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Article

Monitoring the Aroma Profile During the Production of a Pea Protein Isolate by Alkaline Solubilization Coupled with Isoelectric Precipitation

Yara L. Benavides-Paz,* Baraem P. Ismail, and Gary A. Reineccius

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ABSTRACT: The aroma profile was monitored during an optimized pH-extraction method (alkaline solubilization coupled with isoelectric precipitation) to produce pea protein isolates (PPIs). Samples were taken at different steps throughout the protein extraction. The aroma compounds were isolated from these samples using solvent-assistant flavor evaporation (SAFE) and were identified by gas chromatography-mass spectrometry-olfactometry (GC-MS-O) and gas chromatography-time-of-flight-mass spectrometry (GC-TOF-MS). A sensory evaluation of pea flour (PF) and PPI aqueous solutions was also conducted. From the instrumental analysis, 13 compounds were found to be likely the main contributors to the aroma profile of the samples examined. This hypothesis was also supported by the sensory data, which showed that the PF and PPI aqueous solutions were described with some of the odor descriptors used during the instrumental analysis. No new aroma compounds appear to be produced via the optimized pH-extraction and no existing compounds were completely removed from making a sensory contribution as determined by the olfactory analysis.

KEYWORDS: *pea flour, off-flavor, volatiles, pH-based extraction, SAFE*

INTRODUCTION

The interest in developing novel plant-based protein isolates has grown remarkably in recent years. This increased interest is mainly due to concerns over the welfare of animals and the environment as well as the perception among consumers that plant-based foods are a healthier source of protein when compared to animal products.[1] Although peas (*Pisum sativum* L.) have been studied for years,[2,3] it is not until recently that peas have been recognized as an important source of protein. Peas have a high protein content (20−30%), are not genetically modified, and have a low occurrence of allergenicity.[4] However, pea protein ingredients also possess strong beany, grassy, and green notes, which have limited their utilization in food applications.[5] A few studies have reported the effect of specific processing treatments on the flavor profile of peas. Azarnia et al. analyzed the effect of dry milling, cooking, and dehulling on volatile aroma compounds in peas. They found that the concentration levels of aldehydes increased after milling and dehulling and decreased after cooking the dehulled and whole seeds. Additionally, an increase in the ketones levels was observed after cooking the whole and dehulled seeds.[6] Trikusuma et al. characterized the changes in the volatile aroma composition of pea protein beverage during ultrahigh temperature (UHT) processing. The authors found that UHT processing significantly changed the sensory profile and aroma composition of the pea protein beverage.[7] The flavor profile of food may be influenced by heat treatment, water activity, pH, salts, and oxidation. These conditions often lead to the formation of aroma compounds via the Maillard reaction, oxidation, or fermentation.[8]

Another factor that may be extremely important in determining the aroma profile is food composition. Food components such as proteins, lipids, and carbohydrates can modify aroma perception due to specific interactions with aroma compounds. Unlike lipids and carbohydrates, proteins may possess very complex structures.[9] Proteins are known to interact with aroma compounds and these interactions are influenced by intrinsic factors such as the protein structure, amino acid profile, and nature of the aroma. Numerous studies on the binding of aroma compounds to leguminous proteins have been reported in the literature.[10,11] Additionally, extrinsic factors including temperature, pH, and ionic strength lead to conformational changes of the proteins, which can also impact the binding of aroma compounds. Wang and Arntfield studied the effect of pH and salts on the binding properties of aroma compounds to pea protein.[12] Similarly, in another study, Wang and Arntfield investigated the impact of heat treatment on the flavor binding of pea protein.[13]

Soybeans have traditionally been the dominant source of protein in plant-based foods. However, soy protein has some limitations. Soy protein is one of the "Big Eight" allergens and it is sourced from genetically modified (GM) crops.[14] To make

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pea protein more competitive with soy protein in the market, the functional properties of pea protein have to be improved. These properties as well as protein purity and yield may be improved by optimizing the method and conditions used during protein extraction. Maintaining the native protein structure during manufacturing may be extremely important as this structure tends to have better functionality compared to a denatured protein. Alkaline solubilization coupled with isoelectric precipitation is the most common method for manufacturing pea protein isolates. In preliminary work within our research group, pea protein extraction conditions were optimized to maximize protein purity and yield following alkaline solubilization coupled with isoelectric precipitation (pH-extraction).[15] Currently, there is a lack of knowledge regarding the effect of the processing conditions used during an optimized pH-extraction on the aroma profile of pea protein isolates. Therefore, the main purpose of this study was to identify aroma compounds at each step of the optimized pH-extraction process and monitor any changes in the aroma profile.

MATERIALS AND METHODS

Samples and Chemicals. Yellow field pea (*P. sativum* L.) flour was kindly supplied by AGT Foods (Regina, Canada). The flour was stored at room temperature in closed glass jars until analysis. Chemical standards of methyl hexanoate (99%), (Z)-4-heptenal (98%), (Z)-6-nonenal (95%), methional (98%), 1-octen-3-ol (95%), (E)-2-nonenal (97%), 3-methylbutanoic acid (isovaleric acid) (99%), hexanoic acid (99%), and maltol (98.5%) were purchased from Sigma-Aldrich (St. Louis, MO). 2-isobutyl-3-methoxypyrazine (IBMP) (97%) and (Z)-2-octanol (98%) were obtained from AstaTech (Bristol, PA) and (E)-2-octenoic acid (98%) was purchased from TCI America (Portland, OR). A homologous series of straight-chain alkanes (C_5−C_{27}) and anhydrous magnesium sulfate (99.5%) were purchased from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM) (GC Resolv) (99.9%) was obtained from Fisher Scientific (Fair Lawn, NJ).

Extraction of the Pea Protein Isolate by pH-Extraction and Sample Collection. The optimized alkaline solubilization/isoelectric precipitation process developed for pea protein extraction[15] is shown in Figure 1. The pea flour was first solubilized in deionized (DI) water, adjusted to pH 7.5 with concentrated NaOH (6.25 M) for 1 h at room temperature (23 °C), and agitated in a jacketed bulk tank equipped with a stirrer (Vektor Series, Lightnin, Rochester, NY). The solution was passed through a decanting centrifuge (Westfalia Separator AG, CA 220-01-30, Oelde, Germany) and clarified with a desludging centrifuge (Westfalia Separator AG, SB 7-06-076, Oelde, Germany) to remove insoluble materials, such as starch and fibers. The separated liquid containing protein was set aside. The insoluble material was resuspended in DI water and the pH of the solution was adjusted to 7.5 with NaOH (6.25 M). The solution was again agitated in a jacketed tank for 1 h and passed through a decanting centrifuge and desludging centrifuge. The combined liquid fractions were placed in a jacketed tank, the pH was adjusted to 4.5 with HCl (4 M) and agitated for 10 min. The solution was again passed through a decanting centrifuge and the precipitate (protein solution) was collected. The precipitate was then transferred to a jacketed tank with a mixer and resolubilized in DI water. Due to the duration of the extraction process (2 days), the sample had to be held overnight. The pH was adjusted to 3 with HCl (4 M) and held in a cold room (6−8 °C) to ensure no microbial growth and prevent protein denaturation.

The next day, the protein solution was neutralized (pH 7) and agitated for 1 h at room temperature (23 °C). The protein solution was then ultrafiltered (103−138 kPa inlet, 70−103 kPa outlet, PTI Advanced Filtration, PTI Technologies, St. Louis, MO) with tangential (cross) flow and a spiral wound membrane (3 kDa MWCO) and diafiltered to further concentrate the proteins. The high solids retentate was pasteurized by passing the solution through a high-temperature, short-time (HTST; 73 °C for 15 s) processing system (MicroThermics Electric Model 25HV Hybrid, 60−170 L/h, MicroThermics Inc., Raleigh, NC), followed by a two-stage homogenizer (Gaulin 125 L; 17,200 kPa, 230 L/h, Manton-Gaulin Mfg. Co. Inc., Everett, MA). The homogenized retentate was then spray-dried using an SPX Flow Anhydro Spray Dryer (9.5% TS, 180 °C inlets, 90 °C outlets, ca. 15 kg water evaporation per hour) with a wheel-type atomizer (24 500 rpm) (SPX Flow Inc., Charlotte, NC).

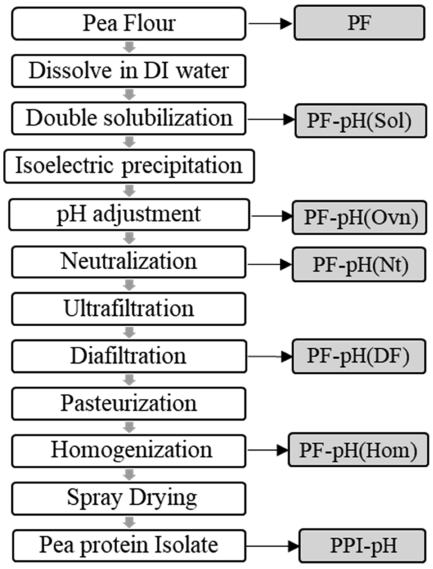

Figure 1. Optimized pea protein extraction by following alkaline solubilization with isoelectric precipitation. Sampling points are listed in the second column.

Samples for flavor analysis were collected at different processing steps where we expected that the process may alter the aroma profile (Figure 1) (i.e., pea flour (PF), after double solubilization (PF-pH(Sol)), after pH was adjusted to 3 and stored overnight (Ovn) (PF-pH(Ovn)), after neutralization (PF-pH(Nt)), after diafiltration (PF-pH(DF)), after homogenization (PF-pH(Hom)), and the final product or pea protein isolate (PPI-pH)). The samples were collected in glass jugs (3.8 L) and stored at −18 °C until further analysis.

Isolation of Volatile Aroma Compounds by Solvent-Assisted Flavor Evaporation (SAFE). Volatiles were extracted by SAFE following the protocol and parameters described by Benavides-Paz et al., without modifications.[16] Briefly, the amounts of the sample used in flavor extraction were 100.0 g for PF, 59.8 g for PF-pH(Sol), 281.3 g for PF-pH(Ovn), 20.1 g for PF-pH(Nt), 23.8 g for PF-pH(DF), 22.0 g for PF-pH(Hom), and 22.2 g for PPI-pH. For the extraction of dry samples (i.e., PF and PPI-pH), the sample and 250 mL of DCM were transferred into an Erlenmeyer flask. Methyl hexanoate solution (100 μL, 0.2 mg/mL DCM) was added as an internal standard (ISTD). The suspension was stirred for 1 h at room temperature (23 °C) and was then filtered to recover the DCM fraction (with extracted volatiles). The method was modified slightly to work with a liquid sample. For liquid samples (PF-pH(Sol), PF-pH(Ovn), PF-pH(Nt), PF-pH(DF), and PF-pH(Hom)), DCM was added to the noted sample, the slurry was stirred for an hour, the DCM (with extracted volatiles) was collected and set aside. The extracted pea slurry underwent a second solvent extraction process following the same process as in the first extraction. The pooled solvent fractions obtained were introduced into a SAFE apparatus. SAFE extraction was carried out at 45 °C under vacuum (1.4×10^{-5} mbar). The SAFE extract was then concentrated to 50 μL using a gentle stream of high-purity nitrogen.

Gas Chromatography-Mass Spectrometry-Olfactometry (GC-MS-O) Analysis. The GC-MS-O analysis was conducted following the procedure and parameters previously described by Benavides-Paz et al.[16] An Agilent 6890N gas chromatograph-5973

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Table 1. Volatile Aroma Compounds Extracted by SAFE and Identified by Instrumental Analysis in Samples Collected at Different Points During the Production of Pea Protein[a]

average RT (min)	aroma compound	calculated RI[c]	log P_{ow}[d]	steps[b]							identification[e]
				PF	PF-pH (Sol)	PF-pH (Ovn)	PF-pH (Nt)	PF-pH (DF)	PF-pH (Hom)	PPI-pH	
				Aldehydes							
3.70	pentanal	978	1.31	+	+	+	+	+	+	+	R, MS, O
5.63	hexanal	1079	1.78	+	+	+	+	+	+	+	R, MS, O
6.62	(E)-2-pentenal	1119	1.28	+	−	+	+	+	+	−	R, MS
7.05	(Z)-3-hexenal	1135	1.43	+	+	+	+	+	+	−	R, MS, O
8.29	heptanal	1177	2.44	+	+	+	+	+	+	+	R, MS, O
9.27	(E)-2-hexenal	1208	1.79	+	−	+	+	+	+	+	R, MS
10.13	(Z)-4-heptenal	1231	2.17	+	+	+	−	−	−	+	R, MS, S, O, TOF
11.83	octanal	1279	2.95	+	+	+	+	+	+	+	R, MS, O
12.99	(E)-2-heptenal	1310	2.30	+	+	+	+	+	+	+	R, MS, O
15.76	nonanal	1384	3.46	+	+	+	+	+	+	+	R, MS, O
16.89	(E)-2-octenal	1416	2.81	+	+	+	+	+	+	+	R, MS, O
17.27	(Z)-6-nonenal	1428	3.11	+	+	+	−	+	+	+	R, MS, S, O, TOF
17.70	methional	1441	0.44	+	+	+	+	+	+	+	R, MS, S, O, TOF
18.87	(E,E)-2,4-heptadienal	1477	1.89	+	+	+	+	+	+	+	R, MS
19.65	benzaldehyde	1502	1.48	+	+	+	+	+	+	+	R, MS
20.19	(E)-2-nonenal	1521	3.32	+	+	+	+	+	+	+	R, MS, S, O, TOF
24.42	(E, E)-2,4-nonadienal	1683	2.91	+	+	+	+	+	+	+	R, MS, O
25.94	(E,E)-2,4-decadienal	1749	3.18	+	−	+	−	−	−	+	R, MS,
27.12	tridecanal	1805	5.49	−	−	−	+	+	+	−	R, MS
33.46	hexadecanal	2121	7.03	−	−	−	−	+	+	−	R, MS
40.50	vanillin	2539	1.58	+	+	+	+	+	+	+	R, MS
				Alcohols							
4.71	2-methylbut-3-en-2-ol	1034	0.66	+	−	+	+	−	+	+	R, MS
7.65	1-penten-3-ol	1154	0.99	+	+	+	+	+	+	+	R, MS, O
8.01	3-penten-2-ol	1166	0.99	+	+	+	+	+	+	+	R, MS
10.70	1-pentanol	1247	1.51	+	+	+	+	+	+	+	R, MS
13.20	(Z)-2-penten-1-ol	1316	1.15	−	+	+	+	+	+	+	R, MS
13.22	3-methylbut-2-en-1-ol	1317	1.06	+	+	−	+	+	−	+	R, MS, O
14.50	1-hexanol	1350	2.03	+	+	+	+	+	+	+	R, MS
15.57	(Z)-3-hexen-1-ol	1381	1.69	−	+	+	+	−	−	−	R, MS
17.13	2-octanol	1423	2.90	+	−	+	−	−	−	+	R, MS, S, O, TOF
17.94	1-octen-3-ol	1449	2.52	+	+	+	+	+	+	+	R, MS, S, O, TOF
18.11	1-heptanol	1454	2.62	+	+	+	+	+	+	+	R, MS
19.18	2-ethylhexan-1-ol	1487	2.82	+	+	+	+	+	+	+	R, MS, O
21.11	1-octanol	1554	3.00	+	+	+	+	+	+	+	R, MS
23.70	1-nonanol	1656	3.77	+	+	−	+	+	+	−	R, MS
28.28	1-undecanol	1857	4.40	+	+	−	+	+	+	+	R, MS
28.40	benzyl alcohol	1861	1.10	+	+	+	+	+	+	+	R, MS
29.10	phenethyl alcohol	1893	1.36	+	+	+	−	+	−	+	R, MS, O
30.36	1-dodecanol	1958	5.13	+	+	+	+	+	+	+	R, MS
36.07	1-pentadecanol	2265	6.44	−	−	−	−	−	−	+	R, MS
				Carboxylic Acids							
22.81	butanoic acid	1618	0.79	+	−	+	+	+	+	+	R, MS, S, O, TOF
23.80	isovaleric acid	1658	1.16	+	+	+	+	+	+	+	R, MS, S, O, TOF
25.43	pentanoic acid	1727	2.55	+	−	+	+	−	−	+	R, MS, O
26.05	methyl salicylate	1754	1.39	+	+	+	+	+	+	+	R, MS, O
27.82	hexanoic acid	1834	1.92	+	+	+	+	+	+	+	R, MS, S, O, TOF
30.03	heptanoic acid	1939	2.42	+	−	+	+	−	−	+	R, MS
30.06	(E)-3-hexenoic acid	1941	1.34	−	−	+	−	−	−	−	R, MS
32.11	octanoic acid	2050	3.05	+	+	+	+	+	+	+	R, MS

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Table 1. continued

average RT (min)	aroma compound	calculated RI[c]	log P_{ow}[d]	steps[b] PF	PF-pH (Sol)	PF-pH (Ovn)	PF-pH (Nt)	PF-pH (DF)	PF-pH (Hom)	PPI-pH	identification[e]
34.10	nonanoic acid	2161	3.42	+	+	+	+	+	+	+	R, MS, O
35.99	n-decanoic acid	2263	4.10	+	+	+	+	−	+	+	R, MS
					Ketones						
5.02	2,3 pentanedione	1049	−0.85	+	+	+	+	+	+	+	R, MS, O
8.22	2-heptanone	1174	1.98	+	+	+	+	+	+	+	R, MS
11.71	2-octanone	1275	2.37	+	+	+	−	+	−	+	R, MS
15.60	2-nonanone	1380	3.14	+	−	−	−	−	−	+	R, MS
16.19	3-octen-2-one	1395	2.18	−	−	+	−	+	−	+	R, MS, O
26.98	2-tridecanone	1798	5.05	−	+	−	+	+	+	−	R, MS, O
27.99	(E)-geranyl acetone	1842	4.13	+	−	+	−	+	+	+	R, MS, O
33.66	hexahydrofarnesyl acetone	2128	7.13	−	−	+	−	−	−	−	R, MS
39.19	benzophenone	2451	3.18	+	+	+	+	+	+	+	R, MS
					Lactones						
24.30	γ-caprolactone	1678	0.41	+	+	+	+	+	+	+	R, MS
31.33	γ-nonalactone	2005	1.94	+	+	+	+	+	+	+	R, MS, O
					Terpenes						
8.68	D-limonene	1189	4.57	+	+	+	+	+	+	+	R, MS, O
					Furans						
9.90	2-pentylfuran	1225	3.82	+	+	+	+	+	+	+	R, MS, O
					Pyrazines						
20.07	2-isobutyl-3-methoxypyrazine (IBMP)	1517	2.55	+	+	+	+	+	+	+	R, MS, S, O, TOF
					Esters						
25.54	ethyl undecanoate	1732	5.37	+	+	−	+	+	+	+	R, MS
					Sulfur Compounds						
29.85	benzothiazole	1930	2.01	+	+	+	−	+	+	+	R, MS
					Pyran						
30.13	maltol	1944	0.09	+	+	+	+	+	+	+	R, MS, S, O, TOF
					Others						
34.50	(E)-2-octenoic acid	2175	2.70	+	−	−	+	+	+	+	R, MS, S, O, TOF
31.70	unknown	2024		+	+	+	−	+	+	+	R, MS, S, O, TOF

[a]PF: pea flour, PF-pH(Sol): after double solubilization, PF-pH(Ovn): after adjusting the pH to 3, PF-pH(Nt): after neutralization, PF-pH(DF): after diafiltration, PF-pH(Hom): after homogenization, and PPI-pH: pea protein isolate. [b]"+" compounds detected by GC-MS in the sample; "−" compounds not detected by GC-MS in the sample. [c]Retention indices. [d]log P_{ow} values from The Good Scents Company Information System.[30] [e]Identification was done for each compound based on the following: R, retention index of the analyte matched the retention index reported in the literature; MS, mass spectra of the analyte matched the NIST library spectra; S, mass spectra and retention index of the analyte matched those of an authentic standard; O, odor of the analyte matched the authentic standard and the description reported in the literature; and TOF, GC-MS-TOF was used to identify a compound and match its identity to the NIST library.

MSD (mass selective detector) mass spectrometer equipped with a sniffing port was used for GC-MS-O analysis. The separation of volatile compounds was performed using a fused silica capillary column DB-WAX (30 m length × 0.25 mm I.D. × 0.25 μm film thickness, serial #UST510456H, Agilent Technologies, Inc.). High-purity helium was used as carrier gas at a constant flow of 3.5 mL/min. The sample (2 μL) was injected in a splitless mode. The oven temperature was programmed from 40 to 85 °C at a rate of 3 °C/min and from 85 to 220 °C at a rate of 5 °C/min and a final hold time of 3 min. The column effluent was split (1:1) using a fused silica Y to feed the MS and the olfactometry port equal volumetric flows. The sniffing port transfer line from the splitting Y (fused silica) was enclosed in a heated line maintained at 220 °C. The injection port and MS transfer line temperatures were 220 and 250 °C, respectively. The ionization energy was 70 eV and the quality scan range was programmed to m/z 29−550.

Three panelists, which were all experienced with GC-O analysis, were recruited and instructed to record the retention time, the sensory descriptors of the volatile aroma compounds detected through the olfactometry port, and to rate the odor intensity of each odorant using a general labeled magnitude scale (gLMS) where "no sensation" is at the left end and "strongest imaginable sensation" at the right end.[17]

The aroma compounds were tentatively identified using MS library (NIST, National Institute of Standards and Technology, version 2.2) matching and by comparison of the calculated retention index (RI) with published values. The retention indices were calculated by spiking the sample extracts with a series of *n*-alkanes (C_5−C_{27}). Absolute identification was performed only for the aroma compounds that were rated with an average odor intensity ≥16.2 (corresponding to the descriptor "moderate") and detected by at least two panelists. Absolute identification was conducted by comparing mass spectra, the RI of the compounds in the sample with those of the pure aroma standards, and odor descriptors with their corresponding standards and with the literature.

Relative quantification was carried out by integrating the area under the curve (AUC) for each identified aroma compound. The area of each aroma compound was then normalized using the average area of the ISTD across all samples. Each aroma isolate was run in triplicate in the GC-MS-O.

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Gas Chromatography-Time-of-Flight-Mass Spectrometry (GC-TOF-MS) Analysis. To confirm the identity of the aroma compounds that had an average odor intensity ≥16.2 and detected by GC-MS-O, a GC-TOF-MS analysis was also carried out. The GC-TOF-MS analysis was conducted following the procedure and parameters previously described by Benavides-Paz.[16] The SAFE extracts obtained from each sample PF, PF-pH(Sol), PF-pH(Ovn), PF-pH(Nt), PF-pH(DF), PF-pH(Hom), and PPI-pH were combined and concentrated to 50 μL using a gentle stream of high-purity nitrogen. An Agilent 7890A Gas Chromatographic system (Agilent Technologies, Santa Clara, CA), coupled to Pegasus 4D TOF-MS (LECO Corporation, St. Joseph, MI), was used. The separation of volatile compounds was performed using a fused silica capillary column DB-WAX (30 m length × 0.25 mm I.D. × 0.25 μm film thickness, serial #US0570343H, Agilent Technologies, Inc.). High-purity hydrogen was used as carrier gas at a constant flow of 3 mL/min. The sample (1 μL) was injected in a splitless mode. The oven temperature was programmed from 40 to 85 °C at a rate of 3 °C/min and from 85 to 220 °C at a rate of 5 °C/min and a final hold time of 3 min. The injection port and transfer line temperatures were 220 and 250 °C, respectively. The ionization energy was 70 eV and the quality scan range was programmed to m/z 29−400 at a scan rate of 20 scan/s. Data processing was carried out with ChromaTOF software (version 3.4).

The compounds were tentatively identified by comparison with mass spectrometric data from the NIST (National Institute of Standards and Technology) library version 2.2.

Sensory Evaluation. The sensory evaluation was conducted in compliance with the University of Minnesota Institutional Review Board (STUDY00011991). The samples used for the sensory evaluation were 10% pea flour (PF) aqueous solution and 10% pea protein isolate (PPI-pH) aqueous solution. Thirty milliliters of each aqueous solution was placed in a clear 120 mL sample cup with a lid and was served at room temperature (28 °C). The samples were assigned 3-digit codes. Eight panelists (37% men, 63% women) from the Department of Food Science and Nutrition at the University of Minnesota served as judges, all of whom have experience in sensory analysis. The sensory evaluation of the samples occurred over one session for 1 h. Participants were provided the two solutions and were instructed to smell the samples and record the odor descriptors as well as each odor descriptor's intensity for each sample. The intensity of each attribute was rated using a general labeled magnitude scale (gLMS) where 0 corresponds to "no sensation" (at the left end of the scale) and 100 corresponds to "strongest imaginable sensation" (at the right end of the scale).[17]

Statistical Analysis. Analysis of variance (ANOVA) was performed using RStudio software version 1.4.1103 (RStudio, Inc., Boston, MA). Significant differences ($p \leq 0.05$) between the mean ($n = 3$) values of three injections of the same aroma isolate in the GC-MS-O were determined using a Tukey−Kramer honest significant difference (HSD) multiple means comparison test.

RESULTS AND DISCUSSION

Volatile Aroma Compounds Identified by GC-MS-O During the Pea Protein Extraction. The aroma compounds detected by GC-MS-O in the samples collected by the extraction process are shown in Table 1. The most represented chemical families in all of the samples were aldehydes, alcohols, carboxylic acids, and ketones, whereas only two lactones, one terpene, one furan, one methoxypyrazine, one ester, one sulfur compound, and one pyran were detected. In total, 59 compounds were detected in PF-pH(Ovn), 58 in PF and PPI-pH, 54 in PF-pH(DF), 52 in PF-pH(Nt) and PF-pH(Hom), and 50 in PF-pH(Sol). Aldehydes, ketones, and alcohols are commonly detected in green peas.[18,19] Similarly, Murat et al. found that most of the aroma compounds identified in pea flour and pea protein belong to the alcohols, ketones, carboxylic acids, and aldehydes families.[20] Although the other chemical groups are less frequently detected, they might have an important contribution to the aroma profile of peas and pea ingredients. Methoxypyrazines, for instance, with a significantly low olfactory threshold, are known to be important contributors to the perceived "green pea, bell pepper" aroma. However, the low concentrations of methoxypyrazines in peas often create difficulty for their isolation in sufficient amounts to be detected by instrumental analysis.[21]

In this study, it was hypothesized that the aroma compounds rated with a high odor intensity are likely to be the most significant contributors to the aroma profile of the samples. Therefore, efforts were focused on identifying the aroma compounds that were detected by at least two panelists, rated with an average odor intensity ≥16.2 ("moderate") and detected in at least one of the samples. In total, 13 aroma compounds had these characteristics and are reported in Table 2. Out of the 13 compounds, the identity of one compound (labeled "Unknown") could not be determined.

Table 2. Aroma Compounds Detected and Rated With an Average Odor Intensity ≥16.2 by At Least 2 Panelists in At Least One Step of the pH-Extraction

no.	compound	description by panelists	description found in the literature
1	(Z)-4-heptenal	oily, fatty, fishy, oxidized oil	oily, fatty, cream-like, fishy[30,31]
2	2-octanol	grassy, musty, moldy, earthy	green, woody, herbal, earthy[30]
3	(Z)-6-nonenal	raw cucumber, celery, beany	green, cucumber, vegetable[30]
4	methional	raw potato	potato, vegetable, musty[7,20,30]
5	1-octen-3-ol	mushroom, brothy	mushroom, fungal, musty[20,29]
6	2-isobutyl-3-methoxypyrazine (IBMP)	bell pepper, earthy, soil	green bell pepper, pea[18,30]
7	(E)-2-nonenal	cucumber, nutty	fatty, cucumber[30,32]
8	butanoic acid	cheesy, spoiled milk, saliva	sharp, acetic, cheese[30,33]
9	isovaleric acid	cheesy, sour, pungent	cheesy, sweaty[7,20]
10	hexanoic acid	cheesy, pungent, rancid	fatty, cheesy, sewer[20,30,32]
11	maltol	sweet, caramel	sweet, caramellic[7,30]
12	unknown	woody, floral, sweet, toasty	
13	(E)-2-octenoic acid	musty, moldy, dirty	musty, fatty, dirty, cheesy[30]

Relative Amounts of the Key Aroma Compounds at Various Processing Steps. Figure 2 shows how the key aroma contributors changed in recovered amounts during the protein isolation steps. Significant increases in the levels of some of the aroma compounds were observed after the PF was double-solubilized (PF-pH(Sol)); however, the concentrations of methional, isovaleric, and hexanoic acid and the "Unknown" remained constant. 2-Octanol, butanoic acid, and (E)-2-octenoic acid dropped below the detection limit of the instrument. This apparent increase in some volatiles on solubilization of the pea flour is likely explained by the fact that some aroma compounds were not readily extracted from the solid cellular material structure of the PF.

There is a great deal of literature reporting on how aroma compound−protein binding is influenced by changes in

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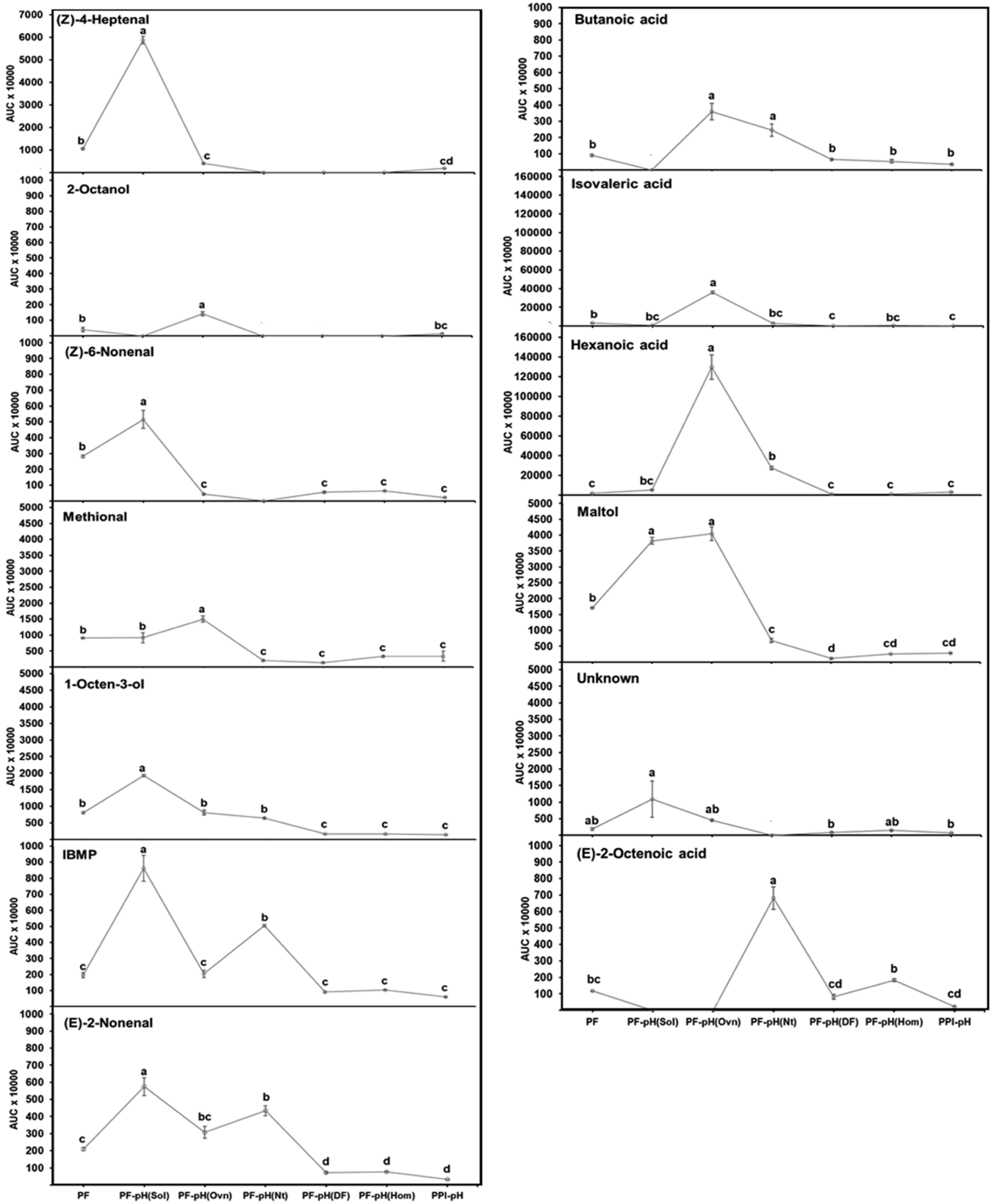

Figure 2. Area under the curve (AUC) of aroma compounds present in samples collected at different steps of the pea protein extraction process. Error bars represent the standard error of the mean (n = 3) values of three injections of the same aroma isolate in the GC-MS-O. Different lowercase letters above the bars indicate significant differences of each aroma compound across processing steps and according to the Tukey–Kramer multiple means comparison test ($p < 0.05$).

temperature, sample pH, and/or protein denaturation.[11] Therefore, data interpretation at various processing steps is complicated as the variation in measured volatile concentration may reflect issues in volatile extraction from the protein

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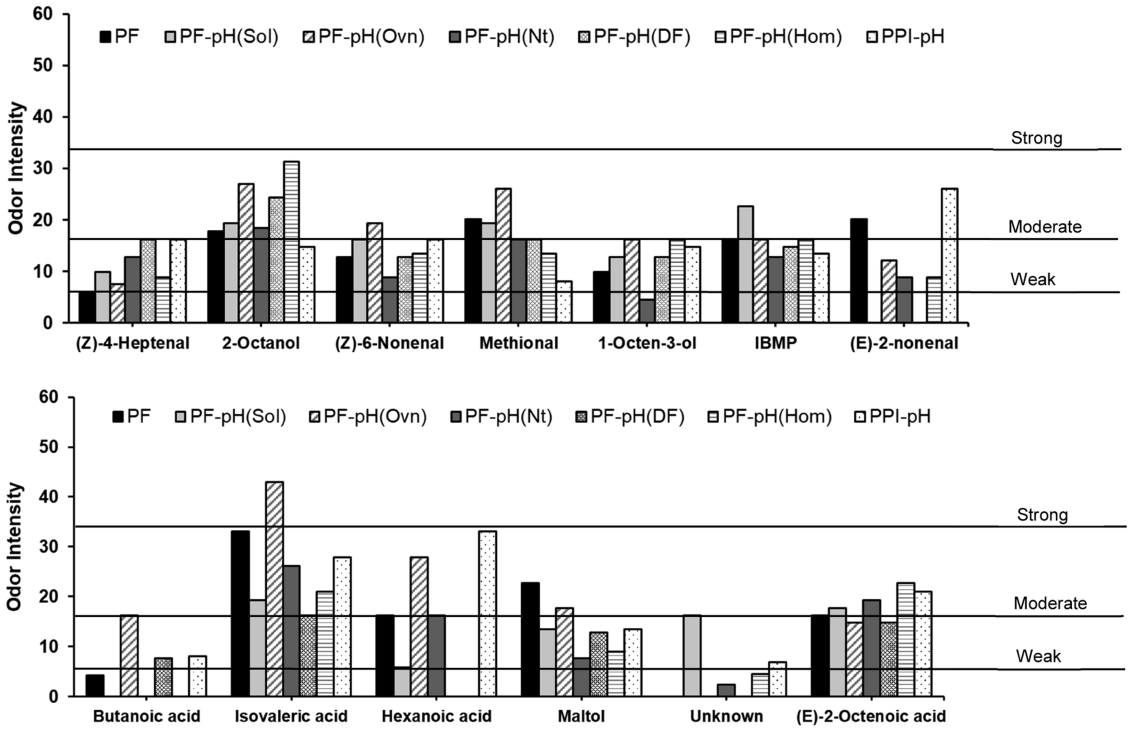

Figure 3. Mean intensity ratings of the 13 most potent aroma contributors extracted by SAFE and found during the isolation of pea protein. Intensity ratings are from 100-point general labeled magnitude scale (gLMS); a rating of 5.8 corresponded to the descriptor "weak", a rating of 16.2 corresponded to the descriptor "moderate", and a rating of 33.1 corresponded to the descriptor "strong".

(solution) rather than the absolute amount of aroma compounds in the sample. To the best of our knowledge, there has been no research done on investigating the ability of SAFE extraction to recover aroma compounds when bound to proteins. Since there is no way to correct this potential analytical complication, we will continue the discussion of the data as obtained.

In the two steps following the double solubilization, substantial changes in pH were made. Figure 2 shows that the pH adjustments caused a significant increase or decrease in the levels of the aroma compounds. These fluctuations may be due to the changes in the net charge and in the structure of the protein, which may have impacted the interactions with the aroma compounds. Dumont and Land reported that decreasing pH leads to a decrease in the binding of diacetyl to pea protein.[22] Wang and Arntfield investigated the effect of pH on the binding properties of the salt-extracted pea protein to selected monoketones and saturated aldehydes and they found that binding decreased in the following order: pH 5 > pH 7 > pH 9 > pH 11 > pH 3. They suggested that the strong hydrophobic associations between proteins at pH 5 could have created additional flavor binding sites, which increased flavor retention. Additionally, they explained that at extreme pH values (pH 3 and 11), the protein is heavily denatured or unfolded, which could have caused loss of flavor binding sites and therefore reduction in flavor retention.[12]

Part of this apparent disagreement in results may now be explained by the work of Anantharamkrishnan and Reineccius.[23] Dumont and Land were studying the interaction of diacetyl with pea protein,[22] while Wang and Arntfield considered a ketone mixture (2-hexanone, 2-heptanone, and 2-octanone).[12] Diacetyl, as a diketone, reacts very rapidly with proteins via covalent bond formation, whereas monoketones do not undergo covalent bond formation. Thus, studies considering only monoketones would measure hydrophobic interactions, which would be strongly influenced by protein folding, while studies with diacetyl (diketone) would not be influenced by protein denaturation (i.e., folding). Also, covalent bonds would not be formed at low pH explaining the low reactivity of the diacetyl at low pH as well. According to Anantharamkrishnan and Reineccius,[23] at low pH, the intermediate step for forming covalent interactions (by the Schiff base mechanism) does not happen, as the amine groups would be protonated.

The levels of isovaleric acid and hexanoic acid significantly increased when the pH was adjusted to pH 3 (PF-pH(Ovn)). This phenomenon may be an artifact of the volatile isolation process. At this pH, most of the acids are in their neutral form (not ionized) and most of the protein is positively charged. Therefore, one would expect that little to no interactions take place between the acids and the protein, which makes the acids more accessible to be extracted by DCM. Additionally, in the neutral form, acids are more likely to be soluble in a nonpolar solvent like DCM and, consequently, they will be easily isolated from the sample. When the pH was increased from 3 to 7.5, a significant decrease in the levels of these acids (isovaleric and hexanoic acid) was observed. This again could be explained by the fact that at pH 7.5, most of the acid molecules are in the ionized form, and therefore, they do not have a strong affinity with DCM hindering their extraction.

After neutralizing the pH, the solution was concentrated following a two-step filtration: ultrafiltration and diafiltration. This step corresponds to the PF-pH(DF) sample. During the two-step filtration, small components like salts and sugars are removed. Along with these components, some of the aroma compounds may also be eliminated. This may explain the significant decrease in the levels of most of the aroma compounds: 1-octen-3-ol, IBMP, 2-nonenal, butanoic acid,

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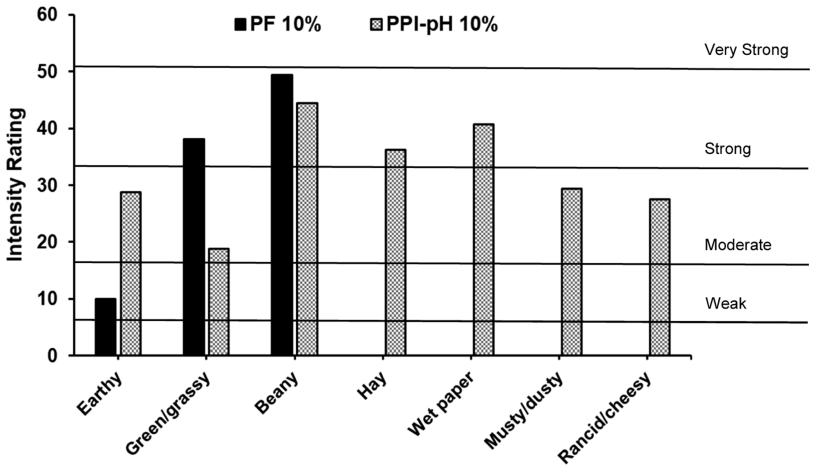

Figure 4. Mean intensity ratings of PF and PPI-pH solutions tested for aroma. Intensity ratings are from 100-point general labeled magnitude scale (gLMS); a rating of 5.8 corresponded to the descriptor "weak", a rating of 16.2 corresponded to the descriptor "moderate", a rating of 33.1 corresponded to the descriptor "strong", and a rating of 50.1 corresponded to the descriptor "very strong".

hexanoic acid, maltol, and (E)-2-octenoic acid. (Z)-4-Heptenal and 2-octanol were below detection levels, while the other measurable volatile compounds remained constant. The volatiles removed from the system in this manner would be dependent upon their competitive binding with the respective protein fractions.

After the two-step filtration step, the protein solution was pasteurized (to kill pathogens) and homogenized. The pasteurization and homogenization steps were performed in a close system, and therefore, one might expect no significant changes in the levels of the aroma compounds. While there were no significant changes in the levels of most of the aroma compounds from the filtration through the pasteurization/homogenization step (PF-pH(Hom) sample), the concentrations of (E)-2-octenoic acid decreased and (Z)-4-heptenal and 2-octanol dropped below the instrument's detectable levels.

After homogenization, the protein solution was spray-dried. Figure 2 ((PPI-pH) sample) shows that the levels of most of the aroma compounds remained ca constant, except for (E)-2-octenoic acid, which decreased. Spray drying involves both heat treatment and loss of water via evaporation. Due to the high boiling point of (E)-2-octenoic acid, one would not expect a significant loss of this compound during spray drying.

When comparing the starting material (PF) and the final product (PPI-pH), the concentrations of the aroma compounds either decreased or remained constant, which suggests that the pH-extraction method would remove some proportion of the aroma compounds originally present in the PF.

Odor Description and Intensity of Aroma Compounds Identified During the Production of Pea Protein by pH-Extraction. The odor intensity of the aroma compounds detected by panelists sniffing at the GC-MS-O sniffing port in each sample is presented in Figure 3.

2-Octanol, (Z)-6-nonenal, methional, IBMP, (E)-2-nonenal, isovaleric acid, hexanoic acid, maltol, and (E)-2-octenoic acid were detected and had an average odor intensity >16.2 ("moderate") in at least one of the sampling points. 2-Octanol was detected at each step of the process and was described as having "grassy, musty, moldy, and earthy" notes by the panelists. This compound was also found in unblanched green peas by Murray et al.[18] (Z)-6-Nonenal was detected in all of the samples and contributed to a "raw cucumber, celery, and beany" aroma. This compound is reported for the first time. Methional was detected in all of the samples and was described as having a "raw potato" aroma by the panelists. In a previous study by Murat et al., methional was found in the pea protein extract.[20] Similarly, Trikusuma et al. found methional in a pea protein beverage.[7] These two studies agreed that methional contributed to a "potato and boiled potato" aroma in the samples. IBMP was described by the panelists with "bell pepper, earthy, soil" notes. Several reports have noted the presence of this methoxypyrazine in frozen green peas, blanched green peas, pea flour, and in a pea protein beverage.[7,18,24] (E)-2-Nonenal was described with "cucumber and nutty" notes and was detected in all of the samples by panelists, except in PF-pH(Sol) and PF-pH(DF). This compound has been previously reported in frozen green peas and pea flour.[18,25] Isovaleric acid was perceived at each step of the process by panelists, whereas hexanoic acid was detected in all of the samples except in PF-pH(DF) and pF-pH(Hom). These compounds were both described as having a "cheesy, pungent, and rancid" aroma. Murat et al. reported the presence of isovaleric acid in pea flour and hexanoic acid in the pea protein extract.[20] In their study, isovaleric acid was described with "animal" notes and hexanoic acid with "feces, meat broth, and sewer" notes. Maltol was perceived at each step of the process and was described as having "sweet and caramel" notes similar to what it was found by Trikusuma et al. in a pea protein beverage.[7] (E)-2-Octenoic was perceived in all samples and was described as "musty, moldy, and dirty". To the best of our knowledge, the presence of this compound in peas or pea ingredients has not been reported in the literature.

The other compounds (Z)-4-heptenal, butanoic acid, 1-octen-3-ol, and "Unknown" were perceived with an odor intensity of 16.2 ("moderate") in at least one of the samples. (Z)-4-Heptenal was detected at each sampling point and was described as having "oily, fatty, and fishy" notes. (Z)-4-Heptenal is reported for the first time in this study. Butanoic acid was only detected in PF, PF-pH(Ovn), PF-pH(DF), and PPI-pH and was described as "cheesy, spoiled milk, and saliva". The presence of butanoic acid was previously reported by Kryachko et al. in fermented pea protein-enriched flour but no olfactory analysis was conducted in this study.[26] 1-Octen-3-ol was described with "mushroom and brothy" notes and was perceived in all of the samples by the panelists. Murat et al.

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reported the presence of 1-octen-3-ol in both pea flour and the pea protein isolate.[20] Panelists were able to detect the "unknown" compound in most of the samples except in PF, PF-pH(Ovn), and PF-pH(DF) and was described as having a "woody, floral, sweet, and toasty" aroma.

Sensory Evaluation. To examine the relationships between the GC-MS-O data and overall perception, a sensory evaluation of aqueous solutions of PF (starting material) and PPI-pH (final product) was conducted. Seven odor descriptors were used by panelists to characterize the aroma of the PF and PPI-pH solution samples (Figure 4).

The majority of these descriptors were also used to describe the aroma compounds eluting from the sniffing port coupled to the GC-MS system. Some of these odor descriptors could be linked to individual aroma compounds (Table 2). For instance, the aroma compounds primarily responsible for the "earthy" notes in the tasting solutions were likely 2-octanol and IBMP. The "green/grassy" aroma was likely due to 2-octanol. The "beany" odor character could be attributed to (Z)-6-nonenal. The "musty/dusty" aroma was likely from the presence of 2-octanol and (E)-2-octenoic acid, and the "rancid/cheesy" notes were likely due to butanoic acid, isovaleric acid, and hexanoic acid. Figure 4 also illustrates that unlike PPI-pH, PF was characterized only with three out of the seven odor descriptors (earthy, green/grassy, and beany). The other descriptors (hay, wet paper, musty/dusty, and rancid/cheesy) were used to describe the aroma of PPI-pH. Based on the analytical data, no compounds having hay and wet paper notes were identified in this study. It may be possible that the compound(s) responsible for these notes were not extracted in adequate quantities to be noted by panelists sniffing at the GC-MS-O sniffing port. Regarding the musty/dusty and rancid/cheesy descriptors, these aromas might have been formed during the pH-extraction of pea protein (pH extremes) or their concentrations might have been increased to the point that they were detectable by the panelists in PPI-pH.

Theoretical Pathways of Formation of Aroma Compounds. The theoretical pathway of formation of the aroma compounds found in this study was discussed in a previous study by Benavides-Paz et al.,[16] except for butanoic acid. The theoretical formation pathway of butanoic acid is discussed as follows. The presence of butanoic acid in pea protein has been reported by Kryachko et al.[26] In this study, they evaluated the potential antimicrobial(s) produced by *Lactobacillus plantarum* during fermentation of pea protein-enriched flour. The authors found that butanoic acid was one of the most predominant organic acids produced by *L. plantarum* and indicated that this compound might have been produced as a result of amino acid catabolism. Shukla et al. found butanoic acid in different samples of Doenjang, a traditional Korean fermented soybean paste. The authors suggested that butanoic acid might be synthesized by lactate bacterium, especially *L. plantarum*, which converts lipids into butanoic acid through intracellular enzyme activity.[27]

In conclusion, the majority of the odor descriptors used during the sensory evaluation to describe the PF and PPI-pH aqueous solutions were also used during the GC-MS-O analysis. This supports our hypothesis that the 13 aroma compounds identified through instrumental analysis are likely to be significant contributors to the aroma profile of the samples examined. Additionally, in this study, it was found that the processing steps used during an optimized pH-extraction of pea protein tended to alter the concentration of the volatiles and some of these variations appeared to affect the odor intensity perceived by panelists through the sniffing port. While no new aroma compounds appear to be produced via the protein isolation process, no existing compounds were completely removed from making a sensory contribution as determined by the olfactory analysis. This is not a surprise in the sense that most of the aroma compounds tended to be hydrophobic, and thus, they would stay with the protein through the extraction process rather than be lost to aqueous washes.

These findings suggest that most of the aroma compounds identified in the samples may come from the normal metabolism or during the storage of peas. Therefore, approaches to reducing the inherent undesirable aroma compounds from the peas could involve plant breeding programs or processing approaches that include the extraction of the lipids to remove them as precursors and/or supercritical extraction[28,29] at the beginning or end of the protein isolation process.

AUTHOR INFORMATION

Corresponding Author

Yara L. Benavides-Paz − *Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108, United States;* orcid.org/0000-0002-2830-7659; Phone: 651-208-0791; Email: benav025@umn.edu

Authors

Baraem P. Ismail − *Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108, United States*

Gary A. Reineccius − *Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108, United States;* orcid.org/0000-0003-1817-8758

Complete contact information is available at: https://pubs.acs.org/10.1021/acsfoodscitech.1c00412

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Notes

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ABBREVIATIONS

UHT, ultrahigh temperature; GC-MS-O, gas chromatography-mass spectrometry-olfactometry; GC-TOF-MS, gas chromatography-time-of-flight-mass spectrometry; ISTD, internal standard; NaCl, sodium chloride; PF, pea flour; Sol,

solubilization; Nt, neutralization; DF, diafiltration; Hom, homogenization; Ovn, overnight; PPI, pea protein isolate; SAFE, solvent-assisted flavor evaporation; RI, retention index; gLMS, general labeled magnitude scale; IBMP, 2-isobutyl-3-methoxypyrazine

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