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Validation of a paper-disk approach to facilitate the sensory evaluation of bitterness in dairy protein hydrolysates from a newly developed food-grade fractionation system

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Abstract

Casein-hydrolysates (NaCaH) are desirable functional ingredients, but their bitterness impedes usage in foods. This study sought to validate a paper-disk approach to help evaluate bitterness in NaCaHs and to develop a food-grade approach to separate a NaCaH into distinct fractions, which could be evaluated by a sensory panel. Membrane filtration generated <0.2-μm and <3-kDa permeates. Further fractionation of the <3-kDa permeate by flash-chromatography generated four fractions using ethanol (EtOH) concentrations of 5, 10, 30 and 50%. As some fractions were poorly soluble in water, the fractions were resolubilzed in EtOH and impregnated into paper-disks for sensory evaluation. Bitterness differences observed in the membrane fractions using this sensory evaluation approach reflected those observed for the same fractions presented as a liquid. The flash-chromatography fractions increased in bitterness with an increase in hydrophobicity, except for the 50% EtOH fraction which had little bitterness. Amino acid analysis of the fractions showed enrichment of different essential amino acids in both the bitter and less bitter fractions.

Practical Applications—The developed food-grade fractionation system, allowed for a simple and reasonably scaled approach to separating a NaCaH, into physicochemically different fractions that could be evaluated by a sensory panel. The method of sensory evaluation used in this study, in which NaCaH samples are impregnated into paper-disks, provided potential solutions for issues such as sample insolubility and limited quantities of sample. As the impregnated paper-disk samples were dehydrated, their long storage life could also be suitable for sensory evaluations distributed by mail for large consumer studies. The research, in this study, allowed for a greater understanding of the physicochemical basis for bitterness in this NaCaH. As some essential amino acids were enriched in the less bitter fractions, selective removal of bitter fractions could allow for the incorporation of the less bitter NaCaH fractions into food products for added nutritional value,

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without negatively impacting sensory properties. There is potential for this approach to be applied to other food ingredients with undesirable tastes, such as polyphenols.

Keywords

hydrolysates; bitter; fractionation; paper-disk; sensory

Introduction

There is now a greater focus on the use of functional foods for the prevention of nutrition-related diseases (Bigliardi and Galati 2013). For this reason much attention has been focused on dairy protein hydrolysates (DPHs) which often contain peptides with health benefits, including anti-inflammatory, antioxidant, antimicrobial, antihypertensive effects (Hernández-Ledesma *et al.* 2014) and insulin secretion (Brennan *et al.* 2015). Within the parent protein the peptides are not active, however, during enzymatic hydrolysis, proteolysis or fermentation of dairy products they are released and become active (Mohanty *et al.* 2015).

There are, however, major challenges hindering the incorporation of DPHs into food products, as they often have an unpleasant taste. A sensory study on the development of a sensory lexicon for characterizing DPHs identified bitterness as the strongest taste attribute in casein hydrolysates (NaCaH) (Newman *et al.* 2014b). Whey protein hydrolysates (WPH) were also bitter, but their flavor profile attributes also included “wet dog”, “cooked milk” and “vanilla” notes, as well as “potato/brothy” and “malty” flavors (Leksrisompong *et al.* 2010). While bitterness in foods such as coffee, beer and certain cheeses can be a desirable attribute, it is generally deemed an undesirable attribute for most food products. Consumer acceptance of a product is vital and the taste often outweighs other factors such as the health benefits of the food (Verbeke 2006). Therefore, in order for DPHs to be marketable, this challenge of poor taste must be overcome; in doing so, the bioactivity must not be compromised, which is a difficult task.

In order to overcome this challenge, a greater understanding of bitterness in DPHs is needed. Studies have investigated the bitterness of synthesized peptides and reported links between low molecular weight, hydrophobic peptides and increased bitterness (Ney 1979), as well as the positioning of amino acids within the peptide chain (Ishibashi *et al.* 1987a, b; Ishibashi *et al.* 1988). However, the bitterness of DPHs is much more complicated as they contain hydrolyzed proteins, so hundreds or thousands of peptides may be present in the DPH, in combination with free amino acids, ash and traces of fat. A recent study (Newman *et al.* 2014a) showed that bitterness in DPHs was best described in a statistical model that considered both the hydrophobicity (determined by reverse phase (RP)-high performance liquid chromatography (HPLC)) and size (determined by size exclusion chromatography (SEC)-HPLC) of the peptides present.

Studies have employed a “sensomics” or sensory-directed approach to concentrate and ultimately isolate bitter peptides from foods, such as cheeses (Toelstede and Hofmann 2008; Karametsi *et al.* 2014) or WPHs (Liu *et al.* 2013). The sensory evaluation of fractions generated from these “sensomics” schemes can be a challenge. For the most part these studies used analytical grade fractionation systems, in which multiple fractionations with

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fraction pooling was needed, especially since each fractionation step further reduces the remaining sample quantity. As previous studies employed a non-food grade fractionation approach, the fractions were evaluated in liquid form using a "sip and spit" method of sensory evaluation (Toelstede and Hofmann 2008; Liu, Jiang and Peterson 2013). Fractions generated from a RP-fractionation scheme (such as RP-HPLC or solid phase extraction (SPE)) in which peptides are eluted at a high percentage of organic solvent (such as acetonitrile (ACN), methanol (MeOH) or ethanol (EtOH)) could lead to issues in trying to resolubilise the samples in water for sensory evaluation. Therefore, a suitable method for the sensory evaluation of such samples is needed. A paper disk approach could provide a potential solution for this problem, as samples could be resolubilised in food-grade EtOH and impregnated into the disks; the EtOH is then evaporated from the disks and the bitterness of the samples is evaluated. The paper disk approach has mainly been used to detect the bitterness of 6-*n*-propylthiouracil (PROP), a single bitter compound, (Zhao *et al.* 2003; Baranowski *et al.* 2011; Feeney *et al.* 2014) and has never been used before for more complex foods, such as a DPH. However, there are many other potential advantages of this paper disk approach including dealing with issues associated with limited quantities of expensive samples, generated from fractionation schemes or new product development.

These fractionation schemes were not food-grade, as toxic solvents such as ACN, MeOH, trifluoroacetic acid (TFA), formic acid and ammonium acetate were used to fractionate peptides (Toelstede and Hofmann 2008; Liu *et al.* 2013; Karametsi *et al.* 2014). Although safety precautions can be taken to ensure samples generated by such schemes are safe for sensory evaluation, methods for fractionations that are designed to be fully food-grade are needed. Craig-Petsinger (1992) created a non-sequential food-grade peptide fractionation method for a chymosin-digested casein hydrolysate using solid-phase extraction (SPE), low-pressure chromatography and preparative RP-HPLC, using potable EtOH to elute fractions for a sensory panel to taste. Geisenhoff (2009) fractionated a soy protein hydrolysate using a food-grade gel filtration approach. However, multiple fractionations were needed in both studies and small sample quantities were reported (Geisenhoff 2009) as an issue, which proved difficult to overcome. A relatively fast, simple and reasonably scaled fractionation approach combined with a suitable method of sensory evaluation to screen DPHs for bitterness is needed in order to gain a greater understanding of the major influencers of bitterness in DPHs.

The aims of the present study were to validate the paper disk method, for the sensory evaluation of bitterness in a NaCaH. In doing so, due to the complexity of the peptides present in the NaCH, both in terms of size and hydrophobicity, there was a need to separate the NaCaH into physicochemically different fractions which vary less in terms of size and hydrophobicity. Therefore this study also aimed to create a food-grade fractionation system to separate a bioactive NaCaH. This NaCaH was chosen as it is shown to increase insulin secretion and alter glycemic management in mammals (Brennan *et al.* 2015) but its intense bitterness hinders its potential to be sold in the marketplace. The study also aimed to characterize the amino acid profiles of fractions to help determine the nutritional value of the fractions and whether selective removal of bitter fractions, while maintaining the nutritional value of the NaCaH, is possible.

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Materials and Methods

Protein Sample

A bitter bioactive NaCaH (Brennan *et al.* 2015) was provided by a research partner (Moorepark Technology Ltd., Fermoy, Co. Cork). The NaCaH was stored at room temperature (20 °C) and was used for this study less than a year after its production.

Chemicals

All solvents used to fractionate the NaCaH for the sensory panel were food grade: EtOH (200 proof, absolute ACS/USP grade; Aper Alcohol & Chemical Co: Shelbyville, KY, USA) and CH_3COOH (Sigma-Aldrich, St. Louis, MO, USA). All sensory analysis standards were food grade: sucrose (C&H Sugar Crockett), potassium aluminum sulfate (McCormick), sodium chloride (Morton Salt), citric acid (EMD Millipore, Billerica, MA, USA) and caffeine (USP, Fisher Scientific, NJ, USA). For analytical HPLC, HPLC-grade acetonitrile (ACN), trifluoroacetic acid (TFA) and protein standards (β-casein, β-lactoglobulin, α-lactalbumin, cytochrome C, insulin, insulin β-chain (oxidized), uridine and sodium azide) were obtained from Sigma-Aldrich (Ireland).

Generation of NaCaH Fractions

Membrane Fractionation—The NaCaH was first fractionated on the basis of size, generating two membrane fractions: a <0.2-μm permeate and a <3-kDa permeate. To generate the <0.2-μm permeate fraction, 20 g of NaCaH were solubilized in 1 L of distilled water on a stirplate for 1 h at room temperature and then applied to a 0.2-μm polyethersulfone Vivaflow 200 membrane (Sartorius Stedim Ireland Limited, Dublin, Ireland) using a Millipore peristaltic pump (Millipore Technology, Bedford, MA, USA) at a pressure of ~2.5 bar and temperature of ~15 °C. When the volume of >0.2-μm retentate reached 200 mL (approximately a five-fold concentration), 800 mL of deionized water were added to the >0.2-μm retentate for diafiltration against the same 0.2-μm membrane; the retentate volume was again reduced to 200 mL. The process of generating ~1.8 L of the <0.2-μm permeate took ~2 h.

A larger quantity of the <3-kDa permeate was generated using a pilot-scale tangential membrane filtration system (Model L, GEA Filtration, Hudson, WI, USA). Three kilograms of NaCaH were solubilized in 150 L of distilled water using constant recirculation and impeller agitation through the membrane filtration system for 70 min at 40 °C. The solubilized NaCaH was processed first using a spiral-wound 0.2-μm 2.5″ × 40″ polyvinylidene difluoride membrane (model no: 2540M; GEA Process Engineering Inc., Hudson, WI, USA) using a 6-L/min feed flow at 37 °C and 1 bar transmembrane pressure. The permeate (<0.2-μm) was applied to a spiral-wound 3-kDa polyethersulfone 2.5″ × 40″ membrane (model no: 2540F; GEA Process Engineering Inc., Hudson, WI, USA) at 28 °C, using the same feed flow rate (6-L/min) at 8 bar transmembrane pressure. A DOW/Filmtec thin film composite 2.5″ × 40″ reverse osmosis membrane (part no: 101312; GEA Process Engineering Inc. Hudson, WI, USA) was subsequently used to concentrate 90 L of <3-kDa permeate to 9 L. This process was carried out at 42 °C and 15 bar using a feed flow rate of

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6-L/min. The concentrated <3-kDa permeate was subsequently freeze-dried (Virtis, model no: 50SRC).

Flash Chromatography Fractionation—The <3-kDa permeate was further fractionated by flash chromatography. Two grams of freeze-dried sample were solubilized (2% w/v) in 5% EtOH, 0.1% CH_3COOH in nanopure water using a stirplate, for 40 min at room temperature. Solubilizing in 5% EtOH was required to prevent a phase collapse of the column during sample loading. The solubilized <3-kDa permeate was fractionated using a Combiflash RF200 UV/Vis system (Teledyne ISCO, Lincoln, NE, USA) fitted with a Redisep Rf, 100 g C18 Gold column. The stepwise gradient used two solvents: solvent A, 1% EtOH, 0.1% CH_3COOH in nanopure water; and solvent B, 99.9% EtOH, 0.1% CH_3COOH. Eluting peptides were detected at 280 and 205 nm. A 60-mL/min flow rate was used for sample loading and peptide elution. To avoid loss of the small amount of sample that the pump could not aspirate (due to physical limitations), an additional 1 L of 5% EtOH, 0.1% CH_3COOH in nanopure water was added to the sample flask towards the end of the sample loading, and this solution was loaded onto the column. A stepwise gradient was then used to elute the peptides at increasing concentrations of EtOH: 5, 10, 30, and 50% EtOH (all with 0.1% CH_3COOH). This choice of EtOH concentration for each step gradient was chosen on the elution profile of the unfractionated sample in the analytical RP-HPLC (Figure 4). Each step of the gradient was maintained until the UV trace returned to the baseline before the next step commenced, to ensure that all peptides soluble in each fraction had been eluted.

The solvents in the eluted fractions (ranging from ~ 0.5 L to 1 L) were concentrated using a rotary evaporator (model no: R-3000; Buchler instruments, Switzerland) at 50 °C. The internal rotavap pressure was initially held at -50.8 cmHg and slowly increased to -68.6 cmHg (a slow increase in pressure was necessary to avoid bumping). The temperature of the fluid (EtOH) circulating the evaporation zone was set at ~5 °C. The time required to dry the samples using the rotavap was dependent on the percentage of EtOH in the fractions (those with more EtOH evaporated more rapidly). Generally, fraction drying required 1.5–3 h. Residual solvent was removed via vacuum centrifugation (miVac, Genecav Inc, Gardiner, NY, USA) at 36 °C. A flow diagram of the overall NaCaH fractionation process is presented in Figure 1.

Characterization of Samples

SEC-HPLC Analysis—To assess the molecular weight distributions of the parent hydrolysate and membrane fractions (<0.2-μm permeate and <3-kDa permeate), SEC-HPLC analysis was performed using an Agilent 1200 HPLC system with a variable wavelength detector (Agilent Technologies, Palo Alto, CA, USA). The samples were resolubilized at 2 g/L in nanopure water. Twenty microliters of sample (40 μg) were loaded onto a 4 × 3 mm gel filtration chromatography guard column and then onto a Yarra 3-μm SEC-2000 (300 mm × 7.8 mm) gel filtration chromatography analytical column (Phenomenex, Cheshire, UK). Chromatography was performed with a 20-min isocratic elution at 30 °C and a flowrate of 0.8 mL/min. Peaks were detected at 214 nm. The mobile phase was 45% ACN, 0.1% TFA (w/w) in nanopure water. A calibration curve for molecular weight based on retention time

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was constructed for peptides within the range of 42–24,000 Da. The standards used were β-casein (24,000 Da), β-lactoglobulin (18,400 Da), α-lactalbumin (14,175 Da), cytochrome C (12,384 Da), insulin (5,808 Da), insulin β-chain (oxidized) (3,496 Da), uridine (244 Da) and sodium azide (42 Da). Comparison with the elution times of the standards allowed determination of estimated molecular size distributions of the samples. The absorbance percentage was calculated by: ((absorbance*100)/sum of the absorbance)), and the molecular weight was calculated from the standard curve.

RP-HPLC Analysis—RP-HPLC was employed to characterize the parent NaCaH and the <3-kDa permeate to gain a greater understanding of the hydrophobicity profiles and complexity of these samples. This information helped determine an appropriate EtOH step gradient for the RP flash chromatography fractionation of the <3-kDa permeate in order to clearly fractionate the sample. RP-HPLC was also employed to assess the separation efficiency of the RP flash chromatography. RP-HPLC analysis was performed on the fractions (5, 10, 30 and 50% EtOH) with the Agilent 1200 HPLC system. The fractions were resolubilized at a concentration of 20 μg/μL at the concentration of EtOH at which they were eluted from the RP flash column. Aliquots containing 100 μg of the fractions were injected onto a C18 wide-pore guard column and then onto a Zorbax Eclipse XB-C18 analytical column (4.6 mm × 150 mm, particle size 5 μm, Phenomenex, Cheshire, UK). The binary solvent system was made up of solvent A (90% ACN, 0.095% TFA (w/w) in nanopure water) and solvent B (nanopure water containing 0.1% TFA (w/w)). The separations were performed at 30 °C by gradient elution at a flow rate of 1 mL/min. The gradient used was 5% ACN for 0–5 min, 5 to 50% ACN from 5–60 min, 65% ACN from 60–65 min, and 5% ACN from 65–70 min. The eluting peaks were detected at 214 nm.

Total amino acid analysis—Total amino acid analysis was performed on the NaCaH and each of the flash chromatography fractions (5, 10, 30 and 50% EtOH) using an Hitachi L-8800 amino acid analyzer (Hitachi High Technologies America, Dallas, TX 75261, USA). The fractions were solubilized at 1 mg/mL in 2% ACN and 0.1% TFA by sonification (Ultrasonic processor Q55, Qsonica, Newtown, CT) at 60% amplitude, for 1.5 min. Aliquots (50μL) of the solubilized 5 and 10% EtOH flash chromatography fractions and 200μL of the solubilized NaCaH <3kDa permeate, NaCaH and 30 & 50% EtOH flash fractions were hydrolyzed with 200 μL of 6 N HCl/ 1% phenol at 110 °C for 24 h and then dried using a speed vac (Labconco). NorLeu dilution buffer was added as an internal standard. The samples were vortexed and centrifuged (Eppendorf) for 5 min at 16 g-force. The sample (50 μL) was then loaded onto a strong cation exchange column (Transgenomic). Cysteine, methionine and tryptophan could not be calculated as they are destroyed during the hydrolysis of the amino acid analysis. The analysis was unable to distinguish between glutamine and glutamic acid, so these were combined as Glx, in the results. Similarly, asparagine and aspartic acid are also combined as Alx. Evaluation of amino acid composition of the fractions was calculated based on the weight percentage of amino acids relative to the parent NaCaH, to identify the amino acids enriched in the fractions. The weight percentage of amino acids relative to the parent NaCaH was calculated by: (weight % of amino acids in the flash fraction / weight % of amino acids in the NaCaH)*100.

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Preparation of Samples for Sensory Testing—In order to test the validity of filter paper disks as a method of sensory evaluation of hydrolysates, the membrane samples (<0.2-μm permeate and <3-kDa permeate) were presented to the sensory panel in two different forms—in liquid form (resolubilized in water) and also impregnated into filter paper disks. To avoid a learning effect, half the panel evaluated the liquid samples first, and the other half evaluated the sample-impregnated filter paper disks first. The flash chromatography fractions were evaluated using paper disks only.

Preparation of the Membrane Fractions in Liquid Form—The dried membrane fractions (<0.2-μm permeate and <3-kDa permeate) and NaCaH were rehydrated with bottled water (Arrowhead Mountain Spring Water, USA) at a 2% (w/w) concentration and mixed for 40 min, at room temperature, using a stirplate. Five-milliliter samples were placed in 1-oz polystyrene cups (Solo Foodservice) coded with random three-digit numbers and served to panelists in individual sensory booths under a red light to avoid visual bias. The samples were prepared on the same day of sensory evaluation and all samples were served at room temperature.

Preparation of the Membrane Fractions Impregnated into Paper Disks—The panel members were also presented with the NaCaH and its membrane fractions impregnated into paper disks. The samples were prepared as described above and sample aliquots (50 μL) were pipetted into the center of 1.6-cm filter paper disks (Whatman quantitative filter paper, ashless, Grade 40) and allowed to dry at room temperature for ~1 h. A further 50 μL of sample was similarly applied onto the reverse side of the filter paper disks and the dehydration step was repeated. The paper-disk samples were also served in 1-oz polystyrene cups (Solo Foodservice) coded with random three-digit numbers. The paper disks were served to panelists under a white light as there were no differences in appearance between the disks. The fraction-impregnated paper disks were all prepared on the same day and stored in air-tight containers until needed for sensory evaluation. The paper disks were used for analysis within two weeks of preparation.

Preparation of the Flash Chromatography Fractions Impregnated into Paper Disks—As preliminary work showed that some of the flash chromatography fractions were too poorly soluble in water for sensory evaluation, the fractions were rehydrated at a 2% (w/w) concentration in the original percentage of EtOH:water (Arrowhead Mountain Spring Water, USA) in which they were eluted and sonicated for 15 min. The 0.1% (w/w) CH_3COOH used for fractionation was not used to resolubilize the samples for sensory testing, as its intense sourness could alter the taste of the paper disk-impregnated fraction. Aliquots (50 μL) of these flash chromatography fractions were impregnated into each side of the filter paper disks, using the same technique as described above. The unfractionated <3-kDa permeate and the parent hydrolysate (NaCaH) were also tested on filter paper disks within the same sensory evaluation of the flash chromatography fractions.

Sensory Analysis

Panel selection—The University of California at Davis Institutional Review Board granted approval to conduct sensory analysis. Panelists (n=17) were screened for their ability

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to distinguish the different basic tastes based on the following standards dissolved in potable water (Arrowhead Mountain Spring Water, USA): sweet, 1% (w/w) sucrose; salty, 0.2% (w/w) sodium chloride; astringent, 0.05% (w/w) potassium aluminum sulfate; sour, 0.03% (w/w) citric acid and bitter, 0.03% (w/w) caffeine (ISO8586 2012). Panelists were also screened on their ability to rank bitter solutions of caffeine at three concentrations: 0.08% (w/w), 0.15% (w/w) and 0.2% (w/w) (Meilgaard *et al.* 2006). As the ability to distinguish between samples of differing concentrations of bitterness was essential for the study, panelists who failed to correctly rank the bitter test solutions were excluded from the study. In conjunction with correctly ranking bitter solutions, panelists were also expected to correctly answer at least 80% of the basic taste test (sweet, salty, sour, bitter and astringent); however, if panelists could not distinguish between bitter and sour, they were also excluded from the study. A final group of 12 panelists (4 men and 8 women), were selected to evaluate the membrane fractions (<0.2-μm permeate and <3-kDa permeate) and flash chromatography fractions (5, 10, 30 and 50% EtOH).

Panel training—During three 1-h training sessions the panel primarily focused on one taste attribute: bitterness. The panel were presented with a DPH lexicon (Newman, O'Riordan, Jacquier and O'Sullivan 2014b) to gain a greater understanding of flavors, other than bitterness, that may also be present in DPHs. The panel were instructed to focus on bitterness alone and no other attributes. The panel were introduced to NaCaH-impregnated filter paper disks and practiced bitterness evaluations on paper disks by simple ranking tests of paper disks impregnated with different amounts (1, 2 and 3-mg) of NaCaH. Some panelists described the paper disks as exerting a drying effect, so various techniques such as evaluating the disks along with 5 mL of water were investigated. However, the panel decided no water should be taken with the disks, but instead to thoroughly rinse the mouth with water, served at room temperature, after each paper-disk evaluation.

Sensory Evaluation of Fractions—An unstructured line scale (10 cm) ranging from 'not bitter' to 'very bitter' was employed to rate the bitterness of the fractions. The bitterness ratings were carried out on a computer and panelists used a mouse to select the bitterness ratings of the fractions. For the liquid samples, the panelists were instructed to place the entire 5-mL sample into their mouth and swirl the sample around for at least 5 s before expectorating the sample and rating the bitterness intensity. For the samples impregnated into paper disks, panelists were instructed to place the disk onto their tongue for at least 5–7 s, wetting the disk with their saliva before removing the disk from their mouth and then rating the bitterness intensity of the samples on the line scale. Evaluations of the fractions were replicated three times. The membrane samples (three samples) were evaluated in a simultaneous multiple presentation with a 1-min break in between each repetition. The flash chromatography fractions (six samples–including the two control samples) were also evaluated in a simultaneous multiple presentation with at least a 30-min break in between each of the repetitions to avoid panel fatigue. The order of fractions within each of the replicates was randomized using a Latin square design. Panelists were requested not to smoke or drink/eat strong-flavored foods (e.g., caffeine-rich beverages and spicy foods) for 1 h prior to each sensory session. Panelists were provided water and unsalted crackers to

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cleanse their palates between samples. The sensory results were collected and compiled using Fizz version 2.45A (Biosystèmes, Couternon, France).

Statistical Analysis—All statistical analyses were performed using SPSS version 20 (IBM). The differences between the membrane fractions and test methods (liquid vs. paper) were assessed using a 2-way ANOVA with bitterness as the dependent variable, fractions and test method as independent variables and panelist, gender and replicate as covariates with *post hoc* least significant difference (LSD) calculations. The bitterness scores generated from the membrane fractionation were normally distributed. The bitterness values generated from flash chromatography were not normally distributed and were transformed using the Johnson equation from Minitab: $0.111520 + 0.359181 \times \text{Ln}((X + 0.0382809) / (10.0710 - X))$; where X = bitterness values.

Results and Discussion

The study aimed to validate a paper-disk approach for evaluating the bitterness of NaCaH fractions. This sensory method has great potential in dealing with issues such as poorly soluble samples and limited quantities of samples. In order to validate this paper-disk approach distinctively different NaCaH fractions must first be generated. The fractionation approaches previously employed to identify bitter peptides in foods such as cheeses (Toelstede and Hofmann 2008) and WPH (Liu, Jiang and Peterson 2013) were not food-grade and required multiple/pooled fractionations to generate enough sample for sensory evaluation. This can be a long and expensive process and from an industry perspective it may not be practical to identify the bitter peptides in every DPH produced. A faster approach to identifying the most bitter fractions of DPHs would be beneficial, allowing for the potential removal or targeted bitterness masking of such fractions. Therefore, another objective of this study was to create a relatively fast and food-grade fractionation approach that could be applied to screen DPHs for bitterness. To achieve this objective, we aimed to fractionate a NaCaH into fractions based on 1) molecular weight using membrane filtration, and 2) hydrophobicity using flash chromatography, and to also generate fractions in sufficient amounts for evaluation of bitterness by a sensory panel.

NaCaH Fractionation

1) Membrane fractionation—SEC-HPLC was carried out on the parent hydrolysate (NaCaH), the <0.2-μm permeate and the <3-kDa permeate to determine their molecular weight profiles (Figure 2). The SEC-HPLC chromatogram showed some peptides >3 kDa (~27%) were present in the <3-kDa permeate. Nongonierma *et al.,* (2013) also observed ~20% of the peptides in a <1-kDa NaCaH permeate had a molecular weight of 1–5 kDa. As peptides can have a globular or linear structure perhaps the shape of a peptide can have an effect on whether it is retained by a membrane. The loose tertiary structure of casein could allow for linear peptides to sinuously move through the ultrafiltration membrane in a way that would retain globular peptides of the same molecular weight (Revchuk and Suffet 2009). In this present study, the SEC chromatogram (Figure 2) of the absorbance percentage plotted against molecular weight showed the <3-kDa permeate had a higher concentration of smaller peptides and a lower concentration of larger peptides than the NaCaH and <0.2-μm

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permeate, demonstrating sufficient differences between the fractions for sensory evaluation and further fractionation by flash chromatography.

2) Flash chromatography fractionation—The large numbers of eluting peaks in the RP-HPLC chromatogram of the NaCaH (Figure 3) highlights the complexity of the starting sample and emphasizes the need for simplifying the hydrolysate to facilitate the understanding of bitterness in this NaCaH. RP-HPLC was also employed to assess the hydrophobicity profile of the <3-kDa permeate (Figure 4) and determine an appropriate step gradient for the flash chromatography fractionation. Despite having been fractionated already the <3-kDa permeate was almost as complex as the NaCaH (Figure 3) with many early eluting peaks (between 0–20 min) suggesting the presence of free amino acids or hydrophilic peptides, and also a lot of later eluting peaks (between 30–50 min) suggesting the presence of hydrophobic peptides. Therefore a step gradient of 5, 10, 30 and 50 % EtOH was chosen in attempt to obtain reasonably discrete peptide fractions (Figure 4).

During the flash chromatography fractionation, the flow of each elution in the step gradients were monitored until the UV absorbance returned to the baseline (see the elution profile, Figure 5) to ensure, in so far as possible, that all peptides soluble in each fraction were eluted. At a 60-mL/min flow rate, the 5% EtOH fraction eluted in 18 min (~1,080 mL of solvent); the 10% EtOH fraction eluted between ~18–32 min (~840 mL); the 30% EtOH fraction eluted between ~32–50 min (~1,080 mL) and the 50% EtOH eluted between 50–59 min (540 mL). The 5 and 30% EtOH fractions eluted as much broader peaks than the 10 and 50% EtOH fractions. Although the UV elution profile (Figure 5) of the flash chromatography fractions indicated a good separation, RP-HPLC was also carried out on the fractions to characterize its relative hydrophobicity and to verify that the fractions for sensory evaluation were substantially different (Figure 6). The 5% EtOH fraction was the most hydrophilic fraction, with all the peaks eluting during the first 20 min of the chromatogram. The majority of the 10 and 30% EtOH fractions eluted between 20 and 45 min. The most hydrophobic fraction (50% EtOH) eluted latest (after 40 min). Based on the RP-HPLC profiles, it appears that the flash chromatography produced good separation. While there are still many peaks present in each of the fractions, they are less complex than the original starting <3-kDa permeate. Although there is some overlap between the fractions the degree of overlap is relatively small suggesting that distinct different fractions were produced for sensory evaluation. To our knowledge this is the first report of a food-grade fractionation system whereby no multiple/pooled fractionations were needed and fractions were produced in sufficient quantities which were safe for sensory evaluation. This is therefore a relatively fast approach to fractionate DPHs into fractions which have different physicochemical characteristics in relation to molecular weight and hydrophobicity. Characterizing the fractions by RP-HPLC and sensory evaluation of such fractions may aid the understanding of the physicochemical characteristics of bitterness in DPHs.

Validation of Paper-disk Method

Two different methods of sensory evaluation were utilized for the sensory evaluation of the NaCaH and its membrane filtration sub-fractions: 1) 100 mg of sample in a 5 mL aqueous serving and 2) 2 mg of sample embedded onto filter paper disks. The bitterness scores of the

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samples presented on the paper disks were consistently lower than those of the liquid samples, however the differences were not significant (p=0.05) (Figure 7). This lower bitterness is most likely due to the lower sample amount delivered via the paper disks. Most critically, however, the significant differences in bitterness observed between the membrane fractions presented as liquids were also observed when the same samples were presented as paper disks, showing that this is a valid method for comparison of samples, despite the slightly lower taste intensity.

The paper disk approach has previously been used to evaluate reasonably pure preparations of individual compounds, such as PROP and sodium chloride (Zhao *et al.* 2003; Zhao and Tepper 2007; Baranowski *et al.* 2011; Feeney *et al.* 2014). The present study is the first application of paper disks for the sensory evaluation of more complex mixtures. In previous studies, the paper disks were immersed into PROP solutions and MeOH extractions were used to quantify the amount of PROP impregnated into the paper-disks (Zhao *et al.* 2003). However, in this present study a known amount of sample was aliquoted onto the disks. Only 50 μL of sample was aliquoted onto the disks to avoid an overflow of sample from the disks, which would have led to an increased variation of sample quantity impregnated into the paper-disks. An equal amount of sample was aliquoted onto both sides of the disks to again avoid any concentration differences or variation among the disks depending on which side of the paper-disk was placed onto the tongue.

There are a number of advantages of the paper disks over evaluating samples in liquid form using a "sip and spit" method. Firstly, only 72 mg of each fraction was required in total for a panel of 12 to evaluate the paper disk-impregnated fractions in triplicate, compared to the liquid samples which required a total of 3.6 g of sample; a 50 fold decrease in the amount of sample required. This is therefore an ideal method of sensory evaluation where sample quantity is limited. Using a paper-disk approach as a method of sensory evaluation may also be advantageous in overcoming issues associated with sample solubility, for example in the present study, the samples from flash chromatography were insoluble in water and needed to be solubilized in the percentage of EtOH in which they were eluted. As differing percentages of EtOH would influence the perceived bitterness of the fractions, applying samples to paper disks and evaporating the solvent presented a potential solution to this problem. A further advantage is that when sample quantity is limited it is often difficult to prepare samples fresh on each day of sensory evaluation (for example, in cases such as this present study), impregnating the samples into paper-disks allows for large numbers of samples to be prepared all on the same day which could then be stored in air-tight containers until they were needed. As samples are dehydrated and therefore relatively stable against microbial growth, this paper-disk approach of sensory evaluation could have great potential for sensory evaluations distributed by mail for large consumer studies, both nationally and worldwide (Feeney *et al.* 2014). There is potential for this paper disk approach to be applied to other foods with undesirable tastes such as polyphenols, which have strong bitterness and astringency sensory properties (Gonzalo-Diago *et al.* 2014). Many polyphenol extractions are carried out in EtOH or MeOH/water mixtures (Ajila *et al.* 2011) and therefore may also give rise to issues in resolublising these fractions in water for sensory evaluation so impregnating the polyphenol fractions into paper disks would be advantageous.

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Sensory Evaluation of Fractions

Evaluation of Bitterness in the Membrane Filtration Fractions—The NaCaH and its two membrane filtration sub-fractions, the <0.2-μm permeate and the <3-kDa permeate, were presented to a sensory panel who rated the bitterness of each sample. The <3-kDa permeate was deemed the most bitter fraction, followed by the <0.2-μm permeate and the NaCaH, respectively. These differences were all significant ($p<0.05$) (Figure 7). Although there was some overlap between the molecular weights of the membrane fractions (Figure 2), the <3-kDa permeate contained the highest concentration of lower molecular weight peptides which may contribute to its increased bitterness of that fraction. The increased bitterness of the <0.2-μm permeate compared to the NaCaH could suggest that the aggregates or material >0.2-μm present in the NaCaH were slightly masking the bitterness of that sample. These findings are consistent with previous reports by Leksrisompong *et al.,* (2010) between hydrolysates with a higher concentration of lower molecular weight peptides and increased bitterness. A statistical model, developed by Newman *et al.,* (2014a), determined SEC-HPLC to be a good indicator for DPH bitterness. A previous study on the fractionation of a WPH also identified a <3-kDa permeate fraction as the most bitter fraction compared with a larger molecular weight fraction (>3-kDa retentate) (Liu *et al.* 2013).

Evaluation of Bitterness in the Flash Chromatography Fractions—The sensory evaluation of the four flash chromatography fractions (5, 10, 30 and 50% EtOH), the parent hydrolysate (NaCaH) and the most bitter membrane sample (<3-kDa permeate) showed differences in bitterness intensities between the fractions (Figure 8). The 30% EtOH fraction was the most bitter, followed by the 10, 50 and 5% EtOH fractions. The 30% EtOH fraction was significantly ($p<0.05$) more bitter than all other samples, including the unfractionated NaCaH and the <3-kDa permeate. There were no significant differences in bitterness between the <3-kDa permeate and the 10% EtOH fraction; however, these fractions were significantly more bitter than the 5 and 50% EtOH fractions. The 50% EtOH fraction was significantly more bitter than the 5% EtOH fraction (Figure 8). The reproducibility of the paper disk method was again demonstrated when the same mean bitterness values were obtained for the NaCaH and <3-kDa permeate were obtained when evaluated with the flash chromatography fractions (Figure 8), as the previous sensory evaluation of the membrane fractions impregnated into paper disks (Figure 7).

A statistical model identified a combination of both SEC-HPLC and RP-HPLC as potential indicators for DPH bitterness (Newman *et al.* 2014a) so it was therefore important to include a fractionation step whereby fractions of different levels of hydrophobicity were also generated. The flash chromatography fractions increased in bitterness with increased hydrophobicity up to the 30% EtOH fraction, however a remarkably low level of bitterness was evident for the 50% EtOH fraction. As the very hydrophobic NaCaH fraction (50% EtOH) in the present study was derived from a <3-kDa permeate, it had a high concentration of lower molecular weight peptides. As both hydrophobicity and low molecular weight are considered important attributes for determining peptide bitterness (Ney 1979), the low bitterness score of this most hydrophobic fraction (50% EtOH fraction) was surprising. However, in a previous "sensomics" study (Toelstede and Hofmann 2008) in which gouda cheese was fractionated by SPE using a step gradient of 1–40% ACN (with 1% TFA) no

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bitterness was observed in the most hydrophobic fraction (40% ACN) and the slightly more hydrophilic fractions eluting at 10 and 30% ACN (with 1% TFA) were deemed the most bitter. Newman *et al.* (2014a) also observed a lack of bitterness in two very hydrophobic NaCaHs. The two NaCaHs characterized by Newman *et al.* (2014a) and the 50% EtOH flash chromatography fraction in the present study had similar hydrophobicities, as they were all eluted after 40 min in the RP-HPLC chromatogram (with nearly identical gradient conditions). The majority of the bitterness in the <3-kDa permeate (in this present study) was found in the flash chromatography fractions eluting between 20–45 min in the RP-HPLC chromatogram, with the more bitter peptides eluting between 30–45 min; very little bitterness was detected in the peptides eluting after 45 min. Although hydrophobicity does play an important role in peptide bitterness perhaps there may not be a direct relationship between DPH bitterness and hydrophobicity and a certain degree of hydrophilicity may be needed for bitterness to be elicited. The very hydrophobic nature and lower water-solubility of the 50% EtOH fraction's peptides compared with other fractions may mean that they have reduced solubility in saliva, leading to less interaction with the TRCs and a decreased perceived bitterness.

Amino acid analysis

Total amino acid analysis was performed on the parent hydrolysate (NaCaH) and each of the flash chromatography fractions (Figure 9). The analysis showed differences in the amino acid profiles of the fractions and in most cases, enrichment of certain essential amino acids (EAAs) in the bitter and less bitter fractions was observed. The isolation of reduced bitter NaCaH fractions, with enrichment of EAAs is of interest as EAAs are not naturally produced in the body but are necessary for good health.

The least bitter fraction (5% EtOH) was enriched in the EAAs lysine and histidine, by over 100% and 50%, respectively. Many EAAs, including lysine and histidine, are important for protein synthesis in mammals (Rasmussen *et al.* 2000; Tomé and Bos 2007). The 5% EtOH fraction was also enriched arginine which has been reported to enhance exercise performance (Sharma *et al.* 2016). The 50% EtOH fraction, which also lacks in bitterness, was enriched in different essential amino acids: threonine, valine, leucine and phenylalanine and the non-EAAs acids: proline and glycine. Enrichment of leucine in the diet can aid the reversal of the decline in muscle protein synthesis in elderly people (Katsanos *et al.* 2006). The most bitter fractions (30% and 10% EtOH) were enriched in proline, leucine, tyrosine and phenylalanine. Interestingly, phenylalanine and tyrosine can increase peptide bitterness (Ishibashi *et al.* 1987b), which may account for some of the bitterness in these fractions. The 10% EtOH fraction was also enriched in threonine and arginine, while the 30% EtOH fraction was highly enriched in glycine. The 10% and 30% EtOH fractions are therefore still beneficial for nutritional purposes but their intense bitter taste may be a deterrent for their incorporation into food products. Debittering (FitzGerald and O'Cuinn 2006), or encapsulation of just the bitter fractions would be cheaper than treating the whole hydrolysate. However, there is also great interest in selectively removing bitter fractions from NaCaHs (Soussan *et al.* 2016; Soussan and Marzorati 2016) to allow for the incorporation of less bitter NaCaHs, enriched in EAAs, into food products without negatively impacting the sensory properties of the food.

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Conclusion

Bitterness of DPHs is complex due to the diversity of the peptides present in DPHs, both in terms of size (from low to high molecular weight peptides) and hydrophobicity (from highly water soluble to non-water soluble peptides), therefore the causes of bitterness may well be specific for each DPH. From an industry point of view, it may not be practical nor indeed possible to identify the individual peptides responsible for bitterness in every DPH produced due to the high cost and time associated with this. The method in this present study is an ideal alternative as it is a food-grade, relatively fast and reasonably scaled approach for facilitating the understanding of the physicochemical characteristics most responsible for bitterness in DPHs. However evaluating the bitterness of such fractions can be a challenge. The novel application of impregnating NaCaH fractions into paper disks proved a suitable method for the sensory evaluation of samples available in limited quantities, and also for the NaCaH fractions which are poorly soluble in water. There are many opportunities where this food-grade fractionation approach combined with the paper-disk sensory evaluation method could be employed to help identify bitterness or other undesirable tastes in other complex food mixtures. The type of research in this study could, depending on the desired end use of the DPH, allow for the bitter fractions of the DPH could be potentially removed and, if desired, subsequently debittered or masked. The amino acid profiles of the flash chromatography fractions showed differences between the fractions and enrichment of certain EAAs, necessary for good health, in both the bitter and less bitter fractions. This could allow for the selective removal of bitter fractions from the NaCaH without compromising the levels of EAAs and nutritional value of the NaCaH.

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Abbreviations Used

DPH	dairy protein hydrolysates
NaCaH	sodium caseinate hydrolysate
WPH	whey protein hydrolysate
EAAs	essential amino acids
TRC	taste receptor cell
SEC	size-exclusion chromatography
RP	reverse-phase
HPLC	high performance liquid chromatography
EtOH	ethanol
SPE	solid phase extraction
ACN	acetonitrile
TFA	trifluoroacetic acid
PROP	6-*n*-propylthiouracil

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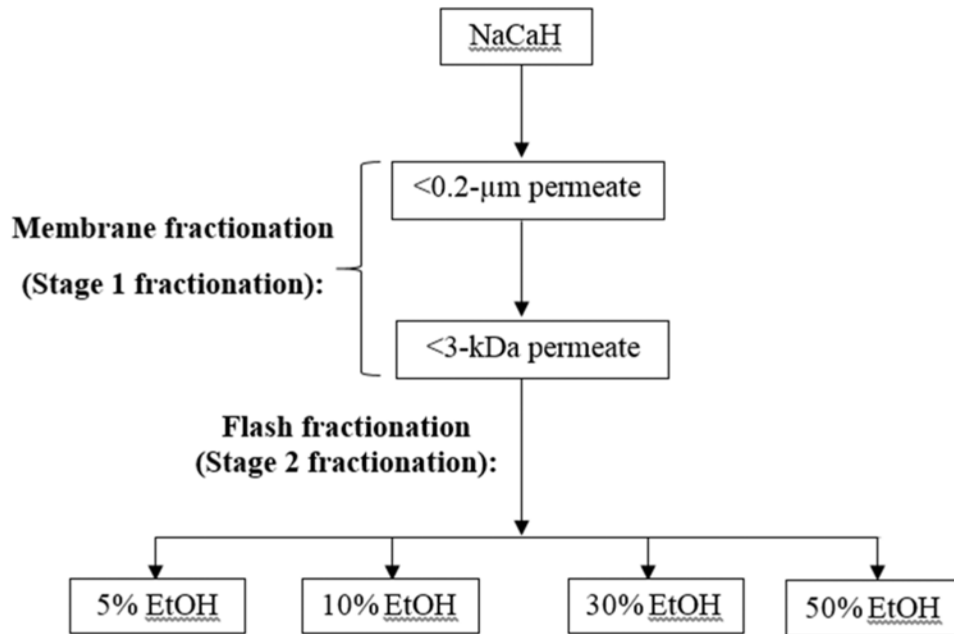

Figure 1.
Fractionation process for the NaCaH sample.

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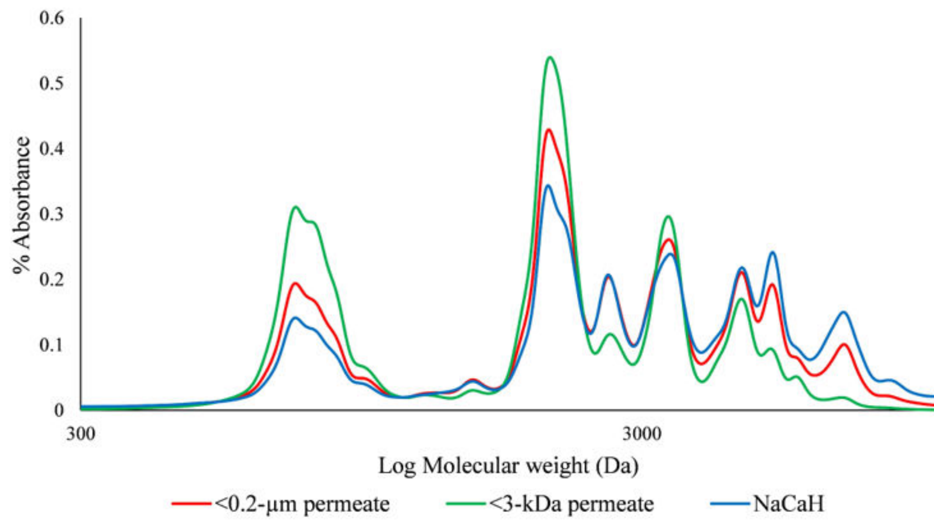

Figure 2.

SEC-HPLC chromatogram of the parent hydrolysate (NaCaH) and the membrane fractions: <0.2-µm permeate and <3-kDa permeate.

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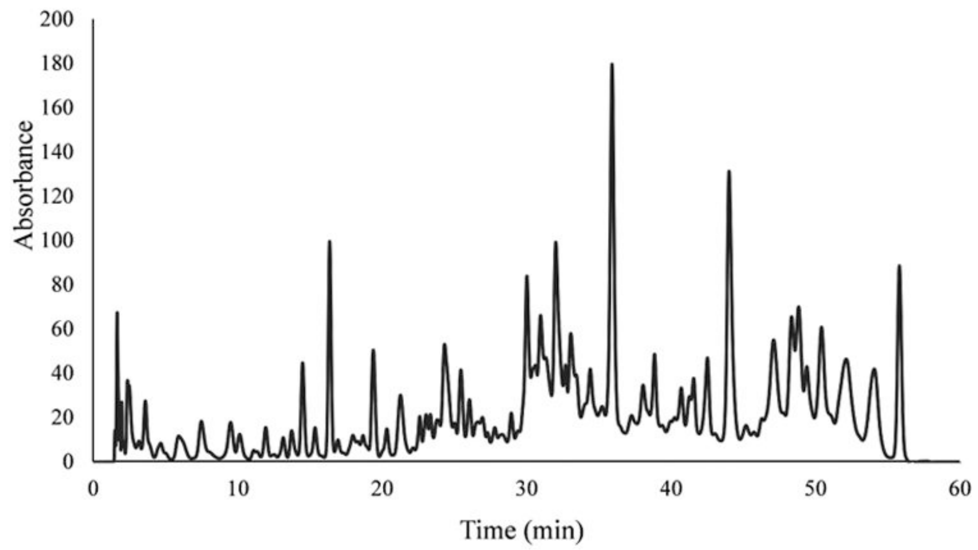

Figure 3.
RP-HPLC chromatogram of the parent hydrolysate (NaCaH).

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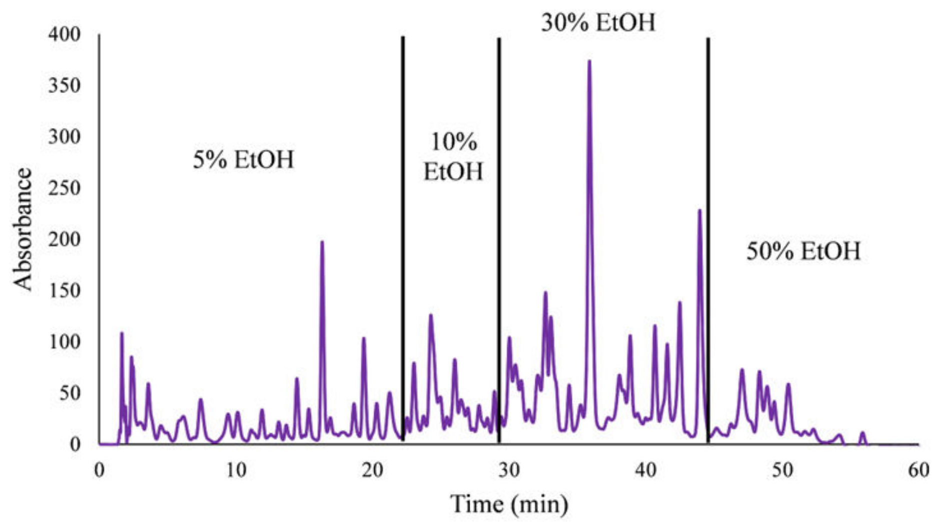

Figure 4.
RP-HPLC chromatogram of the <3-kDa permeate–the black lines indicate the flash chromatography separation of the <3-kDa permeate into the 0, 10, 30 and 50% EtOH fractions also shown in Figure 7.

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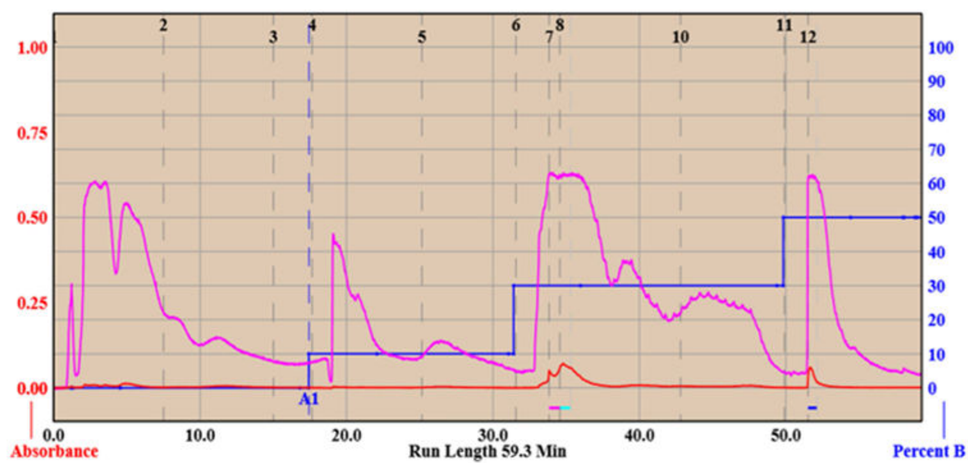

Figure 5.

Elution profiles of the flash chromatography fractions, with the 5% EtOH fraction eluting during the first 18 min of the run, the 10% EtOH fraction eluting between ~18–32 min, the 30% EtOH fraction eluting between ~32–50 min and finally the 50% EtOH eluting between 50–59 min. Absorbance was measured at 280nm (highlighted in red) and 205nm (highlighted in pink).

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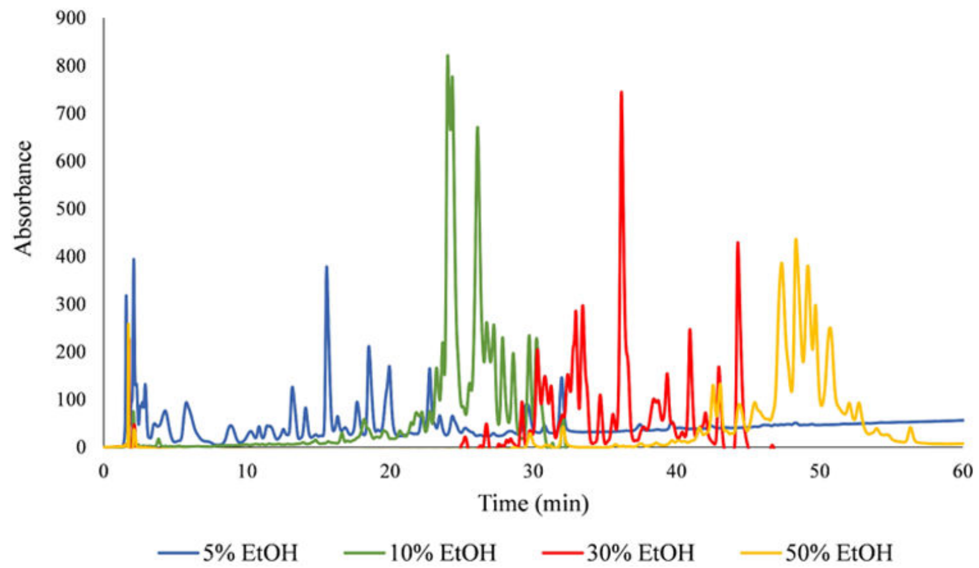

Figure 6.

RP-HPLC chromatogram of the <3-kDa permeate separated into 4 fractions by flash chromatography using 5, 10, 30 and 50% EtOH.

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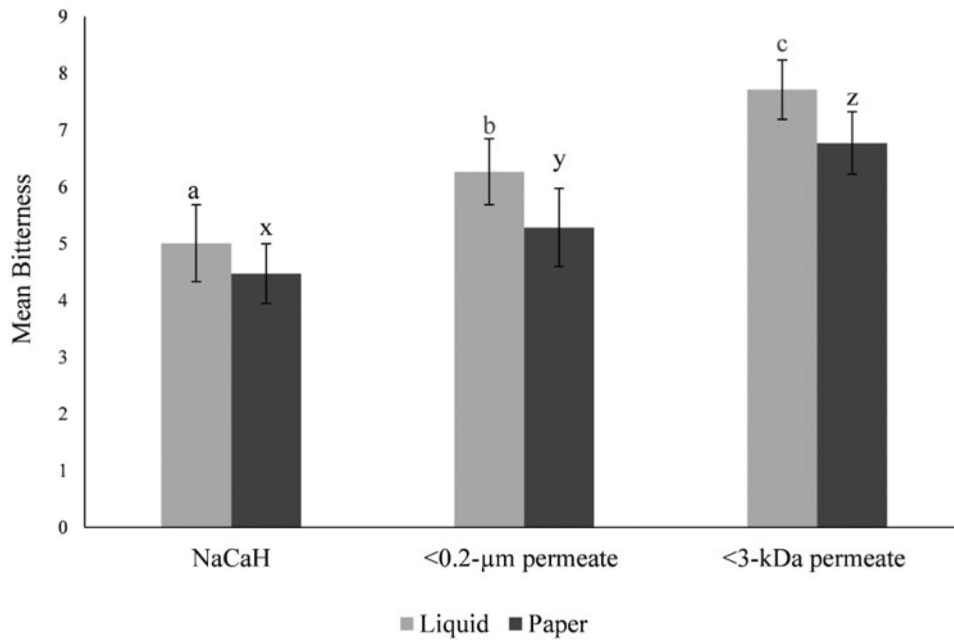

Figure 7.

Mean bitterness scores of the NaCaH and membrane fractions (<0.2-μm permeate and <3-kDa permeate) evaluated in liquid form and impregnated into the paper disks, with standard error bars. Bitterness intensities of samples were rated on a 10-cm unstructured line scale. Evaluations were replicated three times. Data presented on n=12 panelists. Means bearing different letters, a, b, c for the liquid samples, or x, y, z for the paper samples were significantly different ($p<0.05$).

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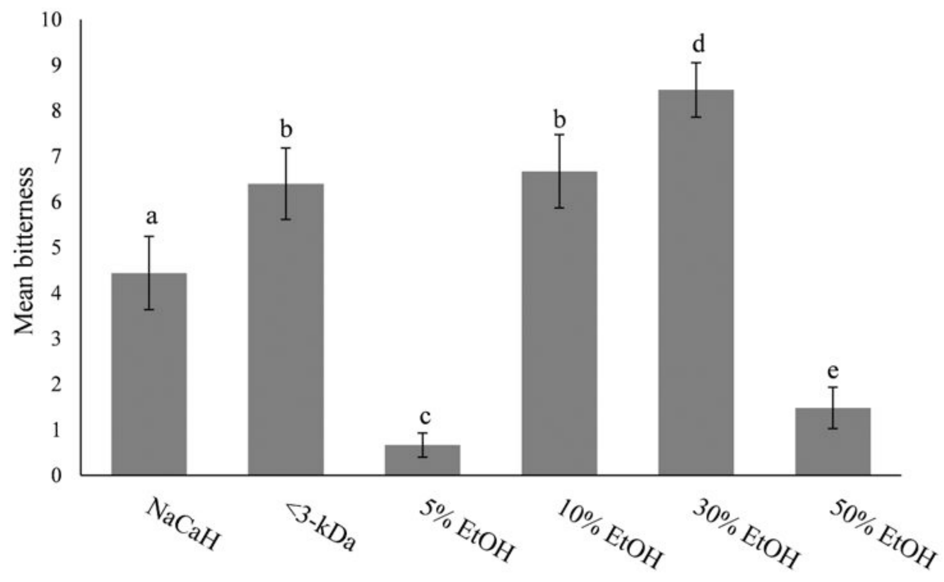

Figure 8.

Mean bitterness scores of flash chromatography fractions (5, 10, 30 and 50% EtOH), NaCaH and the <3-kDa permeate evaluated impregnated into the paper disks, with standard error bars. Bitterness intensities of samples were rated on a 10-cm unstructured line scale. Evaluations were replicated three times. Data presented on n=12 panelists. Means bearing different letters a, b, c, d, e were significantly different ($p<0.05$).

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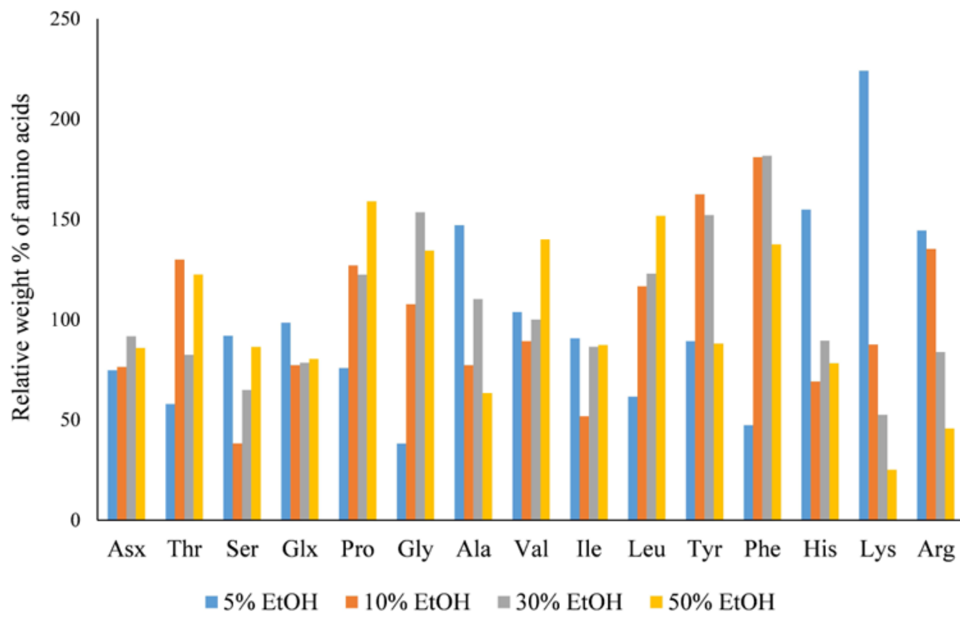

Figure 9.

Total amino acid analysis of the flash chromatography fractions (0, 10, 30 and 50% EtOH) expressed as weight % of amino acids relative to the parent hydrolysate (NaCaH).