processes

MDPI

Article

Using Peptidomics and Machine Learning to Assess Effects of Drying Processes on the Peptide Profile within a Functional Ingredient

Sweeny Chauhan, Sean O'Callaghan, Audrey Wall *, Tomasz Pawlak, Ben Doyle, Alessandro Adelfio, Sanja Trajkovic, Mark Gaffney and Nora Khaldi

Nuritas Ltd., Joshua Dawson House, Dawson St. D02 RY95 Dublin, Ireland; chauhan.sweeny@nuritas.com (S.C.); info@nuritas.com (S.O.); pawlak.tomasz@nuritas.com (T.P.); doyle.ben@nuritas.com (B.D.); adelfio.alessandro@nuritas.com (A.A.); trajkovic.sanja@nuritas.com (S.T.); gaffney.mark@nuritas.com (M.G.); n.khaldi@Nuritas.com (N.K.)

* Correspondence: wall.audrey@nuritas.com; Tel.: +353-1-430-1290

Abstract: Bioactive peptides are known to have many health benefits beyond nutrition; yet the peptide profile of high protein ingredients has been largely overlooked when considering the effects of different processing techniques. Therefore, to investigate whether drying conditions could affect the peptide profile and bioactivity within a functional ingredient, we examined the effects of spray (SD) and freeze (FD) drying on rice natural peptide network (NPN), a characterised functional ingredient sourced from the *Oryza sativa* proteome, which has previously been shown to effectively modulate circulating cytokines and improve physical performance in humans. In the manufacturing process, rice NPN was either FD or SD. Employing a peptidomic approach, we investigated the physicochemical characteristics of peptides common and unique to FD and SD preparations. We observed similar peptide profiles regarding peptide count, amino acid distribution, weight, charge, and hydrophobicity in each sample. Additionally, to evaluate the effects of drying processes on functionality, using machine learning, we examined constituent peptides with predicted anti-inflammatory activity within both groups and identified that the majority of anti-inflammatory peptides were common to both. Of note, key bioactive peptides validated within rice NPN were recorded in both SD and FD samples. The present study provides an important insight into the overall stability of the peptide profile and the use of machine learning in assessing predicted retention of bioactive peptides contributing to functionality during different types of processing.

Keywords: functional ingredient; spray-dry; freeze-dry; bioactive peptide; hydrolysate; peptidomics; machine learning

Citation: Chauhan, S.; O'Callaghan, S.; Wall, A.; Pawlak, T.; Doyle, B.; Adelfio, A.; Trajkovic, S.; Gaffney, M.; Khaldi, N. Using Peptidomics and Machine Learning to Assess Effects of Drying Processes on the Peptide Profile within a Functional Ingredient. *Processes* **2021**, *9*, 425. https://doi.org/10.3390/pr9030425

Academic Editors: Jue-Liang Hsu and Olaniyi Amos Fawole

Received: 21 January 2021
Accepted: 20 February 2021
Published: 26 February 2021

1. Introduction

High protein ingredients have increasing gained commercial interest over the past number of years due to the nutritional and functional benefits associated with their consumption [1]. The processing of high protein ingredients, and the enzymatic hydrolysates derived from them, is often technically problematic, particularly during drying, which can induce protein denaturation and peptide aggregation [2].

Freeze drying (FD) and spray drying (SD) are conventional methods used in the food industry to convert an ingredient into a powdered version of itself, which typically increases product stability, facilitates more efficient transport options, and prolongs product shelf life due to reduced water activity [3]. SD is the process of transforming a solution from a liquid to a dry state in a single operation, where a liquid suspension is atomised into a chamber with hot dry air, evaporating the droplets and resulting in fine particles with a relatively low moisture content [4]. This technique is commonly used in the food industry as it is rapid, simple, and comparatively inexpensive [5]. However, drying processes such

as SD, studied by Chong et al. (2015) and Schmitz-Schug et al. (2013) [6] can lead to the loss of key actives, nutritional benefits that exist in the raw source, and proteins that are not heat resistant [7–9]. In addition, this process is technically challenging and requires continuous surveillance to ensure optimal product quality.

Conversely, an alternative drying process is FD, which tends to be more expensive and time-consuming but does not require the same level of process surveillance and management as SD [10]. Furthermore, this process has also shown to help retain the stability of physiochemical and biological activities of peptides [11,12]. In this process, water is removed by sublimation from a frozen state under reduced pressure [5]. Factors considered for utilising either process include scale and end-product usage. SD, for example, would be preferred if low cost and higher scale production throughput are required. FD would be preferable if product stability was a concern and smaller product volumes were required. Other considerations include physical and chemical attributes such as solubility, taste, density, colour, etc., which need to be assessed for the different drying methods to best align with the desired end-product formulation. In the case of protein hydrolysates, retention of their bioactive constituents and properties must also be a priority when deciding which processing method to choose.

When assessing the effects of FD and SD processing on hydrolysate production, previous studies have largely concentrated on the effects of drying processes on physical attributes and protein content, while the peptide content has been largely overlooked [11,13]. However, bioactive peptides, which are amino acid chains released from protein sources during hydrolysis, are an integral part to the functionality of an active ingredient. These key actives, latent in food proteomes, present the scientifically intriguing concept that they can exert functional effects beyond nutrition [14]. Specific activities associated with natural peptides include anti-aging, anti-cancer, anti-hypertensive, anti-inflammatory, anti-microbial, anti-oxidant, cholesterol lowering, glycaemic management, and immune modulation [15–17]. Importantly, many peptides have been shown to possess more than one bioactivity, making them truly dynamic ingredients [18]. However, there have been few investigations examining if conditions associated with common drying processes can affect the bioactivity and composition of food-derived peptides or whether this class of food molecules is more robust to the manufacturing process. Additionally, as SD and FD are the common processing approaches behind many food products that we consume daily [19], understanding whether these processes affect food-derived bioactive peptides is an important question that remains unanswered. Therefore, it is of interest to investigate the influence of drying techniques on the physicochemical attributes of constituent peptides rather than just protein, as well as the evaluation of the effects of these techniques on functionality [20–23]. To that end, machine learning has recently been shown to predict novel peptides with functionalities such as anti-aging and anti-inflammatory [24–27], which indicates that this novel approach offers a prime opportunity to evaluate functionality retention within food products.

This study aims to evaluate the effects of drying approach on the characteristics of constituent peptides within an enzymatic plant protein hydrolysate. Additionally, using a machine learning approach, we investigate if different drying processes could affect the retention of predicted efficacy and key constituent bioactive peptides in a previously validated functional ingredient derived from the *Oryza sativa* proteome, rice Natural Peptide Network (NPN). To date, the primary focus of published literature has been reporting the effects of either SD or FD on the functional properties and characteristics of the material being processed. To the best of the authors' knowledge, an in-depth peptidomic and bioinformatic assessment using hyphenated mass spectrometric techniques to elucidate the effects of high and low temperature on food peptides has not previously been reported in literature.

2. Materials and Methods

2.1. Hydrolysate Preparation and Drying

Protein hydrolysis was adapted from Rein et al. (2019) and Kennedy et al. (2020) [25,26]. Briefly, brown rice protein (80% organic rice protein powder commercially sourced from China) was solubilised in an alkalising buffer and sequentially hydrolysed with food grade serine protease in a pH and temperature controlled aqueous solution. After the reaction mixture was homogenised in a water bath under constant agitation, the pH was adjusted to 6 using potassium hydroxide, the protease was added, and hydrolysation carried out for several hours. The hydrolysis reaction was terminated by heating the solution to 85 °C for 10 min. Immediately after, the solution was rapidly cooled by putting on ice and stored overnight at 4 °C.

The soluble fraction was separated by centrifugation and divided into two separate aliquots. The first aliquot was SD using a laboratory scale dryer (Mini Spray Dryer Buchi B290, Flawil, Switzerland). The operational conditions of the drying process were inlet air temperature of 175 °C, outlet temperature ranging from 90–100 °C, and flow rate 15 mL/min. Moisture content was not specifically assessed once dried, but samples were stored in a desiccator prior to analysis. The second aliquot was FD using a laboratory scale dryer (BUCHI Lyovapor L-200 Freeze Dryer, Flawil, Switzerland). It was operated under a constant temperature of −50 °C at a chamber pressure of 0.1 mbar. The FD process lasted 48 h. Moisture content was not specifically assessed once dried, but samples were stored in a desiccator prior to analysis. The resulting dried rice protein enzymatic hydrolysate is herein referred to as a rice NPN and differentiated between drying approach as either SD or FD.

2.2. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis

FD and SD samples were each prepared as follows: 200 mg of hydrolysate was weighed and dissolved in 4 mL of Optima grade LC/MS water (Fisherbrand, Thermo Fisher Scientific, Inc., Canoga Park, CA, USA) and then vortexed. The sample was centrifuged at 4000 rpm for 20 min and supernatant filtered through 0.2 µm sterile filter (Fisherbrand, Thermo Fisher Scientific, Inc., Canoga Park, CA, USA). Protein content for both FD and SD was determined using Bicinchoninic Acid Assay (BCA) assay (Thermo Fisher Scientific, Inc., Canoga Park, CA, USA).

Aliquots containing 5 mg of proteins and peptides were acidified with 0.2% formic acid (Sigma-Aldrich, St. Louis, MO, USA). Desalting and peptide enrichment was done using Oasis HLB prime Solid-Phase Extraction (SPE) cartridges (Waters Corporation, Milford, MA, USA). Dried eluates were resuspended in 100 µL Optima grade LC/MS water (Fisherbrand, Thermo Fisher Scientific Inc., Canoga Park, CA, USA). Peptide content was determined using BCA assay. Sample volume, containing 21 µg of peptides, was resuspended in 21 µL of 0.1% Trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO, USA), containing 1% solution of Pierce™ Peptide Retention Time Calibration Mixture (ThermoFisher Scientific, Inc., Canoga Park, CA, USA), yielding a final concentration of 1 mg/mL.

Samples were analysed by nano LC-MS/MS Dionex UltiMate 3000 coupled to a ThermoFisher Q Exactive (ThermoFisher Scientific, Inc., Canoga Park, CA, USA) in positive polarity mode. Trapping column was utilised for loading peptides, which were subsequently eluted over a 25 cm analytical column PepMap RSLC C18 (ThermoFisher Scientific, Inc., Canoga Park, CA, USA) with a 60 min gradient at a flow rate of 300 nL/min. The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 full width at half maximum (FWHM) and 17,500 FWHM resolution, respectively.

From the MS scan, the 15 most intense ions were selected for fragmentation. PEAKS software (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used for peptide identification with the following parameters: enzyme, none; peptide mass tolerance, 10 ppm; fragment mass tolerance 0.05 Da; variable modifications, oxidation (M), Deamidation (NQ), Pyro-glu from Q; false discovery rate (FDR) 1%; activation method CID.

2.3. Bioactivity Prediction

Previously, a predictive machine learning approach was described to predict peptides with anti-inflammatory activity [26,27]. Briefly, sources of structured data (bioactivity databases, signalling pathways) and unstructured data (scientific literature, patents) were used to identify peptides with experimental measurements for anti-inflammatory activity across multiple bioassays. After manual curation, a non-redundant dataset of labelled anti-inflammatory peptides, which included ~10^4 data points, was compiled. Contextualized embeddings were computed for all the peptides in the set, to get latent features that would encode the biophysical properties of peptides. Similarly, computed features were shown to enhance the performances for several in-house downstream classification tasks. An artificial neural network model, based on a stack of bidirectional Long Short Term Memory and dense layers, was trained on the computed embeddings in 10-cross fold validation. The resulting best models were then refined against a smaller set of peptides (~10^2 data points), which were previously validated for anti-Tumour Necrosis Factor Alpha (TNF-α) activity in our laboratory.

The ensemble of the best resulting models was used to predict potential anti-inflamm atory activity, with bias towards TNF-α inhibition, within sets of common and unique peptides to FD and SD samples. Here, only peptides with a confidence higher than 75% were classified as anti-inflammatory.

2.4. Visualisation

Peptide count and peptide prediction graphs were generated using the ''ggplot2'' R package [28]. Custom python scripts were used to create the visualisations of peptide characteristics, using the libraries Matplotlib, Seaborn, Pandas, BioPython, and SciPy.

2.5. Statistics

Data is presented as mean ± standard deviation (STD) of at least three independent experiments, and where required, Student's t test was performed. Statistical analyses are indicated on relevant graphs, all analyses were performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com (accessed on 18 February 2021). Where $p < 0.05$, results were considered significant.

3. Results

3.1. Peptide Count and Physical Characteristics of FD and SD Samples

LC-MS/MS data for FD and SD samples were analysed for peptide content. Peptide count was consistently higher in the FD samples compared to the SD samples (Figure 1). However, basic t-test statistical analysis indicated that despite a considerably numerical difference in the number of MS peptides detected, these results were not significant ($p = 0.0773$). Samples produced by SD resulted in a fine, small particle powder with a light beige colour. FD samples on the contrary, were of a flaky and lightweight consistency, with a slightly lighter colour. The list of unique peptides identified in bout sample groups are supplied in Supplementary Tables S1 and S2.

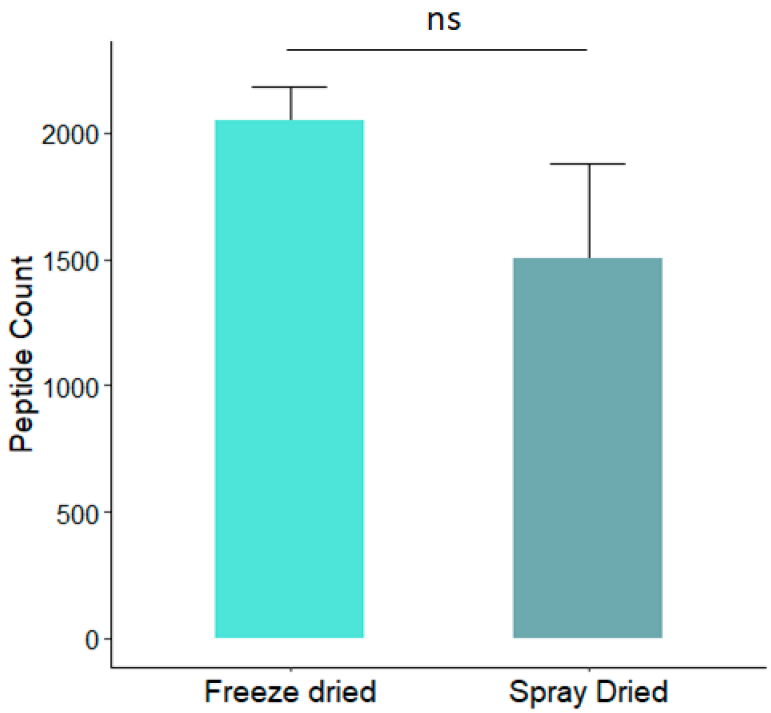

Figure 1. Effect of FD and SD on peptide count of rice NPN. The peptide counts as determined by LC-MS/MS in a bar chart representation of rice NPN FD (teal) and SD (blue) samples. Data represented at mean ± STD. Analysis was carried out using a Student's *t* test; ns, not significant; $p = 0.0773$.

3.2. Effects of FD and SD on Constituent Peptide Characteristics

We analysed several physicochemical properties within the representative peptide profiles of both FD and SD samples. Properties such as amino acid distribution, length, weight, charge, and hydrophobicity can not only be informative on the effects of drying processes on peptide content, but also on potential peptide bioactivity.

3.2.1. Amino Acid Distribution

Amino acid distribution for each peptide preparation was normalised based on peptide number for each drying process and depicted to illustrate the differing trends between SD and FD preparations. Findings suggest little difference between SD and FD preparations, with both amino acid distributions comparative to each other (Figure 2a). To elucidate if the drying process had an influence on amino acid distribution, the peptides exclusive to each of the FD and SD processes were also assessed (Figure 2b). These findings were in general agreement with the global amino acid distribution analysis, with trends comparative to each process in both cases; however, following statistical analysis, we found that Asp ($p < 0.05$), His ($p < 0.05$) and Lys ($p < 0.05$) residues were significantly greater in peptides exclusive to FD samples.

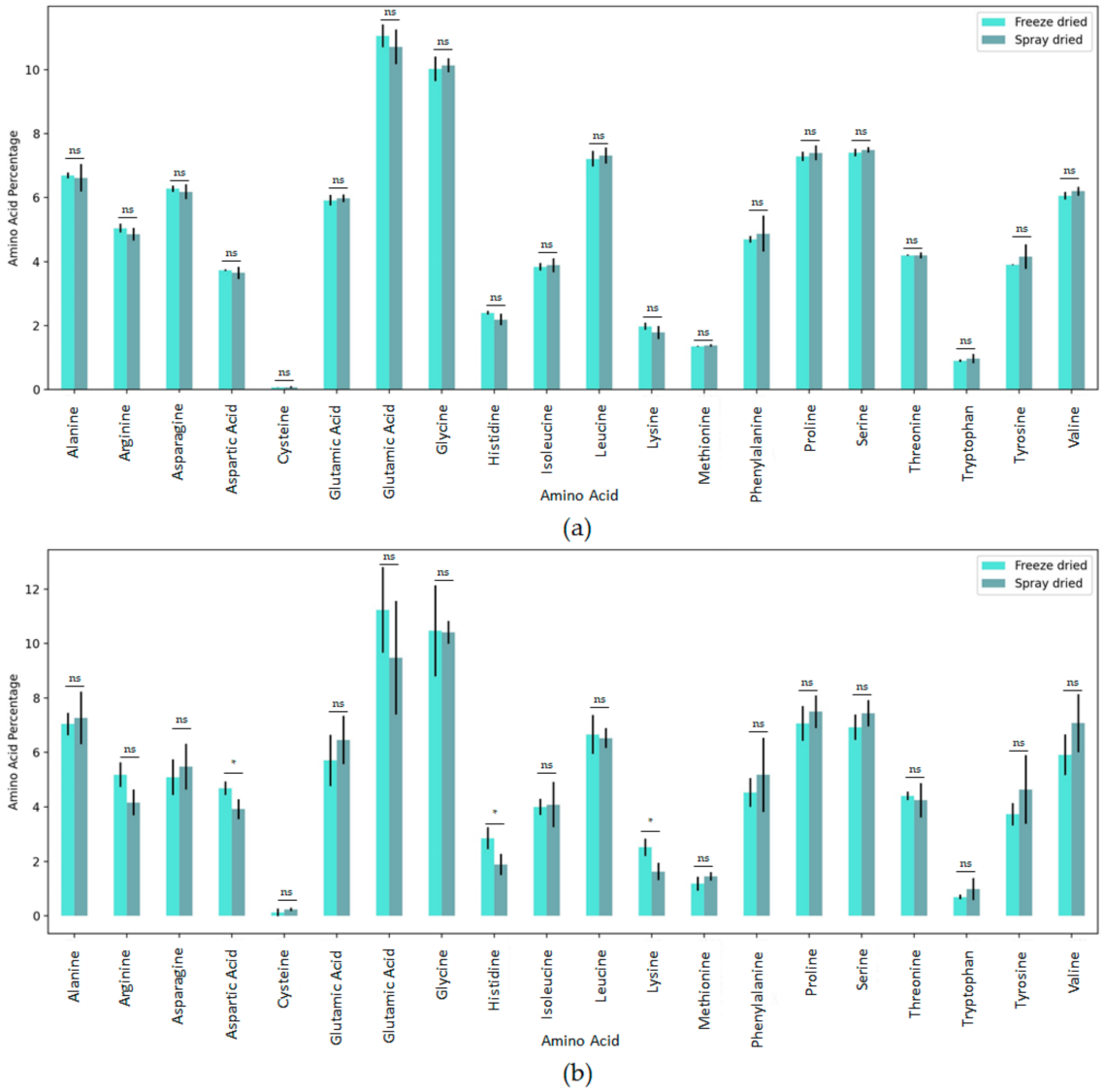

Figure 2. Amino Acid distribution in FD and SD samples of rice NPN. Total amount of amino acid (%) for each amino acid within (**a**) peptides present in full samples and (**b**) peptides exclusive in either FD (teal bar) or SD (blue bar) samples. Analysis was carried out using a Student's t test (data represented at mean ± STD; ns, not significant; * $p < 0.05$).

3.2.2. Peptide Length and Weight

Differences in peptide length were minor across the global peptide profile for each process (Figure 3a). There was a greater trend towards longer peptides in the FD preparation when only the peptide profiles exclusive to each drying process were assessed (Figure 3b). A similar analysis was carried out for peptide molecular weight on the full peptide profile for each process (Figure 3c) and for those peptides exclusive to each drying process (Figure 3d). Molecular weight of peptides in FD samples correlated to peptide length, whereby the FD preparation had a slight trend towards peptides with a higher molecular weight (Figure 3d), correlating to the longer peptides observed in Figure 3b.

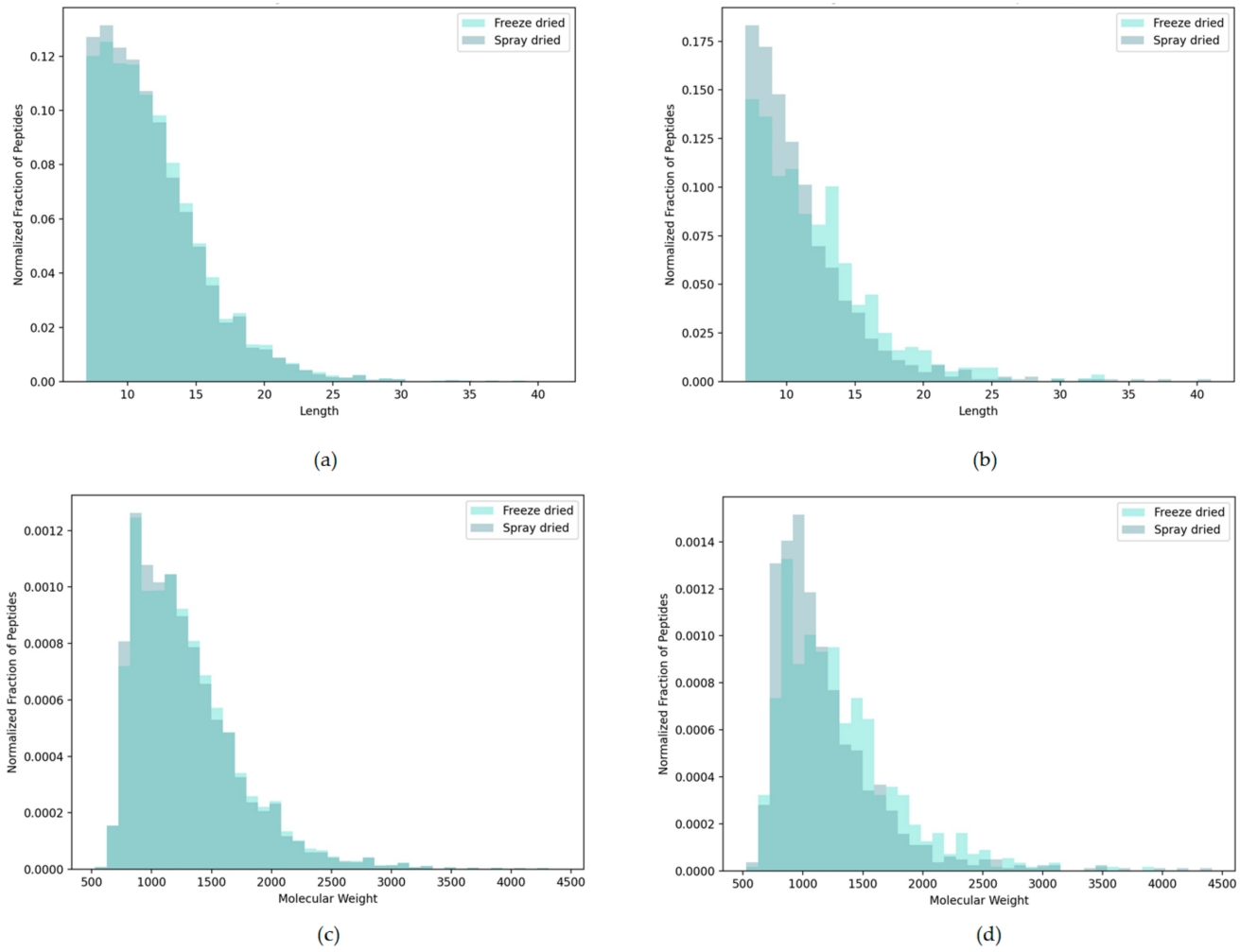

Figure 3. Peptide distribution according to length and molecular weight in FD and SD samples of rice NPN. The peptide profile as determined by LC-MS/MS in a histogram representation of rice NPN samples. Peptide distribution is according to length of amino acid chain in (**a**) peptides present in the total samples or (**b**) peptides exclusive in either FD (teal bar) or SD (blue bar) samples and according to weight (Da) in (**c**) peptides present in full samples or (**d**) peptides exclusive in either FD or SD samples, all data is normalised for count.

3.2.3. Peptide Charge

The inherent charge of peptides within each preparation was also calculated and depicted in Figure 4a for total peptide profiles of SD and FD samples and in Figure 4b for peptides exclusive to either the FD or SD preparations. There was no difference in charge between the drying process at a global peptide level (Figure 4a), with a median charge of −0.24 calculated for both FD and SD preparations. Closer analysis of peptides exclusive to either preparation indicated a slight trend towards a greater proportion of negatively charged peptides in the SD preparations, with a median value of −0.44 compared to the −0.24 of FD preparations.

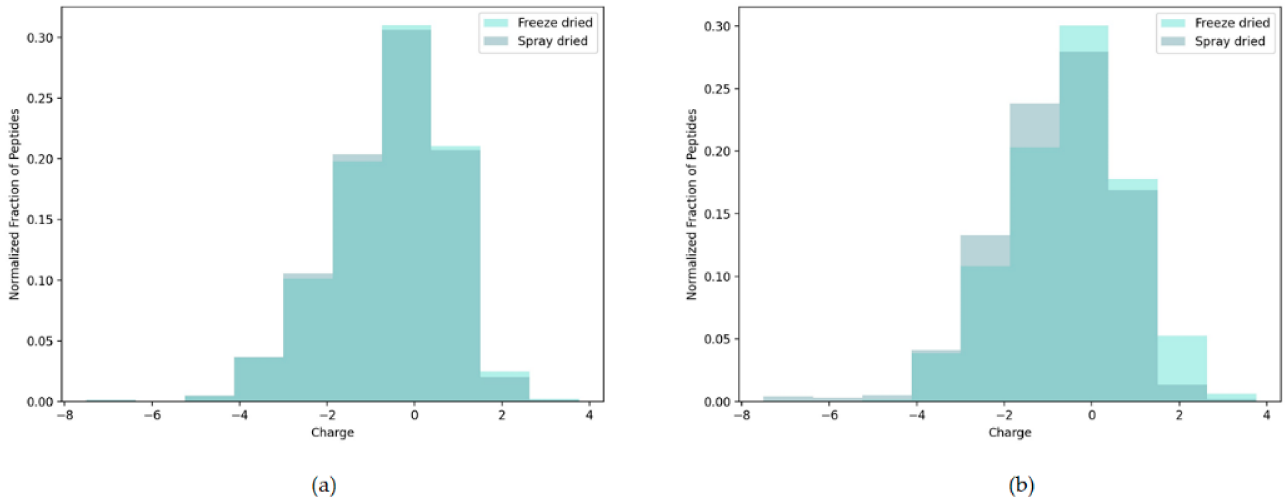

Figure 4. Peptide distribution according to charge in FD and SD samples of rice NPN. The peptide profile as determined by LC-MS/MS in a histogram representation of rice NPN samples; peptide distribution is according to charge in (**a**) peptides present in full samples or (**b**) peptides exclusive in either FD (teal bar) or SD (blue bar) samples. All data is normalised for count.

3.2.4. Peptide Hydrophobicity

To assess peptide hydrophobicity, the percentage of hydrophobic residues (Gly, Ile, Val, Leu, Phe, Cys, Met, Ala, and Trp) within each peptide was calculated. The results are displayed in Figure 5a for the total peptides associated with each process and separately in Figure 5b for peptides exclusive to each drying process. While there were little differences across total peptide profiles (Figure 5a), when peptides exclusive to either drying process were considered, a more diverse distribution of hydrophobic residues was observed (Figure 5b). Median hydrophobicity values for FD and SD samples were 42.45% and 42.85%, respectively, when the global peptide profiles were assessed. Comparatively, when peptides were exclusive to either drying process, the median hydrophobicity values were 42.48% and 44.22% for FD and SD samples, respectively.

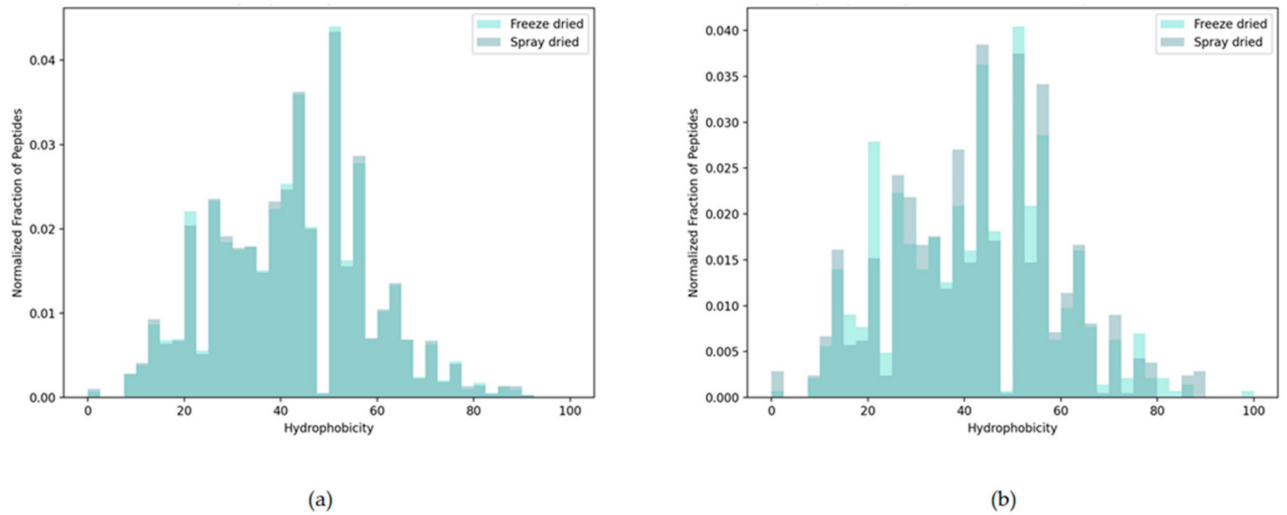

Figure 5. Peptide distribution according to hydrophobicity in FD and SD samples of rice NPN. The peptide profile as determined by LC-MS/MS in a histogram representation of rice NPN samples, peptide distribution is according to hydrophobicity, calculated from % of hydrophobic residues, in (**a**) peptides present in full samples or (**b**) peptides exclusive in either FD (teal bar) or SD (blue bar) samples. All data is normalised for count.

3.3. Key Bioactive Peptide Retention

Seven constituent bioactive peptides have been characterised for rice NPN (Table 1). All peptides were validated in silico for affinity to the TNF-α receptor and/or in vitro with anti-inflammatory activity [26,27]. We used MS/MS ion spectra to confirm if these peptides of interest were present in rice NPN following each different drying processes (Table 1). Here, all 7 bioactive peptides were present in SD samples, and 6 were detected in the FD samples. Peptide FYNEGDAPVVAL was not detected in all samples but an elongated version, with an additional Trp on N terminus, was detected.

Table 1. Key peptides within FD and SD samples.

Peptide Sequence	Bioactivity	FD	SD
TVFDGVLRPGQL	Anti-Inflammatory	Yes	Yes
FYNEGDAPVVAL [+]	Anti-Inflammatory	Yes	Yes
IYGPDTGVDYKDNQMR	Anti-Inflammatory	Yes	Yes
GYYGEQQQQPGMTR	Anti-Inflammatory	Yes	Yes
IDGYDTPVEGR	Anti-Inflammatory	Yes	Yes
NGVLRPGQL	Anti-Inflammatory	Yes	Yes
SEEGYYGEQQQQPGMTR	Anti-Inflammatory	Yes	Yes

[+] Elongated (1 additional N-terminal amino acid).

3.4. Predicted Anti-Inflammatory Activity

The rice NPN detailed in the present study comprised of thousands of peptides, making it difficult to ascribe functionality to seven peptides alone (Supplementary Tables S1 and S2). To assess the retention of predicted functionality, all peptides observed in SD samples and FD samples were combined, respectively, and assessed, using machine learning, to predict the percentage of constituent peptides in FD and SD samples of rice NPN with potential anti-inflammatory bioactivity. The predictor was based on reducing TNF-α, with an accuracy of 85%. In Figure 6, a total of 63.18% of predicted anti-inflammatory peptides (AIPs) were common to both SD and FD samples. Of the total predicted AIPs, 11.76% of peptides were unique to FD samples and 25.05% were unique to SD samples.

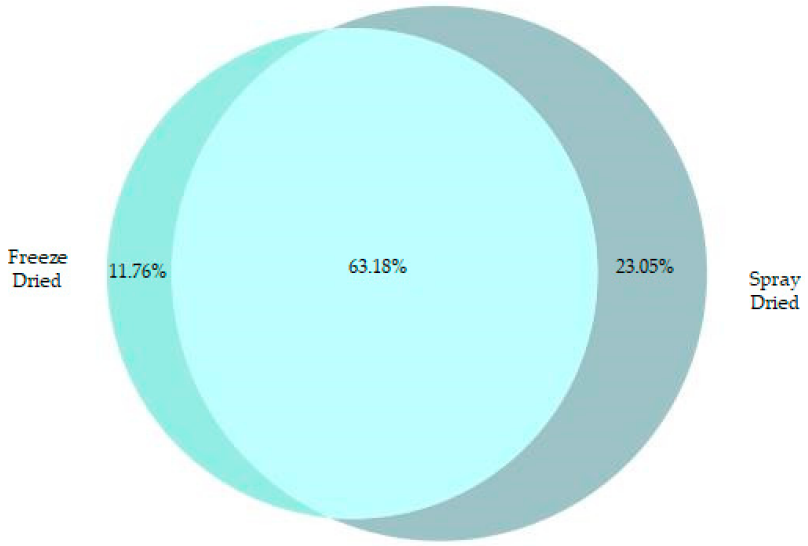

Figure 6. Predicted anti-inflammatory peptides in SD and FD samples. Venn diagram illustrating the percentage of anti-inflammatory peptides common to SD and FD samples (turquoise), unique to SD (blue) samples and unique to FD samples (teal) as a total of all predicted anti-inflammatory peptides.

4. Discussion

Food product processing is the major driving force behind loss of active compounds, health and nutritional benefits present in the original, unprocessed starting material [9,10]. We focus here on differences in drying processes, as the parameters around drying can have a significant effect on end-product quality [8]. We examined whether commonly employed drying processes could affect the overall composition and activity of food peptides.

In this study, we show the effects of drying processes, namely SD and FD, on the peptide content of a previously validated anti-inflammatory functional ingredient [25,26]. A similar peptidomic and bioinformatic approach to peptide content was undertaken when comparing human and bovine milk, whereby peptide count differences were observed across the major proteins in each species [29]. However, to the best of the authors' knowledge, this is the first time this type of bioinformatic approach has been utilised to investigate the peptide differences due to drying processes within a functional ingredient.

Published literature has previously examined the effects of SD and FD techniques on physical characteristics. Here, differences in the physical attributes between FD and SD match previously reported findings in regards to colour, which was observed to be slightly darker in the SD sample [30]. However, there is little published literature documenting the peptidomic response to drying technique. Riveria del Rio et al. (2020) reported that SD plant protein isolates underwent heat induced protein aggregation with increasing drying temperature [2]. The lower number of peptides in SD preparations reported in the present study may be a similar result of heat-induced aggregation. Interestingly, the authors concluded that heating during SD disrupted particle structure, increasing the number of smaller and better digestible particles [2]. Notably, larger particles typically associated with FD preparations allows for better flowability through reduced particle surface area interaction [31] and larger mean particle diameter in FD rice protein isolates have been previously reported [32], consistent with the differences observed in the present study. From a whole protein perspective, Tang et al. (2013) did not report any significant differences in protein content for porcine placenta hydrolysates prepared either by SD or FD [33]. Additionally, Blaise et al. (2017) did not see a difference in protein content across plant proteins spray dried with increasing inlet temperatures [34]. Similarly, previous research did not determine any differences in protein profile as determined by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS PAGE) in rice protein isolates subjected to either SD or FD [32]. However, it is important to consider that these assessments were made on whole proteins using standard protein determination and/or SDS-PAGE techniques. The findings reported within the present study are derived from an in-depth peptidomic and bioinformatic analysis and consider both peptide characterisation and distribution, an approach which has not previously been reported in literature.

By examining the amino acid distribution across FD and SD samples, peptide stability can potentially be assessed. It was hypothesised that due to the thermal conditions associated with SD, peptides identified within these preparations would be composed of amino acids associated with more stable and robust peptides. Peptides with proposed longer half-lives have been reported to be abundant in Ala, Asp, Glu, Gln, Gly, Ser, and Thr [35]. Similarly, in thermophilic proteins, a number of amino acids have been shown to be statistically prevalent above others, including Ala, Arg, Glu, and Thr [36]. Within the present study, no such preference was apparent for these specific amino acids when whole samples of both drying processes were compared, with only minor differences in amino acid distribution between SD and FD preparations. When peptides exclusive to each process were compared, only significant increases in Asp, His, and Lys were detected in FD preparations ($p < 0.05$). Interestingly, Schmitz-Schug et al. (2013) noted that Lys was particularly susceptible to damage during SD, agreeing with the findings of the present study, but that those losses could be mitigated by optimising SD parameters [8]. A standard enzymatic hydrolysate process, as described in the method section, typically includes an enzyme deactivation step at 70–90 °C. This may have contributed some selective thermal pressure in this case, but no additional changes in amino acid distribution were observed

at the elevated temperatures (175 °C) associated with standard SD. Huang et al. (2019) documented the amino acid distribution of different rice cultivars grown over successive years in different growing climates [37]. While the relative amino acid abundance was proportionate to the nitrogen content, the relative amino acid distribution did not change considerably in response to climate temperature. Amino acid distribution was similar to the findings of the present study, bar a reverse trend in the proportion of Ala, Arg, and Asp, amino acids often associated with more stable peptides. As observed in the present study, Gln was the most prevalent amino acid, which has been reported to infer additional protein flexibility, lending to increased stability in extremophilic proteins [38].

Physicochemical characteristics such as differences in biochemical, physical, and structural characteristics have been previously reported for materials subjected to either SD or FD [2,31,32]. When investigating the peptide characteristics associated with each drying process, only minor differences were observed, except for a slight trend towards longer, higher molecular weight, and more positively charged peptides within FD preparations. This may be attributed to the hypothesis that FD is a comparatively gentler drying process to SD. This was further supported by a higher number of peptides found in FD preparations. This trend could be an important consideration for retention of longer bioactive peptides, whereby drying processes could influence their presence in the final dried preparation.

Characteristics such as solubility and certain bioactivities can be attributed to peptide charge. A well-known characteristic of proteins is that negative surface charge is strongly correlated with increased solubility. While protein solubility increases with increasing positive or negative charge, Kramer et al. (2012) concluded that more negatively charged proteins tend to display increased solubility [39]. Our results suggest a similar behaviour with peptides, in some cases carrying a -8 charge. Findings indicated that peptides exclusive to the SD preparations had a greater proportion of negatively charged peptides by comparison, which may be a contributing factor to the increased solubility typically associated with SD powders. Peptides exclusive to the FD preparation carried a slightly less negative charge of -0.24, which when combined with the increased peptide length, molecular weight, and physical characteristics, may further elucidate the reduced solubility associated with lyophilised powders.

Only minor differences in peptide hydrophobicity were observed in the present study, for SD and FD rice protein preparations. Zhao et al. (2013) noted no difference in surface hydrophobicity as determined by fluorescence intensity for rice protein [32]. Similarly, Wang et al. (2020) reported a relatively high but significantly similar sensory bitterness in soybean hydrolysates, which had been either FD or SD, a trait that is typically associated with an increased number of exposed hydrophobic residues [40]. However, taking a peptidomic and bioinformatics approach to elucidate differences in hydrophobicity, a 1.74% increase in hydrophobicity was predicted with SD, which may correlate to the slight but non-significant increases in hydrophobic amino acids (Ala, Met, Phe, Pro, Try, and Val) in peptides exclusive to the SD preparations (Figure 2b). Following the similarity in both groups' physicochemical characteristics, it would be of interest to examine the raw ingredient, pre-hydrolysis, and investigate the effects of temperature during SD and FD processing on the peptide profile and compare to hydrolysed samples.

Following the greater count observed in FD samples but lack of variability in overall physicochemical parameters, we evaluated the effect of drying technique on bioactive peptide retention. It is important to note that the hydrolysate presented here, rice NPN, was previously shown to reduce TNF-α secretion in vitro [25]. This was followed by an in human trial to assess efficacy where it was shown that rice NPN effectively reduced circulating TNF-α in a small aged but otherwise healthy population. Cytokine levels were not only reduced but also showed a concomitant benefit to physical strength. Using machine learning, specific anti-inflammatory bioactive peptides have been previously described for rice NPN [25,26]. These peptides were present in rice NPN, but when synthetically produced, effectively reduced TNF-α in THP-1 cells. As part of the larger rice NPN matrix, the digestibility of these peptides was assessed and found to survive

simulated gastric digestion in vitro [26]. To assess if drying processes effected the presence of these key peptides, we used ion spectra to identify these key actives in FD and SD samples. Importantly, all of the key bioactive peptides, +/− 1 amino acid, were identified in both FD and SD samples, increasing the possibility of retaining functionality regardless of drying processes.

Recently, machine learning approaches have presented the opportunity to identify key peptides within a complex proteome with predicted bioactivity [25,41]. Instead of traditional bioinformatic screening approaches to find bioactive ingredients, the use of machine learning to identify proteomes of interest to produce functional ingredients containing known bioactivities, has proven to be a successful approach, although this approach has yet to be widely adopted [25,26,42]. As key bioactive peptides for rice NPN were identified in FD and SD samples, we decided to investigate if machine learning techniques could be utilised to assess retention of overall functionality within an ingredient following different drying processes. Specifically, if drying techniques would affect the overall predicted anti-inflammatory functionality of other constituent peptides within rice NPN. To investigate the effects of drying processes on bioactivity, we employed a machine learning approach, whereby a training set was built to identify possible AIPs within FD and SD samples. We examined predicted AIPs common to each sample and additionally we examined the bioactivity of unique peptides to either FD samples or SD samples. We found that the majority of predicted peptides were present in both FD and SD samples. Interestingly, previous functional differences have been reported for a porcine placenta hydrolysate, whereby the drying method had a significant effect on antioxidant potential with FD preparations displaying higher antioxidant activity, which the authors attributed to the increased powder solubility [33]. Conversely, Wang et al. (2020) reported no functional antioxidant differences between FD or SD soybean hydrolysates [20], similar to a separate study on the antioxidant capacity of hydrolysed egg white protein [23]. However, further in vitro testing is required to validate the functionality of SD and FD samples used in this study. Rice NPN, produced using SD, has been previously validated for anti-inflammatory activity [25] and as a large proportion of AIPs are common to both SD and FD, it is predicted that the functionality of FD preparation is comparable to the previously determined SD preparation.

5. Conclusions

The present study utilised peptidomic and machine learning techniques to better understand how the drying approach for a plant protein hydrolysate could potentially influence peptide profile and its subsequent predicted functionality. While there is a vast array of published literature detailing the effects of drying on the structural and functional characteristics of powdered food products, the present study elucidates the effects of drying approach on the peptidomic profile of the hydrolysate, a consideration which has gone largely un-investigated in current literature. We observed similar peptide profiles with both spray and freeze-dried processes, suggesting stability of the peptide profile within the hydrolysed material. As peptides and their constituent amino acids have a direct effect on final product characteristics, understanding how the manufacturing process affects end peptide profile, and the subsequent effects on functionality, will be an important and necessary consideration in processing of food and ingredient production going forward. Using machine learning, we observed that validated constituent bioactive peptides and the majority of relevant functional peptides are retained with either drying process, which represents an opportunity for an in silico approach to assessing functionality retention following processing.

Supplementary Materials: The following are available online at https://www.mdpi.com/2227-9717/9/3/425/s1, Table S1: List of unique peptides and their physicochemical characteristics identified in Freeze−dried samples, Table S2: List of unique peptides and their physicochemical characteristics identified in Spray−dried samples.

Author Contributions: Conceptualization, S.C. and M.G.; methodology, S.C., S.T. and S.O.; software, S.O. and A.A.; validation, S.C. and S.O.; formal analysis, S.O., A.W. and A.A.; investigation, S.O., S.T. and M.G.; data curation, A.A. and S.O.; writing—original draft preparation, S.C., A.W., M.G., S.T., T.P. and B.D.; writing—review and editing, M.G., S.T., A.W. and N.K; visualization, S.O., A.A. and A.W.; supervision, N.K.; project administration, A.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in Supplementary Tables S1 and S2.

Acknowledgments: The authors would like to thank Brendan Molloy (Nuritas Ltd.) and Jean Manguy (Nuritas Ltd.) for preparation of samples and useful discussions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Tunick, M. Whey protein production and utilization. In *Whey Processing, Functionality and Health Benefits*; Onwulata, C., Huth, P.J., Eds.; Blackwell Publishing: Oxford, UK; IFT Press: Ames, IA, USA, 2008; pp. 169–184.
2. Rivera del Rio, A.; Opazo-Navarrete, M.; Cepero-Betancourt, Y.; Tabilo-Munizaga, G.; Boom, R.M.; Janssen, A.E.M. Heat-induced changes in microstructure of spray-dried plant protein isolates and its implications on in vitro gastric digestion. *LWT* **2020**, *118*, 108795. [CrossRef]
3. Östbring, K.; Sjöholm, I.; Rayner, M.; Erlanson-Albertsson, C. Effects of storage conditions on degradation of chlorophyll and emulsifying capacity of thylakoid powders produced by different drying methods. *Foods* **2020**, *9*, 669. [CrossRef]
4. Silva, J.; Freixo, R.; Gibbs, P.; Teixeira, P. Spray-drying for the production of dried cultures. *Int. J. Dairy Technol.* **2011**, *64*, 321–335. [CrossRef]
5. Barbosa, J.; Borges, S.; Amorim, M.; Pereira, M.J.; Oliveira, A.; Pintado, M.E.; Teixeira, P. Comparison of spray drying, freeze drying and convective hot air drying for the production of a probiotic orange powder. *J. Funct. Foods* **2015**, *17*, 340–351. [CrossRef]
6. Chong, S.Y.; Wong, C.W. Production of Spray-Dried Sapodilla (Manilkara zapota) Powder from Enzyme-Aided Liquefied Puree. *J. Food Process. Preserv.* **2015**, *39*, 2604–2611. [CrossRef]
7. Schmitz-Schug, I.; Foerst, P.; Kulozik, U. Impact of the spray drying conditions and residence time distribution on lysine loss in spray dried infant formula. *Dairy Sci. Technol.* **2013**, *93*, 443–462. [CrossRef]
8. Li, X.; Feng, Y.; Ting, S.; Jiang, J.; Liu, Y. Effect of processing conditions on the physiochemical properties and nutrients retention of spray-dried microcapsules using mixed protein system. *CYTA J. Food* **2019**, *17*, 25–35. [CrossRef]
9. Haque, M.A.; Adhikari, B. Proteins in Spray Drying Process. *Handb. Ind. Dry.* **2015**, *33*, 971–983.
10. Castro-Albarrán, J.; Aguilar-Uscanga, B.R.; Calon, F.; St-Amour, I.; Solís-Pacheco, J.; Saucier, L.; Ratti, C. Spray and freeze drying of human milk on the retention of immunoglobulins (IgA, IgG, IgM). *Dry. Technol.* **2016**, *34*, 1801–1809. [CrossRef]
11. Ma, J.J.; Mao, X.Y.; Wang, Q.; Yang, S.; Zhang, D.; Chen, S.W.; Li, Y.H. Effect of spray drying and freeze drying on the immunomodulatory activity, bitter taste and hygroscopicity of hydrolysate derived from whey protein concentrate. *LWT Food Sci. Technol.* **2014**, *56*, 296–302. [CrossRef]
12. Wang, W. Lyophilization and development of solid protein pharmaceuticals. *Int. J. Pharm.* **2000**, *203*, 1–60. [CrossRef]
13. Fang, Z.; Bhandari, B. *Spray Drying, Freeze Drying and Related Processes for Food Ingredient and Nutraceutical Encapsulation*; Woodhead Publishing: Cambridge, UK, 2012; pp. 73–109.
14. Li-Chan, E.C.Y. Bioactive peptides and protein hydrolysates: Research trends and challenges for application as nutraceuticals and functional food ingredients. *Curr. Opin. Food Sci.* **2015**, *1*, 28–37. [CrossRef]
15. Cicero, A.F.G.; Fogacci, F.; Colletti, A. Potential role of bioactive peptides in prevention and treatment of chronic diseases: A narrative review. *Br. J. Pharmacol.* **2017**, *174*, 1378–1394. [CrossRef] [PubMed]
16. Möller, N.P.; Scholz-Ahrens, K.E.; Roos, N.; Schrezenmeir, J. Bioactive peptides and proteins from foods: Indication for health effects. *Eur. J. Nutr.* **2008**, *47*, 171–182. [CrossRef]
17. Udenigwe, C.C. Mohan Aishwarya Mechanisms of food protein-derived antihypertensive peptides other than ACE inhibition. *J. Funct. Foods* **2014**, *8*, 45–52. [CrossRef]
18. Zhang, S.S.; Han, L.W.; Shi, Y.P.; Li, X.B.; Zhang, X.M.; Hou, H.R.; Lin, H.W.; Liu, K.C. Two novel multi-functional peptides from meat and visceral mass of marine snail Neptunea arthritica cumingii and their activities in vitro and in vivo. *Mar. Drugs* **2018**, *16*, 473. [CrossRef] [PubMed]
19. Samantha, S.; Bruna, A.; Martin, A.; Fabio, B.; Sandro, A.; Aline, R. Drying by spray drying in the food industry: Micro-encapsulation, process parameters and main carriers used. *Afr. J. Food Sci.* **2015**, *9*, 462–470. [CrossRef]
20. Wang, H.; Tong, X.; Yuan, Y.; Peng, X.; Zhang, Q.; Zhang, S.; Xie, C.; Zhang, X.; Yan, S.; Xu, J.; et al. Effect of Spray-Drying and Freeze-Drying on the Properties of Soybean Hydrolysates. *J. Chem.* **2020**, *2020*, 9201457. [CrossRef]

21. Kanwate, B.W.; Ballari, R.V.; Kudre, T.G. Influence of spray-drying, freeze-drying and vacuum-drying on physicochemical and functional properties of gelatin from Labeo rohita swim bladder. *Int. J. Biol. Macromol.* **2019**, *121*, 135–141. [CrossRef]
22. Chen, C.; Chi, Y.J.; Xu, W. Comparisons on the Functional Properties and Antioxidant Activity of Spray-Dried and Freeze-Dried Egg White Protein Hydrolysate. *Food Bioprocess Technol.* **2012**, *5*, 2342–2352. [CrossRef]
23. Udenigwe, C.C.; Aluko, R.E. Food Protein-Derived Bioactive Peptides: Production, Processing, and Potential Health Benefits. *J. Food Sci.* **2012**, *77*, R11–R24. [CrossRef]
24. Kennedy, K.; Cal, R.; Casey, R.; Lopez, C.; Adelfio, A.; Molloy, B.; Wall, A.M.; Holton, T.A.; Khaldi, N. The anti-ageing effects of a natural peptide discovered by Artificial Intelligence. *Int. J. Cosmet. Sci.* **2020**, *42*, 388–398. [CrossRef] [PubMed]
25. Kennedy, K.; Keogh, B.; Lopez, C.; Adelfio, A.; Molloy, B.; Kerr, A.; Wall, A.M.; Jalowicki, G.; Holton, T.A.; Khaldi, N. An Artificial Intelligence Characterized Functional Ingredient, Derived from Rice, Inhibits TNF-α and Significantly Improves Physical Strength in an Inflammaging Population. *Foods* **2020**, *9*, 1147. [CrossRef] [PubMed]
26. Rein, D.; Ternes, P.; Demin, R.; Gierke, J.; Helgason, T.; Schön, C. Artificial intelligence identified peptides modulate inflammation in healthy adults. *Food Funct.* **2019**, *10*, 6030–6041. [CrossRef] [PubMed]
27. Corrochano, A.R.; Cal, R.; Kennedy, K.; Wall, A.; Murphy, N.; Trajkovic, S.; O'Callaghan, S.; Adelfio, A.; Khaldi, N. Characterising the efficacy and bioavailability of bioactive peptides identified for attenuating muscle atrophy within a Vicia faba-derived functional ingredient. *Curr. Res. Food Sci.* **2021**. in review.
28. Wickham, H. *Use R! ggplot2—Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2009; ISBN 9780387938363.
29. Lopez, C.; Adelfio, A.; Wall, A.M.; Molloy, B.; Holton, T.A.; Khaldi, N. Human milk and infant formulae: Peptide differences and the opportunity to address the functional gap. *Curr. Res. Food Sci.* **2020**. [CrossRef] [PubMed]
30. Caparino, O.A.; Tang, J.; Nindo, C.I.; Sablani, S.S.; Powers, J.R.; Fellman, J.K. Effect of drying methods on the physical properties and microstructures of mango (Philippine 'Carabao' var.) powder. *J. Food Eng.* **2012**, *111*, 135–148. [CrossRef]
31. Saifullah, M.; Yusof, Y.A.; Chin, N.L.; Aziz, M.G. Physicochemical and flow properties of fruit powder and their effect on the dissolution of fast dissolving fruit powder tablets. *Powder Technol.* **2016**, *301*, 396–404. [CrossRef]
32. Zhao, Q.; Xiong, H.; Selomulya, C.; Chen, X.D.; Huang, S.; Ruan, X.; Zhou, Q.; Sun, W. Effects of Spray Drying and Freeze Drying on the Properties of Protein Isolate from Rice Dreg Protein. *Food Bioprocess Technol.* **2013**, *6*, 1759–1769. [CrossRef]
33. Tang, W.L.; Zhang, M.; Adhikari, B.; Mujumdar, A.S. Effects of Preparation and Drying Methods on the Antioxidant Activity of Enzymatically Hydrolyzed Porcine Placenta Hydrolysates. *Dry. Technol.* **2013**, *31*, 1600–1610. [CrossRef]
34. Blaise, K.; Nicolas, N.Y.; Clémence, B.; Richard, K. Influence of spray-drying temperature on physicochemical and functional properties of protein isolates of three leguminous plants (Canavalia ensiformis, Vigna unguiculata and Glycine max) from Cameroon. *Cogent Chem.* **2017**, *3*. [CrossRef]
35. Sharma, A.; Singla, D.; Rashid, M.; Raghava, G.P.S. Designing of peptides with desired half-life in intestine-like environment. *BMC Bioinform.* **2014**, *15*, 1–8. [CrossRef]
36. Yakimov, A.P.; Afanaseva, A.S.; Khodorkovskiy, M.A.; Petukhov, M.G. Design of Stable a-Helical Peptides and Thermostable Proteins in Biotechnology and Biomedicine. *Acta Nat.* **2016**, *8*, 70–81. [CrossRef]
37. Huang, M.; Zhang, H.; Zhao, C.; Chen, G.; Zou, Y. Amino acid content in rice grains is affected by high temperature during the early grain-filling period. *Sci Rep.* **2019**, *9*, 2700. [CrossRef] [PubMed]
38. Panja, A.S.; Maiti, S.; Bandyopadhyay, B. Protein stability governed by its structural plasticity is inferred by physicochemical factors and salt bridges. *Sci. Rep.* **2020**, *10*, 1822. [CrossRef] [PubMed]
39. Kramer, R.M.; Shende, V.R.; Motl, N.; Pace, C.N.; Scholtz, J.M. Toward a molecular understanding of protein solubility: Increased negative surface charge correlates with increased solubility. *Biophys. J.* **2012**, *102*, 1907–1915. [CrossRef]
40. Wang, L.; Ding, L.; Du, Z.; Liu, J. Effects of hydrophobicity and molecular weight on the transport permeability of oligopeptides across Caco-2 cell monolayers. *J. Food Biochem.* **2020**, *44*, 1–8. [CrossRef] [PubMed]
41. Kennedy, K.; Cal, R.; Casey, R.; Holton, T.; Adelfio, A.; Wall, A.; Khaldi, N. pep_35E7UW, a natural peptide with cutaneous anti-ageing effects discovered within the Oryza sativa proteome through machine learning. *J. Dermatol. Cosmetol.* **2020**, *4*, 109–116. [CrossRef]
42. Cal, R.; Davis, H.; Kerr, A.; Wall, A.; Molloy, B.; Chauhan, S.; Trajkovic, S.; Holyer, I.; Adelfio, A.; Khaldi, N. Preclinical Evaluation of a Food-Derived Functional Ingredient to Address Skeletal Muscle Atrophy. *Nutrients* **2020**, *12*, 2274. [CrossRef] [PubMed]