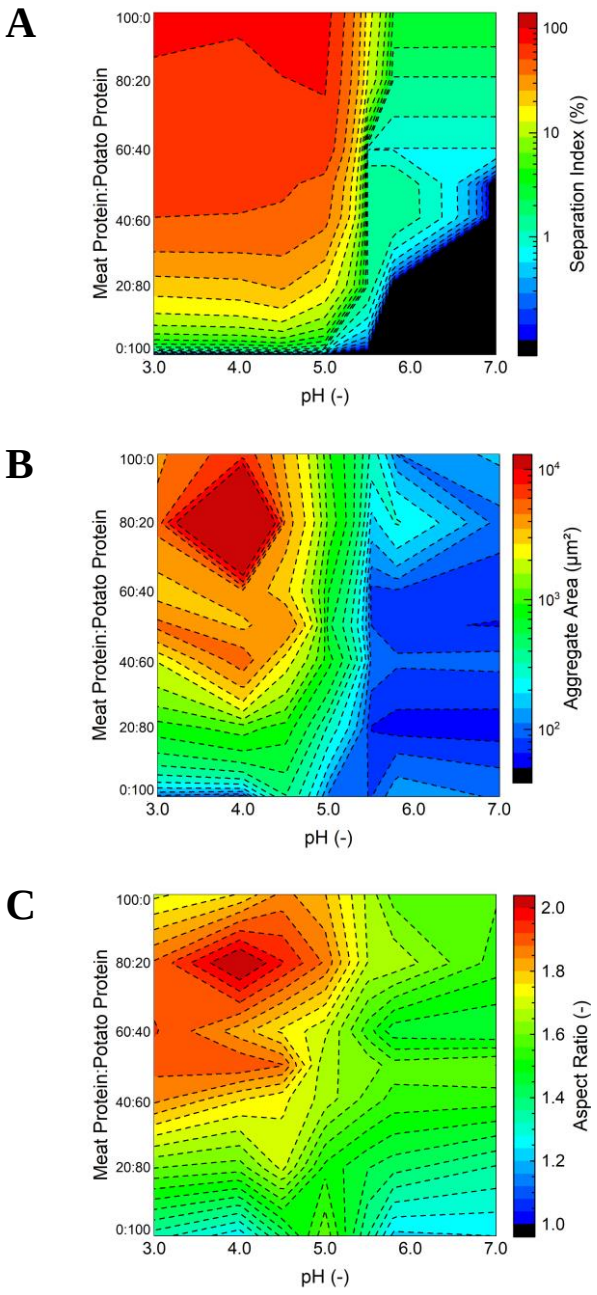


Figure III.4 Separation index (**A**) obtained from test tubes observations, and aggregate area (**B**) and aspect ratio (**C**) obtained from microscopic image analysis of protein solutions at different mixing ratios of meat to potato proteins (MP:PP) and pH (3.0 - 7.0).

FTIR spectroscopy

The functionality of meat proteins as used for the production of meat products involves a complex series of structural changes that involve many intermediates before protein coagulation and finally network formation occurs (Xiong, 2017). FTIR spectroscopy was used to assess if and how solubilized meat proteins are impacted upon their mixture with a potato protein at the tested pH-range based on changes in secondary structural elements in the amide I (1600 - 1700 cm^{-1}) band (**Figure III.5A**) since this defines a protein's backbone conformation and thus a specific structure and folding (Yada, 2017). Additionally, variance analysis was used to describe differences among mixing ratios at individual pH-values (**Figure III.5A**). Second derivative spectra (**Figure III.5A**) of solubilized meat proteins showed a broad minimum in between 1655 and 1610 cm^{-1} with saddle points around 1635 cm^{-1}, 1645 cm^{-1}, and 1655 cm^{-1} indicating the presence of intramolecular β-sheets or turns, random coils, as well as α-helices, respectively (Arrondo et al., 1993; Jiang et al., 2011; Vonhoff et al., 2010). This was in accordance with previous findings on water- and salt-soluble meat proteins (Böcker et al., 2006; Haris & Chapman, 1992; Mitra et al., 2017). For example, myosin, tropomyosin, troponin (salt-soluble), and myoglobin (water-soluble) were found to contain mainly α-helices, while the C- and M-Protein (salt-soluble) were high in β-sheets (Haris & Chapman, 1992; Xiong, 2017). Slight differences among different pH-values for second derivative spectra of meat proteins were further related to unfolding of meat proteins, whereby secondary structures were altered *i.e.* lower peak abundance at 1635 cm^{-1}, 1645 cm^{-1}, and 1655 cm^{-1} in favor structural elements in between 1600 and 1575 cm^{-1}. In contrast, potato proteins possessed a steep minimum in between 1615 and 1650 cm^{-1}, that peaked at 1638 cm^{-1} indicative of a prevalence of β-sheets. This coincided with earlier findings that describe the protease inhibitor fraction primarily as β–II–proteins (Pouvreau et al., 2004; Pouvreau et al., 2005). They represent a subclass in which amino acid residues are mainly involved in β-turns and/or β-sheets. Authors further reported that proteins were able to maintain their secondary structure in between pH 3.0 and 7.5 supporting the consistency of spectra irrespective of pH in our study (**Figure III.5A**). This could also be a reason for the good dispersibility (**Figure III.2**) and low degree of aggregation (**Figure III.3**). Correspondingly, mixtures revealed a decrease of α-helices in favor of β-sheets when solubilized meat were exchanged for potato proteins – a phenomenon that other authors recently also described for mixtures of soy and salt-soluble meat proteins (Gao et al., 2016; Gao et al., 2015c). Furthermore, a rather linear influence of meat-potato-protein mixing ratio on secondary structural elements was determined (**Figure SIII.7**).

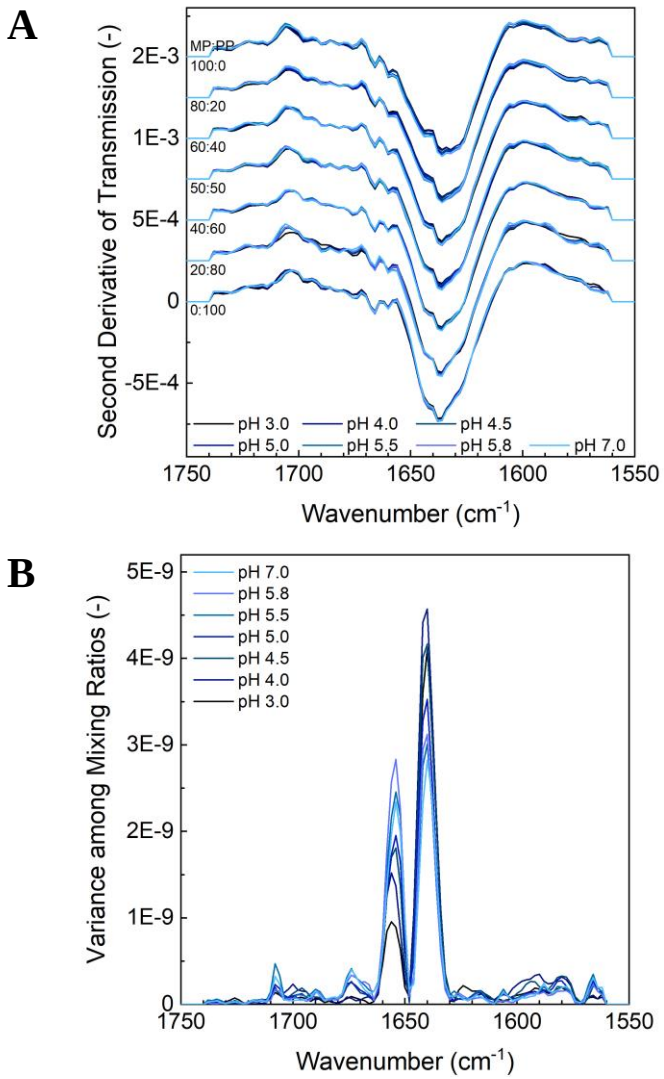


Figure III.5 Second derivative FTIR transmission spectra of protein solutions in between 1600 and 1700 cm^{-1} (A) at various mixing ratios of meat to potato proteins (MP: PP) and pH (3.0 - 7.0) and variance among mixing ratios (B) at a given pH value; different pH-values are marked by differences in color.

Spectra also showed that changes of secondary structure in mixtures were higher than differences among pH-values, especially in samples containing more potato than meat proteins and were linearly related to the meat-potato protein ratio (not shown). Yet, bands assigned to α-helices (1655 cm^{-1}) revealed highest variance at pH 5.5, pH 5.8, and pH 7.0, while β-sheet (1640 cm^{-1}) variation was high at all pH-values but most distinct at pH 4.0, followed by pH 4.5 and pH 3.0 (**Figure III.5B**). This further supported the importance of pH-induced functionality changes of salt-soluble meat proteins, since unfolding of α-helices and the formation of β-sheets were suggested to play an important role in protein network formation (Liu et al., 2008).

Evaluations on secondary structural elements by FTIR spectroscopy highlighted the structural complexity of solubilized meat proteins due to the presence of a variety of structural elements such as β-sheets or turns, random coils, and α-helices especially contrasting to potato proteins with a prevalence of β-sheets. Furthermore, their pH-related changes were particularly contrasting with the inertness of potato protein secondary structures upon pH-changes.

Mechanistic insights

Based on the results obtained we can highlight some mechanistic insights into the impact of mixing ratio and pH on the dispersibility, as well as association and aggregate formation of solubilized meat and potato proteins.

- pH > pI_{MP}: A monophasic regime is discernable. Meat proteins (MP) are present as small, rod-shaped, evenly dispersed particles that decrease in size and abundance with increasing potato protein share (PP). We suggest that PP act as plain co-solutes next to these soluble meat protein complexes resulting in a monophasic and turbid visual appearance.

- pI_{MP} < pH < p$I_{Salt\text{-}soluble\ MP}$: Association of water and salt soluble meat proteins around and in between their individual p*I*s induces the formation of larger, irregularly sized particles, leading to slight phase separation. This sedimentation disappears as soon as a mixing ratio MP:PP of 80:20 is exceeded. Instead, visual appearance resembles monophases observed at pH > pI_{MP} representative for evenly distributed, soluble complexes indicative of a weakening of interactions in between the individual p*I*s of water and salt soluble meat proteins.

- pH < p$I_{Salt\text{-}soluble\ MP}$: A two-phased aggregated regime is induced, and fine-stranded meat protein particulates are formed that precipitate to eventually form coherent networks due to their acid induced coagulation. By increasing potato protein share to more than 20% this structural re-orientation is altered and meat protein network formation is inhibited either by a dilutive effect or unfavorable protein-protein interactions.

Conclusion

This study provided insights into the effect of a small molecular weight protein fraction from potato tuber on the functionality of water-and salt-soluble proteins from pork meat based on an aqueous model system. While meat proteins dominated the macroscopic phase behavior in mixtures due to their pronounced pH-dependency, potato proteins interfered with the

microstructural association and aggregation of meat proteins below their isoelectric point. These results may be of substantial importance to manufacturers wishing to formulate hybrid products and relying on the ability of meat proteins to form stranded gel structures that result in products with well-known bite and mouthfeel. In hybrid products, this ability is likely changed, and results suggest that critical mixing ratios and pH-values exist where large deviations may occur. For example, sliceability may suddenly be lost and spreadability induced, a phenomenon that has previously been described when adding hydrocolloids to meat matrices, but not other proteins (Gibis et al., 2017; Zeeb et al., 2018). Consequently, other characteristics of importance such as handling during industrial manufacturing procedures and consumer acceptance upon end-products may also be affected. Clearly, further investigations need to be carried out to confirm these findings, especially in bulk or product matrices to further assess the influence of plant proteins on the formulation of consumer-accepted matrices from mixtures of meat and plant proteins. Moreover, it may be of interest to look at combinations of meat with other plant proteins, especially those where isoelectric points are less different and to determine if the structural perturbation effect is equally pronounced.

Acknowledgement

This work was supported by the AiF (German Federation of Industrial Research Associations) as part of the CORNET project “Meat Hybrid” (AiF 9783), and funded as part of the Industrial Collective Research (IGF) program of the German Ministry of Economics and Energy (BMWi), based on a resolution of the German Parliament.

Supporting Information

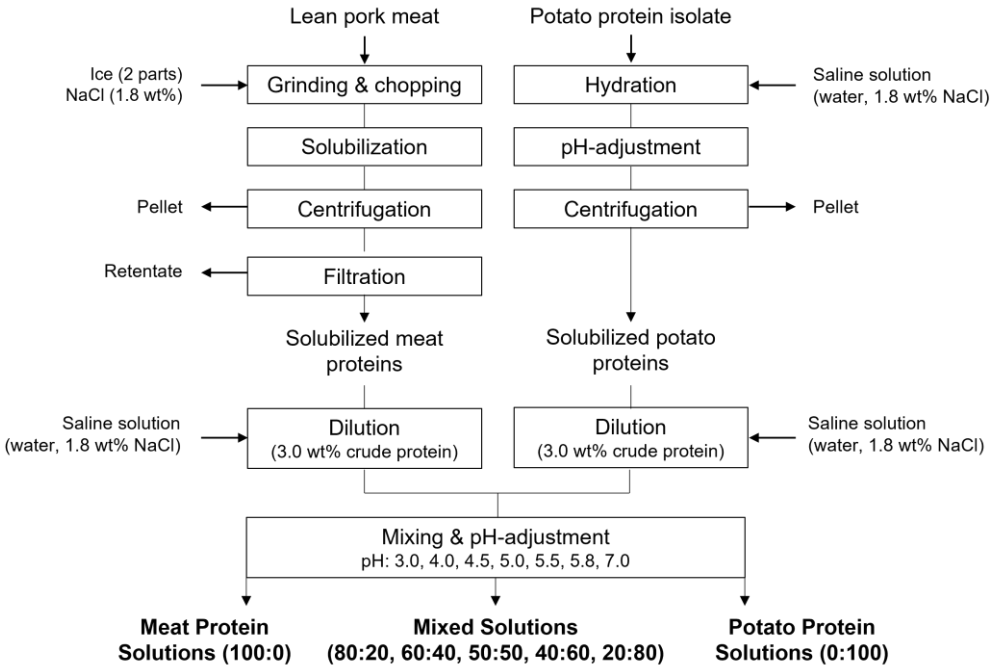


Figure SIII.6 Meat and potato protein solubilization scheme, and preparation of sample solutions

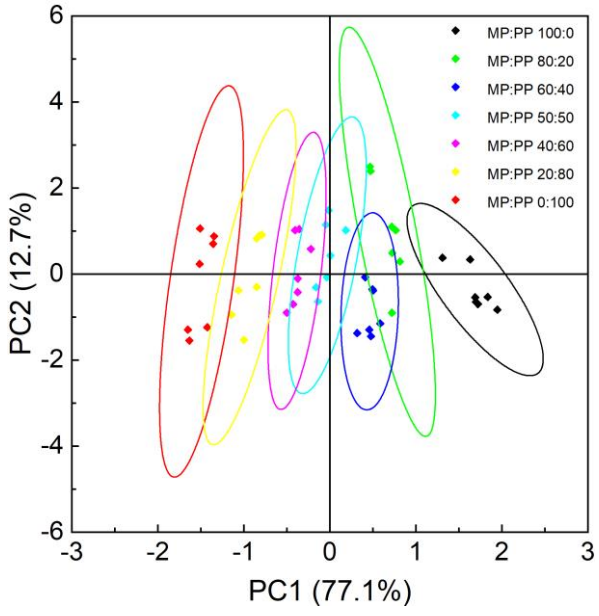


Figure SIII.7 Principle component analysis of second derivative transmission spectra in between 1600 and 1700 cm-1 of protein solutions at varying mixing ratio of meat to potato proteins (MP:PP) and pH; data points with the same color represent samples with the same mixing ratio but varying pH (3.0-7.0), respectively colored ellipses are calculated at a 95 % confidence interval.

Functional and Organoleptic Properties of Plant Protein Extrudates

IV. Chapter

Influence of Protein Extraction and Texturization on Odor-active Compounds in Pea Proteins

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Abstract

Background

The use of plant proteins as food ingredients might be limited due to the presence of foreign or 'off' flavors, which may evolve during extraction and subsequent processing. In this study, the influence of dry (TVP) and wet (WTP) texturization on characteristic volatile compounds of two different pea protein isolates was assessed using gas chromatography–mass spectrometry-olfactometry (GC–MS-O) after direct immersion stir bar sorptive extraction (DI-SBSE).

Results

Twenty-four odor-active compounds were found, with a prevalence of carbonyls from fat oxidation. Nine of these compounds which are also known as major (off-) flavor contributors in peas were distinctively impacted in all texturates: hexanal, nonanal, 2-undecanone, (*E*)-2-octenal, (*E, Z*)-3,5-octadiene-2-one, (*E, E*)-2,4-decadienal, 2-pentyl-furan, 2-pentyl-pyridine, and *γ*-nonalactone. For example, hexanal, a characteristic green odorant, was reduced by up to sixfold by wet texturization, from $3.29 \pm 1.05\%$ (Pea Protein I) to $0.52 \pm 0.02\%$ (Pea WTP I). Furthermore, (*E*,*Z*)-3,5-Octadiene-2-one and (*E*,*E*)-2,4-decadienal were decreased by 1.5- and 1.8-fold when Pea Protein I and Pea TVP I were compared.

Conclusion

An overall reduction in fat oxidation products and of green and fatty odor-active compounds was observed. The results represent a first insight into the process-related modulation of pea protein (off-) flavors to broaden the applicability of pea proteins as food ingredients.

KEYWORDS

Direct Immersion Stir Bar Sorptive Extraction; Extrusion; Off-Flavor; Olfactometry; Pea Proteins.

Introduction

Socio-demographic changes, economic considerations, and health concerns have resulted in an increasing demand for alternative proteins to create vegetarian and vegan products (Asgar et al., 2010). However, many protein sources have limited application due to the presence of strong inherent odor-active and aroma compounds (Hoogenkamp et al., 2017; Kaneko et al., 2011; Nadathur & Carolan, 2017; Roland et al., 2017). Off-flavors are particularly critical in pea proteins where they are often described as green, hay, earthy, and/or beany, making it hard to create food products with high acceptability levels (Schindler et al., 2012; Torres-Penaranda et al., 1998). These flavors develop during maturation, harvesting, and storage, but may also evolve or change during the subsequent processing of raw materials and their proteins (Heng, 2005; Ma et al., 2016; Murat et al., 2013; Roland et al., 2017; Xu et al., 2019). Changes during protein extraction are of particular interest because it is often a combination of several chemical and physical processes followed by a drying step to receive fine powders (Bourgeois et al., 2015b). If they cannot be used in powdered form, pea proteins may be further processed using texturization to provide them with solid properties and the structural integrity to withstand cooking and disintegration by hydration – a property that is crucial for their application as meat extenders or analogues (Asgar et al., 2010). Here, one can generally distinguish between dry/low-moisture and wet/high-moisture texturization. The first results in dry, sponge-like textures, which are rehydrated before their application (Asgar et al., 2010; Harper & Clark, 1979). In contrast, wet texturization enables the formation of hydrated, fibrous textures by modulating the amount of water in the premix, adjusting the process parameters (pressure, temperature), and using an additional cooling unit for protein plastification after texturization (Cheftel et al., 1992; Osen & Schweiggert-Weisz, 2016). A lot of knowledge has been gained regarding the application of pea proteins for these processes but insights into the associated odor changes is mostly limited. However, changes of volatiles from protein powders to texturates are to be expected and may involve modulations that may affect the overall odor profile.

In this study, two pea protein powders and their respective dry and wet texturates were analyzed by gas chromatography–mass spectrometry-olfactometry (GC–MS-O). Direct immersion stir bar sorptive extraction (DI-SBSE) was chosen as a solvent-free extraction method due to its previous application to describe green (off-) flavors in other legumes (Nedele et al., 2021). A comparison of odor-active compounds before and after texturization was used to gain an initial

insight into process-related changes and may help to develop new strategies to improve flavor-related issues associated with pea proteins.

Materials and Methods

Materials

Pea protein isolates Pisane®C9 (Pea Protein I) and M9 (Pea Protein II) were provided by the Cosucra Group (Warcoing, Belgium). Dry (TVP) and wet (WTP) texturized proteins from Pea Protein I and Pea Protein II were manufactured by dry and wet texturization, respectively, and provided by the German Institute for Food Technology (DIL, Quakenbrueck, Germany). The appearance and proximate composition of the powders and texturates are summarized in (**Table IV.1**). The following authentic standards of analytical grade were used: β-pinene (99%), decanal (96%), 2-pentylpyridine (98%), 1-octen-3-one (96%), 3-octen-2-one (97%), benzyl acetate (95%), 3-ethyl-2,5-dimethyl-pyrazine (99%) and 1-nonanol (99%). They were purchased from Alfa Aesar (Karlsruhe, Germany). Nonanal (95%), geraniol (98%), octanal (99%), (E)-2-nonenal (99%), (E,E)-2,4-nonadienal (99%), (E,E)-2,4-decadienal (99%), (E,Z)-2,4-decadienal, (E)-2-decenal (95%), and 2-nonanol (99%) were provided by Sigma-Aldrich (Taufkirchen, Germany). 2-Methoxy-4-vinylphenol (98%), (E)-2-octenal (95%), γ-nonalactone (98%), trimethyl disulfide (98%), 2-undecanone (98%), and lepidine (99%) were purchased from J&K Scientific (Marbach am Neckar, Germany). Carl Roth (Karlsruhe, Germany) provided β-ionone (99%) and eugenol (99%); hexanal (95%) was purchased from Merck KGaA (Darmstadt, Germany). 1-Dodecanol (98%) was purchased from Honeywell (Offenbach, Germany) and (E)-4,5-epoxy-2-(E)-decenal (99%) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA).

Determination of characteristic odor-active compounds in pea protein powders and dry and wet extrudates

Preparation of standard solutions

Stock solution mixtures of 10 to 15 standards (see the previous section) in hexane, with each compound in the same concentration range (4–8 ng μL^{-1}), were prepared and stored at −20 °C in vials sealed with silicone/polytetrafluoroethylene (PTFE) caps.

Table IV.1 Appearance and proximate composition (%) of Pea Protein I and II powders and their respective dry (Pea TVP I, II) and wet (Pea WTP I, II) texturates according to the manufacturers' specifications.

	Pea Protein I	**Pea Protein II**	**Pea TVP I**	**Pea TVP II**	**Pea WTP I**	**Pea WTP II**
Appearance						
Moisture (%)	6.0	5.7	11.9	17.1	63.8	64.6
Crude Protein[a] (%)	76.4	77.1	70.0	67.5	29.0	29.6
Total Fat (%)	9.0	8.0	8.6	7.3	4.3	3.3
- of which SFA[b] (%)	1.7	1.6	1.5	1.3	0.8	0.6
- of which MUFA[c] (%)	2.0	1.8	1.8	1.6	1.0	0.7
- of which PUFA[d] (%)	5.4	4.6	5.3	4.4	2.5	2.0
Ash (%)	5.9	5.9	5.6	5.2	2.3	2.3
Carbohydrates[e] (%)	2.7	3.3	3.9	2.9	0.6	0.2

[a] Total Nitrogen (%)*6.25;
[b] Saturated fatty acids;
[c] Monounsaturated fatty acids;
[d] Polyunsaturated fatty acids;
[e] Carbohydrates (%) = 100 – Moisture (%) – Crude Protein (%) – Total Fat (%) – Ash (%)

Preparation of sample dispersions

Wet texturized proteins and TVP were ground with a Thermomix Type 31–1 (Vorwerk, Wuppertal, Germany) for 15 s (WTP) and 45 s (TVP), respectively, at the highest rotational speed. Pea protein powders (Pea I, II) were used without further pre-processing. Pea Protein I, II, WTP I, II or TVP I, II were suspended in double distilled water at a powder or texturate concentration of 1 % (w/w) based on dry matter - meaning a standardization according to their moisture content. The pH was measured and varied by less than 0.2 pH-units in all samples (pH 7.3 to 7.5). Dispersions were added to airtight glass tubes and stirred for at least 2 h at room temperature to ensure a transfer of volatile components from the samples into the aqueous solution.

Direct immersion stir bar sorptive extraction (DI-SBSE)

Direct immersion stir-bar sorptive extraction (DI-SBSE) was used to isolate volatile sample components from pea protein powders (Pea I, II), and their TVPs and WTPs, as well as from standard solutions. In short, 5 mL of the obtained solution was transferred to a 20 mL vial, equipped with a polymethylsiloxane (0.5 mm PDMS) coated stir bar (Twister, Gerstel, Mülheim an der Ruhr, Germany) and sealed with silicone/PTFE caps. Samples were stirred at

1000 rpm for 2 h at room temperature (25 °C) for the volatile components to adsorb on the coating. Stir bars were subsequently removed, rinsed with double distilled water, dried, and inserted into a conditioned thermal desorption unit (TDU) liner (Gerstel). The temperature was held at 40 °C for 1 min, followed by a temperature rise to 220 °C (rate 120 °C min^{-1}) with a holding time of 10 min to desorb the stir bars in splitless mode. The analytes were cryo-focused in a Cold Injection System 4 (CIS) (Gerstel) in solvent vent mode (40 mL min^{-1}). Then, CIS was heated, starting from −100 °C with an equilibration time of 1.0 min up to 230 °C (rate: 720 °C min^{-1}) and held for 5 min to release the analytes to the GC column.

Gas chromatography–mass spectrometry olfactometry analysis

Gas chromatography analysis of the volatile compounds in pea proteins and standard solutions was performed with an Agilent 7890B gas chromatograph connected to an Agilent 5977B mass spectrometry (MS) detector (Agilent Technologies, Waldbronn, Germany). A polar J&W DB-WAX column (30 m × 250 μm × 0.25 μm) was used with helium (5.0, Westfalen AG, Münster, Germany) as carrier gas with a flow rate of 1.62 mL min^{-1}. Oven temperature was set to 40 °C and held for 3 min, then heated up to 240 °C (rate: 5 °C min^{-1}) and held for 7 min. The total GC run time was 50 min with a solvent delay of 3 min. Cleaning prior to measurement was performed by heating the oven to 240 °C and holding it for 10 min to avoid adulteration of the results due to former samples. A split ratio of 1:2 of MS detector:olfactory detection port (ODP) was used (Gerstel). Other conditions were: a septum purge flow rate of 3 mL min^{-1}; scan mode; a scan range of *m/z*: 40–330; an electron ionization energy of 70 eV, a source and a quadrupole temperature of 230 °C and 150 °C, respectively. Transferline parameter temperatures were 250 °C for GC–MS and 250 and 180 °C (mixing chamber) for GC-ODP. Nitrogen was used as a make-up gas.

Compound identification

Odor-active compounds were detected by gas chromatography-olfactometry (GC-O) according to their characteristic olfactory descriptor (odor). Mass spectra (MS) were assigned on the basis of the NIST MS 2017 library database and retention indices (RI) were calculated with **Eq. IV.1** for non-isothermal temperature programs. Pea protein samples were then compared with values obtained from authentic standards (see above) using their RI and MS profile based on one quantifier and two qualifier ions (**Table SIV.4**) or data published in the literature:

$$RI = 100 * N + 100 * n * \left(\frac{t'_A - t'_N}{t'_{N+n} - t'_N}\right) \quad \textbf{IV.1}$$

N = number of carbon atoms of alkane, eluting prior to analyte;

n = difference in number carbon atoms of analyte to the alkane, eluting after analyte;

A = analyte (unknown substance);

t‘ = adjusted/relative retention time = retention time-time delay.

Compound semi-quantification by peak normalization method

Peak areas in the chromatograms obtained from GC–MS analysis were integrated using Agilent MassHunter Workstation Software (Agilent Technologies). To prevent misinterpretation based on resins released from the column at high retention times found in preliminary experiments (not shown), minute 0 to 38 min were defined as the boundaries of the odor-active zone for peak normalization. The baselines of all peaks was re-checked and hand-integration was done if necessary. Individual peak areas of identified compounds from GC–MS-O analysis were then calculated by the normalized area method (also known as 100% method) to compare samples quantitatively (Kromidas & Kuss, 2008; Turner et al., 2019) by using **Eq. IV.2**:

$$\frac{A_{KAC}}{\sum_{i=0\ min}^{38\ min} A_{Peak}} * 100 \quad \textbf{IV.2}$$

A_{KAC}= Peak area of identified volatile compound

A_{Peak}= Peak area of integrated peak in selected interval

Statistical analysis

Statistically significant differences among samples were tested by a one-way analysis of variance with a Tukey post hoc test (α level of 0.05) using SPSS Statistics V23 (IBM Corp., Armonk, NY, USA) after checking the assumption of normality (Shapiro–Wilk-test, *P*-value to reject ≤0.05) and equal variance ($P \leq 0.05$). All analyses were done in triplicate from freshly prepared samples. Odor impressions at the olfactory port were perceived by an experienced assessor.

Results and Discussion

Pea proteins have gained popularity as alternative protein sources resulting in a broad variety of commercially available flours and isolates that can be used to functionalize or fortify pastry, beverages, dairy or meat products (Ebert et al., 2020; Tulbek et al., 2017). Besides, their dry and wet texturates are represent ingredients for meat mimetics (Asgar et al., 2010; Osen & Schweiggert-Weisz, 2016). However, extraction and texturization may also affect the abundance and composition of odor-active compounds. In this study, two different pea protein isolates (Pea Protein I and II) from the same manufacturer and with similar composition (Table 1) were analyzed by GC–MS-O after the aqueous extraction of volatile components by direct immersion stir bar sorptive extraction (DI-SBSE). Odor-active compounds were identified by their perception at the olfactory port (odor), as well as their respective mass spectra (MS) and retention indices (RI) related to representative standards. Results were then compared to those obtained from their respective dry (Pea TVP I and II) and wet (Pea WTP I and II) texturates.

Characteristic odor-active compounds in pea protein powders

In the first step, Pea Protein I and Pea Protein II isolates were analyzed to identify odor-active compounds and to reveal differences based on modulations in the applied protein extraction approach. For example, peas might be subjected to an additional pasteurization, fermentation and/or ultrafiltration step to modify powder functionality and composition (Bourgeois et al., 2015b). Their respective peak area share was calculated according to the normalized area method after peak integration (**Eq. IV.2**), which is generally applicable for the semi-quantitation of samples with similar composition, concentration, and consistent analysis conditions (Kromidas & Kuss, 2008; Turner et al., 2019). In total, 24 characteristic odor-active compounds were identified based on their RI, odor impression perceived at the olfactory port, and/or MS (**Figure IV.1, Table IV.2**), with a prevalence of carbonyls or unsaturated carbonyl compounds. Perceived odors ranged from pungent, green, and fatty notes – that is from hexanal [**1**], 2-pentylfuran [**2**], (*E*)-2-octenal [**7**], (*E,Z*)-3,5-octadien-2-one [**12**], and (*E,E*)-2,4- nona- and decadienal [**16, 17**] to floral and fruity ones originating from octanal [**3**], nonanal [**5**], decanal [**9**]. Paprika-, mushroom-, and lavender-like, as well as floral/green scents were linked to smaller peaks of 2-nonanol [**10**], 3-octen-2-one [**6**], geraniol [**18**] and *β*-ionone [**19**], respectively. Sweetish heterocycles such as 2-pentyl-pyridine [**13**] and *γ*-nonalactone [**22**] were also found and compounds with earth-like, floral [**21**], spicy, perfume-like [**23**], and stall-like odors [**24**] were perceived but not identified according to MS or their retention index from

known standards. Upon comparison, Pea Protein I consisted majorly of carbonyls with a green (74% peak area of all identified peaks) and floral (20% peak area of all identified peaks) scent, while Pea Protein II was high in longer chain ($C \geq 8$) and branched, unsaturated carbonyl compounds with green, fatty odor (83% peak area of all identified peaks) and some additional volatiles (2-nonanol [**10**], geraniol [**18**], beta-ionone [**19**], 1-dodecanol [**20**]) with lower peak area shares. Accordingly, overall odor impressions of sample dispersions (not shown) and volatiles sensed at the olfactory port could be described as being more intense in Pea Protein II than in Pea Protein I, meaning a strong green, beany, acidic, and earthy scent compared to a less beany and rather flour-like one with some additional oat- and hay-like notes.

The results were in accordance with previous ones on peas and pea flours, where compounds **1–9, 11–12, 14–20**, and **22** were also found (**Table IV.2**). As far as we are aware, 2-nonanol [**10**] and 2-pentyl-pyridine [**13**] were found in pea for the first time. However, the latter, was previously described in soy drinks (Nedele et al., 2021). whose volatile profile has strong similarities with odor-active components identified in this study. These authors further described the presence of trans-4,5-epoxy-(*E*)-2-decenal (green, peapod-like) and eugenol (green, herbal), which might be attributed to unknown components **21** and **23** at retention indices of 1992 and 2158, and a grassy, earth-like and spice, perfume-like odor impression, respectively. Finally, the second and third highest abundances of hexanal ($> 350\ \mu g\ L^{-1}$) and decadienal ($100\ \mu g\ L^{-1}$) coincided with large peak area shares of these volatiles in Pea Protein I and II (**Table IV.2**). In general, high peak shares and intensive green odors perceived at the olfactory port found in this study fit descriptions of hexanal [**1**], 2-pentyl furan [**2**], (*E*)-2-heptenal [**4**], (*E*)-2-octenal [**7**], and (*E,E*)-2,4-nona and decadienal [**16, 17**] as major impactors on beany off-flavors in pea flours (Trikusuma et al., 2020; Xu et al., 2019) or strong contributors to volatile green notes associated with pea flavor (Berger, 2007). This influence to the overall green and beany scent of Pea Protein I and II might be particularly distinct for those with low to moderate odor thresholds ($\mu g\ L^{-1}$) such as recently shown *i.e.* for hexanal [**1**] with $4.5\ \mu g\ L^{-1}$, (*E*)-2-heptenal [**4**] with $1.3\ \mu g\ L^{-1}$, and (*E,E*)-2,4-decadienal [**17**] with $0.03\ \mu g\ L^{-1}$ (Leffingwell & Leffingwell, 1991; Nedele et al., 2021). Similarly, a perception of some compounds at the olfactory port (**Table IV.2**) irrespective of their low peak area share (**Figure IV.1**) such as 2-nonanol [**10**], 2-pentyl-pyridine [**13**] and (*E,E*)-2,4-nonadienal [**16**] may be related to their low odor thresholds of $0.4\ \mu mol L^{-1}$, $0.6\ \mu g\ L^{-1}$ and $0.07\ \mu g\ L^{-1}$ in water, respectively (Leffingwell & Leffingwell, 1991; Schnabel et al., 1988).

Table IV.2 Odor-active compounds in Pea Protein I and II detected at the olfactory detection port via SBSE (5 mL, 2 h, room temperature) and subsequent GC-MS-O analysis.

No.	RI		Compound	Perceived odor[a]	Peak Area (%)[b]		Identification[c]	Previously described in pea (proteins)
	Sample[d]	Std / Lit			Pea I	Pea II		
1	1073	1079	hexanal	grassy, green	L	L	MS, RI, odor	Cui et al. (2020); Heng (2005); Ma et al. (2016); Murat et al. (2013); Murray et al. (1976); Schindler et al. (2012); Trikusuma et al. (2020); Wang et al. (2020); Xu et al. (2019)
2	1220	1221	2-pentylfuran	earthy, green	L	L	MS, odor	Heng (2005); Schindler et al. (2012); (Trikusuma et al., 2020); Wang et al. (2020); Xu et al. (2019)
3	1278	1286[e]	octanal	floral, fruity	M	M	MS, RI, odor	Cui et al. (2020); Heng (2005); Murray et al. (1976); Schindler et al. (2012); Wang et al. (2020)
4	1314	1310	(*E*)-2-heptenal	grassy, fatty, green	M	*n. d.*[f]	MS, RI, odor	Heng (2005); Murray et al. (1976)
5	1385	1392	nonanal	fruity, floral, green	L	L	MS, RI, odor	Cui et al. (2020); Heng (2005); Murat et al. (2013); Murray et al. (1976); Schindler et al. (2012); Trikusuma et al. (2020); Xu et al. (2019)
6	1399	1389[g]	3-octen-2-one	mushroom-like, floral	M	*n. d.*	RI, odor	Cui et al. (2020); Schindler et al. (2012); Wang et al. (2020); Xu et al. (2019)
7	1421	1428	(*E*)-2-octenal	fatty, green	M	M	MS, RI, odor	Cui et al. (2020); Murat et al. (2013); Murray et al. (1976); Trikusuma et al. (2020)
8	1442	1449[h]	acetic acid	acid, sour	S	S	MS, odor	Murray et al. (1976); Xu et al. (2019)
9	1492	1498	decanal	floral, fruity	S	*n. d.*	MS, RI, odor	Cui et al. (2020); Heng (2005); Wang et al. (2020); Xu et al. (2019)
10	1516	1513	2-nonanol	paprika-like	*n. d.*	T	MS, RI, odor	--
11	1526	1532	(*E*)-2-nonenal	fatty, green	S	*n. d.*	MS, RI, odor	Heng (2005); Murat et al. (2013); Murray et al. (1976)
12	1562	1562	(*E,Z*)-3,5-octadien-2-one	green, herbal[i]	L	L	RI, odor	Cui et al. (2020); Murray et al. (1976); Xu et al. (2019)
13	1570	1565	2-pentyl-pyridine	sweetish	T	S	MS, RI, odor	--
14	1591	1589	2-undecanone	ethereal, fatty	M	M	MS, RI, odor	Cui et al. (2020); Murray et al. (1976); Schindler et al. (2012); Xu et al. (2019)
15	1655	1653	1-nonanol	fruity, citrus-like	S	M	MS, RI, odor	Cui et al. (2020); Heng (2005); Murat et al. (2013); Murray et al. (1976); Schindler et al. (2012); Wang et al. (2020); Xu et al. (2019)
16	1690	1697	(*E,E*)-2,4-nonadienal	earthy, green, fatty	T.	S	MS, RI, odor	Murray et al. (1976); Trikusuma et al. (2020); Xu et al. (2019)
17	1800	1808[j]	(*E,E*)-2,4-decadienal	fatty, green grassy	L	L	MS, RI, odor	Murat et al. (2013); Xu et al. (2019)
18	1841	1839	geraniol	ethereal, lavender-like	S	S	MS, RI, odor	Schindler et al. (2012); Xu et al. (2019)
19	1930	1928	*β*-ionone	floral, green	*n. d.*	S	MS, RI, odor	Murray et al. (1976)
20	1961	1959	1-dodecanol	warm, fruity, green	*n. d.*	L	MS, RI, odor	Schindler et al. (2012)
21	1992	--	unknown	grassy, floral, earth-like	--	--	--	--
22	2015	2015	*γ*-nonalactone	sweetish	M	L	MS, RI, odor	Murat et al. (2013); Schindler et al. (2012); Trikusuma et al. (2020); Xu et al. (2019)
23	2157	--	unknown	spice, fruity, perfume-like	--	--	--	--
24	2166	--	unknown	stall-like	*n. d.*	--	--	--

[a] Perceived odor impression at the olfactory detection port;
[b] Calculated based on normalized area method Turner et al. (2019); L = Large (> 1 %), M = Moderate (0.6 – 1 %), S = Small (0.6 %), T = Traces (not integrable; peak area < 65 000 1/min);
[c] For identification, odor-active compounds were suggested on the basis of NIST MS 2017 library database (MS); suggested odorants were identified by the characteristic odor impressions (odor), the retention indices (RI) on a polar column, and the mass spectra in comparison with authentic standard compounds using one quantifier and two qualifier ions (Appendix A1) as well as data published in literature (references marked with superscript letters);
[d] Means of sample RI using polar DB-WAXms column
[e] Bianchi et al. (2007);
[f] Not detectable in total ion current chromatogram;
[g] Shimoda et al. (1995);
[h] Valim et al. (2003);
[i] Odor: www.thegoodscentscompany.com;
[j] Babushok et al. (2011)

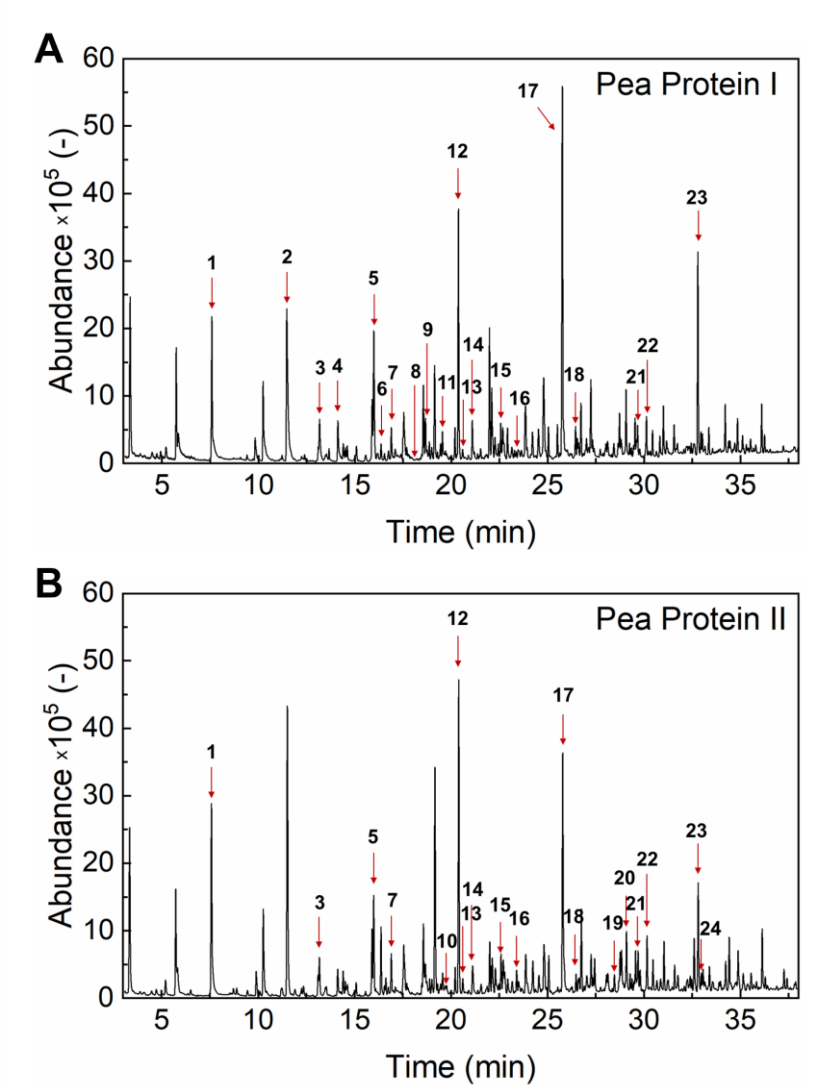

Figure IV.1 Chromatograms of pea protein isolates I and II analyzed by SBSE (5 mL, 2 h, room temperature) and subsequent GC–MS-O analysis; detected odor-active compounds numbered in ascending order.

Acetic acid [**8**] was perceived strongly at the olfactory port and identified according to MS, but lay below the threshold for peak integration and pea-specific 1-octen-3-one (Trikusuma et al., 2020; Tulbek et al., 2017; Xu et al., 2019) was not clearly identified due to overlapping peaks, regardless of its characteristic intense mushroom-like odor. As shown by other authors (Frankel et al., 1981; Li & Wang, 2016; St. Angelo et al., 1980), the majority of odorants identified via GC–MS-O (aldehydes, ketones, unsaturated carbonyl compounds) are fat oxidation products that arise from the degradation of hydroperoxides from (*i*) oleic acid (octanal [**3**], nonanal [**5**], 3-octen-2-one [**6**], decanal [**9**], (*E*)-2-nonenal [**11**]); (*ii*) linoleic acid (hexanal [**1**], 2-pentylfuran [**2**], (*E*)-2-heptenal [**4**], (*E*)-2-octenal [**7**], (*E*,*E*)-2,4-nona- and decadienal [**16, 17**]); and (*iii*) arachidonic acid ((*E*,*Z*)-3,5-octadien-2-one [**12**], 2-undecanone [**14**]). This is linked to a fairly

high content of unsaturated fatty acids content (**Table IV.2**), which are prone to lipid oxidation (Frankel et al., 1981). More precisely, radical-induced autoxidation and light-induced lipid oxidation result in the formation of hydroperoxides that may then be decomposed via enzymes (lipoxygenase), or by non-enzymatic decomposition with a catalyst (e.g., iron) (Frankel, 1980; Frankel et al., 1981). In contrast, compounds with a sweet (2-pentyl-pyridine [**13**]) or roasted odor (γ-nonolactone [**22**]) are secondary Maillard products that might evolve during protein extraction using pasteurization or spray drying (Bourgeois et al., 2015b; Trikusuma et al., 2020; Xu et al., 2019). Finally, geraniol [**18**] and β-ionone [**19**] are inherent plant compounds or originate from the degradation of carotenoids (Murray et al., 1976). Although the exact procedure to extract proteins was kept confidential by the manufacturer, differences between Pea Proteins I and II underlined the importance of understanding process-related changes of odor-active components during protein purification.

Pea Protein I possessed a variety of carbonyls and unsaturated carbonyl compounds (C6 to C12) that result from fat oxidation, whereas Pea Protein II contained a predominance of longer chained, intermediate fat oxidation products, as well as some alcohols and additional odor-active compounds that are characteristic of peas. Furthermore, substantial lipid oxidation of raw materials prior to protein extraction, or an incomplete thermal deactivation of oxidation triggers can be assumed, that could have degraded lipids during storage, in particular in Pea Protein I.

Characteristic odor-active compounds in texturates compared to pea protein powders

Besides storage effects on raw materials and changes during protein extraction, volatiles in plant proteins may change as a result of further processing steps before they are used as food ingredients. Due to their increasing relevance especially in the field of meat analogues dry (TVP) and wet (WTP) texturates of Pea Proteins I and II were analyzed by GC–MS-O after DI-SBSE. Sample dispersions were prepared at 1 % (w/w) based on dry matter (standardized according to moisture content), thus resulting in comparable absolute amounts of protein and fat as their respective pea protein powders (**Table IV.1**). Thus, semi-quantification by the normalized area method (Chen et al., 2014) was used to compare total and partial peak areas of odor-active compounds among all pea proteins. Data were grouped according to the structural classes of carbonyls, unsaturated carbonyls, alcohols, heterocycles, and terpenes/terpenoids, reflecting the results for Pea Protein I and II (**Table IV.3**).

Table IV.3 Peak area (%) of identified odor-active compounds in Pea Protein I and II and their dry (Pea TVP I, II) and wet (Pea WTP I, II) extrudates, calculated by the normalized area method after detection at the olfactory detection port by SBSE (5 mL, 2 h, room temperature) and subsequent GC-MS-O analysis

No.	Compound	Pea Protein I	Pea TVP I	Pea WTP I	Pea Protein II	Pea TVP II	Pea WTP II
	carbonyls						
1	hexanal	3.29 ± 1.05^{bc}	3.16 ± 0.03^{b}	0.52 ± 0.02^{a}	4.40 ± 0.33^{c}	2.37 ± 0.06^{b}	3.50 ± 0.13^{bc}
3	octanal	0.88 ± 0.27^{a}	1.04 ± 0.05^{ab}	*n. i.*[a]	0.76 ± 0.01^{a}	0.78 ± 0.02^{a}	1.32 ± 0.19^{b}
5	nonanal	2.36 ± 0.58^{bc}	2.20 ± 0.11^{bc}	0.58 ± 0.07^{a}	1.69 ± 0.14^{b}	1.88 ± 0.18^{b}	2.57 ± 0.05^{c}
9	decanal	0.59 ± 0.26	0.98 ± 0.02	*n. i.*	*n. d.*[b]	*n. d.*	*n. d.*
14	2-undecanone	0.83 ± 0.16^{bc}	0.48 ± 0.15^{a}	1.06 ± 0.05^{c}	0.74 ± 0.07^{ab}	*n. i.*	0.47 ± 0.01^{a}
	unsaturated carbonyls						
4	(*E*)-2-heptenal	0.89 ± 0.04^{c}	0.72 ± 0.05^{b}	0.48 ± 0.04^{a}	*n. d.*	*n. d.*	*n. d.*
6	3-octen-2-one	0.61 ± 0.40^{a}	0.48 ± 0.02^{a}	*n. i.*	*n. d.*	*n. d.*	*n. d.*
7	(*E*)-2-octenal	0.64 ± 0.02^{b}	0.65 ± 0.05^{b}	*n. i.*	0.76 ± 0.04^{c}	0.42 ± 0.01^{a}	0.77 ± 0.04^{c}
11	(*E*)-2-nonenal	0.53 ± 0.02	*n. i.*	*n. i.*	*n. d.*	*n. d.*	*n. d.*
12	(*E,Z*)-3,5-octadiene-2-one	4.36 ± 0.12^{c}	2.89 ± 0.11^{b}	4.80 ± 0.01^{d}	5.72 ± 0.35^{e}	1.98 ± 0.07^{a}	4.11 ± 0.01^{c}
16	(*E,E*)-2,4-nonadienal	*n. i.*	*n. i.*	*n. i.*	0.44 ± 0.01	*n. i.*	*n. i.*
17	(*E,E*)-2,4-decadienal	4.71 ± 3.54^{abc}	2.61 ± 0.37^{ab}	3.15 ± 1.07^{ab}	5.15 ± 0.38^{bc}	0.94 ± 0.11^{a}	7.85 ± 0.04^{c}
	alcohols						
10	2-nonanol	*n. d.*	*n. d.*	*n. d.*	*n. i.*	*n. i.*	*n. i.*
15	1-nonanol	0.57 ± 0.01^{a}	0.53 ± 0.06^{a}	1.00 ± 0.02^{c}	0.71 ± 0.01^{b}	0.57 ± 0.02^{a}	0.74 ± 0.1^{b}
20	1-dodecanol	*n. d.*	*n. d.*	*n. d.*	3.76 ± 3.75^{a}	1.97 ± 0.26^{a}	0.88 ± 0.28^{a}
	heterocycles						
2	2-pentyl furan	5.42 ± 0.61^{c}	3.28 ± 0.46^{b}	5.95 ± 1.08^{cd}	5.96 ± 0.08^{cd}	1.45 ± 0.03^{a}	7.30 ± 0.47^{d}
13	2-pentyl-pyridine	*n. i.*	0.48 ± 0.15^{a}	*n. i.*	0.34 ± 0.02^{a}	0.43 ± 0.01^{a}	0.43 ± 0.03^{a}
22	γ-nonalactone	0.77 ± 0.02^{ab}	1.03 ± 0.03^{bc}	1.12 ± 0.23^{c}	1.01 ± 0.11^{bc}	1.82 ± 0.01^{d}	0.70 ± 0.03^{a}
	terpenes/ terpenoids						
18	geraniol	0.30 ± 0.01^{a}	*n. i.*	0.72 ± 0.09^{b}	0.32 ± 0.06^{a}	*n. i.*	0.28 ± 0.03^{a}
19	β-ionone	*n. d.*	*n. d.*	*n. d.*	0.35 ± 0.01^{ab}	0.32 ± 0.01^{a}	0.39 ± 0.02^{b}
	Total peak area $(x10^6)^3$	247.49 ± 7.93^{b}	187.16 ± 1.40^{a}	216.72 ± 33.45^{ab}	241.14 ± 40.38^{ab}	183.82 ± 11.43^{a}	249.95 ± 9.29^{b}

Small capital raised letters indicate significant difference ($p \leq 0.05$) among samples in one row,
[a] Not integrable (peak area < 65 000 1 min^{-1}), [b] Not detectable in total ion current chromatogram;
[c] Area sum of all peaks in the chromatogram after normalization method (100 % reference value).

As shown for Pea Protein I volatiles numbered with [**1**] to [**9**], [**11**] to [**18**] and [**21**] to [**23**] were sensed and identified by odor, RI and/or MS in Pea TVP I and Pea WTP I (**Figure IV.1** and **Figure IV.2**). Furthermore, Pea TVP II and Pea WTP II possessed the 20 odor-active compounds found in Pea Protein II. Besides, compounds shown in **Table IV.2** one (WTP I and II) or three (TVP I and II) pyrazines were additionally identified at retention times >14 min (see stars, **Figure IV.2**). As a result, overall odor impressions perceived at the olfactory port were similar to the respective powders and described as beany/cereal-like (Pea TVP I and WTP I) and pungent green/peaty-like (Pea TVP II and WTP II) due to some additional roasty, nutty, and sweet scents. However, a decrease in the overall odor intensity of green and fatty scents detected at the olfactory port in both dry and wet extrudates was perceived and beany odors were lower in TVPs. In accordance with this, the peak height (**Figure IV.1** and **Figure IV.2**) and area of most identified volatiles (**Table IV.3**) were reduced. For example, Pea TVP I and II had significantly ($p < 0.05$) smaller total peak areas (sum of all volatiles up to min 38) than Pea I and II. The main reason for the observed differences among samples was a change in (unsaturated) carbonyl compounds. As such, (*E,Z*)-3,5-octadien-2-one [**12**] was significantly lower ($p \leq 0.05$) in both Pea TVP I (2.89 ± 0.11 %) and TVP II (1.98 ± 0.07 %), as well as in Pea WTP II (4.11 ± 0.01 %) when compared with their respective powders (4.36 ± 0.12 % and 5.72 ± 0.35 %). Similarly, nonanal [**5**] decreased significantly ($p \leq 0.05$) from 2.36 ± 0.58 % (Pea Protein I) to as low as 0.58 ± 0.07 % (Pea WTP I) and 1-dodecanol [**20**] was reduced by more than fourfold if Pea Protein II and Pea WTP II were compared. Octanal [**3**], (*E*)-2-octenal [**7**], 3-octen-2-one [**6**], and decanal [**9**] dropped below the integration limit in Pea WTP I, which meant that there was a strong impact of wet texturization on these carbonyls in Pea Protein I (**Figure IV.2**), which in turn remained mostly unchanged or even increased in Pea TVPs (**Table IV.3**). Moreover, secondary Maillard products such as 2-pentyl-pyridine [**13**] and γ-nonalactone [**22**] increased distinctively in Pea TVP I and II, where they were also sensed most intensively. Finally, the natural pea compound geraniol [**18**] dropped below integration limit in both TVPs (**Figure IV.2**), but not in their respective WTPs.

This decrease in carbonyl compounds from lipid oxidation could be linked to thermal breakdown due to heat application (Zamora et al., 2015) during texturization. Compounds resulting from this might, then, be less odor active, or no longer odor active – especially those with high odor thresholds such as 1-nonanol [**15**], geraniol [**18**], 1-dodecanol [**20**], and octanal [**3**], where values of 50, 40–74, and 7 $\mu g\, L^{-1}$, respectively, were described (Leffingwell & Leffingwell, 1991; Trikusuma et al., 2020).

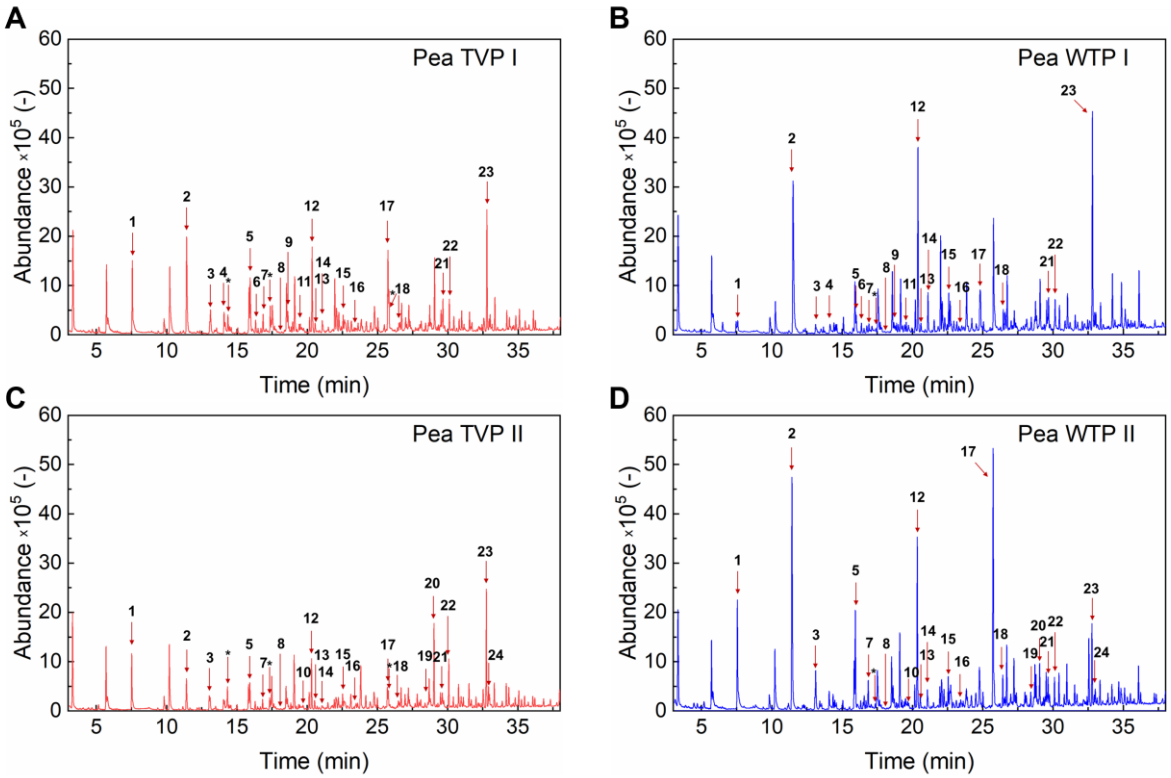

Figure IV.2 Chromatograms of dry (TVP) and wet texturized (WTP) extrudates from pea protein isolate I (A, B) and II (C, D) analyzed by SBSE (5 mL, 2 h, room temperature) and subsequent GC–MS-O analysis; detected odor-active compounds numbered in ascending order.

Other authors have recently proposed that elevated temperatures might also increase volatiles based on the Maillard reaction and/or a degradation of lipids, carotenoids, and vitamins (Xu et al., 2019). As such, pyrazines with a nutty, roasted odor were identified, in particular in TVP I and II (see stars in **Figure IV.2**). This included 2,5-dimethyl-pyrazine, 3-ethyl-2,5-dimethyl-pyrazine, and 2-butyl-3,5-dimethyl-pyrazine, which were previously described in peas (Cui et al., 2020; Ma et al., 2016; Murray et al., 1976; Schindler et al., 2012; Trikusuma et al., 2020). Moreover, a reduction of some compounds might go along with the formation of new ones as recently shown for a decrease in 2-alkenals in favor of the aldehydes propanal and hexanal (Zamora et al., 2015). At the same time, 2-alkenals can arise from a thermal degradation of unsaturated carbonyls such as (*E,E*)-2,4-decadienal (Zamora et al., 2015). Authors further described a mathematical correlation in between the degradation of unsaturated carbonyls, heating time, and temperature in favor of a creation of these shorter chained aldehydes. As a result of these numerous reactions, some odorants increased or decreased when Pea Proteins I and II and their respective texturates were compared (**Figure IV.1** and **Figure IV.2, Table IV.3**). For example, the characteristic green odorant

hexanal [**1**] was reduced by up to sixfold after wet texturization of Pea Protein I (3.29 ± 1.05 % compared with 0.52 ± 0.02 %), whereas other texturates showed no significant ($p > 0.05$) change (**Table IV.3**). Moreover, we observed decreasing percentages of (*E*)-2-heptenal [**4**] and (*E*)-2-nonenal [**11**] for dry and wet texturates of both pea proteins (**Table IV.3**) but increases in octanal [**3**] from 0.76 ± 0.01 % (Pea Protein II) to 0.78 ± 0.02 % (Pea TVP II) to 1.32 ± 0.19 % (Pea WTP II). At the same time, degradation of (*E,E*)-2,4-decadienal in Pea Protein I in favor of 3,5-octadien-2-one [**12**] or Maillard products 2-pentyl-pyridine [**13**] and/or *γ*-nonalactone [**22**] might have occurred (Im et al., 2004; Kim et al., 1996). However, the overall amount of Maillard products was rather low compared with green and floral volatiles, possibly related to the small amounts of residual carbohydrates (**Table IV.1**) and the short residence time at high temperatures in the extruder. Nonetheless, Maillard products, possess lower odor thresholds than many fat oxidation products, for example 2-pentyl-pyridine ($0.6\ \mu g\ L^{-1}$) (Berger, 2007), compared to hexanal ($4.5\ \mu g\ L^{-1}$) (Leffingwell & Leffingwell, 1991). This makes them notable odor contributors that might also influence the overall taste of proteins.

Influence of dry in comparison to wet texturization on odor-active compounds in pea proteins

Results from GC–MS-O after DI-SBSE showed that the number of odor-active compounds in Pea Protein I and II remained mainly unaffected after dry (TVP) and wet (WTP) texturization (**Figure IV.1** and **Figure IV.2**). However, chromatograms, odor impressions, and peak area shares from normalization revealed changes in fat oxidation products, in particular (**Table IV.3**). There is still an open question on the influence of dry compared to wet texturization. Irrespective of variations among volatiles, eight compounds were shown to be distinctively affected in both powders and their respective dry (Pea TVP I, II) and wet (Pea WTP I, II) texturates (**Figure IV.3**): Six (unsaturated) carbonyl compounds, hexanal [**1**], nonanal [**5**], 2-undecanone [**14**], (*E)*-2-octenal [**7**], (*E, Z)*-3,5-octadiene-2-one [**12**], (*E,E)*-2,4-decadienal [**17**], as well as the heterocycles 2-pentylfuran [**2**], 2-pentyl-pyridine [**13**] and *γ*-nonalactone [**22**]. Based on these volatiles, the majority of which are known to contribute to (off) flavors in peas (Murray et al., 1976; Trikusuma et al., 2020; Xu et al., 2019), process-related changes of odor-active compounds in pea proteins are proposed.

Dry texturized proteins Pea TVP I and II were altered in most of the selected carbonyl compounds (**Figure IV.3**, **Table IV.3**).. For example, (*E,Z*)-3,5-octadien-2-one [**12**] decreased from 4.36 ± 0.12 % (Pea I) and 5.72 ± 0.35% (Pea II) to 2.89 ± 0.11 % (Pea TVP I) and 1.98 ± 0.07 % (Pea TVP II) and (*E,E*)-2,4-decadienal [**17**] was reduced by up to 1.8-fold.

(*E)*-2-Octenal [**7**] was significantly ($p \leq 0.05$) reduced in Pea TVP II, and 2-undecanone [**14**] was lowered by 40% in Pea TVP I and even dropped below integration limit in Pea TVP II. The 'green' fat oxidation intermediate 2-pentylfuran [**2**] was reduced by 40 % (Pea TVP I) and 75 % (Pea TVP II). This resulted in lower odor intensities for green volatiles sensed at the olfactory port. However, roasted and nutty volatiles were perceived additionally around minutes 14, 17, and 25, and were identified as 2,5-dimethyl-pyrazine, 3-ethyl-2,5-dimethyl-pyrazine, and 2-butyl-3,5-dimethyl-pyrazine, respectively (see stars in **Figure IV.2**).

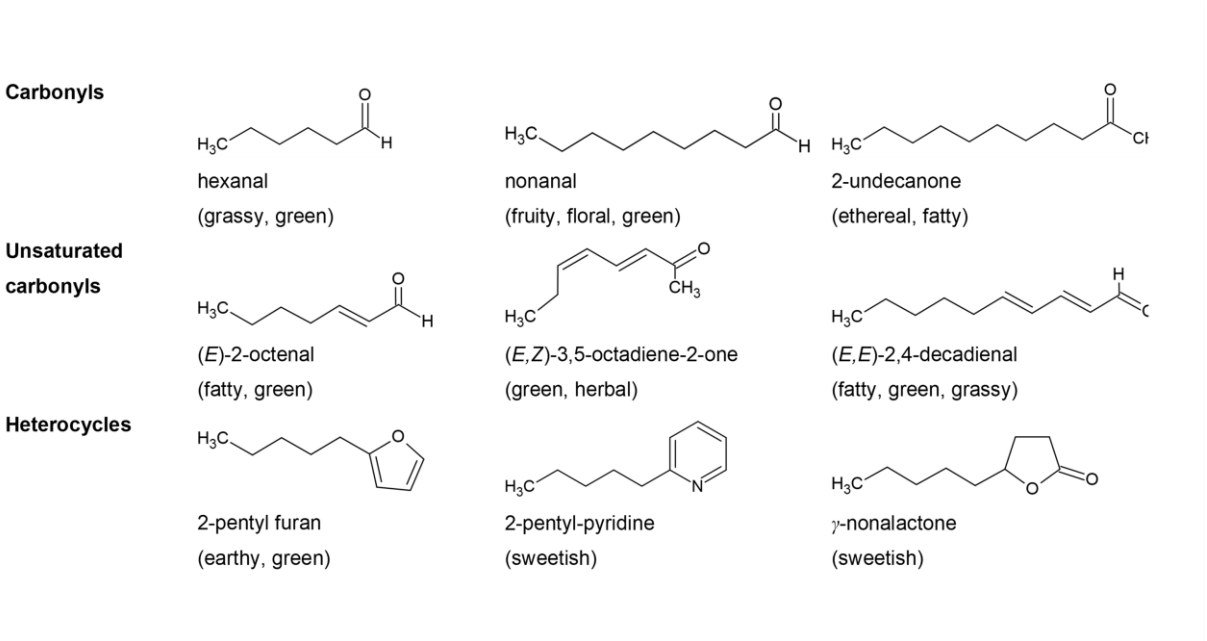

Figure IV.3 Chemical structure of characteristic odor-active compounds substantially affected by dry (TVP) or wet texturization (WTP) of Pea Protein I and II.

In contrast, only 3-ethyl-2,5-dimethyl-pyrazine was found in Pea WTP I and II, which might be related to lower temperatures and/or shorter residence times during the wet texturization process (Harper & Clark, 1979; Osen & Schweiggert-Weisz, 2016). Moreover, wet texturates differed in most peak area shares of selected odor-active compounds (**Table IV.3, Figure IV.3**) with no clear decreasing or increasing content. First, hexanal [**1**] varied distinctively - for example by factor 7 if Pea WTP I (0.52 ± 0.02 %) and Pea WTP II (3.50 ± 0.13 %) were compared - irrespective of similar amounts in Pea Proteins I and II. Second, peak area shares of 2-undecanone [**14**], 1-nonanol [**15**], and *γ*-nonalactone [**22**] were > 1 % in Pea WTP I, but were present in Pea WTP II in much smaller amounts. In turn, 0.77 ± 0.04% of (*E*)-2-octenal [**7**] and 0.43 ± 0.03 % of 2-pentyl-pyridine [**13**] were found in Pea WTP II, while areas lay below the integration limit in Pea WTP I. Finally, (*E,Z*)-3,5-octadien-2-one [**12**] was lowered in Pea WTP II, but increased if Pea WTP I and their respective powder were compared, whereas

the opposite effect was shown for peak area shares of nonanal [**5**]. Based on these results, no clear effect of wet texturization could be deduced. All in all, texturization reduced odor-active carbonyl compounds in favor of new volatiles or was related to degradation to shorter chained carbonyls. This was particularly true for 'green' fat oxidation products, which reduced their overall odor intensity.

Conclusion

The analysis of pea protein isolates and their respective dry (TVP) and wet (WTP) texturates by gas chromatography–mass spectrometry-olfactometry (GC–MS-O) after direct immersion-stir bar sorptive extraction (DI-SBSE) provided valuable insights into characteristic odor-active components and their changes upon texturization. Based on this, it can be generally proposed that (*i*) green, fatty odors can be reduced by dry texturization; (*ii*) new odor impressions can be created through a cross-reaction of inherent compounds (e.g., conjugation of aldehydes and reducing sugars through Maillard reaction); (*iii*) the composition of the initial pea protein powder determines the odor-active compounds of their respective texturates. As a result, dry texturization can be suggested as a promising technique to reduce (off-) flavors in pea proteins, while wet texturization might even intensify odor-active compounds. Future studies should be conducted to assess volatiles quantitatively in powdered and texturized plant proteins in connection with their effect on flavor and taste. This might also include an evaluation of temperature-induced protein degradation that may induce the release of peptides or affect protein functionality. Moreover, further research should be undertaken to remove unfavorable odor-active compounds or to increase the oxidation stability of plant proteins prior to their application in foodstuffs, to obtain products with reproducible and acceptable organoleptic properties and high shelf-stability.

Acknowledgments

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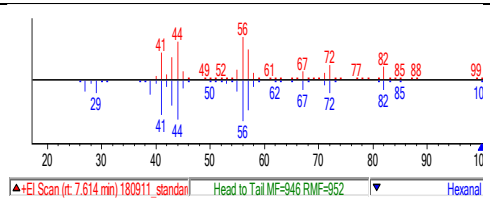
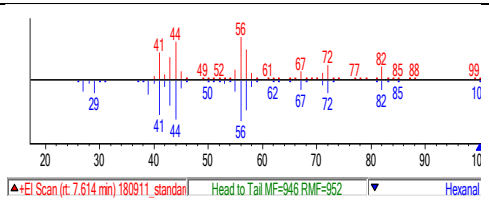
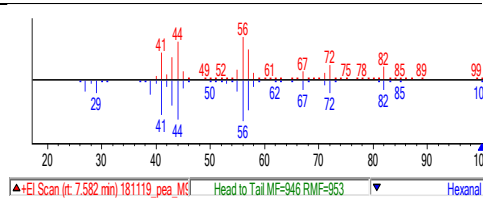
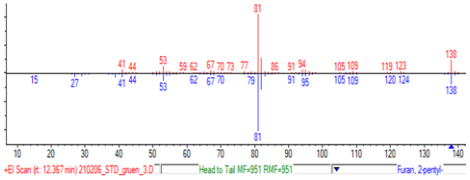
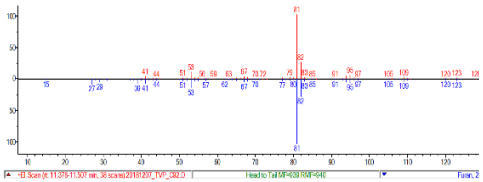
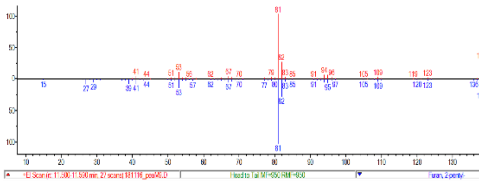
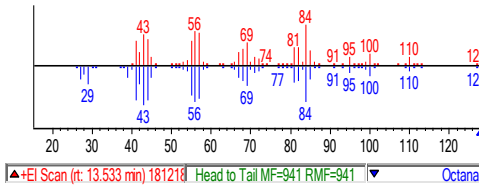
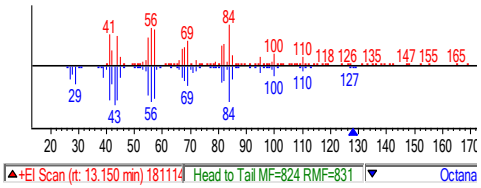
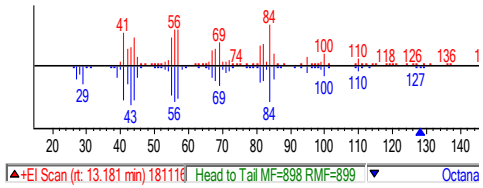
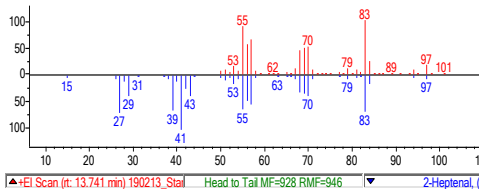
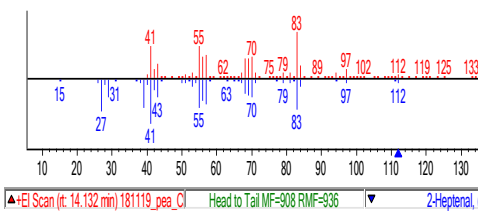
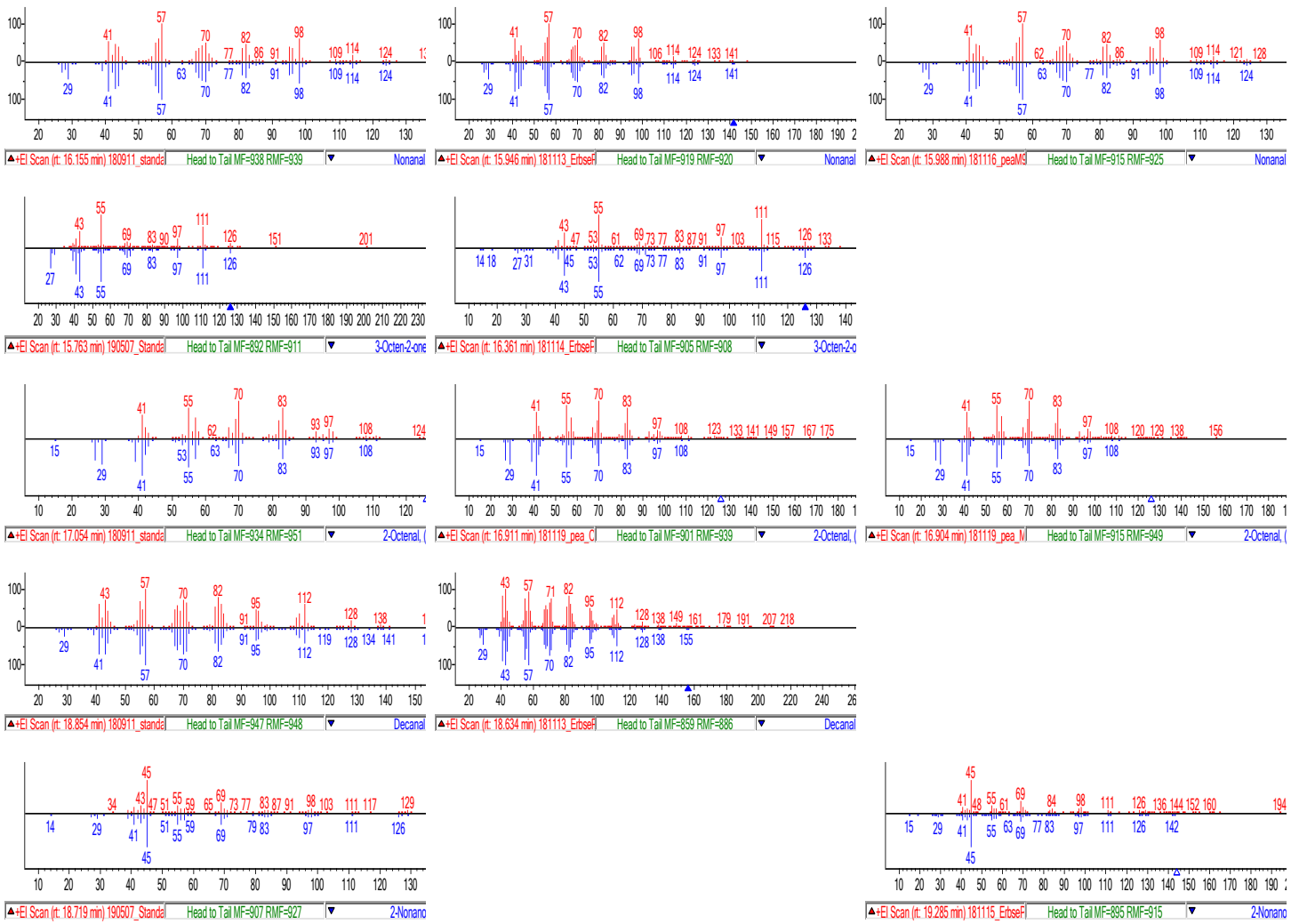
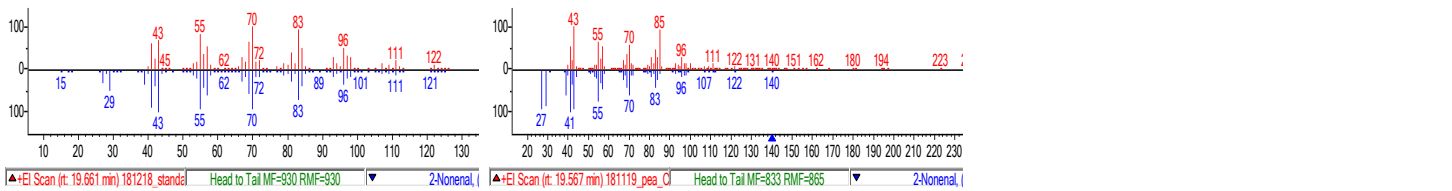
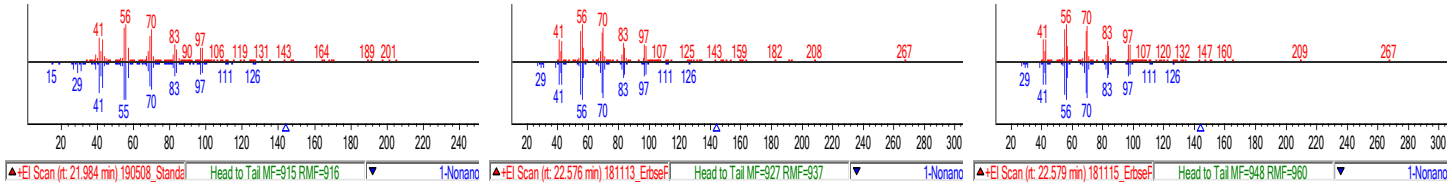
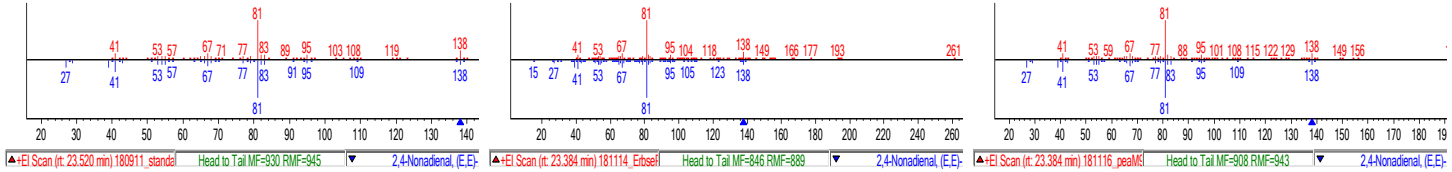
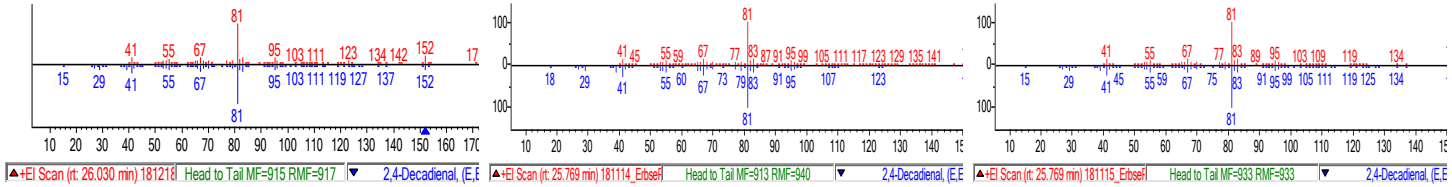
Supporting Information

Table SIV.4 Mass spectra obtained for odor-active substances detected in pea protein powders I & II and authentical standards thereof; Determination by comparison with authentic standard compounds using one quantifier and two qualifier ions

Compound	Ion			MS Standard	MS Pea Protein I	MS Pea Protein II
	1	2	3			
hexanal	56	44	41			
2-pentyl furan	81	13	53			
octanal	84	56	43			
(*E*)-2-heptenal	83	55	57			

nonanal	57	98	41
3-octen-2-one	55	11	43
(*E*)-2-octenal	70	55	83
decanal	57	82	43
2-nonanol	45	69	43



(*E*)-2-nonenal	70	83	55	
2-undecanone	58	43	71	
1-nonanol	56	55	70	
(*E*,*E*)-2,4-nonadienal	81	13	41	
(*E*,*E*)-2,4-decadienal	81	67	83	

V. Chapter

Buffering Capacity of Wet Texturized Plant Proteins in Comparison to Pork Meat

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Abstract

There is an increasing demand to develop and characterize high moisture extrudates from alternative plant proteins due to their increased use in various foods. In this study, wet texturized proteins from two pea isolates and four oilseed flours from pumpkin and sunflower were subjected to an acid titration to gain insights into their buffering capacity. Results were compared to pork meat with a special emphasis on compositional differences. Wet texturized pumpkin and sunflower proteins had the highest buffering capacity, especially in between pH 7.0 and pH 4.5, while pea protein extrudates and pork meat were more prone to acidification and similar in buffering capacity. A multiple linear regression model further revealed that ash and select minerals and amino acids are key influencing factors on the overall buffering capacity, while the effect of protein and non−protein nitrogen depends on the evaluated pH−regime. The obtained results underline the importance for a more in-depth physicochemical characterization of texturized plant proteins and their raw materials and suggest a need for recipe and process adjustment to achieve stable pH values.

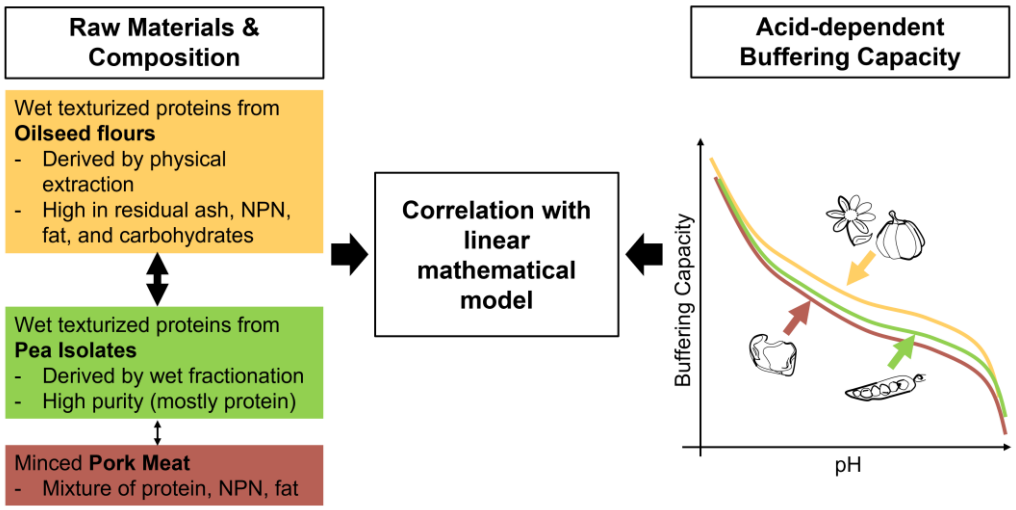
HIGHLIGHTS

- Compositional differences account for varying buffering capacities of oilseed texturates
- Texturized pea proteins resemble meat proteins in their pH-dependent behavior
- A mathematical model describes the relation of composition and buffering capacity

KEYWORDS

Texturized Proteins; High Moisture Meat Analogues; Meat Proteins; pH-Dependency; Hybrid Meats; Mathematical Model

GRAPHICAL ABSTRACT



INTRODUCTION

The importance of alternative proteins is steadily increasing due to a growing consumer demand for a more sustainable and plant-based diet (Aschemann-Witzel et al., 2020). This has resulted in considerable research developments in protein extraction from plants, microalgae, and insects, as well as the characterization of their derived protein fractions (Bourgeois et al., 2015b; Dossey et al., 2016; Ebert et al., 2020; González-Pérez & Vereijken, 2007; Grossmann et al., 2018; Grossmann et al., 2019b; Rezig et al., 2013). Along with this, high moisture extrusion technology has gained importance to overcome limitations of these alternative proteins in terms of functionality and structure formation making them promising replacements for traditional, animal–based protein sources from dairy and meat *i.e.* in meat analogues(Asgar et al., 2010; Osen & Schweiggert-Weisz, 2016). However, their use in foods requires a knowledge of their functional and physicochemical characteristics. This also includes their pH-dependent behavior - a property that is crucial in many food production processes. In example, dry-cured meat products are generally obtained through ripening, which includes acidification (target pH < 5.0) and drying (water activity a_w < 0.90) to obtain a shelf-stable product with a sliceable texture (Feiner, 2006). In other words, acid-induced denaturation of meat proteins promotes the formation of a coherent matrix. As a result, knowledge on the pH-dependent behavior of wet texturized protein may be crucial to create high-quality meat hybrids or analogues.

The pH-dependency can be related to the buffering capacity which has an effect on final pH as it generally describes the response of an ingredient upon addition with acid or base (Van Slyke,

1922) and/or microbial fermentation. While a lot of studies have described buffering capacity of different animal- and plant-based foods and their ingredients in the context of human digestion (Luo et al., 2018; Maher et al., 2010; Mennah-Govela & Bornhorst, 2021; Mennah-Govela et al., 2020; Mennah-Govela et al., 2019; van der Sman et al., 2020), knowledge from a food technology point of view is still scarce and limited to traditional protein sources (Goli et al., 2007; Honikel & Hamm, 1974; Kylä-Puhju et al., 2004; Puolanne & Kivikari, 2000; Tan et al., 2014). However, it is already clear, that the buffering capacity can be related to various compositional elements. This includes protein, non-protein nitrogen (NPN), fat, ash, and its constituents, namely phosphorous, sodium, potassium, and calcium (Mennah-Govela et al., 2020; Mennah-Govela et al., 2019; Salaün et al., 2005), as well as some amino acids with a pKa in the range of foodstuff, such as aspartic (pKa_2 3.86) and glutamic acid (pKa_2 4.25) and histidine (pKa_2 6.0) (Bhagavan & Ha, 2011; Mennah-Govela et al., 2020; Mennah-Govela et al., 2019; Salaün et al., 2005; van der Sman et al., 2020).

In this study, wet texturized proteins from pea isolates (Pea I, Pea II) and four oilseed flours from roasted (Pumpkin I, Pumpkin II) and unroasted pumpkin (Pumpkin III) and sunflower seeds (Sunflower) were analyzed in their proximate, mineral, and amino acid composition. Following this, wet texturized proteins were assessed in their capability to withstand fluctuations and changes in pH upon acidification starting-from a pH value of 8.0. The area under the curve and buffering capacity were calculated over the whole and a partial pH-regime to compare samples qualitatively and quantitatively and to describe the effect of compositional elements based on a mathematical model. Results were then compared to lean pork meat to better understand differences and assess the suitability of wet extrudates as ingredients for meat analogues and hybrids and to predict effective recipe or process modulations upon their use.

Materials and Methods

Materials

Lean pork meat was purchased from MEGA (Stuttgart, Germany) and standardized to SI quality (approx. 75 % water, 20 % protein) according to GEHA standards (Brombach et al., 2003). Wet texturized plant proteins were obtained from the German Institute for Food Technology (DIL, Quakenbrueck, Germany). Respective raw materials for Pea I and II were wet fractionated pea isolates (Bourgeois et al., 2015b) Pisane®M9 (81.7 % protein, 4 % fat, 3.7 % salt, 3.2 % total carbohydrates) and Pisane®C9 (81.7 % protein, 4 % fat, 3.7 % salt, 3.2 % total carbohydrates) from the Coscura Group (Warcoing, Belgium). Pumpkin seed flour (55 % protein, 12.3 % fat,

5.1 % salt, 19.8 % total carbohydrates) from Ölmühle Fandler GmbH (Sonnhofen, Austria) was used for Pumpkin I and represented a dried and milled pomace from vegetable oil production as shown by Fruhwirth and Hermetter (2008). Similarly, protein flours Pumpkin®60 Roasted (60 % protein, 12.8 % fat, 0.01 % salt, 15.5 % total carbohydrates) for Pumpkin II and Unroasted (60 % protein, 9 % fat, 0.01 % salt, 17 % total carbohydrates) for Pumpkin III, and Heliaflor®45 (45 % protein, 10 % fat, 0.002 % salt, 25 % total carbohydrates) for Sunflower represented oilseed pomaces from All Organic Treasures GmbH (Wiggensbach, Germany) that were milled with their corporate *kryonert*® technology. In short, powders were dosed into a double-screw extruder (ZSK 27MV, Coperion GmbH, Stuttgart) at a water to powder ratio of 6 to 4 (Pisane®C9, M9), 7 to 7 (Pumpkin seed flour), 7 to 8 (Pumpkin®60, Roasted), 9 to 7 (Pumpkin®60, Unroasted), and 6 to 6 (Heliaflor®). The inlet temperature of 40 °C was stepwise increased to 145 °C. Protein plastification was done in the subsequently attached cooling-die (FKD-750, DIL, Quakenbrück). The obtained wet extrudates were cut into stripes, packed airtight, and stored at - 18 °C until further use.

Tris (hydroxymethyl) aminomethane (THAM, purity ≥ 99.9 %), sodium hydroxide (purity ≥ 98.0 %), hydrochloric acid HCl (concentration 32 %) Dichloromethane (ROTIPURAN® ≥ 99.5 %), sulfuric acid (ROTIPURAN® 98 % and standardized solution at 0.5 mol/l), trichloro acetic acid (purity ≥ 99 %), petroleum ether (40 – 60°C), Tashiro indicator (0.75 g/l methyl red sodium salt, 0.375 g/l methyl blue in ethanol 50 % (v/v)) magnesium acetate tetrahydrate ($(CH_3COO)_2Mg * 4 H_2O$, purity ≥ 99.5 %), folded filters (ROTILABO®113P), and boiling stones (Type B) were acquired from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Boric acid (purity ≥ 99.5 %), Kjeldahl tablets (47.7 % K_2SO_4, 47.7 % Na_2SO_4, 1.8 % $CuSO_4$, 2.8 % TiO_2; 5 g) and dithiodiglycolic acid (purity ≥ 98 %), were purchased from Merck KGaA (Darmstadt, Germany). Double deionized (DI) water was used for all experiments.

Preparation of pork meat and wet texturized plant proteins

Lean pork meat was minced with a meat grinder (Type WD114, Seydelmann, Aalen, Germany) using a 3 mm punch disk, packet airtight, and stored at 4 °C before analysis. Wet texturized plant proteins were thawed, cut into squares of 4 x 4 cm, and chopped in a bowl chopper (Type MTK 661, Maschinenfabrik Dornhan GmbH, Dornhan, Germany) to receive evenly distributed particles of approximately 0.2 cm. Chopped extrudates were packed airtight and stored at 4 °C until further analysis.

Proximate and chemical composition of pork meat and wet texturized plant proteins

Moisture content according to sea sand method

The moisture content of pork meat and wet texturized plant proteins was calculated from the amount of dry matter analyzed according to the sea sand method AS § 64 L 06.00-3 (BVL, 2005a) and calculated with data from back weighing.

Total nitrogen content according to Kjeldahl and specific protein content

The total nitrogen content was analyzed by an acidic digestion, followed by a distillation and back titration by using the Kjeldahl method AS § 64 L 06.00-7 (BVL, 2005a). The specific protein content was calculated from **Eq. V.1**.

$$Specific\ protein\ (\%) = Total\ nitrogen\ (\%) * F_{Lit} \qquad \textbf{V.1}$$

F_{Lit} = Nitrogen to protein conversion factor from literature
Pork meat = 6.25 according to Keeton et al. (2014);
Pea = 5.36 according to Mariotti et al. (2008);
Pumpkin = 5.50 according to Milovanović et al. (2014);
Sunflower = 5.29 according to Mariotti et al. (2008)

Non-protein nitrogen (NPN) content

The amount of NPN was determined by using the Kjeldahl method after a precipitation of proteinogenic nitrogen with dichloromethane based on AS § 64 L 07.00-41 (BVL, 2005a).

Total fat content according to Weibull-Stoldt

Total fat was analyzed by applying the method of Weibull-Stoldt AS § 64 L 07.00-6 (BVL, 2005a) followed by a low-boiling Soxhlet extraction with petroleum ether.

Total ash content

The total ash content was analyzed after a complete combustion at 600 °C according to AS § 64 L 06.00-4 (BVL, 2005a) after pre-incineration. A sample amount of around 5 g was used.

Phosphorus content

The phosphorus content of samples was determined according to AS § 64 L 06.00-9 (BVL, 2005a). In short, sample ash (from 2.3.5) was hydrolyzed with nitric acid and a color complexation with ammonium vanadate and ammonium heptamolybdate was detected at

430 nm. The obtained value expressed as phosphor pentoxide (mg/100 g) was converted to phosphorus (mg/100 g) by a multiplication with a conversion factor f = 0.4364 (Murf, 2008).

Composition of selected atoms by inductively coupled plasma optical emission spectrometry (ICP-OES)

Minced pork meat and wet texturized plant proteins were freeze-dried, milled, and analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) to quantify amounts of the selected atoms sodium, potassium, calcium, magnesium, iron, copper, manganese, and zinc according to Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten e.V. (2007). In short, 0.2 g of sample were subjected to a microwave pressure digestion (UltraClave Fa. MLS Leutkirch, Germany) in nitric acid. Following this, samples were sequentially extracted, sprayed into argon plasma, and vaporized at 10,000 K to dissociate atoms (Agilent 5110 SVDV from Agilent Technologies GmbH, Waldbronn, Germany). The selected emission wavelength was separated over a polychromator and the light intensity of the respective light line was detected. Detection wavelength were 214 nm (zinc), 258 nm (manganese), 260 nm (iron), 280 nm (magnesium), 318 nm (calcium), 327 nm (copper), 589 nm (sodium), 766 nm (potassium). Quantitation was done according to a calibration with the respective standard solutions.

Amino acid composition by ion exchange chromatography

Wet texturized plant proteins were freeze-dried and ground with an ultracentrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) equipped with a 0.75 mm sieve. Sample preparation and analysis of amino acids was done according to VO (EG) Nr. 152/2009 Annex III G (tryptophan) and F (all other amino acids) with a Biochrom 30 (Biochrom Ltd., Cambridge, UK). Anion exchange columns were used for purification and separation (Grain size 4 μm), namely a PEEK pre-column (100 x 4.6 mm) and a PEEK separation column (200 x 4.6 mm) and determination was done after a reaction with ninhydrin at 570 nm.

Acid titration and buffering capacity of pork meat and wet texturized plant proteins

Preparation of sample suspensions

Minced pork meat or chopped wet texturized plant proteins were suspended in double deionized water at a concentration of 2 wt% and 0.03 wt% of sodium azide were added to prevent microbial spoilage. Samples were homogenized with an Ultra Turrax (Silent Crusher M, Heidolph, Schwabach, Germany) for 2 min at 10,000 rpm and were adjusted from their native

pH (pH 6.2 to 8.0) to pH 8.0 through the addition of sodium hydroxide (0.02 N to 2 N) . After 2 h of stirring, homogenization was repeated to obtain a coherent particle size and to ensure hydration in all samples and pH was adjusted if necessary.

Sample titration (total) buffering capacity

The titration procedure and analysis of the total buffering capacity was adapted from Mennah-Govela et al. (2019). First, several HCl stock solutions (0.01 to 3.0 N) were prepared. To prevent dilutional effects of acid addition on the protein concentration, 5 ml aliquots of 2 wt% sample suspensions (see 2.5.1) were transferred into 20 different test tubes equipped with ascending amounts of HCl stock solutions (50 to 100 μl of 0.01, 0.05, 0.25, 0.5, 1.0, 2.0, or 3.0 N, representative for acid concentrations in between 1 to 5882 mmol H^+/kg sample). A control sample without acid was prepared in addition. Samples were stirred overnight and pH was measured after 12 h.

Buffering capacity (BC) and area under the curve (AUC)

The area under the curve (AUC) and buffering capacity (BC) were calculated to enable a quantitative comparison of pork meat and plant protein suspensions. The procedure was modified from Mennah-Govela et al. (2019). First, AUC was calculated by plotting measured pH-values against the respective HCl-concentration in mmol H^+/kg sample. Curves were then integrated with Origin Pro (Version 2018b, OriginLab Corporation, Northampton, MA, USA) to obtain the total AUC (pH 8.0 to final pH) and partial AUC (pH 7.0 to 4.5). The BC at each acid concentration was calculated according to **Eq. V.2** and plotted as a function of acid concentration (mmol H^+/kg sample). The total BC (tBC) was calculated from **Eq. V.3**. Finally, the partial BC (pBC) in between pH 7.0 and 4.5 was obtained from **Eq**. **V.4**.

$$BC\ (mmol\ H^+/(kg * pH)) = c(H_i^+)/(pH_0 - pH_i) \quad \textbf{V.2}$$

$$tBC\ (mmol\ H^+/(kg * pH)) = c(H_{total}^+)/(pH_0 - pH_{final}) \quad \textbf{V.3}$$

$$pBC\ (mmol\ H^+/(kg * pH)) = c_{\Delta pH}/\Delta pH \quad \textbf{V.4}$$

$c(H_i^+)$ = Concentration of HCl added to sample tube (mmol H^+/kg sample)

$c(H_{total}^+)$ = Total amount of HCl added = 5882 mmol H^+/kg sample

pH_0 = Titration starting point = pH 8.0

pH_i = Measured sample pH at $c(H_i^+)$

pH_{final} = Final pH-value at titration end = addition of 5882 mmol H^+/kg sample

$c_{\Delta pH}$ = Acid concentration used in the tested pH-range

Statistical and data analysis

Statistically significant differences among samples were tested by a one-way analysis of variance with a Tukey posthoc-test (α-level of 0.05) using SPSS statistics V23 (IBM Corp., Armonk, USA) after checking the assumption of normality (Shapiro-Wilk-test, p-value to reject ≤ 0.05) and equal variance ($p \leq 0.05$). All analyses were done at least in duplicates from freshly prepared samples. A multiple linear regression with backwards selection of variables was carried out using SPSS statistics V23 (IBM Corp., Armonk, USA) to identify factors that influence total and partial BC and AUC (dependent variables) at a significance level of $p \leq 0.1$ (Dufner et al., 2013). Independent variables were protein, NPN, fat, ash, and the phosphate content calculated as P_2O_5 (phosphorus*2.2914 according to Murf (2008)), as well as the sum of sodium, potassium, and calcium, and the sum of glutamic and aspartic acid and histidine related to their relevance noted in literature (Mennah-Govela et al., 2020; Mennah-Govela et al., 2019; Salaün et al., 2005). All independent variables were converted to g/kg.

Results and Discussion

Composition of pork meat and wet texturized plant proteins

Proximate and chemical composition

Proximate composition (moisture, total nitrogen, fat, ash), non-protein nitrogen (NPN), phosphorus and individual minerals of Pork Meat and wet texturized proteins from pea isolates (Pea I, Pea II) and oilseed flours from roasted (Pumpkin I, Pumpkin II) and unroasted pumpkin (Pumpkin III) and sunflower seeds (Sunflower) are summarized in **Table V.1**. All tested samples contained water as a primary constituent but with significant ($p \leq 0.05$) variations between samples. As shown in **Table V.1**, moisture contents ranged between 50.0 ± 0.1 % (Pumpkin II) and 73.7 ± 0.8 % (Pork Meat). Wet texturized plant proteins were significantly ($p \leq 0.05$) higher in total nitrogen than pork meat (3.61 ± 0.11 %) with nitrogen contents decreasing in the order of Pumpkin II (4.65 ± 0.01 %), Pea II (4.61 ± 0.03 %) and Pea I

(4.55 ± 0.11 %), Pumpkin I (4.38 ± 0.11 %), Pumpkin III (3.84 ± 0.05 %), and Sunflower (3.73 ± 0.06 %). A calculation of the specific protein content based on individual nitrogen-to-protein conversion factors (**Table V.1**) revealed the same trend with values in between 19.7 ± 0.3 % (Sunflower) and 25.6 ± 0.1 % (Pumpkin II). Carbohydrates were negligible in meat but made up as much as 18.7 % in extrudates, where residuals of ash and fat were also noticeably high. There was no clear correlation of specific protein – and thus purity – and NPN. NPN was highest in lean pork meat (2.89 g/100g) and lowest in Pea I and II (0.348 g/100g and 0.430 g/100g), while oilseed extrudates contained 1.2 to 1.7 % of NPN. Moreover, determination of selective minerals by ICP-OES revealed an abundance of sodium and/or potassium in pork meat as well as in all wet texturized plant proteins, followed by phosphorus, magnesium, and calcium, but low amounts of copper, manganese, and zinc.

Proximate and chemical composition of pork meat was in accordance with literature values obtained from porcine top cuts that contain around 74.8 % of moisture, 22.2 % of protein, 1.90 % of fat, and 1.20 % of minerals (Souci et al., 2008). In contrast to meat, wet texturized plant proteins varied according to the respective plant-source, initial powder purity, and/or handling during extrusion. For example, raw materials for Pumpkin I, II, and III and Sunflower represent dried and milled pomaces that are obtained as side-streams from vegetable oil production. The respective flours contained 45 % (Heliaflor®45 for Sunflower), 55 % (Pumpkin seed flour for Pumpkin I), or 60 % of protein (Pumpkin®60, Roasted/Unroasted for Pumpkin II/III) depending on the technology used by the manufacturer. In example, some may (Pumpkin seed flour for Pumpkin I and Pumpkin®60, Roasted for Pumpkin II) or may not (Pumpkin ®60, Unroasted for Pumpkin III, Heliaflor®45 for Sunflower) involve roasting and salt addition to modulate oil yield and product safety (Panic et al., 2013; Pickardt et al., 2017). In contrast, pea isolates Pisane®M9 and C9 (82 % protein) for Pea I and II are generally obtained by a multi-step wet fractionation that includes acid and base addition for protein precipitation and re-solubilization and results in high purity (Bourgeois et al., 2015b). As such, variations in the original protein content of powders, but a similar water to powder ratios during high moisture extrusion cooking (see materials and methods), caused considerably higher total nitrogen contents in Pea I and II than in most oilseed extrudates and lean pork meat. Meanwhile NPN was > 4-times lower in Pea I and II compared to Pumpkin I, II, III, and Sunflower.

Table V.1 Proximate composition, non-protein nitrogen (NPN), and selected minerals in pork meat and wet texturized plant proteins from pea isolates and oilseed flours.

Composition (%)	**Pork Meat**	**Pea I**	**Pea II**	**Pumpkin I**	**Pumpkin II**	**Pumpkin III**	**Sunflower**
Moisture	73.7 ± 0.8^{e}	63.3 ± 0.1^{d}	63.8 ± 0.2^{d}	52.9 ± 0.2^{b}	50.0 ± 0.1^{a}	61.6 ± 0.7^{c}	54.3 ± 0.1^{b}
Total Nitrogen	3.61 ± 0.11^{a}	4.55 ± 0.11^{cd}	4.61 ± 0.03^{d}	4.38 ± 0.11^{c}	4.65 ± 0.01^{d}	3.84 ± 0.05^{b}	3.73 ± 0.06^{ab}
Specific protein[1]	$22.6 \pm 0.6^{2,c}$	$24.4 \pm 0.6^{3,de}$	$24.7 \pm 0.2^{3,de}$	$24.1 \pm 0.6^{4,d}$	$25.6 \pm 0.1^{4,e}$	$21.1 \pm 0.2^{4,b}$	$19.7 \pm 0.3^{5,a}$
NPN	2.89 ± 0.01^{g}	0.348 ± 0.014^{a}	0.430 ± 0.040^{b}	1.50 ± 0.01^{e}	1.69 ± 0.03^{f}	1.33 ± 0.01^{d}	1.23 ± 0.01^{c}
Total Fat[6]	0.800 ± 0.002^{ab}	0.154 ± 0.018^{a}	0.747 ± 0.046^{ab}	3.41 ± 0.06^{c}	2.64 ± 0.72^{c}	1.45 ± 0.24^{b}	3.61 ± 0.02^{c}
Total Ash	1.15 ± 0.02^{a}	2.62 ± 0.04^{b}	2.65 ± 0.02^{b}	6.05 ± 0.01^{f}	4.36 ± 0.03^{e}	3.51 ± 0.06^{d}	3.72 ± 0.05^{d}
Total Carbohydrates[7]	1.80	9.50	8.07	13.6	17.4	12.3	18.7
Minerals (mg/100g)							
Phosphorus	167 ± 4^{c}	74.6 ± 2.9^{a}	71.9 ± 3.7^{a}	224 ± 4^{e}	205 ± 4^{d}	240 ± 1^{f}	116 ± 2^{b}
Sodium	29.3 ± 0.4^{a}	455 ± 2^{c}	464 ± 21^{c}	571 ± 17^{d}	154 ± 1^{b}	1.92 ± 0.12^{a}	1.79 ± 0.39^{a}
Potassium	251 ± 1^{b}	54.0 ± 0.2^{a}	46.8 ± 2.5^{a}	497 ± 16^{e}	348 ± 3^{c}	450 ± 4^{d}	451 ± 6^{d}
Calcium	1.75 ± 0.11^{a}	12.1 ± 0.1^{b}	10.1 ± 0.5^{b}	33.5 ± 1.0^{e}	26.4 ± 0.3^{c}	30.9 ± 0.2^{d}	96.1 ± 1.5^{f}
Magnesium	16.0 ± 0.1^{a}	12.4 ± 0.2^{a}	11.0 ± 0.6^{a}	279 ± 8^{d}	203.1 ± 1.4^{b}	350 ± 2^{e}	217 ± 4^{c}
Iron	0.262 ± 0.021^{a}	4.47 ± 0.05^{d}	3.97 ± 0.17^{c}	5.52 ± 0.37^{e}	3.97 ± 0.02^{c}	5.27 ± 0.05^{e}	3.04 ± 0.07^{b}
Copper	$< 0.087^{a}$	0.284 ± 0.013^{c}	0.192 ± 0.026^{b}	0.772 ± 0.017^{f}	0.544 ± 0.006^{d}	0.686 ± 0.001^{e}	1.39 ± 0.03^{g}
Manganese	$< 0.087^{a}$	0.221 ± 0.001^{b}	0.194 ± 0.008^{b}	2.29 ± 0.07^{d}	1.80 ± 0.02^{c}	3.47 ± 0.02^{e}	1.76 ± 0.03^{c}
Zinc	0.982 ± 0.008^{a}	2.12 ± 0.01^{c}	1.71 ± 0.08^{b}	5.06 ± 0.15^{f}	3.63 ± 0.04^{d}	5.26 ± 0.06^{f}	4.12 ± 0.07^{e}

Different small raised letters indicate significant ($p \leq 0.05$) differences among samples in one row;
[1] Nitrogen (%) * Factor F; [2] F = 6.25 (Keeton et al., 2014);
[3] F = 5.36 (Mariotti et al., 2008);
[4] F = 5.50(Milovanović et al., 2014);
[5] F = 5.29 (Mariotti et al., 2008);
[6] Based on fresh weight
[7] Calculated from 100 % - (Specific Protein + Moisture + Ash + Fat).

In Pork Meat, NPN is mainly represented by carnosine, anserine, creatine, creatinine, taurine, as well as free amino acids (Aristoy & Toldrá, 1998; Mora et al., 2008). From a more general point of view, NPN describes nitrogenous compounds that are not amino acid building blocks of proteins such as free peptides and amino acids, nucleotides, urea, and glycolytic enzymes (Mariotti et al., 2008). These compounds possess a high solubility and may be washed out during wet extraction of pea isolates Pisane®M9 and C9 which resulted in low amounts in their respective wet extrudates Pea I and II (**Table V.1**). In contrast, intermediate amounts in Pumpkin I, II, and III and Sunflower reflect the lower purification degree of their respective flours (physical extraction only). To our knowledge no evaluation of proximate composition and NPN compounds in wet texturized pea or oilseed proteins has been done. However, data from their respective plant protein powders might allow a rough estimation of differences also among the resulting wet extrudates. In example, pea flours were shown to contain around 0.5 to 1.0 % of NPN (Periago et al., 1998; Vidal-Valverde et al., 2003), which fits to around 0.97 % of NPN in Pea I and II on a dry matter basis (calculated from **Table V.1**). Moreover, Balasaraswathi and Sadasivam (1997) found 35 % of protein and 2.3 % of NPN in sunflower seeds - comparable to 2.7 % of NPN *i. d. m* in Sunflower (calculated from **Table V.1**). Authors also described variations depending on the degree of seed germination. Similarly, data on pumpkin seed flour varied according to their processing *e.g.* if ungerminated/unfermented (0.4 % NPN) and germinated (1.5 % NPN) or fermented (1.7 % NPN) fluted pumpkin seed flours were compared (Giami, 2004; Giami et al., 1999). High amounts of phosphorus, magnesium, calcium, and potassium agree with literature data on oilseed crops (Alfawaz, 2004; Lazos, 1992; McKevith, 2005; Sotillo & HettlArachchy, 1994), while the addition of sodium chloride to improve the yield during oil extraction (Pumpkin seed flour for Pumpkin I and Pumpkin®60, Roasted for Pumpkin II) or the neutralization with sodium hydroxide (Pisane®M9/C9 for Pea I/II) resulted in noticeable quantities of sodium (up to 464 mg/100g) in these extrudates. Finally, residual carbohydrates in oilseed extrudates might be mostly fiber due to the high amount in their originating powders (see materials and methods).

Amino acid composition

The amino acid composition is not only important to predict the nutritional quality of proteins but may also affect the buffering capacity due to the presence or absence of specific amino acids with a pKa in the range of foodstuff , such as aspartic ($pKa_2 = 3.86$) and glutamic acid ($pKa_2 = 4.25$) and histidine ($pKa_2 = 6.0$) (Bhagavan & Ha, 2011; Maher et al., 2010). Therefore, Pork Meat and wet texturized plant proteins were analyzed with ion exchange chromatography

and results were converted to g/100 g fresh weight to ease comparison among samples. In general, contents of individual amino acids in Pea I, II, Pumpkin I, II, III, and Sunflower ranged from as little as 228 mg/100 g (cysteine in Pork Meat) to as high as 3796 mg/100 g (glutamic acid in Pumpkin I) (**Table V.2**). All tested protein sources were highest in non-polar amino acids (mainly valine and leucine), followed by acidic (both glutamic and aspartic acid), and basic (mainly lysine in Pork Meat or arginine in extrudates) and/or neutral ones (similar contents but very low in cystine/cysteine). When compared to meat, plant extrudates were lower in the essential amino acids methionine and histidine, but similar in alanine and cysteine/cystine, and considerably higher in all other amino acids. In example, Pea I and II contained 3196 and 3207 mg/100 g of aspartic acid, while Pork Meat possessed only 1952 mg/100 g. Moreover, Pea I and II were significantly ($p \leq 0.05$) higher in phenylalanine, tyrosine, leucine, threonine, proline, aspartic acid, and lysine than Pork Meat and oilseed extrudates and Pumpkin I and II contained the largest amounts of glycine, arginine, and tryptophan among all samples.

Table V.2 Amino acids composition (mg/100 g) of pork meat and wet texturized plant proteins from pea isolates and oilseed flours

Amino Acid	Pork Meat	Pea I	Pea II	Pumpkin I	Pumpkin II	Pumpkin III	Sunflower
Phenylalanine	826 ± 15^{a}	1585 ± 3^{f}	1586 ± 3^{f}	1295 ± 1^{d}	1362 ± 18^{e}	1074 ± 5^{c}	1003 ± 10^{b}
Tyrosine	724 ± 22^{b}	1001 ± 5^{d}	1042 ± 5^{d}	838 ± 13^{c}	860 ± 7^{c}	687 ± 1^{b}	537 ± 10^{a}
Leucine	1671 ± 4^{c}	2368 ± 5^{f}	2358 ± 15^{f}	1799 ± 7^{d}	1917 ± 25^{e}	1538 ± 5^{b}	1382 ± 16^{a}
Methionine	574 ± 1^{e}	266 ± 3^{a}	271 ± 5^{a}	447 ± 1^{b}	507 ± 11^{d}	483 ± 1^{c}	498 ± 6^{cd}
Isoleucine	1000 ± 1^{b}	1444 ± 5^{c}	1407 ± 31^{c}	1010 ± 10^{b}	1045 ± 21^{b}	852 ± 22^{a}	918 ± 19^{a}
Valine	1384 ± 4^{a}	2447 ± 65^{a}	2340 ± 31^{a}	2098 ± 50^{a}	2314 ± 42^{a}	1849 ± 5^{a}	1713 ± 32^{a}
Threonine	905 ± 7^{d}	1017 ± 3^{e}	1053 ± 10^{f}	744 ± 1^{b}	792 ± 4^{c}	631 ± 3^{a}	797 ± 16^{c}
Serine	771 ± 1^{a}	1376 ± 3^{ef}	1449 ± 23^{f}	1255 ± 3^{d}	1352 ± 18^{e}	1072 ± 30^{c}	925 ± 42^{b}
Alanine	1157 ± 28^{bc}	1195 ± 1^{cd}	1205 ± 10^{cd}	1128 ± 3^{b}	1209 ± 14^{d}	953 ± 3^{a}	928 ± 6^{a}
Proline	776 ± 48^{a}	1219 ± 3^{c}	1237 ± 20^{c}	899 ± 13^{b}	950 ± 7^{b}	765 ± 3^{a}	914 ± 1^{b}
Glycine	917 ± 20^{a}	1155 ± 1^{b}	1158 ± 5^{b}	1394 ± 7^{d}	1454 ± 14^{e}	1185 ± 11^{b}	1286 ± 10^{c}
Glutamic acid	2608 ± 1^{a}	3416 ± 16^{c}	3455 ± 67^{cd}	3796 ± 60^{e}	3581 ± 88^{cd}	2916 ± 11^{b}	3637 ± 32de
Aspartic acid	1952 ± 9^{b}	3196 ± 5^{e}	3207 ± 49^{e}	2171 ± 20^{c}	2309 ± 7^{d}	1820 ± 3^{a}	1919 ± 26^{b}
Cystine/Cysteine	228 ± 2^{a}	253 ± 5^{b}	271 ± 5^{c}	297 ± 1^{d}	315 ± 7^{e}	274 ± 3^{c}	336 ± 3^{e}
Histidine	946 ± 17^{b}	682 ± 1^{ab}	684 ± 5^{ab}	584 ± 1^{ab}	617 ± 11^{ab}	489 ± 8^{a}	758 ± 297^{a}
Arginine	1328 ± 32^{a}	2384 ± 8^{c}	2389 ± 3^{c}	3826 ±30^{e}	3988 ± 35^{f}	3161 ± 49^{d}	1853 ± 23^{b}
Lysine	1782 ± 33^{c}	2078 ± 5^{d}	2085 ± 3^{d}	895 ± 1^{b}	935 ± 14^{b}	800 ± 8^{a}	827 ± 6^{a}
Tryptophan	274 ± 9^{a}	243 ± 10^{a}	243 ± 2^{a}	429 ± 16^{d}	436 ± 6^{e}	367 ± 8^{c}	313 ± 1^{b}

Different small raised letters indicate significant ($p \leq 0.05$) differences among samples in one row;

Results on the amino acid composition of Pork Meat fit to those shown by Souci et al. (2008) with an abundance of glutamic acid and shares 7 % of arginine, 5 % of threonine, 4 % of phenylalanine and tyrosine, 3 % of methionine, and 1 % of cysteine/cystine related to the sum of all amino acids. All other amino acids were in accordance by +/- 1 %, except for aspartic acids whose deviation might be related to a different slaughter age of the pig (Gan et al., 2020) or pork breed. As expected from similarities in their proximate composition (**Table V.1**), wet texturized proteins from Pea I and II coincided in their overall amino acid distribution *i.e.* tryptophan, phenylalanine, and histidine varied by < 2 mg/100 g (**Table V.2**). Furthermore, a prevalence of glutamic acid (~3435 mg/100g), aspartic acid (~ 3650 mg/100 g), and basic amino acids such as arginine (~2687 mg/100g) and lysine (~2082 mg/100g) fits to results from peas, their isolates, and dry extrudates (Boye et al., 2010; Gorissen et al., 2018; Samard & Ryu, 2019) since amino acid composition might not be changed during extrusion (Samard & Ryu, 2019). Similarly, high amounts of glutamic acid, arginine, aspartic acid, and leucine in Pumpkin I, II, and III fit to those recently presented for pumpkin seed kernels (Alfawaz, 2004; Pereira & Adeola, 2016). These authors also described low amounts of methionine, histidine, and cysteine coinciding with **Table V.2**. While absolute values obtained from Pumpkin III were much lower than those for Pumpkin I and II, percentages of individual amino acids on the total amino acid amount were quite similar *e.g.* around 15 % of arginine, 9 % of aspartic acid, 5 % of phenylalanine and serine, and 6 % of glycine. Major amino acids found in Sunflower were comparable to those of all pumpkin extrudates which underlined the compositional similarity of oilseed crops as shown by **Table V.1**. When it comes to the abundance of the amino acids with proposed buffering capacity *i.e.* aspartic and glutamic acid and histidine, pea extudates were highest, followed by Pumpkin I, Pumpkin III, Sunflower, Pumpkin II, and Pork Meat (Bhagavan & Ha, 2011; Mennah-Govela et al., 2020; Mennah-Govela et al., 2019; Salaün et al., 2005; van der Sman et al., 2020).

Upon comparison of their proximate, chemical, and amino acid composition, wet texturized proteins differed distinctively from the composition of Pork Meat in particular in moisture, protein, NPN, ash, and total carbohydrates and buffering amino acids were distinctively higher. This was particularly true for Pumpkin I, II, III and Sunflower which were produced from less purified oilseed flours. These oilseed extrudates were generally higher in total carbohydrates and significantly ($p \leq 0.05$) higher in NPN, total fat, and ash as well as the ions potassium and magnesium. However, values obtained from extrudates derived from protein powders from the same genus and/or a similar extraction method (wet fractionation or physical extraction) were mostly coinciding which might ease their comparison for subsequent analysis.

Acid titration and buffering capacity of pork meat and wet texturized plant proteins

The pH-dependency and buffering capacity of plant protein extrudates and pork meat was determined by titration with a strong acid. First, minced pork meat and chopped plant extrudates were homogenized with water at a concentration of 2.0 wt% and different amounts of HCl stock solutions - representative for acid concentrations in between 1 and 5882 mmol H^+/kg - were added. A pH of 8.0 was used as a consistent starting point to enable a proper comparison among samples. Acid dependent pH-drop and buffering capacity calculated according to **Eq. V.2** are shown in **Figure V.1 A-D** and **Figure V.2 A-D**, respectively. Total buffering capacity tBC from **Eq. V.3** and the total area under the curve tAUC after integration of **Figure V.1 A-D** are summarized in **Table V.3**. Besides this overall investigation, partial buffering capacity (pBC) and area under the curve (pAUC) in between pH 7.0 to 4.5 were assessed to evaluate effects of wet texturized plant proteins when used as food ingredients or even as substitutions for pork meat in meat analogues or hybrids (**Table V.3**).

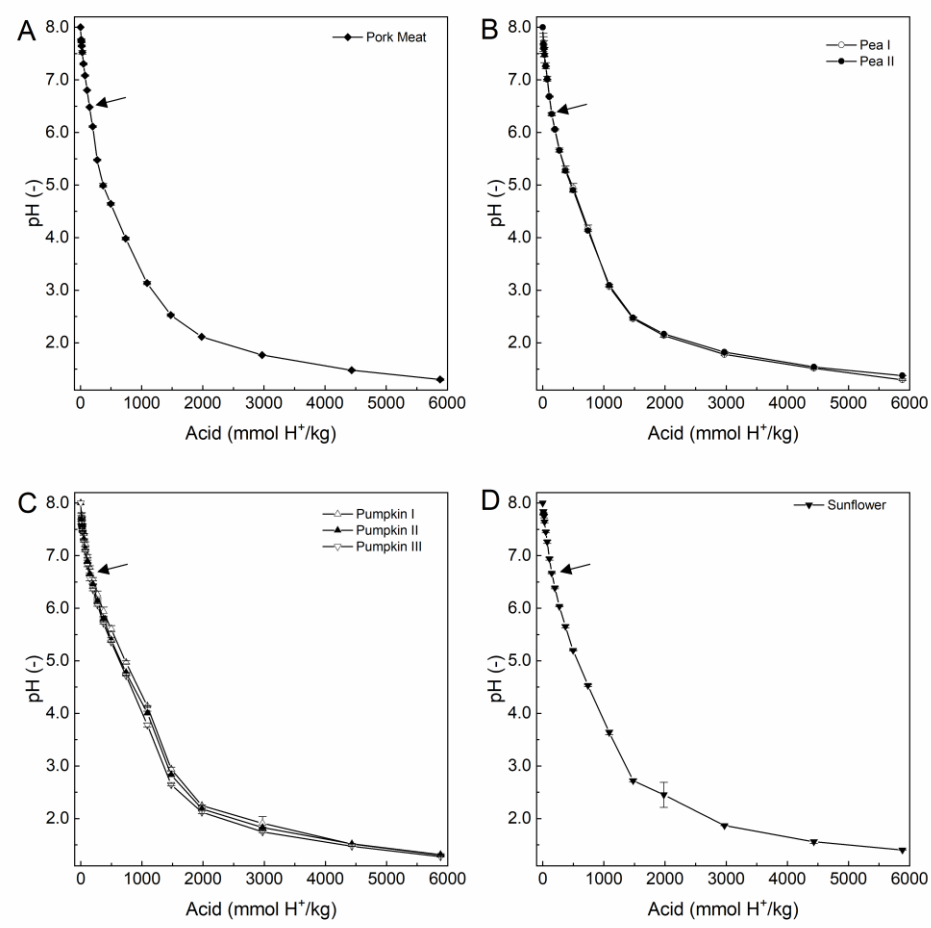


Figure V.1 Titration curve of 2 wt% pork meat (A) and wet texturized proteins from pea isolates (B) or oilseed flours (C, D) in water; Titration with HCl at concentrations from 0 to 5882 mmol H^+/kg sample; Arrow marks end of linear region ($R^2 \geq 0.99$) at 148 mmol H^+/kg.

Figure V.1 A-D shows the various behavior of samples as a function of pH and acid concentration. Titration curves of all sample dispersions were similar with two distinct regimes that described a steep pH-decrease (arrows in Figure V.1 **A-D**), followed by a gradual one with final values in between pH 1.28 (Pumpkin III) and pH 1.39 (Sunflower) at 5882 mmol H+/kg. The first linear pH-decrease ($R2 \geq 0.99$, Figure SV.3), included acid concentrations of up to 148 mmol H+/kg where pH of Pork Meat, Pea I, II and Pumpkin III was quite comparable, but slightly higher for Pumpkin I, II and Sunflower e.g. at 73.9 mmol H+/kg pH 7.01 ± 0.01 (Pea II) and 7.08 ± 0.01 (Pork Meat) compared to 7.22 ± 0.04 (Pumpkin I) and 7.26 ± 0.03 (Sunflower). In the first part of the gradual regime (> 148 to approx. 2000 mmol H+/kg) extrudate dispersions revealed distinct differences in their pH-response with smallest values for Pork Meat followed by Pea I, II, Sunflower, and Pumpkin I, II, III e.g. at approximately 272 mmol H+/kg: pH 5.48 ± 0.02 (Pork Meat) < 5.65 ± 0.02 (Pea II) < 5.67 ± 0.04 (Pea I) < 6.03 ± 0.01 (Sunflower) < 6.06 ± 0.02 (Pumpkin III) < 6.14 ± 0.02 (Pumpkin II) < 6.26 ± 0.07 (Pumpkin I). These pH-variations among samples were overcome at acid concentrations > 3000 mmol H+/kg. Along with the acid-dependent pH-decline buffering capacity was increasing in particular in between pH 7.0 and 4.5 and below pH 2.5 (Figure V.2 **A-D**). Upon comparison, Pea I and II were similar to Pork Meat in having both a sigmoidal curve shape and in terms of the magnitude of their pH-dependent buffering capacity (Figure V.2 **A-B**). In contrast, values of all oilseed extrudates increased in a rather degressive manner (Figure V.2 C-D) and possessed higher buffering capacity e.g. at pH 4.5 ± 0.1 with 213 mmol H+/kg*ΔpH (Sunflower) compared to 148 mmol H+/kg*ΔpH (Pork Meat, Figure V.2 **A**).

The total area under the curve tAUC ranged from around 6100 pH*(mmol H^+/kg) (Pork Meat, Pea II) to as much as 7663 ± 133 pH*(mmol H^+/kg) (Pumpkin I) (**Table V.3**). Finally, pAUC of all samples differed significantly ($p \leq 0.05$) except among Pea I and II with the same trend observed in **Figure V.1 A-D** *i.e.* for pAUC (pH*(mmol H^+/kg)) of Pumpkin I (907 ± 20) > Pumpkin II (810 ± 3) > Pumpkin III (783 ± 1) > Sunflower (678 ± 6) >> Pea I (549 ± 26) > Pea II (543 ± 7) > Pork Meat (455 ± 3). Accordingly, the amount of acid for an acidification from pH 7.0 to 4.5 was lowest for Pork Meat *i.e.* 67 % and 56 % of the amount needed to achieve the same pH-drop in Sunflower and Pumpkin I, respectively. Upon comparison, Pork Meat and Pea I and II were similar in their pH-dependent acidification and buffering behavior but differed distinctively from wet texturized oilseeds.

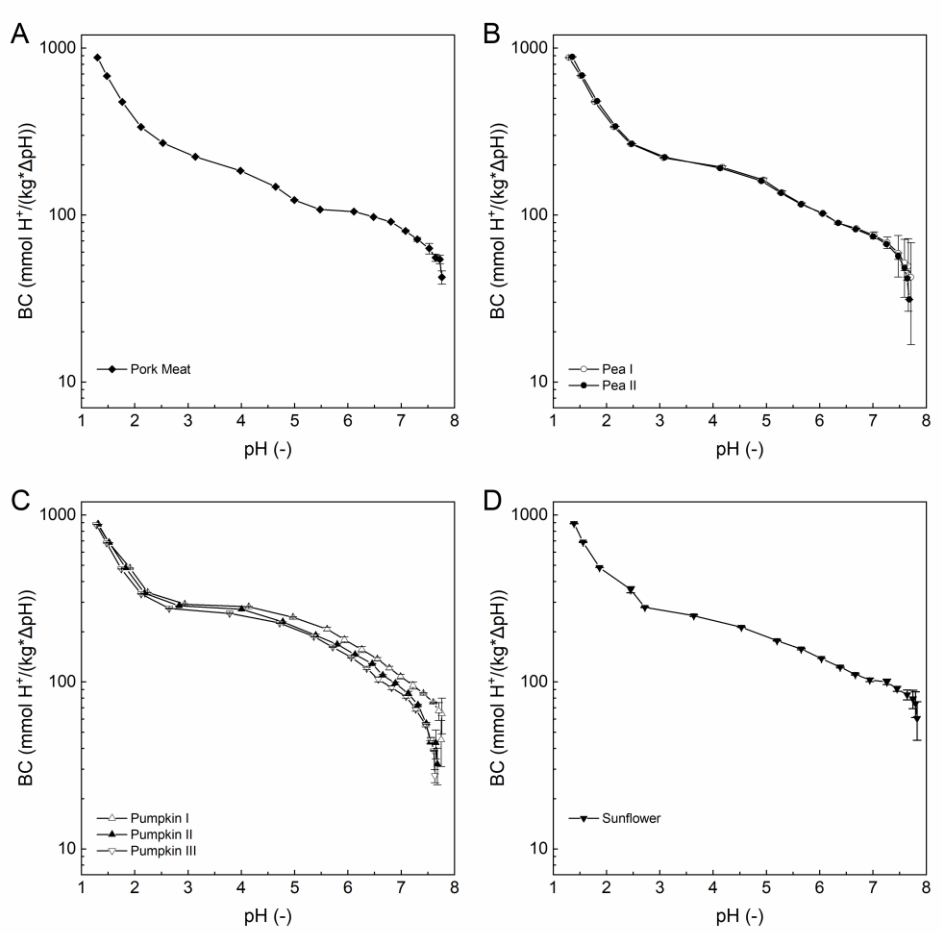


Figure V.2 Buffering capacity (mmol H^+/(kg*ΔpH)) of 2 wt% pork meat (A) and wet texturized proteins from pea isolates (B) or oilseed flours (C, D) in water; titration with HCl at concentrations from 0 to 5882 mmol H^+/kg sample.

The observed acid-dependent behavior of sample dispersion from pork meat and wet texturized proteins fit to curve shapes for proteins from egg, dairy, legumes, and soy protein gels when subjected to a strong acid and in between pH 8.0 to 1.5, as well as to those shown for more complex, multi-ingredients food products (Maher et al., 2010; Mennah-Govela et al., 2020; Salaün et al., 2005; Tanford & Roxby, 1972; van der Sman et al., 2020). More precisely, **Figure V.1** resembled titration curves of beef, minced turkey, or chicken meat products that were found to be separated into two parts: *i*) a rapid pH-drop and a *ii*) rectilinear part with a lower slope (Goli et al., 2007; Honikel & Hamm, 1974; Mennah-Govela et al., 2020). Additionally, buffering capacity in pork sirloin between pH 6.5 and 7.5 and pH 7.0 and 8.0 was determined as 77.5 and 53.1 mmol titrant/(pH*kg) (Okuma & Abe, 1992) which fit to averages calculated from data in this study namely 81 and 57 mmol H^+/(kg*ΔpH) (**Figure V.2**). Furthermore, Goli et al. (2012) recently published buffering capacity of beef and chicken breast cuts with approximately 150 mmol H^+/(kg*ΔpH) in between pH 3.0 and 5.0 thus being

supportive of an average value of 151 mmol H^+/(kg*pH) for the pork meat sample (**Figure V.2**). However, it is important to note that differences in the sample composition, preparation, or titration procedure, as well as the presentation of data and the respective pH-regime used for the calculation complicate data comparison among different studies. In example, Puolanne and Kivikari (2000) have shown that pork meat from different cuts and/or different sample dilution showed different buffering capacity in between pH 5 and 6 *i.e.* BC_{min} of pork *m. longissimus* was 38.9 and 48.9 mmol H^+/(kg*pH) at a sample to water ratio of 1:10 and 1:1, respectively, while values were 32.2 and 40.3 mmol H^+/(kg*pH) for *m. triceps brachii*. Moreover, average buffering capacity (pH 5 to 3.5) of whole pieces of beef sirloin was around 150 mmol H^+/(kg*pH) (Goli et al., 2012), while pureed beef baby food possessed a total buffering capacity (pH 6 to 1.5) of 54.4 μmol H^+/(g*ΔpH) (Mennah-Govela et al., 2020) and homogenized beef meat had a mean buffering capacity (pH 7.0 to 5.0) around 50 mmol H^+/(kg*pH) (Puolanne & Kivikari, 2000). Finally, different pH-staring points and protein contents were shown to affect the pH-response. As such, steamed lentils (native pH 6.3 and 7 % of protein) possessed a tBC of 28.2 μmol H^+/g*ΔpH, while black beans (native pH 6.1 and 6 % of protein) were at 39.3 μmol H^+/g*ΔpH when acidified to pH 1.5 with 134 and 180 μmol H^+/g, respectively. In our study, all samples were similar in protein (**Table V.1**), titrated from the same starting pH of 8.0 and with the same acid concentrations. As a result, tBC of Pork Meat and wet texturized proteins was comparable with values in between 87.6 mmol H^+/(kg*ΔpH) (Pumpkin III) and 89.0 mmol H^+/(kg*ΔpH) (Sunflower) (**Table V.3**).

Table V.3 Influence of acid addition on suspensions of 2 wt% pork meat and wet texturized plant proteins from pea isolates and oilseed flours related to the total buffering capacity (tBC) and total area under curve (tAUC) in between 0 to 5882 mmol H^+/kg sample, and partial area under curve (pAUC), respective acid addition (mmol H^+/kg sample), and partial buffering capacity (pBC) in between pH 7.0 to 4.5.

	Pork Meat	**Pea I**	**Pea II**	**Pumpkin I**	**Pumpkin II**	**Pumpkin III**	**Sun-flower**
tAUC (pH *(mmol H^+/kg))	6103 ± 41^{a}	6305 ± 16^{a}	6079 ± 10^{a}	7663 ± 133^{c}	7226 ± 81^{b}	6987 ± 65^{b}	6835 ± 203^{b}
tBC (mmol H^+/(kg*ΔpH))	878 ± 1^{a}	877 ± 2^{a}	886 ± 3^{bc}	878 ± 1^{a}	880 ± 3^{ab}	876 ± 1^{a}	890 ± 2^{c}
pAUC (pH *(mmol H^+/kg))	455 ± 3^{a}	549 ± 26^{b}	543 ± 7^{b}	907 ± 20^{e}	810 ± 3^{d}	783 ± 1^{d}	678 ± 6^{c}
c(acid) pH 7.0-4.5 (mmol H^+/kg)	469 ± 1^{a}	563 ± 16^{b}	555 ± 2^{b}	833 ± 4^{f}	772 ± 2^{e}	740 ± 4^{d}	648 ± 6^{c}
pBC (mmol H^+/(kg*ΔpH))	188 ± 1^{a}	225 ± 7^{b}	222 ± 1^{b}	333 ± 2^{f}	309 ± 1^{e}	296 ± 1^{d}	259 ± 2^{c}

Different small raised letters indicate significant ($p \leq 0.05$) differences among samples in one row

Relation of compositional components and buffering behavior

In most studies related to buffering capacity, the protein content is suggested to be the key influencing factor in food and feedstuff (Mennah-Govela et al., 2020; Mennah-Govela et al., 2019; Montañez-Valdez et al., 2013; Puolanne & Kivikari, 2000; Salaün et al., 2005). Based on this, Mennah-Govela et al. (2020) recently proposed a subsumption of food stuff by their protein content (respective mean protein content of class ± 2.3 %). In our study, the average specific protein content was 23.2 ± 2.1 % (**Table V.1**) fitting to their proposed average class 6 which included tuna and chicken whose respective acid-titration curve was similar to **Figure V.1 A**. Besides the overall protein content, their inherent and free amino acids as part of non-protein nitrogen NPN (Bhagavan & Ha, 2011; Luo et al., 2018; Mennah-Govela et al., 2019; Okuma & Abe, 1992; Righetti et al., 2001; van der Sman et al., 2020) may also impact buffering capacity. For example, as substances generally buffer best around their pka (Henderson, 1908), amino acids generally affect the buffering behavior mostly around pH 2.34 (pka_1, α-COOH) and pH 8.95 (pka_3, NH_3). While the latter was not within the pH-range tested in our study, the influence of α-COOH-groups supported sharply increasing buffering capacity below pH 2.4 (**Figure V.2**). Furthermore, amounts of aspartic and glutamic acid and histidine may additionally affect the buffering capacity due to the pKa_2 of their side-chains, namely at pH 3.86, 4.25, and 6.0 respectively (Bhagavan & Ha, 2011). As such, their sum in wet texturized plant proteins (4736 to 6661 mg/100g) in comparison to Pork Meat (4560 mg/100g) (**Table V.2**) may boost the buffering capacity *e.g.* at pH 4.25 BC of Pork Meat was as low as ~ 200 mmol H^+/(kg*ΔpH) but around 280 mmol H^+/(kg*ΔpH) in Pumpkin I. Moreover, pBC (pH 7.0 to 4.5) of all wet texturized proteins was significantly ($p \leq 0.05$) higher than the one obtained from Pork Meat (**Table V.3**). However, the influence of these ionizable side groups also depends on the spatial organization of proteins that may re- or unfold during processing and interactions of side chains with minerals such as sodium, potassium, and calcium that affect the acid-base equilibrium (Maher et al., 2010). The pKa of amino acid side-chains may vary by up to 0.5 pH-units due to electrostatic interactions or external influences such as enzymes and the influence of aspartic and glutamic acid and histidine was recently suggested to range from pH 3.4 to 6.7 (van der Sman et al., 2020). Besides buffering amino acids, amounts of total ash and the presence of select ions may play a role. In example, phosphate, sodium, potassium, and calcium were described to impact buffering capacity (Kivikari, 1996; Maher et al., 2010). Kivikari (1996) and Puolanne and Kivikari (2000) even described phosphates as one of the most influential compounds on the buffering capacity of meat between pH 5.5 and 7.0 due to their pKa-values (pH 6.1-7.1). Thus, high quantities of these ash, constituents, total ash and high

amounts of buffering amino acids may play an important important role, in particular if Pumpkin I, II, III and Pork Meat were compared (**Table V.1**).

In the end, the overall buffering capacity results from an interplay of various compositional elements whose individual contribution may further depend on external factors such as the tested pH-value or regime (Honikel & Hamm, 1974; Mennah-Govela & Bornhorst, 2021; Okuma & Abe, 1992). Therefore, a multiple linear regression with backwards elimination of variables was done to narrow down key influencing parameters on the buffering capacity of the tested proteins (**Table V.4**). tAUC and tBC represented the dependent variables over the whole acid concentration and pH-range (0 - 5882 mmol H^+/kg and pH 8.0 to final pH), while pAUC and pBC were evaluated for a representative pH-regime of pH 7.0 to 4.5 (**Table V.3**) due to its significance for food stuff and processing. According to the multiple linear regression model ash and phosphate content, and the sum of the amino acids glutamic (G) and aspartic acid (A) and histidine (H) content were statistically significant ($p < 0.1$) for all dependent variables (**Table V.4**).

Table V.4 Parameter estimates and their adjusted coefficient of determination (adjusted R^2) from the regression model for all the total and partial buffering capacity (BC) and area under the curve (AUC) as dependent variables; NS = not statistically significant ($p > 0.1$)

	Parameter estimates			
Terms	**tBC (mmol H^+/(kg*ΔpH))**	**tAUC (pH* (mmol H^+/kg))**	**pBC (mmol H^+/(kg*ΔpH))**	**pAUC (pH *(mmol H^+/kg))**
Intercept	849.93	7231.08	230.93	648.45
Specific Protein	NS	NS	NS	1.434
NPN	NS	NS	NS	-3.715
Total Fat	0.429	NS	-1.203	NS
Total Ash	-1.510	93.11	4.074	12.71
Phosphate content[1]	-6.896	278.73	-9.420	NS
Sum ions NaKCa	7.311	-345.97	NS	-16.55
Sum Amino Acids GAH	0.911	-42.50	-0.836	-9.477
Adjusted R^2	0.770	0.973	0.990	0.993

[1] Calculated as P_2O_5 (Phosphorus * 2.2914 according to Murf (2008))

As for the pH-regime in between pH 7.0 and 4.5 it became apparent that pAUC and pBC may be additionally influenced by the content of protein and non-protein nitrogen. Corrected coefficients of determination R^2 were ≥ 0.973 expect for tBC with 0.770. Both tBC and pBC showed negative correlations with the fat contents while tAUC and pAUC did not and the influence of phosphate varied when t/pBC and t/pAUC were compared. Interestingly, total ash had a negative effect on tBC, but a positive one on tAUC, pBC, and pAUC, whereas the

opposite trend was observed for the sum of GAH and the sum of sodium, potassium, and calcium. As for amino acids, the pKa_2 possibly promoted pBC in between pH 7.0 and 4.5, while all α-carboxyl groups may affect tBC when values down to pH 1.5 were evaluated. The effect of phosphorous might be highly dependent on the way it is bound *e.g.* in phosphates, phosphoproteins, phospholipids, or nucleic acids. Moreover, the solubility and buffering capacity of calcium and potassium varies depending on the pH-regime evaluated (Najafi & Jalali, 2016). Similarly, the overall protein solubility was shown to be affected by the extrusion process as recently shown for pea isolates in comparison to their dry extrudates (Samard & Ryu, 2019). All in all, the content of buffering amino acids, ash and its constituents is one of the main contributors to the buffering capacity over a great pH-range. The abundance of protein and non-protein nitrogen may play an important role at pH-values typical food stuffs in particular if contents among samples vary more than those evaluated here. Results were in line with previous observations (Kivikari, 1996; Kylä-Puhju et al., 2004; Maher et al., 2010; Mennah-Govela et al., 2020; Mennah-Govela et al., 2019; Salaün et al., 2005) and underline the need to carefully evaluate novel food ingredients in their proximate and pH-related behavior. When it comes to extrudates and their pH-response in food products, care should be taken with respect to residual ash constituents of their originating powders especially sodium, potassium, calcium, and phosphate, as well as the amounts of acidic amino acids and histidine. The area under the curve proves to be useful to describe dependencies of compositional elements and the buffering behavior of meat and plant proteins not only over the whole pH spectrum, but also within specific pH windows.

Conclusion

The pH-dependent behavior of pork meat was compared to wet texturized plant proteins from pea isolates and oilseed flours to ascertain observed differences in food formulations with respect to pH and to estimate effects upon their use as food ingredients based on their proximate and chemical composition. Differences among samples were most distinct between pH 7.0 and pH 4.5. Buffering capacity ranked in the order of Pork Meat < Pea I, II << Pumpkin III and Sunflower < Pumpkin I, II. The dominance of wet texturized oilseeds proteins in comparison to Pork Meat and Pea I, II was likely related to higher residuals of ash and its constituents, as well as distinctively higher amounts of buffering amino acids. The lower degree of purification of oilseed flours prior to extrusion likely contributes to this. More precisely, original powder properties are related to the extraction method applied. As a result, physical extraction (oilseed flours) results in higher amounts of buffering components than wet fractionation (pea isolates).

This influence needs to be taken into account when using such compounds for the manufacture of meat alternatives or hybrids and requires that recipe and process adjustments are being made in order to ensure microbial safety, sensory performance, and structural stability. All in all, meat is more prone to acid-induced pH-changes than wet texturized oilseed proteins, but similar to highly purified pea extrudates. This possibly explains the current rise of pea proteins to fabricate meat analogues alongside with their high abundance and lower price. Moreover, results showed that starting at a standardized sample amount and the same pH in combination with similar acid concentration steps enables a qualitative and quantitative evaluation and comparison of data from different samples irrespective of their origin, not only for the overall pH-spectrum but also for partial pH-windows. Future research should now focus on validating the observed behavior in more complex matrices and at higher protein concentrations.

Acknowledgement

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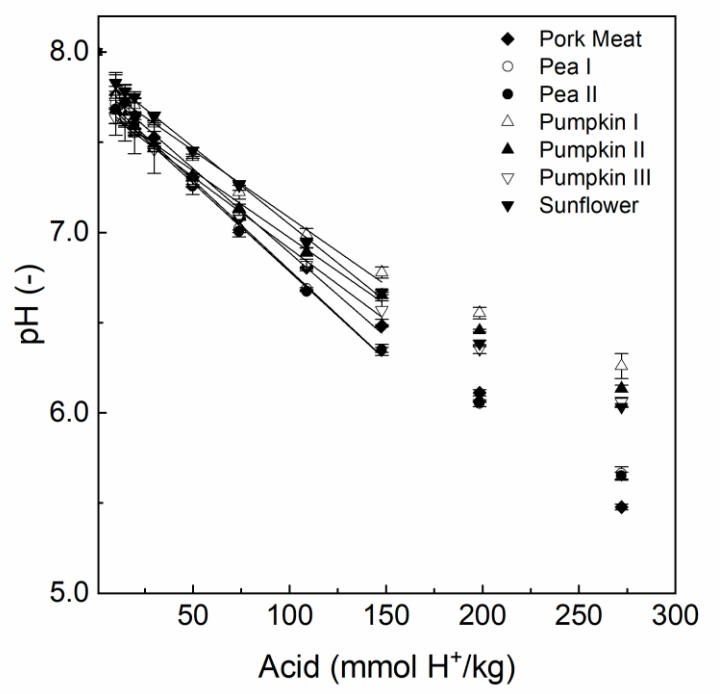
Supporting Information



Equation	y = a + b*x						
Sample	Pork Meat	Pea I	Pea II	Pumpkin I	Pumpkin II	Pumpkin III	Sunflower
Intercept y-axis	7.82 ± 0.02						
Slope	-0.01 ± 3.1	-0.01 ± 1.	-0.01 ± 1.9	-0.01 ± 3.2	-0.01 ± 2.6	-0.01 ± 2.3	-0.01 ± 1.6
Sum of error squares	0.01	0	0	0.01	0.01	0.01	0
Pearson R	-1	-1	-1	-0.99	-1	-1	-1
R-Squared (R2)	0.99	1	1	0.99	0.99	0.99	1
Cor. R2	0.99	1	1	0.99	0.99	0.99	1

Figure SV.3 Linear fits and respective result table of sample dispersions with 2 wt% pork meat or wet texturized proteins from pea isolates and oilseed flours from **Figure V.1** from 0 to 148 mmol H^+/kg; Data shown up to an acid concentrations of 272 mmol H^+/kg sample.

Effect of Plant Proteins on Product Development and Manufacture of Hybrid Meats

VI. Chapter

Acidification Behavior of Mixtures of Pork Meat and Wet Texturized Plant Proteins in a Minced Model System

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Abstract

The increasing use of wet texturized plant proteins as meat substitutes requires characterization of their functional properties, especially in terms of pH-behavior when being mixed with meat proteins to create so-called hybrid products. In this study, a minced model systems containing pork meat, curing salt, and various amounts (0 to 100 wt%) of wet extruded proteins from pea (Pea I, II), pumpkin (Pumpkin I, II, III), and sunflower was used to evaluate the effect of mixing on pH and time dependent pH-changes upon the addition of Glucono-delta-lactone (GDL). Increasing concentrations of plant extrudates resulted in a linear increase of the initial (pH_{0h}), intermediate (pH_{6h}), and final pH_{48h} for all samples and higher slopes at higher native pH of extrudates were found. Acidification kinetics of all samples were similar with a distinct pH drop by 0.3 to 0.8 pH-units per wt% GDL in the first 6 h, followed by a plateau where pH remained constant. At extrudate concentrations of 5 wt% (Pea I, II, Pumpkin I, II) or 15 wt% (Pumpkin III, Sunflower), a sufficient acidification with typically used GDL-amounts (= 1 wt%) could be achieved, while higher plant protein contents required higher GDL concentrations in order to reach a pH value of 5.0; a common target value in dry cured sausages. A mathematical model was proposed to correlate pH, time, acidifier, extrudate concentration and plant protein origin, to aid in the adjustment of dry cured hybrid meat formulations, and to describe thresholds of feasible extrudate and acidifier concentrations.

Practical application

Despite increasing relevance of texturized plant proteins as meat mimetics, little is known about their functional and process related properties. This study shows that plant protein origin, the level of meat replacement, and the amount of acidifier are linked to the time dependent pH value on the basis of a mathematical model. This brings food developers one step closer in creating tailored formulations and estimating effects of these novel ingredients in the final product characteristics of hybrid meats and analogues.

HIGHLIGHTS

- Addition of wet extrudates linearly increase the mixing pH in minced model systems.
- Pea extrudates have no influence on the acidifcation kinetics in dry-cured meat hybrids.
- Traditional amounts of GDL yield sufficient acidification at low extrudate contents.
- Higher GDL concentrations are required for an acidificaiton at high extrudate contents
- Recipe adjustments to achieve a certain pH may rely on a proposed mathematical model

KEYWORDS

Plant proteins; Alternative proteins; Meat proteins. Hybrid meats. pH-dependency. Glucono-delta-lactone.

Introduction

Legumes and oilseeds are promising protein sources and alternatives to meat as due to their high protein content, sustainability, and wide availability as side-streams from carbohydrate and oil production (Nadathur et al., 2016). However, the use of these ingredients in meat products and hybrids often demands for an additional extrusion step that transforms them from an unstructured powder to a structured solid state (Asgar et al., 2010; Joshi & Kumar, 2015). While texturized vegetable proteins from low moisture extrusion have been well established since the 1980s (Mussman, 1974), wet texturized proteins from high moisture extrusion have gained importance in the last decade due to their ability to create meat analogues with appealing textural properties and fibrous structures without the need of extensive pre-hydration. In this process, extruders are additionally equipped with a cooling die after the mixing and heating unit, that enables protein plastification and limits the evaporation of water (Osen & Schweiggert-Weisz, 2016). However, there are still open questions on how these novel ingredients perform in traditional formulations and how their functionality is affected by changes in temperature, salt concentration, or pH (Sha & Xiong, 2020). This is particularly important for hybrid products consisting of both meat and protein extrudates. These products are becoming more common as meat proteins may provide some beneficial sensorial and

functional properties to create an appealing taste, texture, and appearance, while plant-based proteins tackle health and sustainability concerns on meat products.

One example of meat products are dry-cured sausages, a product class with high raw material requirements and rather complex manufacture that consists of acidification and drying. Acidification is generally done by adding a starter culture-dextrose mixture or a chemical acidifier such as Glucono-delta-lactone (GDL). It aims to provide microbial safety against pathogens and spoilage, and a firm, sliceable texture with well-known bite and mouthfeel due to the coagulation of meat proteins (Feiner, 2016). Thus, controlling the acidification process of dry-cured hybrid products displays a crucial starting point to create high value end products. To date though, there is only little information available on how minced textured vegetable proteins affect the acidification behavior of such concentrated mixed systems, especially with respect to final pH-values and time-dependent pH-declines. As it has been previously shown that plant extrudates and pork meat may differ in their acid susceptibility in dilute aqueous systems (Ebert et al., 2021a), assessing their behavior in mixed, bulk matrices and by using traditional acidifiers displays a next step in establishing the product class of dry-cured hybrid meats.

Therefore, this study aimed at evaluating the time-dependent pH-behavior of minced pork meat combined with increasing amounts of a variety of high moisture extrudates from pea protein isolates or oilseed flours (pumpkin and sunflower) after adding GDL. To that purpose, the pH of minced model systems was compared to that of pure pork meat and pure extrudate samples over the course of 48 h and after addition of different amounts of GDL. Results were intended to provide formulation guidelines for hybrid dry-cured sausages, where wet extrudates are used as ingredients (Deliza et al., 2002; Omwamba et al., 2014; Rao et al., 1984; Zepeda Bastida et al., 2018). A mathematical model was established to aid in this by correlating key parameters, namely extrudate concentration (0 to 100 wt%) and origin, amount of acididier (1.0, 2.0, 3.0 wt% GDL) and time (0 to 48h). Based on this, suitable GDL-concentrations were suggested and mixing thresholds for the addition of plant extrudates in dry-cured hybrid meats proposed.

Materials and methods

Materials

Lean pork meat SI (GEHA standard) was obtained from MEGA (Stuttgart, Germany). Wet texturized plant proteins from pea (Pea I, II), pumpkin (Pumpkin I, II, III), and sunflower

(Sunflower) were provided by the German Institute for Food Technology (DIL, Quakenbrueck, Germany). Respective raw materials for high moisture extrusion consisted of Pisane®M9 (81.7 % protein, 4 % fat, 3.7 % salt, 3.2 % total carbohydrates) and Pisane®C9 (81.7 % protein, 4 % fat, 3.7 % salt, 3.2 % total carbohydrates) from the Coscura Group (Warcoing, Belgium), Pumpkin seed flour (55 % protein, 12.3 % fat, 5.1 % salt, 19.8 % total carbohydrates) from Ölmühle Fandler GmbH (Sonnhofen, Austria), Pumpkin®60 Roasted (60 % protein, 12.8 % fat, 0.01 % salt, 15.5 % total carbohydrates) and Unroasted (60 % protein, 9 % fat, 0.01 % salt, 17 % total carbohydrates), and Heliaflor®45 (45 % protein, 10 % fat, 0.002 % salt, 25 % total carbohydrates) from All Organic Treasures GmbH (Wiggensbach, Germany). For high moisture extrusion, powders were dosed into a double-screw extruder (ZSK 27MV, Coperion GmbH, Stuttgart) at a water to powder ratio of 3 to 2 (Pisane®C9, M9), 1 to 1 (Pumpkin seed flour, Heliaflor®), 7 to 8 (Pumpkin®60, Roasted), and 9 to 7 (Pumpkin®60, Unroasted) at an inlet temperature of 40 °C. Temperature was increased stepwise to 145 °C, followed by a protein plastification in the attached cooling die (FKD-750, DIL, Quakenbrück) at around 100 °C. The obtained wet extrudates were cut into stripes, packed airtight and stored at -18 °C until further use. Glucono-δ-lactone (GDL) was acquired from Roquette Frères (Lestrem, France). Nitrite curing salt (99.6 % sodium chloride, 0.4 % sodium nitrite) was obtained from Gilde (Profi Line, Zentralgenossenschaft des europäischen Fleischergewerbes eG, Frankfurt, Germany).

Preparation of pork meat and wet texturized plant proteins

Lean pork meat was minced with a meat grinder (Type WD114, Seydelmann, Aalen, Germany) using a 3 mm punch disk, packet airtight, and stored at 4 °C until further analysis. Extrudates were thawed, cut into squares (40 x 40 mm), and chopped in a bowl chopper (Type MTK 661, Maschinenfabrik Dornhan GmbH, Dornhan, Germany) until a uniform particle size of approximately 20 mm was obtained. Temperature during chopping was monitored and stays well below 10 °C. Chopped extrudates were packed airtight and stored at 4 °C before analysis.

Preparation of minced model systems

Minced model systems were obtained by partially replacing minced pork meat with chopped extrudates from pea, pumpkin, or sunflower at 5, 15, 20, 40, 60, and 70 wt% plant protein. The salt concentration was adjusted to 2.8 wt% with nitrite curing salt representative of traditionally used amounts in dry-cured sausages (Feiner, 2016) according to the obtained specifications (**Table 1**). GDL was added as chemical acidifier at concentrations of 1.0, 2.0, or 3.0 wt%. Samples were hand mixed until a uniform distribution of meat and extrudate particles was

achieved. Pure pork meat (0 %wt%) and pure plant protein samples (100 wt%) were equally salted, acidified, and mixed as control samples. Samples were packed into plastic cups, closed, and stored at 4 °C during 48 h of acidification. Each mixing concentration was prepared at least in duplicates.

Table VI.1 Proximate composition and native pH of lean pork meat and plant protein extrudates from pea, pumpkin, and sunflower proteins

	Pork Meat[1]	**Pea I**[2]	**Pea II**[2]	**Pumpkin I**[2]	**Pumpkin II**[2]	**Pumpkin III**[2]	**Sun-flower**[2]
Moisture (%)	73.7	64.6	63.8	49.9	52.9	60.8	53.0
Crude Protein (%)[3]	22.6	30.8	31.4	31.5	30.2	26.2	23.7
Fat (%)	0.8	3.3	4.3	7.2	5.1	4.9	5.4
Ash (%)	1.2	2.3	2.3	4.4	5.8	4.1	3.4
Carbohydrates (%)[4]	1.85	< 1.0	< 1.0	1.8	1.0	0.9	7.7
Native pH (-)[5]	5.45	7.35	7.25	6.17	6.06	6.70	6.41

[1](Ebert et al., 2021a)
[2] From manufacturer's specification
[3] Nitrogen to protein conversion factor = 6.25;
[4] Calculated from 100 % - (Protein + Moisture + Ash + Fat)
[5] Measured before mixing with a pH-meter

Acidification

The acidification course of samples was monitored by measuring the pH with a pH meter Lab 720 with temperature adjustment (WTW, Weilheim, Germany) at four timepoints: 0 h, 6 h, 24 h, and 48 h. Individual pH values were obtained from three separate measurement points in the minced model systems and averaged.

Statistical and data analysis

Samples were subjected to a one-way analysis of variance with a Duncan posthoc-test (α-level of 0.01) using SPSS statistics V23 (IBM Corp., Armonk, USA) to describe statistically significant differences. The assumption of normality and equal variance were checked with a Shapiro-Wilk-test (p-value to reject ≤ 0.05). All analyses were done at least in duplicates from freshly prepared samples. A multivariate regression (GLM Procedure) was carried out using the SAS statistics software V9.4 (SAS Institute Inc, North Carolina, USA) to obtain parameter estimates to describe the pH. A regression model was used with backward selection of the variables for pH as dependent variable. A level of $p \leq 0.1$ was used (Dufner et al., 2013) to avoid removing significant terms during the backwards selection of variable. Independent variables were plant protein concentration, time and GDL and their interaction terms.

Results and Discussion

Mixing pH of minced model systems

Minced meat was mixed with increasing concentrations 5, 15, 20, 40, 60, 70 wt% of chopped extrudates from pea (Pea I, II) and oilseed proteins (Pumpkin I, II, III, Sunflower) and results were compared to those obtained with pure meat (0 wt%) and pure extrudates (100 wt%) as lower and upper mixing boundaries. The mixing pH of minced model systems was analyzed at three different times directly after (pH_{0h}), in between (pH_{6h}) and at the end (pH_{48h}) of the chemically induced acidification with 1.0 wt% GDL and at a constant (curing) salt concentration of 2.8 wt% (**Figure VI.1**). The lowest initial pH_{0h} was determined for the pure meat sample with a value of pH_{0h} 5.39 ± 0.18, while the highest one was observed for 100 wt% of Pea I (pH_{0h} 6.97 ± 0.08), followed by Pea II (pH_{0h} 6.96 ± 0.03), Pumpkin III (pH_{0h} 6.42 ± 0.07), Sunflower (pH_{0h} 6.19 ± 0.01), Pumpkin I (pH_{0h} 5.93 ± 0.02), and Pumpkin II (pH_{0h} 5.91 ± 0.02). All mixtures had pH-values that were in between that of pure meat and the respective pure extrudate, starting from pH_{0h} 5.41 ± 0.14 for 5 wt% Sunflower addition to pH_{0h} 6.54 ± 0.04 when 70 wt% of meat was replaced with Pea II. Concentrations with significant pH-difference ($p \leq 0.01$) to pure meat at pH_{0h} were 15 wt% for Pea I, 40 % for Pea II and 60 % for Pumpkin I, II, III, and Sunflower as indicated by one star in **Figure VI.1**. Regression analysis revealed a positive linear correlation of extrudate concentration (wt%) and pH with R^2 in between 0.84 and 1.0 at pH_{0h}, pH_{6h}, and pH_{48h}. Replacing pork meat with increasing concentrations of extrudates thus led to a higher mixing pH, which was likely related to the more neutral/alkaline native pH of those (**Table VI.1**).

A rapid acidification of minced model systems, pure meat, and extrudates (**Table VI.1, Figure VI.1**) was observed after mixing with the chemical acidifier due to an immediate hydrolysis of GDL to gluconic acid (Li et al., 2021; Totosaus et al., 2000). For instance, the pH of Pea I decreased from pH 7.35 to pH_{0h} 6.97 ± 0.08, Pumpkin II from pH 6.06 to pH_{0h} 5.91 ± 0.02 and pure meat from pH 5.45 to pH_{0h} 5.39 ± 0.18. The pH-drop was more distinct for extrudates than for meat, which is likely due to the higher initial pH of the systems since GDL hydrolysis has been found to be positively correlated with the pH-value (Shimahara & Takahashi, 1970). Based on the same fact, first-order rate constants of GDL hydrolysis were also lower at later timepoints (slopes at pH_{6h} compared to pH_{48h}) due to a declining pH-value. In other words, the higher the extrudate concentrations and the higher their native pH, the higher the pH-drop leading to the observed order of Pea I > Pea II > Pumpkin III > Sunflower > Pumpkin I > Pumpkin II (**Figure VI.1**).

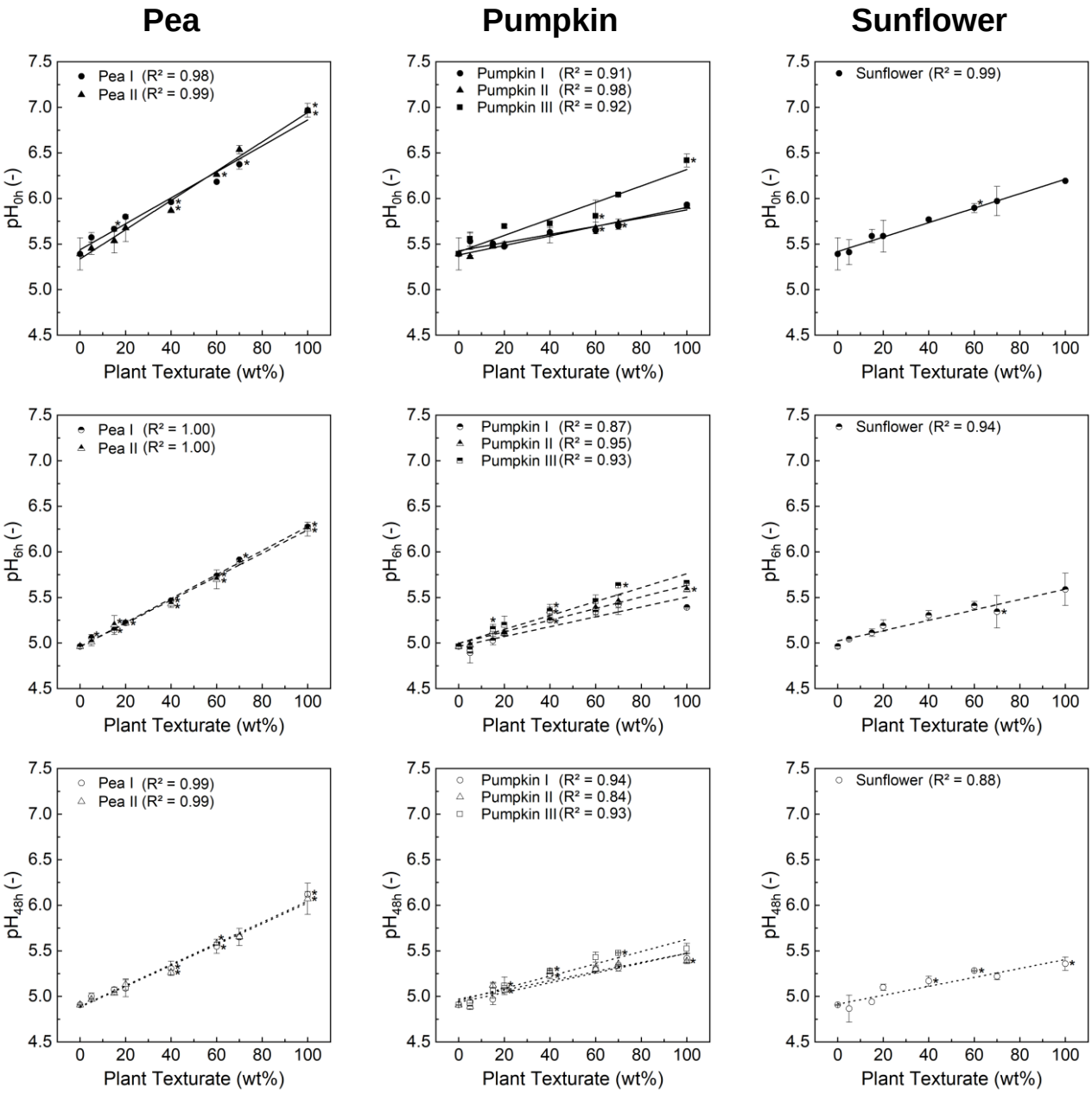

Figure VI.1 Mixing pH of minced meat model systems with lean pork meat and 0 – 100 % plant extrudates from Pea I, Pea II from two pea isolates and Pumpkin I, II, III and Sunflower from oilseed flours acidified with 1.0 wt% GDL after 0h, 6h, and 48 h; star marks significant ($p \leq 0.01$) differences among concentrations of one plant protein; coefficient of determination R^2 from linear regression of plant protein (%) and pH

Results may be attributed to differences in plant extraction method used to obtain protein-rich powders prior to their texturization by extrusion. The higher native pH of pea extrudates for example is due to a wet fractionation process being used that involves a series of protein precipitation and re-solubilization steps at alkaline pH (Bourgeois et al., 2015b). In contrast, raw materials for pumpkin and sunflower extrudates are generally produced via a simple

physical extraction that does not involve any pH-modulations (Panic et al., 2013; Pickardt et al., 2017). Moreover, decreasing slopes for the mixing pH at 0 h, 6 h, and 48 h revealed a decreasing influence of plant extrudates on the mixing pH (**Table SVI.4**) and thus a higher susceptibility towards GDL-mediated acidification. This susceptibility towards acid (or base) is generally described as the buffering capacity (Van Slyke, 1922). It depends on the content, and composition of individual amino acids in proteins, protein folding as well as the presence of select ions and the native pH of the evaluated foodstuff (Mennah-Govela & Bornhorst, 2021; Mennah-Govela et al., 2020). More precisely, a higher number of acidic amino acids on the surface of proteins and an overall higher content of proteins and ash (in particular sodium, potassium, calcium) may positively affect buffering capacity, while re- or unfolding upon denaturation and the burial of these amino acids in the proteins' interior in favor of hydrophobic ones may increase the susceptibility towards acid. As a result, differences in the proximate and in particular in the protein composition and structure (native/denatured) of Pork Meat and extrudates may cause varying acid and pH-dependency of their pure minced models and mixtures. Structural changes are likely to occur at high temperatures during high moisture extrusion of plant proteins which would lead to a higher hydrophobicity and thus a higher acid susceptibility of extrudates in comparison to pork meat. Besides, it was recently shown that residual ash and its constituents and the presence of the buffering amino acids glutamic and aspartic acid and histidine affect the buffering capacity of pork meat, as well as of pea and oilseed extrudates (pumpkin, sunflower) (Ebert et al., 2021a). However, this study evaluated the individual pH-response of proteins in dilute aqueous systems towards the titration with a strong acid from a consistent alkaline starting-pH, which deviates from the concentrated, minced model systems evaluated here, that were mixed at their native pH and acidified with GDL as a traditional sausage additive. At the end, the mixing-pH of minced meat model systems (**Figure VI.1**) might be a result of all these influencing factors noted before and be further affected by the acidification process itself. As such, differences among the different extrudates at the tested concentrations and timepoints are indicators for a high variability in the pH-response of proteins. Minced model systems with Pea I and II (comparable composition and native-pH) had a similar behavior at all timepoints, while those with Pumpkin I, II, and III (varying composition and native-pH) differed from each other at their initial $pH_{0h,}$ but curves converged until pH_{48h} (**Figure VI.1**). In line with this, slopes of pH-declines stayed constant for Pumpkin I – representative for no changes in the concentrations related pH-effects on the mixing pH at 0 h, 6 h and 48 h – but decreased for Pumpkin III. Hence, differences in native pH and the acid susceptibility of plant proteins affected the pH of minced mixed model systems,

with texturized plant proteins shifting the pH towards higher values, but simultaneously making them more prone to GDL-mediated acidification than pork meat.

Acidification course of minced model systems

Next, the acidification behavior of minced model systems, pure meat, and extrudates was monitored during 48 h and at 1.0, 2.0, and 3.0 wt% GDL to evaluate the pH-dependency as a function of time, extrudate, and acidifier concentration. Results from mixtures with Pea I and Pumpkin I are shown exemplarily in **Figure VI.2** due to their highest and lowest influence on the mixing pH, respectively (**Figure VI.1**, **Table SVI.4**)**Figure VI.1**. Results obtained with other samples can be found in **Figure SVI.3**. In general, a high slope of the dashed line indicates a fast pH decline, while more horizontal lines indicate that little to no pH-change took place with increasing acidification time (0 to 48 h). All tested wet extrudates and concentrations – and thus all minced meat model systems – showed a steep pH-decline in the first 6 h (dashed lines vertically oriented) (**Figure VI.2**, **Figure SVI.3**). Higher acidifier concentrations resulted in a faster and more pronounced acidification during the first hours. For example, dashed lines in contour plots for Pea I show an increase in the slope at 2.0 (**Figure VI.2B**) and 3.0 (**Figure VI.2C**) compared to 1.0 wt% GDL (**Figure VI.2A**). There, an ongoing hydrolysis and a greater pH-decline was also observed after 6 h, while samples with 1.0 wt% GDL did not show considerable pH-changes in between 6 and 48 h (-0.1 to -0.2 pH-units). Besides the GDL-concentration, the degree of the first and overall pH-decline was also dependent on the respective plant extrudate type and concentration, caused by initial mixing-pH as described above (**Figure VI.1**). Pure meat (native pH = 5.45) was acidified by 0.4, 0.8, and 0.9 pH-units after 6 h at 1.0, 2.0, and 3.0 wt% acidifier. Effects of GDL addition to pure extrudates during 6 h varied in between 0.3 pH-units (*e.g.* 100 wt% Pumpkin II, native pH = 6.06 at 1.0 wt% GDL) and 1.9 pH-units, representative for 0.6 pH-units per wt% GDL (*e.g.* Pea II, native pH = 7.25, 3.0 wt% GDL). Minced model systems containing both animal- and plant-based proteins had a behavior that was in between these two pure systems.

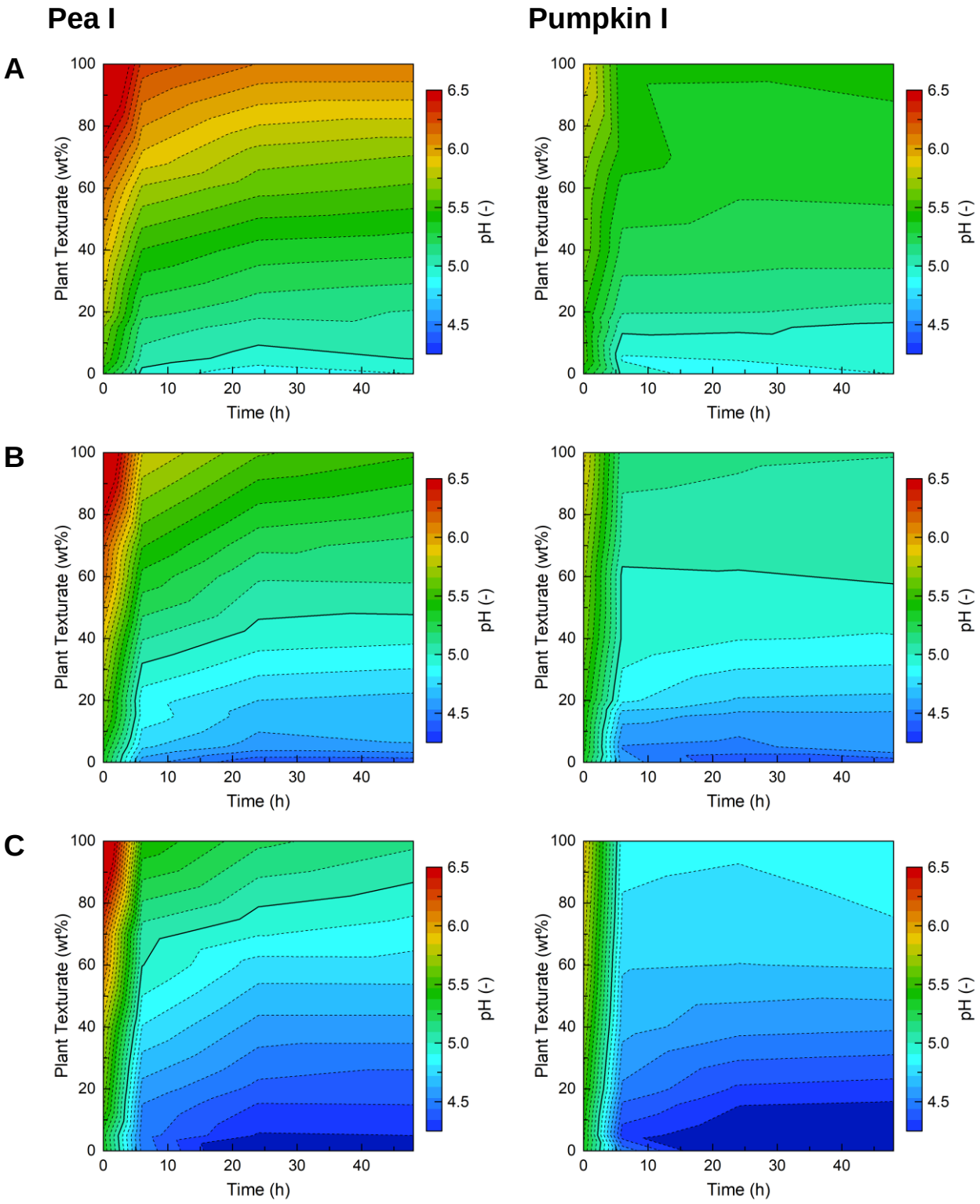

Figure VI.2 Influence of texturate (0-100 wt%) and GDL concentration on the mixing-pH during 48 h of acidification with 1.0 wt% (A), 2.0 wt% (B), and 3.0 wt% (C) exemplarily shown for Pea I and Pumpkin I; solid line marks pH 5.0

Several researchers previously investigated the effect of plant-based ingredients in hybrid meats. For example, Porcella et al. (2001) and Mokni Ghribi et al. (2018) evaluated the effect of soy protein isolate or chickpea protein concentrate in *Chorizos* or *Merguez* and found slight, but no significant ($p > 0.05$) pH-increases, that were suggested to correlate with the native pH of plant proteins. Similarly, 10 to 40 % of texturized soy protein increased the final pH of hybrid raw rabbit meat sausages from pH 4.82 to pH 5.19 (Zepeda Bastida et al., 2018). These results were in accordance with those obtained here, where a higher native pH of wet texturized plant proteins (**Table VI.1**) in comparison to pork meat was found. It is important to note that all authors were using starter cultures for microbial fermentation, which contrasts to a chemical-induced acidification of GDL as investigated here. However it was shown, that similar origins of plant proteins *e.g.* leguminous proteins yielded comparable effects on pH irrespective of their application in powdered or texturized form. These observations are also relevant to other hybrid meat products such as for example cooked products. Here, Kamani et al. (2019) described an increase from pH 6.6 to pH 6.95 and pH 7.01, when chicken meat was partially or fully replaced by a mixture of soy protein isolate, chickpea flour, and gluten in cooked sausages/sausage analogues. Similarly, 5 to 20 % of meat replacement with texturized soy proteins in Turkish *Kofte* caused an increase by 0.14 to 0.19 pH-units (Kilic et al., 2010).

In GDL-acidified products, the lowering in pH is due to a hydrolysis to gluconic acid, which readily occurs in the presence of water (Feiner, 2016; Yim et al., 2015). The compound has therefore also been used in several other ingredients systems (Chen et al., 2016; Grygorczyk & Corredig, 2013; Herz et al., 2021; Li et al., 2021; Sun et al., 2018; Zhu et al., 2011) due to an acidification capability that is mostly independent of matrix composition. As long as GDL and free water are still available (Feiner, 2016; Totosaus et al., 2000), the reaction can proceed. In that context, a positive correlation of reaction speed with pH and acidifier concentration has been reported (Shimahara & Takahashi, 1970). The hydrolysis reaction typically requires a few hours (Feiner, 2016; Totosaus et al., 2000), which is in line with our observations of a distinct pH-drop occurring in all minced model systems, pure meat, and extrudates within 6 h after GDL addition (**Figure VI.2**, **Figure SVI.3**). Dry-cured meat products are generally acidified to a pH of 5.0 in order to ensure microbial stability and formation of a firm gel to facilitate sliceability. To that purpose, typically used concentrations of GDL range from 0.8 to 1.0 g/100 g (Feiner, 2016). Considering this and the solid line that marks pH 5.0 in **Figure VI.2A** and **Figure SVI.3A**, threshold concentrations at 1.0 wt% GDL were already exceeded at > 5 wt% extrudate contents for Pea I, II and Pumpkin I and II, as well as at > 15 wt% for Pumpkin III and Sunflower. Consequently, minced model system containing pea and oilseed extrudates

demand for much higher amounts of acidifier to achieve common target pH-values, *e.g.* 2.0 wt% GDL for ≤ 40 wt% Pea I, II and Pumpkin III, ≤ 60 wt % Pumpkin I and II, or ≤ 70 wt% Sunflower (**Figure VI.2B** , **Figure SVI.3B**).

The replacement of pork meat with Pea I and II resulted in a comparable acidification course of minced meat model systems. Interpolations yielded a plant extrudate concentration of 40 wt% as a mixing threshold whereon GDL was not able to equally acidify minced model systems *e.g.* at 2.0 wt% GDL and 20 wt% of extrudate all samples were around pH_{48h} 4.7, while it ranged from pH_{48h} 4.89 ± 0.04 (Sunflower) to pH_{48h} 5.11 ± 0.07 (Pea I) at 60 wt%.

Relation of various influencing factors and target pH

Figure VI.1 and **Figure VI.2** show the influence of time, acidifier, and extrudate concentration, as well as extrudate origin on the mixing pH and acidification course of a minced model systems, pure meat, and extrudates. While interpolations of pH0h, pH6h, and pH48h and extrudate concentration yielded a linear dependence, there was no direct correlation with the GDL concentration and/or acidification time. Therefore, a multivariate regression with backwards selections of variables was done to relate influencing parameters. The pH (y) was used as the dependent value, while plant extrudate concentration (wt%, x1), time (h, x2), the amount of GDL (wt%, x3) and their interaction terms were tested as independent values of **Eq. VI.1**. **Table VI.2** summarizes parameter estimates (a to i) and significance ($p > 0.1$) of the tested variables and the adjusted R^2 of the proposed mathematical model for all tested plant extrudates.

$$y = ax_1 + bx_2 + cx_3 + dx_1^2 + ex_2^2 + fx_3^2 + gx_1 * x_2 + hx_1 * x_3 + ix_2 * x_3 + t \qquad \textbf{VI.1}$$

First, intercepts among plant proteins from the same genus were similar with $t = 5.604$ for Pea I, 5.526 for Pea II, 5.572 for Sunflower, and 5.396, 5.418, and 5.491 for Pumpkin I, II, and III, respectively (**Table VI.2**). Second, all individual independent parameters had a significant ($p \leq 0.1$) effect on the pH of minced model systems and adjusted R^2 was up to 0.89 representative for a good fit of the mathematical model. The parameter with highest influence was GDL with estimates of -0.136 (Pumpkin I), -0.143 (Pumpkin II), -0.154 (Pea II), -0.155 (Pumpkin III, Sunflower), and -0.191 (Pea I). From this it also became apparent that Pea I might have the highest susceptibility towards increases in GDL, followed by Pumpkin III, Sunflower, Pea II, Pumpkin II, and Pumpkin I which was in accordance with distinct decreases in the slope of the mixing pH (**Table VI.1**, **Table SVI.4**).

Table VI.2 Parameter estimates and their adjusted coefficient of determination (adjusted R^2) from a multiple linear regression model with backward selection of the variables for pH as dependent variable; NS = not statistically significant (dismissed based on $p > 0.1$ during backwards selection of variables)

	Parameter estimates on final pH_{48}					
Terms	**Pea I**	**Pea II**	**Pumpkin I**	**Pumpkin II**	**Pumpkin III**	**Sun-flower**
Intercept	5.604	5.526	5.396	5.418	5.491	5.572
Plant Texturate (wt%)	0.013	0.014	0.011	0.011	0.012	0.007
Time (h)	-0.048	-0.045	-0.039	-0.038	-0.044	-0.046
GDL (wt%)	-0.191	-0.154	-0.136	-0.143	-0.155	-0.155
Plant Texturate² (wt%*wt%)	NS	NS	-4.9E-05	-4.6E-05	-3.7E-05	NS
Time² (h*h)	0.001	0.001	0.001	0.001	0.001	NS
GDL² (wt%*wt%)	NS	NS	NS	NS	NS	NS
Plant Texturate*Time (wt%*h)	-6.7E-05	-8.1E-05	NS	NS	NS	0.001
Plant Textura*GDL (wt%*wt%)	NS	NS	NS	NS	NS	NS
Time*GDL (h*wt%)	-0.005	-0.006	-0.005	-0.006	-0.006	-0.006
Adjusted R^2	0.89	0.88	0.80	0.85	0.86	0.83

This also fit to recent results, where pea extrudates revealed a higher acid susceptibility than those from pumpkin and sunflower seeds (Ebert et al., 2021a) and underlined the relevance of leguminous proteins as meat replacers due to their similar pH-dependency. Additionally, pH was significantly ($p \leq 0.1$) influenced by interaction terms of Time*GDL and Time². This further underlined the importance of time on GDL-mediated pH-declines in particular within the first hours (**Figure VI.2**) since acidification by hydrolysis to gluconic acid is a time-dependent reaction (Feiner, 2016). Finally, interaction terms of Concentration², GDL², Concentration*GDL, and Concentration*Time were mostly not significant ($p > 0.1$) or had a low influence as seen by small parameter estimates *e.g.* for Concentration² and Pumpkin I with $-4.9*10^{-5}$ (**Table VI.2**). In line with this and other studies (Mokni Ghribi et al., 2018; Porcella et al., 2001; Zepeda Bastida et al., 2018), plant protein concentration had a positive influence on the final pH, while GDL, time, and their interaction term had a negative one, as seen in the concentration and time dependency of the acidification (**Figure VI.2, Figure SVI.3**).

Results on the mixing-pH and the time-dependent pH-development after GDL addition (**Figure VI.1, Figure VI.2**) indicate that typical application ranges of GDL for the production of dry-cured sausages of around 1.0 g/100 g (Feiner, 2016) were not sufficient to obtain the

desired acidification in minced model systems to a common pH value around 5.0. Thus, amounts have to be adjusted according to plant protein origin, extrudate concentration, and the desired target pH. **Table VI.3** summarizes needed GDL concentrations to reach pH_{48h} of 5.0 obtained from the proposed mathematical model (**Table VI.2**). Hybrid mixtures containing extrudate concentrations of not more than 40 wt% can be acidified by up to 2.0 wt% of GDL (**Figure VI.2**). All pumpkin extrudates require lower amounts than Pea I and II, while more GDL is needed at higher plant protein contents *i.e.* 1.45 wt% (20 wt% Pea I) compared to 1.36 wt% (20 wt% Pumpkin I) and 1.91 wt% compared to 1.90 wt%, and 2.36 wt% compared to 2.45 wt% at 40 wt% and 60 wt% Pea I and Pumpkin I, respectively. To sum up, required acidifier amounts to reach a specific target pH have to be increased along with the applied extrudate concentration in mixed matrices and the use of pea and pumpkin extrudates in mixtures led to higher cross-over threshold concentrations of GDL to reach the same target-pH, compared to sunflower extrudates that were more prone to acidification at all concentrations.

Table VI.3 Amount of acidifier GDL (wt%) needed to reach target pH48h = 5.0 at different plant extrudate (PT) concentrations calculated according to the proposed model

PT (wt%)	Pea I	Pea II	Pumpkin I	Pumpkin II	Pumpkin III	Sunflower
5	1.11	1.11	0.95	0.98	0.90	0.86
15	1.34	1.33	1.22	1.23	1.19	1.02
20	1.45	1.44	1.36	1.36	1.33	1.10
40	1.91	1.87	1.90	1.86	1.91	1.42
60	2.36	2.30	2.45	2.37	2.48	1.74
70	2.59	2.52	2.72	2.62	2.77	1.90
100	3.27	3.17	3.53	3.38	3.63	2.38

Conclusions

The acidification behavior of minced model systems containing pork meat and wet extrudates from pea or oilseed proteins with the chemical acidifier GDL varied depending on plant origin, native pH, and extrudate concentration. Generally, addition of plant proteins to hybrid products affects the initial and time-dependent pH of mixtures and as a consequence of their different native pH and buffering capacity, the susceptibility to GDL-induced pH is altered. In turn, different concentrations of GDL need to be added to formulations to reach a certain target pH. Manufacturers will need to take the amount and origin of alternative proteins used in meat hybrid products into account rather than relying on traditionally used amounts of GDL used in classical meat formulations. Nevertheless, at appropriate amounts of GDL added, a

chemically-induced acidification can yield a product formulation with a final target pH that ensures microbial safety. Furthermore, ensuring a sufficient coagulation of meat proteins in hybrid matrices constitute an important prerequisite for the formation of a firm, sliceable gel and therefore the creation of the desired texture and mouthfeel in end products. Results suggest that there may also be a need to carry out similar investigations for products that are acidified by microbial fermentations with starter cultures to further support the commercialization of hybrid meat products. However, the obtained findings provide insights to set up hybrid formulations and a basis to work with in future studies to further evaluate process-related, physicochemical, and organoleptic properties in comparison to traditional meat products.

Acknowledgement

We would like to thank Pascal Moll and Hanna Salminen for fruitful discussions and advice on data interpretation. This work was supported by the AiF (German Federation of Industrial Research Associations) as part of the CORNET project “Meat Hybrid” (AiF 196EN), and funded as part of the Industrial Collective Research (IGF) program of the German Ministry of Economics and Energy (BMWi), based on a resolution of the German Parliament.

CONFLICTS OF INTEREST

none.

Supporting Information

Table SVI.4 Mathematical correlation according to linear regression of texturates concentration (wt%) and pH at timepoint 0h, 6h, and 48h

Plant Texturate	Slope (0h/ 6h/ 48h)	Coefficient of determination R^2 (0h/ 6h/ 48h)
Pea I	0.014/ 0.013/ 0.012	0.977/ 0.998/ 0.991
Pea II	0.016/ 0.013/ 0.011	0.988/ 0.996/ 0.989
Pumpkin I	0.005/ 0.005/ 0.005	0.907/ 0.873/ 0.939
Pumpkin II	0.005/ 0.006/ 0.005	0.981/ 0.950/ 0.843
Pumpkin III	0.009/ 0.008/ 0.007	0.923/ 0.930/ 0.934
Sunflower	0.008/ 0.006/ 0.005	0.987/ 0.942/ 0.882

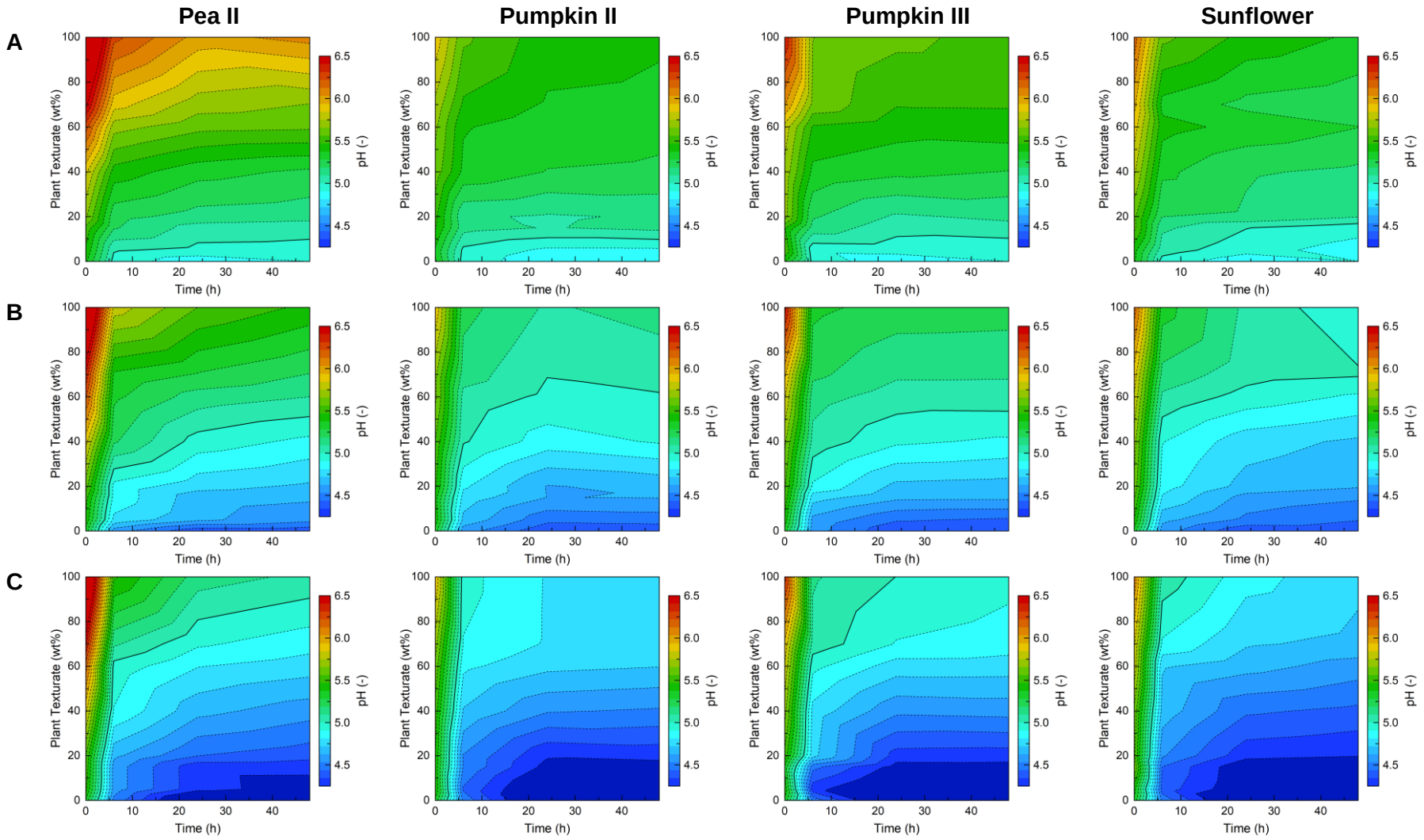

Figure SVI.3 Influence of texturate (0-100 wt%) and GDL concentration on the mixing-pH during 48 h of acidification with 1.0 wt% (**A**), 2.0 wt% (**B**), and 3.0 wt% (**C**) for Pea II, Pumpkin II, III, and Sunflower; solid line marks pH 5.0

VII. Chapter

Influence of Wet Extrudates from Pumpkin Seed Proteins on Drying, Texture, and Appearance of Dry Cured Hybrid Sausages

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Abstract

Hybrid meat products represent a promising, more sustainable alternative to all-meat formulations. However, differences among plant- and animal-based proteins may alter traditional handling and final product properties. In this study, pork meat was partially replaced with texturized pumpkin seed proteins at 12.5, 25, 37.5, and 50 % to obtain dry-cured hybrid meat sausages and their ripening (acidification, drying) during 21 days and final product properties (texture, sensory) were characterized and compared to a control (all-meat formulation). The drying behavior and distribution of moisture and free water of hybrids with extrudate contents of 12.5 and 25% were comparable to the sample made with meat and no significant ($p > 0.05$) differences in proximate composition were found. In contrast, higher meat replacement levels resulted in distinct changes of compositional and textural attributes *i.e.* chewiness was decreasing by up to 70 %. Results suggested 25 % of extrudates as an important threshold in manufacture of hybrid dry-cured sausages due to alterations in their ability to bind or release water. Results may be used to understand the influence of alternative texturized proteins in hybrid formulations and help product developers to understand related process and product relevant changes.

KEYWORDS

Hybrid Meat Products; Texturized Plant Proteins; Ripening; GDL; Drying;

Introduction

The demand for meat has increased over the last decades, primarily due to an increase of the world population (International Food Policy Research Institute, 2017). Livestock production is one of the major drivers of greenhouse gas emissions and affects availability of land and water (Godfray et al., 2018). This together with health-related concerns with regards to consumption of meat and meat products (Key et al., 2019) underlines the need to find alternative, consumer acceptable solutions to reduce meat consumption. One approach is to develop fully plant based products wherein texturized plant proteins (extrudates) are used to imitate the characteristics texture and cooking behavior of meat (Osen & Schweiggert-Weisz, 2016). Despite recent advances in the field though, not all products were readily accepted by consumers (Elzerman et al., 2013) due to challenges to create an appealing texture, appearance, taste, and aroma. These limitations might be overcome if meat and texturized proteins were to be combined to create so-called hybrid meat products. There, the meat content is only partially reduced to still provide some of its unique functional and organoleptic properties. In this context, there are still a number of open questions on how texturized plant proteins behave in hybrid matrices leading to a lack of guidelines when it comes to formulations and processing operations. This is particularly true for dry-cured products, where the quality of raw materials combined with an appropriate process control is of importance to create a coherent, sliceable matrix, that has a good taste and is safe for consumption. For the latter, specific pH and moisture levels have to be achieved (Feiner, 2016; McNeil, 2019). In other words, differences in the proximate composition – especially moisture, protein, and fat content – and native pH of meat and plant extrudates might demand for adjustments in traditional formulations and manufacture.

This study investigates the influence of wet extrudates from unroasted pumpkin seed flour on the ripening behavior and textural and organoleptic properties of the dry-cured hybrid sausages. Four hybrid and a control (all-meat) dry-cured product were manufactured by chemical acidification with Glucono-delta-lactone (GDL) and subsequent drying. Special attention was paid to loss and distribution of water during ripening, and results were related to the textural properties of the final products. Findings were aimed at enhancing the understanding of functionality of texturized plant proteins in hybrid matrices in order to provide guidance on the development of manufacturing protocols that balance degree of acidification and drying to yield products with high consumer acceptance and long shelf life.

Materials and Methods

Materials

Lean pork meat (75 % moisture, 20 % protein, 5 % fat, native pH 5.45) and pork backfat (8 % water, 2 % protein, 90 % fat) were purchased from MEGA Fleisch GmbH (Stuttgart, Germany), standardized to 18 mm (fist-sized), and stored at -18 °C until further use. Wet texturized pumpkin seed proteins (60 % moisture, 25 % protein, 6 % fat, 6 % carbohydrates, native pH 6.70) were provided by Deutsches Institut für Lebensmitteltechnik (Quakenbrück, Germany). There, oilseed flour Pumpkin®60, Unroasted (60 % protein, 9 % fat, 0.01 % salt, 17 % carbohydrates) from All Organic Treasures GmbH (Wiggensbach, Germany) was subjected to high moisture extrusion cooking in a double-screw extruder (ZSK 27MV, Coperion GmbH, Stuttgart) at a water to powder ratio of 9 to 7. The inlet temperature of 40 °C was increased stepwise to 145 °C. Protein plastification was done in the subsequently attached cooling die (FKD-750, DIL, Quakenbrück). The obtained extrudates were cut into stripes, packed airtight and stored at - 18 °C until further use. Curing salt (NPS) was obtained from ZENTRAG eG (Frankfurt, Germany). White pepper and ascorbic acid were purchased from MEGA Fleisch GmbH (Stuttgart, Germany). Glucono-□-lactone (GDL), magnesium acetate (purity ≥ 99.5 % p.a.), and sea sand were obtained from Carl Roth (Carl Roth GmbH & Co KG, Karlsruhe, Germany). Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany).

Production of dry-cured (hybrid) sausages

Part of the lean pork meat was thawed and minced to 3 mm with a meat grinder (model W 114, Maschinenfabrik Seydelmann KG, Stuttgart, Germany). Extrudates were thawed and chopped to particles of approx. 4 mm. Frozen pork meat (- 18 °C, 18 mm), pork backfat (- 18 °C, 18 mm), white pepper, ascorbic acid, and GDL were chopped in a bowl chopper (model K 64 AC8 VAK, Maschinenfabrik Seydelmann KG, Aalen, Germany) at high speed to obtain the desired particle size. Following this, respective amounts of minced meat (4 °C, 3 mm) and chopped extrudates (4 °C, 3 mm), and nitrite curing salt were mixed in to obtain raw meat batters. The formulations are summarized in **Table VII.1**. The meat content (frozen and chilled) was reduced from a total of 80 % (Control) to 70, 60, 50, and 40 % and replaced by 10, 20, 30, and 40% of extrudates, respectively, which was representative for meat reduction levels of 12.5 % (Hybrid 12.5), 25 % (Hybrid 25), 37.5 % (Hybrid 37.5), and 50 % (Hybrid50). The amount of backfat was kept at 20 %. Nitrite curing salt was added at a traditional concentration

of 2.6 g/kg in all recipes for taste and safety reasons (Feiner, 2016). Following this, the batters were filled into sausage casings (d = 50 mm, Naturin Viscofan, Weinheim, Germany), hung onto drying racks and ripened for 21 d in a Unigar 1800 BE chamber (Ness & Co. GmbH, Remshalden, Germany). Relative humidity (RH) and temperature were kept constant at 94 % and 25 °C during the first 24 h. Sausages were then cold smoked (23 °C, RH 75 %) twice for 10 min. Afterwards, RH was stepwise decreased to 85 % (5 d), 80 % (5 d), 75 % (5 d), 72 % (5 d) at 18 °C to ensure homogeneous drying. Ripening parameters were chosen according to preliminary experiments (not shown), that were adjusted from previous studies (Baldini et al., 2000; Colomer Sellas et al., 2021). All samples were prepared from the same raw materials with at least 15 sausages per formulation.

Table VII.1 Formulation of the traditional recipe and dry-cured hybrid sausages.

Ingredient (%)	Control	Hybrid 12.5	Hybrid 25	Hybrid 37.5	Hybrid 50
Pork backfat (18 mm, -18 °C)	20	20	20	20	20
Lean pork meat (18 mm, -18 °C)	45	40	35	30	25
Lean pork meat (3 mm, 4 °C)	35	30	25	20	15
Pumpkin Texturate (3mm, 4 °C)	0	10	20	30	40
Additives (g/kg)					
Nitrite curing salt	2.6	2.6	2.6	2.6	2.6
Pepper, white	3.0	3.0	3.0	3.0	3.0
Ascorbic acid	0.5	0.5	0.5	0.5	0.5
Corrected GDL[1]	7.3	9.8	12.3	14.8	17.4

[1] Calculated according to a mathematical model from preliminary experiments (unpublished results)

Ripening behavior

Acidification

The pH of sausages (control formulation and dry-cured hybrids) was measured during 120 h of ripening at select timepoints (0, 0.5, 1.5, 2.5, 4.5, 5.5, 24, 72, 120 h) with a pH-meter (Microprocessor pH Meter 537 with BlueLine21 electrode, WTW GmbH, Weilheim, Germany). The time-dependent pH-value was obtained as the average from all individual sausages. Each sausage was analyzed at least three-times.

Weight loss and drying rates

The weight of samples was determined daily during 21 d of ripening with a scale (U4100, Sartorius Lab Instruments GmbH & Co. KG, Göttingen, Germany). The relative weight loss related to the sausage weight and the absolute weight loss related to the initial moisture content

were calculated according to **Eq. VII.1** and **Eq. VII.2**. The respective drying rates were calculated according to **Eq. VII.3** and **Eq. VII.4**.

$$Relative\ Weight\ Loss_{day\ i}\ RWL\ (\%) = 100 - \left(\frac{m_{day\ i}}{m_0}\right) \cdot 100 \quad \textbf{VII.1}$$

$$Absolute\ Weight\ Loss_{day\ i}\ AWL\ (\%) = 100 - \left(\frac{\left(\frac{MC_0}{100} * m_o\right) - (m_o - m_{day\ i})}{\left(\frac{MC_0}{100} * m_o\right)} \cdot 100\right) \quad \textbf{VII.2}$$

$$Drying\ Rate\ RWL\ (\%/h) = \frac{RWL_i}{\Delta d_i} \quad \textbf{VII.3}$$

$$Drying\ Rate\ AWL\ (\%/h) = \frac{AWL_i}{\Delta d_i} \quad \textbf{VII.4}$$

where m_0 is the sausage weight at day 0 (g), $m_{day\ i}$ is the Sausage weight at day I (g), MC0 is the moisture content of raw (hybrid) meat batter at day 0 (%), and Δd_i is the elapsed drying time (h). The time-dependent *RWL* and *AWL* and the respective drying rates were obtained as the average from the data obtained from the individual sausages. Each sausage was analyzed at least three-times.

Chemical analysis

Sample preparation

Raw (hybrid) meat batters (day 0) and dried (hybrid) sausages (day 21) were peeled, cut into smaller pieces, chopped with a blender (type 4 171, Braun GmbH, Kronberg im Taunus, Germany) until homogeneous, packed airtight, and stored at 4°C until analysis. Furthermore, (hybrid) sausages at day 3, 5, 8, 14, 21 were subdivided into four different layers using a slicing machine (model VS 8A, Bizerba SE & Co. KG, Bailingen, Germany). Layer I represented the outer 3 mm of sausage, Layer II and Layer III were cut to a thickness of 6 mm each (3 mm to 9 mm and 9 mm to 15 mm). Layer IV represented the remaining Core. Individual layers were chopped and stored as outlined before. Each sausage was analyzed at least in duplicates unless otherwise stated.

Moisture content

The dry matter and moisture content of raw (hybrid) meat batters (day 0), dried (hybrid) sausages (day 21), and their layers (day 3, 5, 8, 14, 21) were determined gravimetrically with the sea sand method (AS § 64 L 06.00-3, BVL (2005a)). Approximately 10 g of sample were used.

Water activity (aw-value)

Water activity (a_w-value) of raw (hybrid) meat batters (day 0), dried (hybrid) sausages (day 21), and their layers (day 3, 5, 8, 14, 21) was determined with a water activity meter (HygroPalm AW1, rotroic AG, Bassersdorf, Switzerland) at room temperature.

Total ash content

The total ash content of raw (hybrid) meat batters (day 0) and dried (hybrid) sausages (day 21) was obtained after pre-incineration, followed by complete combustion at 600 °C (AS § 64 L 06.00-4, BVL (2005a)). Approximately 5 g of sample were used for analysis.

Total nitrogen and crude protein content

The total nitrogen content of raw (hybrid) meat batters (day 0) and dried (hybrid) sausages (day 21) was determined according to Dumas (AS § 64 L 06.00-20, BVL (2005a)). EDTA was used as a standard for calibration. Crude protein content was derived by a multiplication with a nitrogen-to-protein conversion factor of 6.25.

Total fat content

Total fat was determined by applying the method of Weibull-Stoldt (AS § 64 L 07.00-6, BVL (2005a) followed by a low-boiling Soxhlet extraction with petroleum ether. Pre-dried sample residues from moisture determination (see above) were used for analysis.

Appearance and color

Dry-cured (hybrid) sausages (21 d) were sliced to 17 mm thickness and scanned with a scanner (model V100 Photo, Seiko Epson Corporation, Suwa, Japan). The interior color was analyzed with a Chroma-Meter CR-200 (Minolta, Osaka, Japan) using the CIE *L*a*b* colour space and a standard observer at a 2° angle and D_{65} as illumination. Calibration was done with a white standard (Y = 86.9, x = 0.3183, y = 0.3352). The color distance ΔE of dry-cured hybrid sausages from the Control was calculated according to **Eq. VII.5**. Each sausage was analyzed at least in triplicates.

$$\Delta E = \sqrt{(L_C - L_H)^2 + (a_C - a_H)^2 + (b_{C-} b_H)^2} \qquad \textbf{VII.5}$$

where *L*, *a* and *b* were the lightness, red-green balance and yellow-blue balance of the control (subscript *C*) and hybrid (subscript *H*) sausage, respectively.

Texture profile analysis

Textural parameters (hardness, cohesiveness, springiness) of (hybrid) sausages were analyzed with a cyclic double compression test (Instron type 3365, Instron, Darmstadt, Germany) at a 50 % compression. The chewiness was obtained from **Eq. VII.6**. Each sausage sample was analyzed at least 10-times.

$$Chewiness\ (N * mm) = Cohesiveness\ (-) \cdot Hardness\ (N) \cdot Springiness\ (mm) \qquad \textbf{VII.6}$$

Statistical Analysis

Statistically significant differences among samples were tested by a one-way analysis of variance with a Duncan posthoc-test (α-level of 0.05) using SPSS statistics V23 (IBM Corp., Armonk, NY, USA) after checking the assumption of normality (Shapiro-Wilk-test, *p*-value to reject ≤ 0.05) and equal variance ($p \leq 0.05$).

Results and Discussion

The ripening of dry-cured sausages is important for key product characteristics such as microbial stability and texture (Feiner, 2016). It is generally divided into two steps *i)* acidification of raw meat batters at high relative humidity and *ii*) drying at decreasing relative humidity until the desired weight loss/moisture level is reached. For this reason, results pertaining to the chemical acidification with GDL (0 to 120 h) of dry-cured hybrid sausages and a control (all-meat) formulation are discussed first, followed by an evaluation of the drying behavior and moisture distribution during 21 d of ripening. Results were then discussed in relation to the textural properties and appearance of ripened products to gain first insights into the organoleptic aspects of using extrudates in dry-cured formulations. Wet texturized pumpkin seed proteins (extrudates) were used at meat replacement levels of 12.5 %, 25 %, 37.5 %, and 50 % (**Table VII.1**), representative of samples labeled Hybrid 12.5, Hybrid 25, Hybrid 37.5, and Hybrid 50.

Acidification behavior

Start and thus mixing pH-values of raw (hybrid) meat batters were slightly, but not significantly ($p > 0.05$) different with pH 5.55 ± 0.07 (Control), pH 5.75 ± 0.01 (Hybrid 25), pH 5.77 ± 0.01 (Hybrid 37.5), pH 5.81 ± 0.33 (Hybrid 50), and pH 5.85 ± 0.05 (Hybrid 12.5) (**Table VII.2**). The time-dependence of pH in all samples was similar with a steep pH-drop, followed by a further slow and less pronounced acidification. Small differences in initial mixing pH were

compensated by a faster acidification of hybrid samples (0.62 to 0.68 pH-units after 5.5 h) compared to the control (0.50 pH-units after 5.5. h) . After 120 h, final values of pH 5.02 ± 0.02 (Control), pH 5.01 ± 0.01 (Hybrid 12.5), pH 4.96 ± 0.01 (Hybrid 25), pH 5.02 ± 0.02 (Hybrid 37.5), pH 4.96 ± 0.01 (Hybrid 50) were reached.

When it comes to dry-cured sausages, a final pH of 5.0 is considered to be a good target value to achieve safety against food pathogens such as *Salmonella* spp. or *Lysteria monocytogenes* (McNeil, 2019). Moreover this ensures a sufficient denaturation of myofibrillar meat proteins as prerequisite for coherent gel formation in the final product (Barbut, 2014). It can be achieved by the addition of starter cultures and sugar or via a chemical acidifier such as GDL. The latter has recently gained popularity in particular in mixed biopolymer systems (Herz et al., 2021; Li et al., 2021; Sun et al., 2018), since its application quantity can be adjusted according to the formulation and the desired pH-drop. There, intensity and speed of acidification can be modulated since first-order reaction rates of GDL-hydrolysis depend on the matrix pH and/or acidifier amount (Ngapo et al., 1996; Shimahara & Takahashi, 1970).

Table VII.2 Time-dependent pH-course of the traditional recipe and dry-cured hybrid sausages during 120 h acidified with GDL

Time (h)	Control	Hybrid 12.5	Hybrid 25	Hybrid 37.5	Hybrid 50
0	$5.55 \pm 0.08^{a,A}$	$5.85 \pm 0.02^{a,A}$	$5.75 \pm 0.01^{a,A}$	$5.77 \pm 0.07^{a,A}$	$5.81 \pm 0.33^{a,A}$
0.5	$5.52 \pm 0.01^{b,B}$	$5.49 \pm 0.04^{b,B}$	$5.64 \pm 0.01^{ab,B}$	$5.73 \pm 0.06^{a,A}$	$5.79 \pm 0.12^{a,A}$
1.5	$5.46 \pm 0.02^{a,C}$	$5.39 \pm 0.04^{ab,C}$	$5.35 \pm 0.01^{b,C}$	$5.37 \pm 0.05^{ab,B}$	$5.42 \pm 0.05^{ab,B}$
2.5	$5.15 \pm 0.01^{b,D}$	$5.20 \pm 0.01^{a,D}$	$5.19 \pm 0.01^{a,D}$	$5.19 \pm 0.01^{a,C}$	$5.15 \pm 0.02^{b,BC}$
4.5	$5.10 \pm 0.01^{c,DE}$	$5.15 \pm 0.01^{ab,DE}$	$5.19 \pm 0.01^{a,D}$	$5.12 \pm 0.03^{bc,CD}$	$5.14 \pm 0.02^{abc,BC}$
5.5	$5.10 \pm 0.01^{a,DE}$	$5.13 \pm 0.04^{a,DE}$	$5.13 \pm 0.01^{a,DE}$	$5.12 \pm 0.04^{a,CD}$	$5.14 \pm 0.01^{a,BC}$
24	$5.07 \pm 0.01^{ab,EF}$	$5.09 \pm 0.02^{b,EF}$	$5.09 \pm 0.01^{b,E}$	$5.08 \pm 0.01^{b,DE}$	$5.03 \pm 0.03^{a,C}$
72	$5.04 \pm 0.03^{a,FG}$	$5.04 \pm 0.04^{a,FG}$	$5.07 \pm 0.02^{a,E}$	$5.09 \pm 0.03^{a,DE}$	$5.08 \pm 0.01^{a,C}$
120	$5.02 \pm 0.02^{a,G}$	$5.01 \pm 0.01^{a,G}$	$4.96 \pm 0.01^{b,F}$	$5.02 \pm 0.02^{a,E}$	$4.94 \pm 0.01^{b,C}$

Different lower-case letters indicate significant difference ($p \leq 0.05$) among recipes at the same timepoint;
Different upper-case letters indicate significant difference ($p \leq 0.05$) among timepoints from one recipe.

In this study, GDL-concentrations were adjusted according to the extrudate concentration to achieve a final pH of 5.0. This resulted in faster and longer lasting acidification of Hybrids compared to the Control and initial differences in mixing-pH disappeared after 5.5 h. Following this, further pH-declines in hybrids resulted in a final pH of pH 5.00 ± 0.06. As such, the overall time-dependent pH-development was typical for a GDL-induced acidification (Feiner, 2016; Li et al., 2021; Totosaus et al., 2000; Van Krieken & Bontenbal, 2013), indicating that hybrids can

be manufactured with a targeted pH-value in mind using GDL. This may be of interest for product developers since pH-variations of alternative proteins in hybrid formulations as shown by other authors (Kamani et al., 2019; Kilic et al., 2010; Mokni Ghribi et al., 2018; Porcella et al., 2001; Zepeda Bastida et al., 2018) may be overcome.

Drying behavior and proximate composition

The drying behavior was analyzed by monitoring the weight of dry-cured (hybrid) sausages during 21 d/504 h of ripening (24 h of acidification at higher humidity, 20 d/480 h of drying at deceasing humidity). The relative weight loss RWL (**Figure VII.1A**) related to the original sausage weight m_0 and the absolute weight loss AWL (**Figure VII.1B**) based on the original moisture weight MC_o were calculated according to **Eq. VII.1** and **Eq. VII.2**. Respective relative and absolute drying rates were derived from **Eq. 3** and **Eq. 4**. The moisture weight for calculations of the absolute weight loss (**Figure VII.1B**) was obtained from **Table VII.3** where the protein, fat, ash, and the free water content expressed via the water activity value (a_w-value) of raw (hybrid) meat batters and final products (21 d) are additionally shown. Both the relative (**Figure VII.1A**) and the absolute e (**Figure VII.1B**) weight loss curves followed the same course with an initial rapid increase occurring between day one (24 h) and six (144 h), followed by a more gradual weight loss indicating a deceleration of drying. Correspondingly, drying rates increased during the first 96 h, remained high for another 48 h and decreased until the end of the ripening time. For example, the Control formulation had a drying rate of 0.091 %/h after 24 h, 0.144 %/h after 96 h, 0.133 %/h after 144 h and finally 0.070 %/h after 504 h (**Figure VII.1A**). Furthermore, there were differences in both weight loss and rate between the different formulations (**Figure VII.1A**, **Figure VII.1B)**. First, higher meat replacement levels resulted in lower relative weight losses (**Figure VII.1A**) at any given time *i.e.* at day six (144 h) the weight losses were 19.2 ± 0.5 % (Control) > 19.2 ± 0.2 % (Hybrid 12.5) > 18.7 ± 0.2 (Hybrid 25) > 17.7 ± 0.5 % (Hybrid 37.5) > 15.6 ± 0.3 % (Hybrid 50). Statistically, Hybrid 12.5 and Hybrid 25 sausages were not significantly different ($p \leq 0.05$) to the Control from day 15, and the final relative weight loss of these three formulations was comparable (around 35 %). In contrast, Hybrid 37.5 and Hybrid 50 incurred lower relative weight losses of 32.5 ± 0.2 % and 29.2 ± 0.2 %, respectively. If the absolute weight loss was considered (**Figure VII.1B**), Hybrid 25 sausages had the largest values, followed by Hybrid 12.5, Hybrid 37.5, the Control, and the Hybrid 50, *e.g.* samples had lost 41.8 ± 0.5 %, 41.5 ± 0.5 %, 40.9 ± 0.4 %, 40.6 ± 1.1 %, and 36.8 ± 0.3 % at day 10 (240 h), respectively. There, the Control and hybrids having extrudate contents of 12.5 to 37.5 % were comparable, and distinct

differences were found only for the sample with 50% extrudates (Hybrid 50). Replacement of pork meat with extrudates from pumpkin seed proteins resulted in slight elevations in protein and decreases in fat content, but this did not translate into significant differences ($p > 0.05$) in dry-cured (hybrid) sausages (**Table VII.3**). However, small variations in the initial moisture content developed to significant ($p \leq 0.05$) ones in dried end products when meat replacement levels exceeded 12.5 %, and a_w values ranged from 0.890 (Hybrid 37.5) to 0.909 (Control, Hybrid 25).

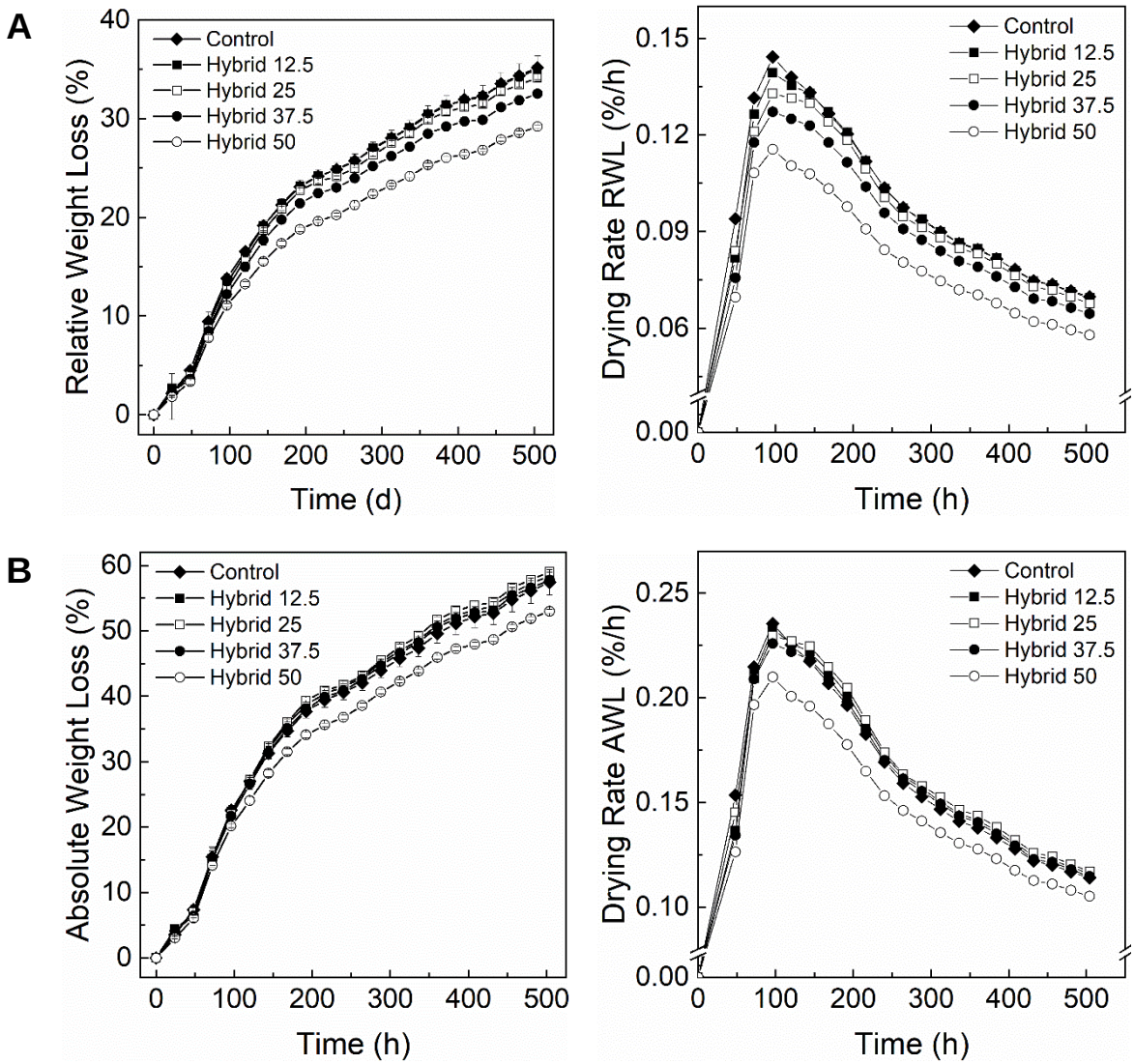

Figure VII.1 Relative weight loss and drying rate related to the sausage weight (A) and absolute weight loss and drying rate related to the moisture content (B) of the control formulation and dry-cured hybrid sausages during 21 d of ripening (RH 94 % (1 d), 85 % (5 d), 80 % (5 d), 75 % (5 d), 72 % (5 d))

Table VII.3 Proximate composition and aw-value of the traditional recipe and dry-cured hybrid sausages at day 0 and day 21

	Time (d)	Control	Hybrid 12.5	Hybrid 25	Hybrid 37.5	Hybrid 50
Moisture (%)	0	61.2 ± 0.7^{a}	59.9 ± 4.7^{ab}	58.0 ± 0.2^{ab}	56.3 ± 0.3^{ab}	55.1 ± 0.1^{b}
	21	44.2 ± 0.7^{a}	43.2 ± 0.9^{a}	40.9 ± 0.5^{b}	34.8 ± 0.9^{c}	34.3 ± 0.1^{c}
Crude Protein[1] (%)	0	17.5 ± 0.3^{a}	18.3 ± 0.5^{a}	18.0 ± 1.6^{a}	18.3 ± 1.3^{a}	19.2 ± 0.7^{a}
	21	25.2 ± 2.1^{a}	27.0 ± 0.9^{a}	26.3 ± 1.8^{a}	26.3 ± 1.1^{a}	27.8 ± 1.7^{a}
Specific Protein (%)[2]	0	17.5 ± 0.3^{a}	18.0 ± 0.4^{a}	17.5 ± 1.6^{a}	17.5 ± 1.2^{a}	18.0 ± 0.7^{a}
	21	25.2 ± 2.1^{a}	26.6 ± 0.8^{a}	25.5 ± 1.8^{a}	25.2 ± 1.0^{a}	26.1 ± 1.6^{a}
Total Fat (%)	0	18.1 ± 0.3^{a}	17.4 ± 2.6^{a}	18.1 ± 0.3^{a}	17.3 ± 0.4^{a}	16.9 ± 0.4^{a}
	21	25.7 ± 0.8^{a}	25.2 ± 1.1^{a}	26.2 ± 0.6^{a}	26.9 ± 1.1^{a}	26.3 ± 0.5^{a}
Total Ash (%)	0	3.49 ± 0.03^{ab}	3.62 ± 0.07^{a}	4.05 ± 0.01^{a}	4.17 ± 0.01^{a}	2.87 ± 0.61^{b}
	21	4.92 ± 0.03^{c}	5.05 ± 0.07^{c}	5.28 ± 0.11^{b}	6.28 ± 0.12^{a}	6.06 ± 0.09^{a}
a_w (-)	0	0.962 ± 0.004^{a}	0.957 ± 0.012^{a}	0.953 ± 0.006^{a}	0.961 ± 0.011^{a}	0.967 ± 0.025^{a}
	21	0.909 ± 0.006^{a}	0.908 ± 0.014^{ab}	0.909 ± 0.001^{a}	0.890 ± 0.001^{b}	0.898 ± 0.004^{ab}

Different lower-case letters indicate significant difference ($p \leq 0.05$) among recipes at the same timepoint
[1] Nitrogen to protein conversion factor = 6.25 (Mariotti et al., 2008)
[2] Calculation based on extrudate share and nitrogen to protein conversion factor for meat = 6.25 (Mariotti et al., 2008) and pumpkin seeds = 5.50 (Milovanović et al., 2014)

Typical drying curves of dry-cured sausages consist of three phases: *i)* an induction period (short time, increase of the surface to the wet bulb temperature), *ii)* a constant rate period (constant drying rate, removal of water from the wet sausage surface), and *iii)* a falling rate period (removal of internal moisture by diffusion) (Grau et al., 2014). In this study, moisture decreased slightly within the first 48 h with average drying rates among all samples (**Figure VII.1B**) of 0.149 ± 0.020 %/h (induction period), followed by a linear 96 h decline in absolute weight loss (not shown) at an average drying rate of 0.218 ± 0.008 %/h, indicating that the drying had entered the constant rate period. The falling rate period began after 168 h (7 d) where average drying rates decreased by 45 %. Along with the pH of around 5.0 (**Table VII.2**), a final moisture content between 30 and 40 %, and a water activity of $a_w < 0.91$ represent common target values in dry and semi-dry sausages (McNeil, 2019; Rahman & Perera, 2007; Toldrá & Flores, 2014). Considering this, all formulations were sufficiently ripened to achieve microbial stability and shelf life (**Table VII.3**). Small differences in proximate composition could be related to differences in their drying behavior (**Figure VII.1A, Figure VII.1B**), which depends on some internal and external parameters, such as the used raw materials and/or additives, the sausage diameter and temperature, the relative humidity, and the air velocity. For example, Walz and coauthors (Walz et al., 2017) dried microbially fermented small caliber (20 to 22 mm) pork meat sausages with different casing materials to a final weight loss of 41.5 %

in as little as 101 h (natural casing) to as long as 134 h (alginate or collagen casing). This resulted in 20 to 21 % of moisture and 5.5 to 7.4 % of ash. In contrast, Yim et al. (2015) combined starter cultures and GDL and beef and pork meat to ferment sausages at 55 mm diameter having a comparable proximate composition as the Control (**Table VII.3**). This yielded samples with final moisture, fat, protein, and ash levels ranging between 29 and 34 %, 31 and 34 %, 28 and 31 %, and 5.1 and 5.4 % after 25 d of ripening. At the end, the proximate composition of products is related to the targeted characteristics of the sausage variety *e.g.* fast vs. medium vs. slow fermented, dry vs. semi-dry or small vs. big diameter sausages (Feiner, 2016). These characteristics impact the drying behavior as fast drying of small calibers generally results in a short, steep drying regime, followed by a long gradual one, while slow drying and/or big diameters prolongs the first and shortens the latter (Walz et al., 2017; Walz et al., 2018; Yim et al., 2015). In this study, relative (**Figure VII.1A**) and absolute weight loss (**Figure VII.1B**) of hybrid sausages and the control formulation were representative for slow drying curves due to their large diameters (50 mm), slowly decreasing relative humidity of the drying air, and low total drying rates after 21 d of ripening, that is 0.105 %/h (Hybrid 50), 0.114 %/h (Control), 0.115 %/h (Hybrid 37.5), 0.116 %/h (Hybrid 12.5), and 0.117 %/h (Hybrid 25) (**Figure VII.1B**). This also shows that lower moisture contents (**Table VII.3**) may correlate with slower relative weight loss and drying rates (**Figure VII.1A**) of Hybrid 37.5 and Hybrid 50 in comparison to the Control, since dry(er) products have a lower moisture migration than wet ones (Krischer & Kast, 1978).

Considering literature data on hybrid meat products, a lot of research has been done on the effect of texturized proteins as meat replacers in boiled meat products (Deliza et al., 2002; Hidayat et al., 2018; Omwamba et al., 2014; Rao et al., 1984; Weiss et al., 2010). The search for suitable texturized fat alternatives in raw ferments has also led to recent investigations (Colomer Sellas et al., 2021) and the effect of meat replacement upon microbial fermentation was assessed (Zepeda Bastida et al., 2018). Despite differences in the assessed type of meat product, extrudates used (dry vs. wet texturized plant proteins), and acidification method applied (starter culture vs. GDL) some results may be of relevance to those obtained here. For example, Zepeda Bastida et al. (2018) assessed the effect of textured soy proteins in raw rabbit meat sausages at replacement levels of 10 to 40 %. Ripening was performed for 14 d which resulted in a final a_w between 0.625 (Control formulation) and 0.945 (40 % hybrid). There, authors concluded that increasing shares of extrudates resulted in an increased water holding capacity. Omwamba et al. (2014) found a similar effect upon beef meat replacement of samosa stuffing using texturized soy proteins, with moisture levels of the final products increasing

along with decreasing protein and fat contents. While both studies used dry texturized instead of wet texturized proteins, differences in the water holding capacity of extrudates compared to meat should be considered in particular for Hybrid 37.5 and Hybrid 50 sausages. As extrudates from pumpkin seed flours have a high dietary fiber content (around 5 % according to the manufacturers specification), results may also be related to those obtained from raw ferments with plant-based fibers. For example, the addition of 1 or 2 % of tiger nut fiber resulted in higher moisture, comparable protein and fat contents, and lower water activity and weight loss of end products (Sánchez-Zapata et al., 2013). Similarly, the addition of a citrus fiber to chorizo formulations decreased the a_w and moisture content in *Longaniza de Pascua* (Sayas-Barberá et al., 2012), and 2 to 4 % of orange fiber decreased the cooking loss of *Sucuk* (Yalınkılıç et al., 2012). In our study, meat replacement levels of 37.5 % and 50 % could be related to a dietary fiber content of around 1.88 % and 2.50 % (according to the manufacturers specifications) which may be one reason for the lower weight loss of the Hybrid 37.5 in comparison to Hybrid 25 and 12.5 sausages and the lagged drying behavior of Hybrid 50. This could also explain the somewhat lower water activity of dried hybrids (**Table VII.3**) with water being sorptively bound to the matrix. Moreover, swelling of fibres may have decreased the pore size of the matrix and thereby reduced the tendency of water to be released through the extrudates' cavities (Chaplin, 2003). However, it is important to say that the degree of acidification and thus the final pH-value also influences the drying behavior of sausages since the water holding capacity of meat is negatively correlated with the pH-value. As a result, literature data on lower or higher weight loss of hybrid meats may also be related to pH-effects of the used plant-based meat replacers (Omwamba et al., 2014; Zepeda Bastida et al., 2018) or additives (Fernández-López et al., 2007; Sánchez-Zapata et al., 2013; Sayas-Barberá et al., 2012).

All in all, meat replacements of up to 25 % resulted in comparable or lower moisture levels after 21 d of ripening, while higher meat replacement levels showed the opposite trend. This might be indicative of a threshold concentration of extrudates in dry-cured hybrid meats above which negative effects become dominant hinting at a complex interplay of compositional elements such as dietary fiber and initial moisture levels on the drying behavior of dry-cured (hybrid) meat matrices.

Distribution of moisture and free water

A more in-depth knowledge of the water distribution in dry-cured sausages during ripening can provide further insights into the drying behavior and quality development in products. Therefore, dry-cured (hybrid) sausages were sectioned into four layers, namely Layer I (outer

3 mm), Layer II (following 6 mm), Layer III (following 6 mm), and a Core Layer (remaining layer), and analyzed as to their moisture (**Figure VII.2, Table SVII.5**) and a_w-values (**Figure VII.3**, **Table SVII.6**) at day 3, 5, 8, 14, and 21 of ripening. Diameters were measured to correct the distance from the core/center according to the shrinkage of the sausages.

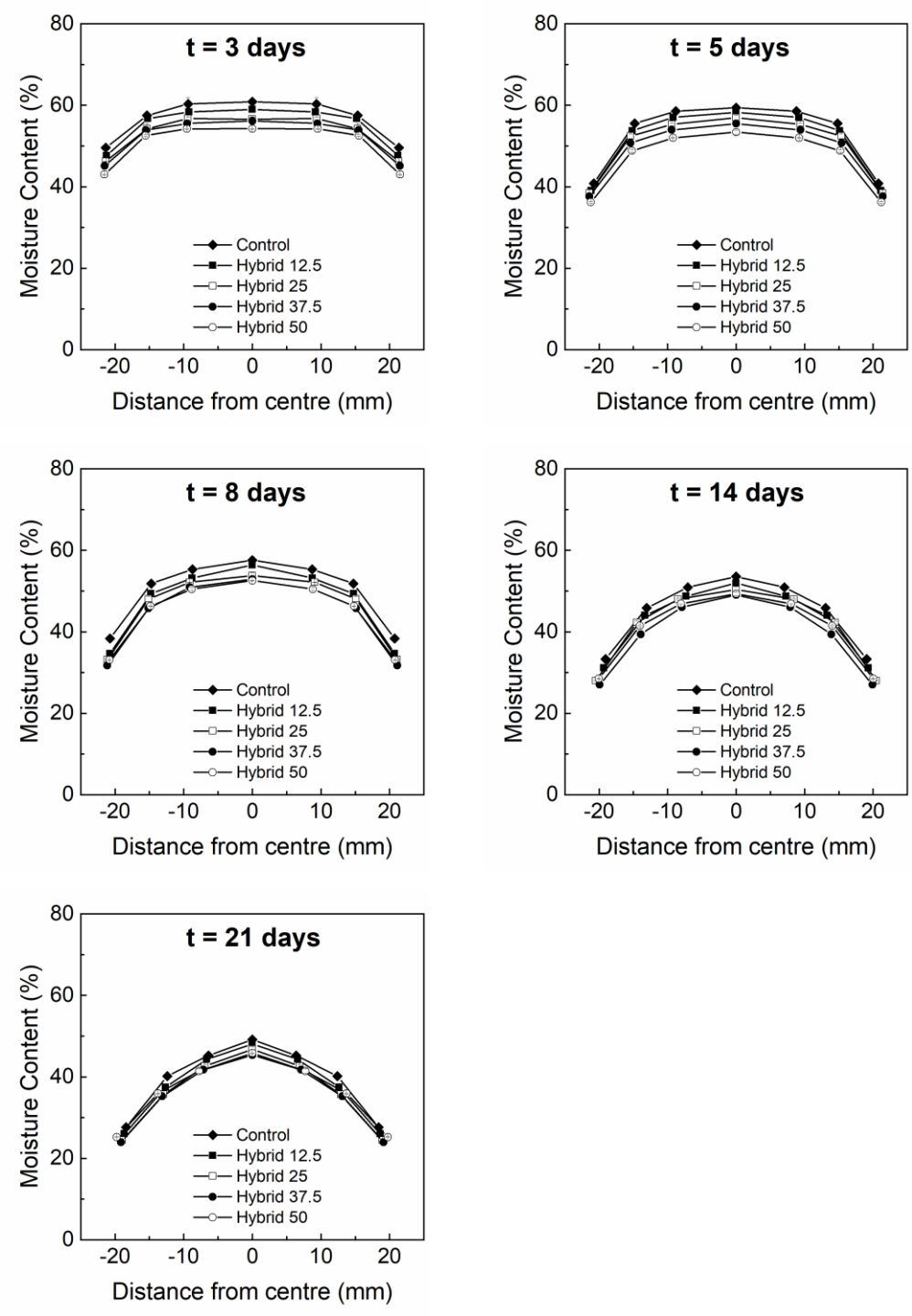

Figure VII.2 Moisture content (%) along the diameter of the control formulation and dry cured hybrids during ripening after 3, 5, 8, 14, and 21 days (RH 94 % (1 d), 85 % (5 d), 80 % (5 d), 75 % (5 d), 72 % (5 d))

All formulations showed an U-shaped moisture versus diameter distribution profile meaning higher contents in the core compared to the outer layers (**Figure VII.2**). Respective moisture gradients were increasing with increasing drying time from an average of 11.1 % (3 d) to 20.6 % (Hybrid 50), 21.5 % (Control, Hybrid 37.5), 22.1 % (Hybrid 12.5), and 22.3 % (Hybrid 25) after 21 d. Furthermore, varying moisture in raw (hybrid) meat batters (**Table VII.3**) resulted in differences among samples in the order of Hybrid 50 < Hybrid 37.5 < Hybrid 25 < Hybrid12.5 < Control. For example, moisture levels after 3 d were 54.2 ± 0.1 % (Hybrid 50), 56.2 ± 0.1 % (Hybrid 37.5), 56.6 ± 0.6 % (Hybrid 25), 59.0 ± 0.1 % (Hybrid 12.5), and 60.8 ± 0.7 (Control) in the Core Layer and 43.0 ± 0.1 %, 45.1 ± 0.1 %, 46.2 ± 0.1 %, 47.6 ± 0.1 %, and 49.5 ± 0.1 % in the outer Layer I, respectively (**Table SVII.5**). Differences among formulations were mostly significant ($p \leq 0.05$) in Layer I, II, and III except for Hybrid 50 sausages where moisture levels were comparable or even significantly ($p \leq 0.05$) higher than in the Hybrid 37.5 in the third drying regime. Moreover, a trend towards lowered differences among all formulations with ongoing ripening time could be deduced (**Figure VII.2**). For example, moisture levels in Layer IV (core) of the Control and Hybrid 12.5 sausages were diverging over time, and values after 21 d were around 49 % and 48 %, respectively. In contrast, Hybrid 25, 37.5, and 50 were similar starting from day 8 and showed no significant ($p > 0.05$) difference at day 14 and 21 (**Table SVII.5**).

The time-dependent moisture changes of hybrids were thus in principal similar to those of dry-cured sausages with gradients developing during drying and equilibration of salt and water occurring later across the matrix during storage (Fabbri & Cevoli, 2015; Feiner, 2016). Fabbri and Cevoli (2015) found that moisture distributions in raw meat batters (day 0) became gradual after 1 d of drying and a distinct gradient over four distinct zones along the diameter of sausages was reached after 28 d of ripening. Baldini and coauthors (Baldini et al., 2000) observed that moisture levels of *Mènage* (50-55 mm diameter) were around 59 % in both the external and the core fraction at day 0 and decreased by 12 and 4 %, respectively until day 7. Accordingly, dry-cured (hybrid) sausages (50-55 mm diameter) had 10 to 13 % of moisture loss in the outer Layer I and 2 to 3 % in the core (Layer IV) after 8 d (**Figure VII.2**). Baldini et al (Baldini et al., 2000) further showed that moisture levels among fractions differed not only depending on the variety, but also when different ripening parameters (temperature, relative humidity) were applied. This underlines the importance of thorough process control in order to balance water diffusion on the inside and evaporation on the surface of the sausages. Otherwise, product defaults such as case hardening may occur, where sausages form a glassy barrier at the surface that may decelerate or even prevent drying yielding products with a high susceptibility towards

microbial spoilage as well as a poor texture (Baldini et al., 2000; Feiner, 2016). Case hardening generally happens when the removal of water from the outside of the sausage is higher than the moisture migration from the inside (Gulati & Datta, 2015). It also affects phenomena such as product shrinkages and appearance of wrinkles or dents. As seen in (**Figure VII.2**), moisture levels of all formulations decreased steadily during drying, but shrinkage of the Hybrid 50 was distinctively lower at later stages of drying (see distance from center after 8, 14, and 21 d). This and observations from **Figure VII.1** may hint at some case hardening at high meat replacement levels possibly due to differences in glass transition temperatures.

While the moisture level describes the whole amount of water in the samples, the a_w-value is representative for the amount of free water and thus the amount that can be dried-off more easily. **Figure VII.3** shows the a_w-values of sausage Layer I to IV at day 3, 5, 8, 14, and 21, and illustrates that differences in the development of the spatial free water distribution profiles of the five formulations develop over time. These different in the final a_w-values after 21 d increase in the order of Hybrid 12.5 ~ Hybrid 25 < Control < Hybrid 37.5 << Hybrid 50. Food products often consist of hygroscopic, porous materials. In such systems, sorptively bound water limits the amount of water that can be removed, since they can only be dried until the so called equilibrium moisture content is reached (Krischer & Kast, 1978). Micro- and macrosized capillaries and pores enable liquid and gas transport through the matrix *e.g.* by capillary motion, hydrodynamic and Knudsen flow, as well as liquid, vapor, and surface diffusion (Trujillo et al., 2007). Differences in these structural features combined with differences in individual water holding capacity of compounds could be an explanation for the observed different drying behavior and end state of solely meat-based and hybrid samples. Cornet and coauthors (2020) recently demonstrated that soy-gluten-based meat analogues possessed water-filled cavities that may enable an easy and fast release of water. They also discussed that the initial moisture release might be more intense than for meat, where the water holding capacity is highly dependent on the spatial organization and state of myofibrillar proteins (Bertram et al., 2002). While they looked primarily at the behavior of meat analogues under compression, the outlined findings could still explain the somewhat faster drying behavior of the Hybrid 12.5 and 25 compared to the Control (**Figure VII.1A**). In addition, presence of dietary fibers in wet extrudates might not only lead to modulated water binding, but may also limit the release of water through the blockage of cavities in the matrix due to swelling (Chaplin, 2003). Likely, a complex interplay of structural features and physicochemical properties is behind the observed differences that will require more detailed studies.

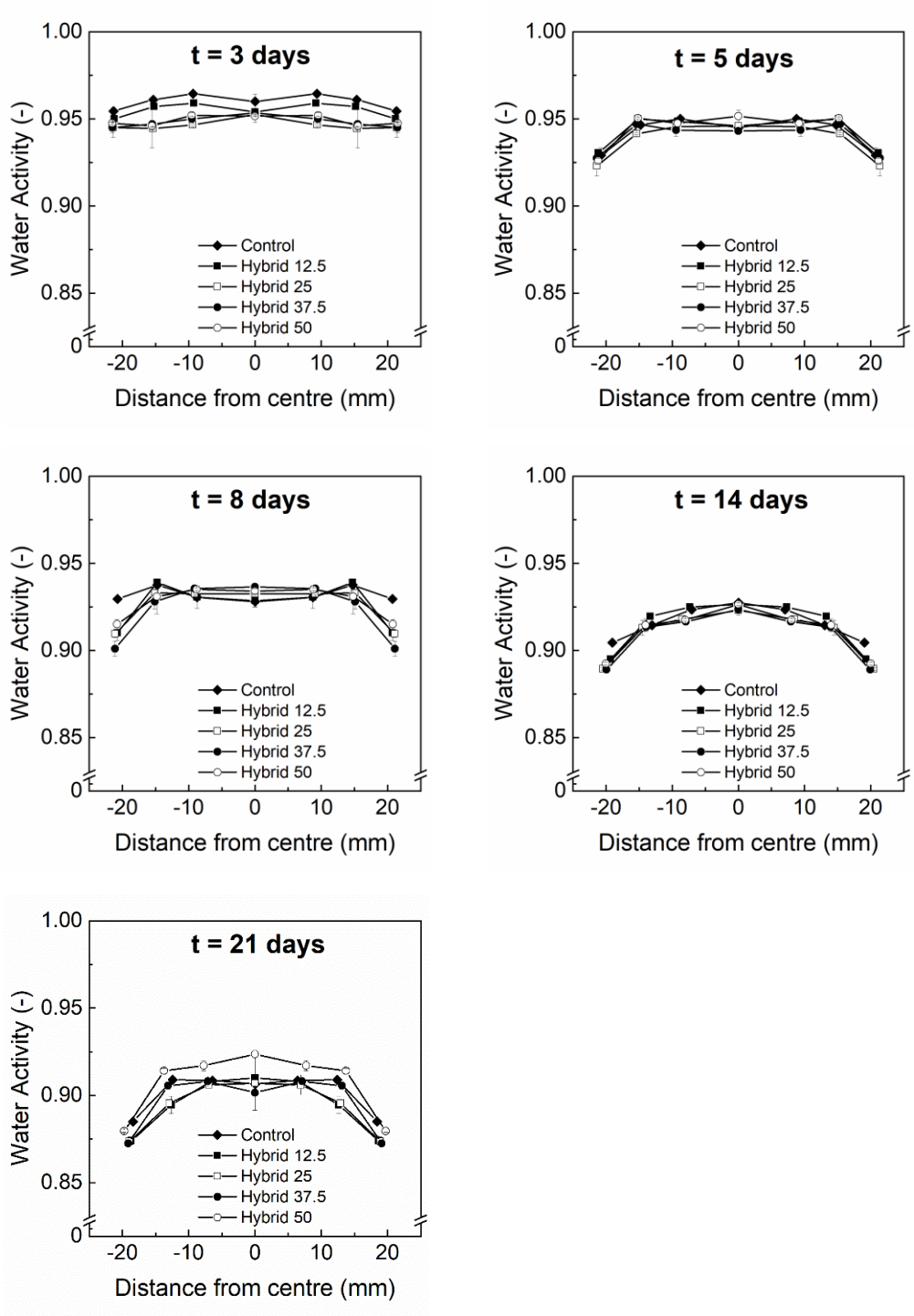

Figure VII.3 Free water content (aw) along the diameter of the control formulation and dry cured hybrids during ripening after 3, 5, 8, 14, and 21 days (RH 94 % (1 d), 85 % (5 d), 80 % (5 d), 75 % (5 d), 72 % (5 d))

Texture and appearance

Key textural attributes of non-heat treated, dry-cured sausages are a good sliceability along with a coherent, elastic texture that evolves through a solidification of the coagulated meat-protein

gel upon drying (Toldrá & Flores, 2014). Moreover, color displays an important quality attribute that influences consumer acceptance of meat analogues. Therefore, dry-cured (hybrid) sausages were analyzed in color (CIE-lab) and subjected to texture profile analysis (double compression test) after 21 d of ripening (**Table VII.4**). The replacement of meat by increasing concentrations of pumpkin seed extrudates resulted in a higher lightness *L** and all dry-cured hybrid sausages were significantly ($p \leq 0.05$) less red (*a**) and more yellow (*b**) compared to the Control. This is also reflected in high color distances ΔE of 4.13 ± 1.38 (Hybrid 12.5), 8.21 ± 1.80 (Hybrid 25), 11.1 ± 1.0 (Hybrid 37.5), and 13.7 ± 1.3 (Hybrid 50). When it comes to texture, hardness of the Control and Hybrid 12.5 and Hybrid 25 were not significantly ($p > 0.05$) different, but hybrid formulations deviated from the all-meat control in all other textural attributes. For example, cohesiveness decreased from 0.487 ± 0.012 (Control Formulation) to 0.408 ± 0.013 (Hybrid 12.5), 0.386 ± 0.011 (Hybrid 25), 0.336 ± 0.008 (Hybrid 37.5), and 0.256 ± 0.005 (Hybrid 50). Similarly, springiness was lower and declined from 4.88 ± 0.16 mm (Control) to as little as 3.88 ± 0.16 mm (Hybrid 50). Effects on the chewiness were similar at meat replacement levels of 12.5 and 25 % with - 139 Nmm, - 146 Nmm, but markedly higher for 37.5 % with - 178 Nmm. Chewiness of Hybrid 50 was substantially lower than all other samples, with only 30 % of the value of the Control.

Table VII.4 Appearance, color values, and parameters derived from texture-profile-analysis (deformation 50 %) of the control formulation and dry cured hybrid sausages after 21 d of ripening

Parameter	Control	Hybrid 12.5	Hybrid 25	Hybrid 37.5	Hybrid 50
Appearance					
Lightness *L** (-)	51.8 ± 1.7^{c}	52.5 ± 2.2^{c}	55.4 ± 2.4^{b}	57.4 ± 0.9^{a}	57.9 ± 0.9^{a}
Red-Green balance *a** (-)	13.2 ± 1.1^{a}	11.7 ± 1.5^{b}	9.53 ± 1.15^{c}	7.30 ± 0.59^{d}	5.91 ± 0.94^{e}
Yellow-blue balance *b** (-)	6.39 ± 0.78^{e}	9.45 ± 0.71^{d}	12.3 ± 1.9^{c}	13.8 ± 1.4^{b}	16.2 ± 0.8^{a}
Color Distance ΔE (-)	0^{e}	4.13 ± 1.38^{d}	8.21 ± 1.80^{c}	11.1 ± 1.0^{b}	13.7 ± 1.3^{a}
Cohesiveness (-)	0.487± 0.012^{a}	0.408 ± 0.013^{b}	0.386 ± 0.011^{c}	0.336 ± 0.008^{d}	0.256 ± 0.005^{e}
Springiness (mm)	4.88 ± 0.16^{a}	4.56 ± 0.18^{b}	4.56 ± 0.17^{b}	4.28 ± 0.24^{c}	3.88 ± 0.16^{d}
Chewiness (Nmm)	614 ± 42^{a}	475 ± 38^{b}	468 ± 44^{bc}	436 ± 37^{c}	180 ± 12^{d}

Different lower-case letters indicate significant difference ($p \leq 0.05$) among recipes and the same textural parameter or color value

It has already been shown that an incorporation of alternative proteins or fibers causes distinct color changes of meat products and most authors found decreasing *a** and increasing *b**-values

especially at high meat replacement levels (Deliza et al., 2002; Fernández-López et al., 2007; Hidayat et al., 2018; Sánchez-Zapata et al., 2013; Sayas-Barberá et al., 2012; Yalınkılıç et al., 2012). The red color of meat products is based on the conversion of myoglobin to the red meat colorant nitrosomyoglobin after its reaction with nitrite, which is characteristic for meat products (Feiner, 2016). As such, decreasing contents of meat proteins lower the number of reactants to form nitrosomyoglobin allowing the light green color of pumpkin seed extrudates to exert an influence (**Table VII.4**). This influence could be correlated with the meat replacement level at 12.5 and 25 % with *a** decreasing by 11.4 and 27.8 %, but not at higher meat replacement levels hinting to a hindrance of the red color formation irrespective of sufficient amounts of nitrite curing salt in the formulation (**Table VII.1**). Moreover, lower amounts of meat protein resulted in a lowered capability to form gels (Toldrá & Flores, 2014), which accounts for changes in the textural parameters (**Table VII.4**), in particular at meat replacement levels of > 25 %. Results also fit to previously reported studies, *e.g.* texturized plant proteins were included into beef sausage (Hidayat et al., 2018) or goat meat patties (Gujral et al., 2002), or meat in frankfurters was partly replaced by rice bran (Álvarez et al., 2012). Kamani and coauthors (Kamani et al., 2019) reported a lowered hardness, chewiness, cohesiveness, and springiness of boiled chicken sausage when meat was partly or fully replaced with a soy-gluten analogue. Based on their findings Alvarez et al (Álvarez et al., 2012) suggested an interference of non-meat ingredients on the heat-induced gelation of frankfurters, while Hidayat et al (Hidayat et al., 2018) proposed an interplay of fat reduction and higher water contents through the replacement of meat with texturized plant proteins.

Figure VII.4 summarizes our findings on the effect of pumpkin extrudates in dry-cured hybrid sausages. It is suggested that 25 % of meat replacement constitutes an important threshold above which distinct effects on the drying behavior, texture, and color of end-products can be observed. This may be related to structural and compositional differences imparted by the extrudates that may lead to an altered water binding and migration behavior. There are still a lot of open questions as to the role of molecular interactions between alternative and meat proteins *i. e.* the role that in particular non-covalent interactions such as hydrophobic or electrostatic ones play, as it has been recently shown that the association of meat proteins is affected by a functional potato protein fraction (Ebert et al., 2021b; Ebert et al., 2021c). Furthermore, it is not clear under what conditions extrudates act as active or purely passive (inert) fillers in the matrix – and depending on this – whether they represent defects in the meat gel matrix or provide additional mechanical strength to the matrix. Some extrudates contain

still soluble proteins that may be capable of binding with meat proteins and/or altering their functionality, while others may not.

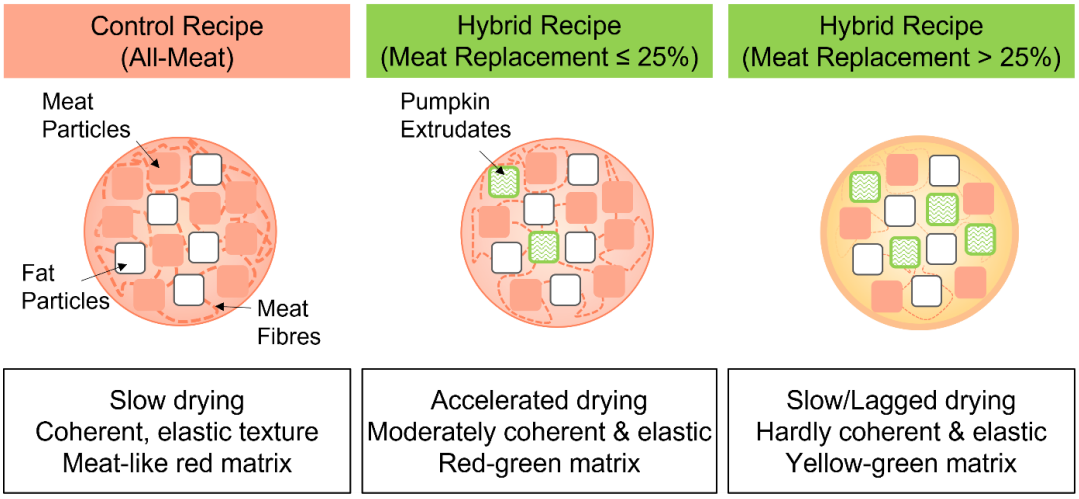

Figure VII.4 Schematic overview of the effect of pumpkin extrudates on the drying behavior and properties of dry-cured hybrids

Conclusion

Analysis on the ripening behavior, color, and texture of dry-cured hybrid sausages and a traditional all-meat recipe provided valuable insights into the effect of adding texturized plant proteins as meat alternatives. A meat replacement that exceeded 25 % altered the drying speed and distribution of moisture and increased the risk of case hardening and undesirable changes in color and texture. This was mostly related to differences in the proximate composition of formulations based on varying extrudates shares, which caused changes in water migration and binding and on the deviation of extrudates from the acid-induced texture formation of meat proteins. Thus, manufacturers of plant-based extrudates should not only aim to generate the macroscopic, fibrous properties of meat, but also play close attention to these physicochemical and functional properties. Nevertheless, the addition of suitable binders that can interact with both meat and/or extrudates might be of key importance to support cohesion and structural integrity in meat hybrids and analogues, which is a prerequisite to creating products with high consumer acceptability. Besides, meat replacers may cause favorable and/or unfavorable organoleptic changes. At the end, both these raw material specific and final product characteristics, as well as consumer-related aspects should be taken into account in order to broaden the commercial relevance of hybrid meat products.

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Statement and declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supporting Information

Table SVII.5 Moisture content (%) of whole sausage and along the diameter of the control formulation and dry-cured hybrids at 3, 5, 8, 14, and 21 days of ripening (RH 94 % (1 d), 85 % (5 d), 80 % (5 d), 75 % (5 d), 72 % (5 d))

	Time (d)	Control	Hybrid 12.5	Hybrid 25	Hybrid 37.5	Hybrid 50
Layer 1	3	$49.5 \pm 0.1^{a,C}$	$47.6 \pm 0.1^{b,D}$	$46.2 \pm 0.1^{c,C}$	$45.1 \pm 0.1^{d,D}$	$43.0 \pm 0.1^{e,C}$
	5	$40.8 \pm 0.4^{a,C}$	$39.1 \pm 0.1^{b,C}$	$38.4 \pm 0.1^{bc,D}$	$37.6 \pm 0.7^{c,D}$	$36.2 \pm 0.1^{d,D}$
	8	$38.3 \pm 0.2^{a,D}$	$34.6 \pm 0.4^{b,D}$	$33.2 \pm 0.2^{c,D}$	$31.7 \pm 0.4^{d,D}$	$33.1 \pm 0.1^{c,D}$
	14	$33.3 \pm 0.8^{a,D}$	$31.1 \pm 0.2^{b,D}$	$28.0 \pm 0.1^{c,D}$	$27.0 \pm 0.1^{d,D}$	$28.5 \pm 0.1^{c,D}$
	21	$27.6 \pm 0.5^{a,D}$	$26.0 \pm 0.1^{b,D}$	$24.4 \pm 0.4^{d,D}$	$23.9 \pm 0.1^{d,D}$	$25.2 \pm 0.1^{c,D}$
Layer II	3	$57.5 \pm 0.2^{a,B}$	$56.7 \pm 0.1^{a,C}$	$54.2 \pm 1.4^{b,B}$	$53.9 \pm 0.1^{b,C}$	$52.6 \pm 0.2^{b,B}$
	5	$55.6 \pm 0.5^{a,B}$	$53.7 \pm 0.1^{b,B}$	$52.4 \pm 0.6^{c,C}$	$50.7 \pm 0.1^{d,C}$	$48.8 \pm 0.3^{e,C}$
	8	$51.8 \pm 0.1^{a,C}$	$49.3 \pm 0.3^{b,C}$	$48.1 \pm 0.7^{c,C}$	$45.8 \pm 0.1^{d,C}$	$46.3 \pm 0.1^{d,C}$
	14	$45.9 \pm 0.3^{a,C}$	$44.0 \pm 0.1^{b,C}$	$42.3 \pm 0.4^{c,C}$	$39.4 \pm 0.1^{e,C}$	$41.4 \pm 0.2^{d,C}$
	21	$40.2 \pm 0.1^{a,C}$	$37.5 \pm 0.3^{b,C}$	$35.7 \pm 0.2^{cd,C}$	$35.2 \pm 0.3^{d,C}$	$35.9 \pm 0.2^{c,C}$
Layer III	3	$60.3 \pm 1.6^{a,A}$	$58.3 \pm 0.1^{b,B}$	$56.7 \pm 0.4^{bc,A}$	$55.5 \pm 0.5^{cd,B}$	$54.2 \pm 0.2^{d,A}$
	5	$58.6 \pm 0.1^{a,A}$	$57.0 \pm 0.4^{b,A}$	$55.3 \pm 0.3^{c,B}$	$53.9 \pm 0.1^{d,B}$	$52.0 \pm 0.1^{e,B}$
	8	$55.3 \pm 0.3^{a,B}$	$53.2 \pm 0.2^{b,B}$	$52.1 \pm 0.1^{c,B}$	$51.0 \pm 0.2^{d,B}$	$50.5 \pm 0.4^{d,B}$
	14	$50.9 \pm 0.2^{a,B}$	$48.8 \pm 0.2^{b,B}$	$48.0 \pm 0.3^{c,B}$	$46.0 \pm 0.1^{e,B}$	$46.8 \pm 0.1^{d,B}$
	21	$45.2 \pm 0.3^{a,B}$	$44.3 \pm 0.4^{a,B}$	$42.6 \pm 0.4^{b,B}$	$41.7 \pm 0.2^{c,B}$	$41.4 \pm 0.6^{c,B}$
Core Layer	3	$60.8 \pm 0.7^{a,A}$	$59.0 \pm 0.1^{b,A}$	$56.6 \pm 0.6^{c,A}$	$56.2 \pm 0.1^{c,A}$	$54.2 \pm 0.1^{d,A}$
	5	$59.4 \pm 0.2^{a,A}$	$58.2 \pm 1.1^{ab,A}$	$56.9 \pm 0.6^{bc,A}$	$55.5 \pm 0.1^{c,A}$	$53.4 \pm 0.5^{d,A}$
	8	$57.6 \pm 0.8^{a,A}$	$56.4 \pm 0.1^{b,A}$	$53.8 \pm 0.6^{c,A}$	$52.9 \pm 0.1^{cd,A}$	$52.6 \pm 0.1^{d,A}$
	14	$53.6 \pm 0.1^{a,A}$	$51.9 \pm 0.3^{b,A}$	$50.4 \pm 0.6^{c,A}$	$49.1 \pm 0.4^{c,A}$	$49.3 \pm 0.5^{c,A}$
	21	$49.1 \pm 0.5^{a,A}$	$48.1 \pm 1.4^{ab,A}$	$46.8 \pm 0.6^{bc,A}$	$45.4 \pm 0.2^{c,A}$	$45.8 \pm 0.1^{c,A}$

Different lower-case letters indicate significant difference ($p \leq 0.05$) among formulations in the same layer and timepoint;
Different upper-case letters indicate significant difference ($p \leq 0.05$) among layers from the same formulation and timepoint.

Table SVII.6 Water activity a_w (-) of whole sausage and along the diameter of the control formulation and dry-cured hybrids at 3, 5, 8, 14, and 21 days of ripening (RH 94 % (1 d), 85 % (5 d), 80 % (5 d), 75 % (5 d), 72 % (5 d))

	Time (d)	Control	Hybrid 12.5	Hybrid 25	Hybrid 37.5	Hybrid 50
Layer I	3	$0.955 \pm 0.002^{a, B}$	$0.950 \pm 0.001^{ab, C}$	$0.945 \pm 0.006^{b, A}$	$0.945 \pm 0.003^{b, B}$	$0.948 \pm 0.001^{ab, A}$
	5	$0.929 \pm 0.004^{a, B}$	$0.931 \pm 0.002^{a, B}$	$0.923 \pm 0.006^{a, B}$	$0.928 \pm 0.004^{a, B}$	$0.926 \pm 0.001^{a, B}$
	8	$0.930 \pm 0.001^{a, B}$	$0.910 \pm 0.004^{b, B}$	$0.910 \pm 0.002^{b, B}$	$0.901 \pm 0.004^{c, C}$	$0.915 \pm 0.003^{b, B}$
	14	$0.905 \pm 0.001^{a, C}$	$0.895 \pm 0.001^{b,C}$	$0.890 \pm 0.001^{d,C}$	$0.889 \pm 0.001^{d,C}$	$0.893 \pm 0.001^{c,C}$
	21	$0.885 \pm 0.001^{a, B}$	$0.874 \pm 0.003^{c, C}$	$0.874 \pm 0.002^{c, B}$	$0.873 \pm 0.001^{c, C}$	$0.880 \pm 0.001^{b, C}$
Layer II	3	$0.961 \pm 0.001^{a, AB}$	$0.957 \pm 0.001^{ab, C}$	$0.945 \pm 0.001^{b, A}$	$0.947 \pm 0.001^{ab, B}$	$0.946 \pm 0.013^{ab, A}$
	5	$0.947 \pm 0.001^{b, A}$	$0.950 \pm 0.001^{a, A}$	$0.942 \pm 0.001^{c, A}$	$0.947 \pm 0.001^{b, A}$	$0.951 \pm 0.001^{a, A}$
	8	$0.938 \pm 0.002^{a, A}$	$0.937 \pm 0.001^{a, A}$	$0.933 \pm 0.004^{a, A}$	$0.928 \pm 0.004^{a, B}$	$0.931 \pm 0.010^{a, A}$
	14	$0.915 \pm 0.002^{a, B}$	$0.920 \pm 0.001^{a,B}$	$0.913 \pm 0.004^{a,B}$	$0.914 \pm 0.002^{a,B}$	$0.915 \pm 0.004^{a,B}$
	21	$0.909 \pm 0.001^{ab, A}$	$0.895 \pm 0.005^{c, B}$	$0.896 \pm 0.002^{c, B}$	$0.906 \pm 0.001^{b, AB}$	$0.914 \pm 0.001^{a, B}$
Layer III	3	$0.965 \pm 0.001^{a, A}$	$0.959 \pm 0.001^{a, B}$	$0.947 \pm 0.001^{b, A}$	$0.950 \pm 0.001^{b, AB}$	$0.952 \pm 0.001^{b, A}$
	5	$0.950 \pm 0.001^{a, A}$	$0.949 \pm 0.002^{ab, A}$	$0.946 \pm 0.002^{ab, A}$	$0.944 \pm 0.004^{b, A}$	$0.948 \pm 0.004^{ab, A}$
	8	$0.931 \pm 0.001^{a, B}$	$0.931 \pm 0.006^{a, A}$	$0.933 \pm 0.001^{a, A}$	$0.936 \pm 0.001^{a, A}$	$0.935 \pm 0.001^{a, A}$
	14	$0.924 \pm 0.002^{a, A}$	$0.925 \pm 0.001^{a, A}$	$0.918 \pm 0.001^{b,AB}$	$0.917 \pm 0.001^{b,B}$	$0.918 \pm 0.001^{b,B}$
	21	$0.909 \pm 0.001^{b, A}$	$0.908 \pm 0.001^{b, A}$	$0.906 \pm 0.006^{b, A}$	$0.908 \pm 0.003^{b, A}$	$0.917 \pm 0.003^{a, B}$
Core Layer	3	$0.960 \pm 0.004^{a, AB}$	$0.954 \pm 0.001^{ab, A}$	$0.953 \pm 0.001^{b, A}$	$0.954 \pm 0.002^{ab A}$	$0.952 \pm 0.004^{b, A}$
	5	$0.945 \pm 0.003^{ab, A}$	$0.946 \pm 0.001^{ab, A}$	$0.946 \pm 0.004^{ab, A}$	$0.943 \pm 0.001^{b, A}$	$0.952 \pm 0.004^{a, A}$
	8	$0.928 \pm 0.003^{b, B}$	$0.929 \pm 0.004^{b, A}$	$0.933 \pm 0.002^{ab, A}$	$0.937 \pm 0.001^{a, B}$	$0.934 \pm 0.001^{ab, A}$
	14	$0.928 \pm 0.001^{a, A}$	$0.927 \pm 0.001^{ab, A}$	$0.923 \pm 0.003^{b,A}$	$0.924 \pm 0.001^{b,A}$	$0.927 \pm 0.001^{ab,A}$
	21	$0.907 \pm 0.004^{ab, A}$	$0.910 \pm 0.001^{ab, A}$	$0.907 \pm 0.016^{b, A}$	$0.902 \pm 0.001^{b, B}$	$0.924 \pm 0.002^{a, A}$

Different lower-case letters indicate significant difference ($p \leq 0.05$) among formulations in the same layer and timepoint;
Different upper-case letters indicate significant difference ($p \leq 0.05$) among layers from the same formulation and timepoint.

Concluding Remarks

This thesis showed that plant-based proteins not only differ from meat-based ones in fundamental ways, but that their mixture also affects the ability of meat proteins to accomplish certain structural organization due to interactions between the two species. Therefore, an in-depth characterization of plant protein formulations based on origin, protein content, mode of addition (*e.g.* as powders or extrudates) is required in order to create hybrid meats with high product safety and quality. Key considerations are solubility and miscibility of both plant and meat proteins in order to determine protein-protein interactions and phase behavior in these mixed matrices. Moreover, adjustments not only of formulations but also of manufacture approaches are needed to successfully manufacture shelf-stable and acceptable products. The development of mathematical correlations that relate raw material characteristics to properties can help ease product development in hybrid meats and thus provides a valuable tool. This was validated in a dry-cured product formulation and shown by observations on the effect of plant proteins on the drying behavior and end-product properties such as texture, appearance, sensory, pH, and water activity.

Solubility and/or pH adjustments are crucial to create high-value emulsified and dry-cured meat, as well as convenience products. However, plant proteins may vary in their solubility depending on the evaluated source. This was shown in a screening of a variety of commercially available plant proteins in terms of their behavior in aqueous media (**Chapter I**). Most plant proteins revealed poor dispersibility and their native pH varied from acidic to alkaline. In contrast to animal-based sources, plants contain mainly storage proteins where only some classes are easily water-soluble. Their prevalence and functionality are further affected by the extraction process used. For example, multi-stage wet fractionations can yield either isolates or concentrates containing varying amounts of carbohydrates and/or fats. The used process also impacts the amount of aggregated and thus difficult to solubilize plant protein particles in the protein preparation. Both protein extraction and the intrinsic composition of plant materials define the overall plant protein solubility. Two potato protein fractions deviated from these findings with a solubility of up to 100 %. This made them promising mixing candidates for meat proteins *e.g.* in model studies from dilute protein solutions, which facilitates an analysis of the phase behavior and yields insights into miscibility and molecular interactions (**Chapter I** and **Chapter III**). Solubility is closely related to mixing ratio and pH, as well as surface hydrophobicity, which deviates from the phenomena described for protein-carbohydrate

systems that are mostly charge-driven. The isoelectric point p*I* of salt-soluble meat proteins (pH ~ 5.5) represents an important boundary value, where co-solubility is lost and phase separation and aggregation occurs. Below the p*I* meat proteins generally start to self-associate into three-dimensional, coherent networks. This can be modulated by even small amounts of potato proteins added, despite the dominance of meat proteins on the overall phase behavior: This shows that plant protein shares in hybrid meats may be limited due to their dilutive or perturbing effect affecting the capability of meat proteins to form stranded gel structures that provide products with well-known fibrous bite and mouthfeel.

Results from **Chapter I** to **Chapter III** provide fundamental knowledge that link molecular scale to bulk properties and indicate challenges in the applicability of powdered plant proteins in hybrid meats. An alternative is to use the product class of extrudates, where powders are converted into viscoelastic fibers, that can withstand disintegration during hydration and cooking and mimic the anisotropic texture of meat. Besides their increasing relevance in meat analogues and a wider availability due to an industrially-scaled production, there is still a lack of knowledge on property changes during texturization, especially pertaining to their later behavior in mixed matrices. In **Chapter IV,** a characterization of dry and wet extrusion on the odor-active compounds of two pea isolates using gas chromatography-mass-spectrometry-olfactometry (GC–MS-O) after direct immersion-stir bar sorptive extraction (DI-SBSE) showed that a conversion of powders to extrudates has a profound effect on the organoleptic properties. It was demonstrated that *i*) green, fatty odors can be reduced by dry texturization; (*ii*) new odor impressions can be created through a cross-reaction of inherent compounds (*e.g.* conjugation of aldehydes and reducing sugars through Maillard reaction); *iii*) the composition of the initial pea protein powder determines the odor-active compounds of their respective texturates. This makes extrusion not only a promising technique to provide a meat-like structure, but also to decrease off-flavors that are currently limiting the application of some plant proteins. As a consequence, it is recommended to rather use extrudates in the formulation of hybrids. As was then done in all subsequent studies of this work.

Changes in and control of pH are crucial for the manufacture of safe and shelf-stable meat products. Typically, formulations are inoculated with acid-generating starter-cultures or chemical acidifiers are added that release acid. This provides characteristic flavors, induces network formation and/or inhibits microbial growth in dry-cured products. Buffering capacities are key here, and when select extrudates from pea isolates or oilseed flours (sunflower, pumpkin) are characterized in their susceptibility towards acidification, differences compared

to pork meat can be observed (**Chapter V**). The buffering capacity (BC) is highest in oilseed extrudates, followed by those obtained from pea and pork meat with largest deviations in between pH 7.0 and pH 4.5. These differences are related to the lower degree of purification of oilseed extrudates *e.g.* a larger amount of buffering ash constituents and the greater share of glutamic and aspartic acid and histidine compared to extrudates from wet extracted pea isolates. Mathematical modelling can be a very useful tool to relate compositional elements and buffering capacity and thus to find optimal formulations. Results underline the need to thoroughly assess all properties of these novel raw materials before their application. Acidic titration may be a promising method to start with, due to the moderate equipment requirements and the ease of qualitative and quantitative data comparison when using consistent sample preparation and procedures and the same calculations basis for proteins from different origins. Findings also provide a first indication on the deviation of extrudates from the pH-dependency of animal-based proteins, which might affect the formulation and handling of hybrid meat products. To move from lab to product scale, a minced meat model system may be a useful intermediate pilot plant scale model, where minced pork meat is mixed with for example chopped extrudates at varying mixing ratios and acidification with traditional compounds such as Glucono-delta-lacton (GDL) is done (**Chapter VI**). Such experiments can for example show that mixing pH and acidification behavior varies depending on plant origin, native pH, and extrudate concentration with distinct differences to purely meat-based systems starting from as low as 5 wt% of extrudates. The respective increase in the mixing and end-point pH in turn requires the use of higher GDL-concentrations at higher extrudate shares in order to reach the desired value for dry-cured meat products and to obtain microbial stability and a sliceable texture. Correlations of acidifier concentration, target pH, and extrudate share again proved to be a useful tool to support in adjusting formulations rather than having to rely on trial and error approaches. It should be noted though, that behavior did not exactly match results obtained on buffering capacity as shown in **Chapter V**, which suggests interactions of pork with texturized proteins upon their mixture and influencing factors that go beyond their individual buffering capacity.

When extrudates from unroasted pumpkin seeds were used to partly replace pork meat in a traditional dry-cured sausage formulation with amounts of chemical acidifier having been chosen based on results obtained from **Chapter VI**, the acidification behavior of hybrids validated the respective correlations and underlined the versatility of GDL for mixed meat matrices (**Chapter VII**). This first step is generally part of any ripening protocol of this product class that also includes a drying process at decreasing relative humidity. However, there are

limits to the addition of plant proteins to meat hybrids, and hybrids with more than 25 % of extrudates may display case hardening and yield end products that deviate from the well-known bite, mouthfeel, and appearance of all-meat dry-cured sausages. Differences in the proximate composition of extrudates such as lower moisture contents along with higher water binding are likely a cause for these changes in drying behavior. At increasing extrudate shares a dilutive effect on the capability of meat proteins to form coherent texture is observed since they provide no/low ability to support a network formation. In the future, this could be overcome if plant-based extrudates were to not only mimic meat texture, but also functionality to achieve acceptable end-products.

To conclude, plant-based proteins are promising ingredients in hybrid meats but their functional and physicochemical properties need to be taken into account to describe feasible application thresholds. This can be done by fundamental analysis on their dispersibility, proximate composition and buffering capacity and by establishing mathematical correlations among these dependent and independent variables. These theoretical correlations may help product developers to adjust formulations and unit operations to these novel matrices of hybrid meat products thereby facilitating the manufacture of products with desired shelf-life and acceptable texture. This work therefore contributes not only to the existing scientific knowledge base of plant-based proteins base but also supports commercialization efforts on hybrid meat products, especially pertaining to the product class of dry-cured sausages. The obtained findings can be used as a basis for raw material and process selection and provide valuable tools to build on. In the end, the properties of all plant protein formulations strongly depend on the extraction process applied, and thus manufactures should supply more information on this to aid users in making appropriate choices. This is because the functionality of powdered plant protein ingredients depends on the presence of soluble and insoluble protein classes, and/or aggregates, which may later result in a set of complex non-covalent interactions with water- and salt-soluble meat proteins in meat hybrids. In contrast, extruded proteins have undergone protein denaturation and plastification at high temperature, pressure, and shear, which renders them rather inert in an otherwise coherent meat matrix. Care has then to be taken when subjecting texturized proteins to unit operations such as acidification and drying since this may result in unexpected changes to end product properties. Taken together this dissertation underlines the need for a holistic characterization of plant proteins prior to their usage in food products in order to come to more rational, mechanistically-guided product development approaches rather than using trial-and-error ones.

Outlook

This study evaluated the suitability of various plant-based proteins for their application in hybrid meats with a special emphasis on dry-cured products. Raw material properties were characterized and set in context with prerequisites to create high-value end products. The obtained findings were then applied in a real product application that involved a traditional ripening scheme. In the following related research areas for further studies are outlined.

Further extrudates in hybrid meat product formulations

The last part of this thesis described the process-related and physicochemical properties of dry-cured hybrid sausages that contained extrudates from pumpkin seed proteins. While the presented findings contribute to increasing the industrial relevance of hybrid meats, more extrudates and meat replacement levels should be screened to establish a holistic knowledge basis that product developers can work with. This may include the remaining pea and oilseed extrudates evaluated within this study, but also other raw material sources in order to test the validity of the proposed mathematical correlations. Furthermore, options on their handling should be tested *e.g.* by pre-soaking/cooking to increase juiciness or by varying the extrudates' particle size in the formulations. When it comes to their organoleptic properties, more research should be done to assess the sensorial properties of extrudates in relation to their originating powders and approaches to possibly alter them before or during the manufacture of hybrid meats. This may also help to improve industrial processes in a way that the presence of unfavorable compounds is lowered, or the oxidation stability of plant proteins is increased.

Functional protein sources and additives for hybrid meats

It has been demonstrated that plant protein powders may negatively affect meat protein functionality and microstructural organization. However, only one highly soluble potato protein fraction was evaluated at a low protein concentration and in a simple system made of water and salt. Future research should now focus on validating the observed behavior in more complex bulk matrices and at higher protein concentrations since interactions with other ingredients may additionally occur and protein-protein interactions are also known to be highly concentration dependent (Saluja & Kalonia, 2008). Furthermore, other plant protein sources should be evaluated for their miscibility with meat proteins and the thereof resulting combined functionality, especially those where isoelectric points are less different to meat proteins. When considering the results on product applications, functional binders will be needed to compensate

lowered protein functionality in meat-reduced hybrids. Clearly defining these functionality deficiencies may help in finding suitable substitutes or technical additives such as modified cellulose or transglutaminase. This may also include process approaches such as a mechanical or chemical disruption of plant protein aggregates.

Starter-cultures for hybrid meats

Evaluations on the acid-dependent behavior of sole extrudates, meat, and their mixtures have resulted in an enhanced understanding of the differences of traditional and hybrid meat matrices. The results were then used to establish recipe recommendations by using the chemical acidifier GDL. However, it is widely known that chemically acidified matrices deviate from those obtained from microbial fermentation with starter cultures from an organoleptic, as well as from a texture and processing point of view. Clearly this represents one of the next steps to commercially establish not only hybrid meats, but also meat analogues. However, starter culture design is not trivial and needs to take into account all intermediate and end products. Therefore, suitable starter cultures may need to be specifically developed for combined meat- and plant-based matrices to optimize product performances and to ensure microbial safety thereby further increasing the commercial relevance and acceptance of hybrid meats.

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Eidesstattliche Versicherung

Eidesstattliche Versicherung gemäß § 18 Absatz 3 Satz 5 der Promotionsordnung der Universität Hohenheim zum Dr. rer. nat.

1. Bei der eingereichten Dissertation zum Thema
   HYBRIDMEAT - PRODUCTS FROM ANIMAL AND PLANT SOURCES
   handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

Die Richtigkeit der vorstehenden Erklärung bestätige ich: Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

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Ort und Datum Unterschrift

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EDUCATION

08/2017 to present	**PhD Student – Food Science** Department of Food Material Science, University of Hohenheim, Stuttgart (Germany) **Project**: HybridMeat - Products from animal and plant sources
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10/2011 – 11/2014	**Bachelor of Science – Food Science and Biotechnology** (Final Grade 1.8) University of Hohenheim, Stuttgart (Germany) **Thesis**: Quantification of the biofilm-formation and the synthesis of surfactin in biofilms from *Bacillus subtilis*
05/2011	**Abitur (University Entrance Diploma)** (Grade 1.5) Karl-Ernst-Gymnasium, Amorbach (Germany)

WORK EXPERIENCE

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02/2020 to present	**CEO and CMO at ZBS Food UG (haftungsbeschränkt)**, Stuttgart (Germany) Consulting industry in food science and technology, developing food products from upcycled ingredients
01/2021 to 12/2021	**Task Lead EIT Food Solutions, University of Hohenheim,** Stuttgart (Germany) Coordinating and supervising student projects for innovations in food product development and packaging design

05/2016 – 10/2016	**Visiting Research Scholar, University of Massachusetts (UMass)**, Amherst, MA (USA) Characterizing a dual-channel microfluidizer for the production of core-shell nanoparticles

FURTHER QUALIFICATION AND PROJECTS

01/2018 – 112/2019	Certificate in European Business Competence License (EBC*L) Level A and B Focus areas: Business administration, accounting and financial forecasting, profitability analysis, marketing and sales, business plan development
11/2017 – 11/2018	EIT Food Project "Foodio" Developing new, innovative solutions to integrate fiber-rich side-streams from juice productions into a dairy-based products. Creation of a business case with high commercial potential.
05/2013 – 08/2013	"Humboldt Reloaded" Research Project, University of Hohenheim, Stuttgart (Germany) Topic: Fat reduction of burger patties with microcrystalline cellulose and its effect on the sensorial quality and microstructure

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2011 to present	Member of Akademische Verbindung Agronomia, Stuttgart (Germany)
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PRIZES AND SCHOLARSHIPS

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Stuttgart, 25.02.2022