JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Quantification and Bitter Taste Contribution of Lipids and Their Oxidation Products in Pea-Protein Isolates (*Pisum sativum* L.)

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Cite This: *J. Agric. Food Chem.* 2021, 69, 8768–8776

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ABSTRACT: An ultra-high-performance liquid chromatography–differential ion mobility (DMS)–tandem mass spectrometry method was developed to quantify 14 bitter-tasting lipids in 17 commercial pea-protein isolates (*Pisum sativum* L.). The DMS technology enabled the simultaneous quantification of four hydroxyoctadecadienoic acid isomers, namely, (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**5**), (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid (**6**), (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**7**), and (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**8**). Based on quantitative data and human bitter taste recognition thresholds, dose-over-threshold factors were determined to evaluate the individual lipids' bitter impact and compound classes. The free fatty acids α-linolenic acid (**10**) and linoleic acid (**13**), as well as the trihydroxyoctadecenoic acids, especially 9,10,11-trihydroxyoctadec-12-enoic (**3**), and 11,12,13-trihydroxyoctadec-9-enoic acids (**4**), were shown to be key inducers to bitterness in the isolates. Additionally, the impact of 1-linoleoyl glycerol (**9**) on the bitter taste could be shown for 14 of the 17 tested pea-protein isolates.

KEYWORDS: *pea, Pisum sativum, bitter taste, sensomics, lipids, fatty acids, oxylipins, differential ion mobility*

INTRODUCTION

Due to their techno-functional properties, the food processing industry uses protein isolates as ingredients, such as emulsifiers, foaming agents, and gel formers. They are also important constituents in the wide field of sports and fitness nutrition.[1−5] For most of these applications, animal-based protein sources, for example, whey, eggs, or casein, are preferred over plant-based protein sources, such as pea (*Pisum sativum* L.), because the latter exhibit a high bitter off-flavor.[6−8] This bitterness appeared to be a significant limiting factor for the usage of pea-protein isolates despite being economically and ecologically relevant alternatives to their animal-based analogues and answering the rising demand in vegan and vegetarian products.[9−12]

Various secondary plant metabolites were discussed to cause the bitter off-flavor by non-covalently sticking to the protein in the past few years. In the past, saponins, especially soyasaponin I and DDMP saponin, have been associated with a bitter and astringent off-taste in pea-protein isolates.[13−16] Bitter amino acids and peptides[17−21]—possibly formed due to hydrolysis during processing—and bitter lipid oxidation products[22−27]—formed by autoxidation or enzymatic pathways—were taken into consideration as well.

Recently, the application of an activity directed sensomics approach involving sequential solvent extraction and chromatographic separation steps [by medium-pressure liquid chromatography (MPLC) and preparative and semi-preparative high-performance liquid chromatography (HPLC)], combined with human sensory experiments, led to the identification of several bitter compounds from a commercial pea-protein isolate.[28] Among these bitter molecules, 14 lipids and lipid oxidation products with human taste thresholds between 0.06 and 0.99 mmol/L were identified by 1D/2D-nuclear magnetic resonance (NMR), liquid chromatography-tandem mass spectrometry (LC-MS/MS), LC–TOF–MS, and MS^E experiments, namely, 9,10,13-trihydroxyoctadec-11-enoic acid (**1**), 9,12,13-trihydroxyoctadec-10-enoic acid (**2**), 9,10,11-trihydroxyoctadec-12-enoic acid (**3**), 11,12,13-trihydroxyoctadec-9-enoic acid (**4**), (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid (**6**), (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**7**), (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**8**), 1-linoleoyl glycerol (**9**), α-linolenic acid (**10**), 2-hydroxypalmitic acid (**11**), 2-hydroxyoleic acid (**12**), linoleic acid (**13**), (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (**14**), and octacosa-6,9,19,22-tetraene (Figure 1).

Although the key bitter molecules of pea-protein isolates have been identified, exact quantitative data to evaluate each substance's overall taste contribution are still missing. The challenge with this was to determine the accurate MS/MS-based quantification of target compound isomers, such as *E,Z*- and *E,E*-hydroxyoctadecadienoic acids (HODEs), which mainly relied on their chromatographic separation previously.[29,30] In the past, the spectral overlap of isobaric and

Received: May 16, 2021
Revised: June 11, 2021
Accepted: June 29, 2021
Published: July 29, 2021

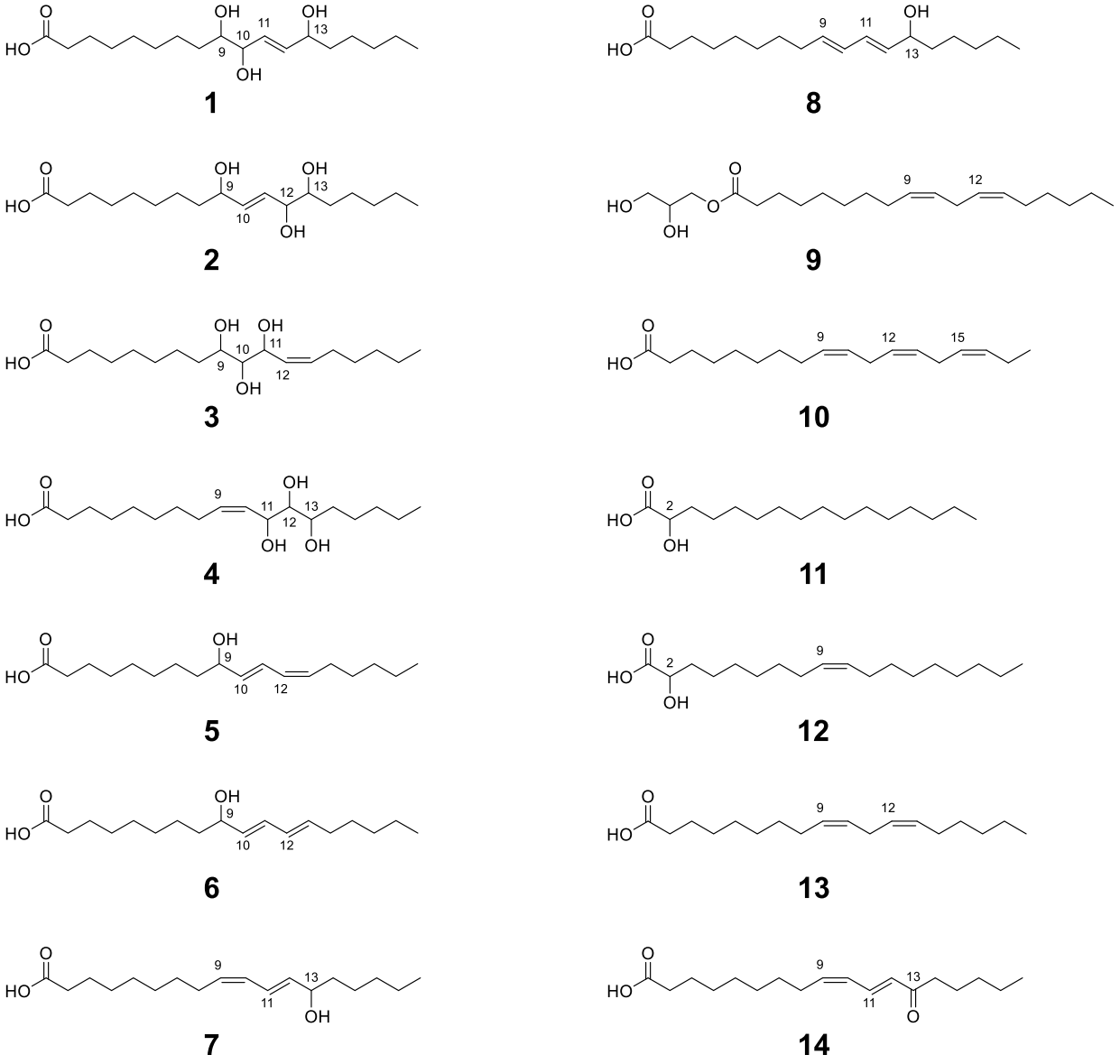

Figure 1. Chemical structures of the analyzed bitter compounds from pea-protein isolates: 9,10,13-trihydroxyoctadec-11-enoic acid (**1**), 9,12,13-trihydroxyoctadec-10-enoic acid (**2**), 9,10,11-trihydroxyoctadec-12-enoic (**3**), 11,12,13-trihydroxyoctadec-9-enoic acid (**4**), (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**5**), (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid (**6**), (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**7**), (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**8**), 1-linoleoyl glycerol (**9**), α-linolenic acid (**10**), 2-hydroxypalmitic acid (**11**), 2-hydroxyoleic acid (**12**), linoleic acid (**13**), and (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (**14**). *Selected peak(s) for quantification.

isomeric analytes could be overcome by introducing differential ion mobility (DMS) into the field of lipidomics.[30−34] Within the DMS cell, the separation voltage (SV) is applied perpendicular to the transport gas flow and prevents the ions, which are transmitted through the DMS cell by the transport gas, from entering the MS. The compensation voltage (CoV)—a compound-specific parameter—counteracts the ion shifts caused by the SV and causes the specific ions to pass the DMS cell into the MS. This CoV can be optimized as an additional, third separation parameter (besides the retention time and MRM transition) for isomeric and isobaric compounds.[30,33,35−37]

Therefore, the present investigation's objective was to develop an accurate LC−MS/MS method based on DMS to provide quantitative data for the key bitter molecules in pea-protein isolates. Furthermore, this study aimed to evaluate each compound's taste impact based on dose−activity considerations.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: acetonitrile (ACN), methanol (MeOH) (J.T. Baker, Deventer, The Netherlands), formic acid (Merck, Darmstadt, Germany), (10*Z*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid, (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, linoleic acid, [$^{13}C_{18}$]-linoleic acid, α-linolenic acid, 1-linoleoyl glycerol, methanol-d_4 (MeOD), 1-myristoyl glycerol, ammonium acetate (NH_4Ac; aqueous solution, 5 mM) (Sigma-Aldrich, Steinheim, Germany), (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid, (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, (9*S*,10*S*,11*R*,12*Z*)-9,10,11-trihydroxyoctadec-12-enoic acid, (9*S*,10*S*,11*E*,13*S*)-9,10,13-trihydroxyoctadec-11-enoic acid, (9*S*,10*E*,12*S*,13*S*)-9,12,13-trihydroxyoctadec-10-enoic acid, (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid, 2-hydroxyoleic acid, 18-hydroxyoleic acid, and 2-hydroxypalmitic acid (Larodan AB, Solna,

https://doi.org/10.1021/acs.jafc.1c02889
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Schweden). Isopropanol and ACN used for ultra-high-performance liquid chromatography (UHPLC)−MS/MS analysis were of LC−MS grade (Honeywell, Seelze, Germany), and all the other solvents were of HPLC grade. The water for chromatography was purified using an Advantage A 10 water system (Millipore, Molsheim, France).

Sample Material. A total of 17 pea-protein isolates were provided by our IGF partners of the FEI project under grant number AiF-18814 N. The following samples were analyzed: Isolate 1: Nutralys S85F, Pea Protein 78% Roquette, Code: 5006541; Isolate 2: Erbotin PF, Protein: 82y.±2y., Dez. 2013, Gustav Parmentier, Batch: 9123631; Isolate 3: Pisane, Cosucra, 2014; Isolate 4: Lysamin GP, 1147355, Pea Protein 78% Roquette, Code: 1147355; Isolate 5: Pea Protein 5005454, Pea Protein Emsland 75%; Isolate 6: Nutralys F85F, Roquette, Batch: WA25J; Isolate 7: Nutralys F85M, Roquette, Batch: WB14J; Isolate 8: Erbsenprotein 80% JYPP, Denk Ingridients, Denk-Artikelnr.: 967569; Isolate 9: Pisane C9 Pea Protein, Cosucra, Lo-no: 2015114923, Date: 16/04/2015; Isolate 10: Pea Protein Conc. Vestkorn; Isolate 11: Prestige; Isolate 12: Pea Pro; Isolate 13: Bio Erbsenprotein; Isolate 14: Empro E86; Isolate 15: Empro E86HV; Isolate 16: Erbsenisolat Döhler; Isolate 17: Bio Erbsenprotein 82% Piowald. All samples were stored in the dark at 4 °C.

LC−DMS−MS/MS Quantification of the Taste-Active Lipids and Oxylipins. *Solvent Extraction for Quantification.* For a triple determination, 3 × 500 mg of each pea-protein isolate, a mixture of MeOH/H_2O (1 + 1, v + v, 5 mL), and the following internal standard solutions were added to a cryogenic tube (10 mL, VWR Chemicals, Fontenay-sous-Bois, France): 25 μL of [$^{13}C_{18}$]-linoleic acid (**IS1**, 1.0 mM in MeOH), 25 μL of 18-hydroxyoleic acid (**IS2**, 1.0 mM in MeOH), and 25 μL of 1-myristoyl glycerol (**IS3**, 1.0 mM in MeOH). The extraction was performed using an Analogue Orbital Shaker 3005 (GFL, Burgwedel, Germany) for 1 h at 300 U/min. The extracts were membrane-filtered (Minisart RC 15, 0.45 μm, Sartorius AG, Göttingen, Germany) and afterward injected into the UHPLC−DMS−MS/MS system.

Calibration Curve. The exact concentration of the analytes (**1**−**3** and **5**−**14**) was verified by quantitative NMR (qNMR), and a stock solution (0.25 mM) was prepared in MeOH. This stock solution was diluted to 0.1, 0.075, 0.05, 0.025, 0.01, 0.0075, 0.005, 0.0025, 0.001, 0.00075, 0.0005, 0.00025, 0.0001, 0.000075, 0.00005, and 0.000025 mM concentrations. To each dilution, the same amount of internal standards was added to reach an end concentration of 0.00493 mM for [$^{13}C_{18}$]-linoleic acid (**IS1**) and 0.00495 mM for 18-hydroxyoleic acid (**IS2**) and 1-myristoyl glycerol (**IS3**). **IS1** was used to quantify analytes **10** and **13**, **IS2** for analytes **1**−**8**, **11**, **12**, and **14**, and **IS3** for compound **9** (Figure 2). The UHPLC−DMS−MS/MS analysis of each sample was run in triplicate. Then, calibration curves were prepared to plot the analyte's peak area ratios to the internal standard ($area_{analyte}/area_{internal\ standard}$) against each analyte's concentration ratios to the internal standards ($c_{analyte}/c_{internal\ standard}$). Finally, a linear regression by the Multiquant software (Version 3.0.2, Sciex, Darmstadt, Germany) was used. The response was linear for chosen molar ratios, and the contents of analytes **1**−**14** in the 17 pea-protein samples were calculated using the respective calibration curve. Compound **4** was quantified using the calibration of the structurally related compound **3**.

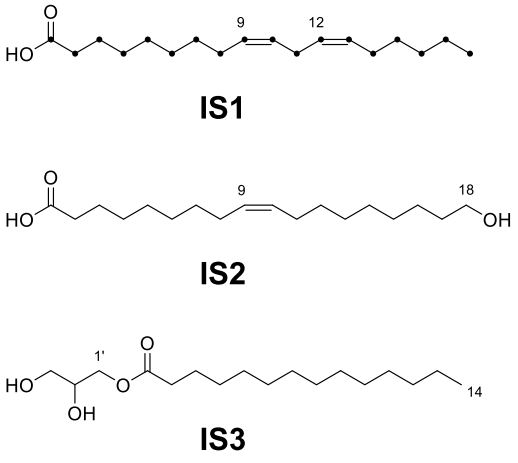

Figure 2. Chemical structures of the internal standards used to quantify bitter-tasting compounds in pea-protein isolates: [$^{13}C_{18}$]-linoleic acid (**IS1**), 18-hydroxyoleic acid (**IS2**), and 1-myristoyl glycerol (**IS3**).

Recovery. To check the accuracy of the method and analyte loss during sample workup, recovery rates were determined. Therefore, pea-protein isolate 5 was spiked with 80 μL of the analyte solutions (**1**−**3** and **5**−**14**) with three different concentration levels (0.0625, 0.125, and 0.25 mM in MeOH) and worked up as described above to determine the recovery of the quantification method. As the control, isolate 5 was worked up without the addition of the analyte solutions. The samples were then quantified through UHPLC−DMS−MS/MS in triplicate.

Inter- and Intraday Precision. Six aliquots of the same pea-protein isolate were analyzed for compounds **1**−**3**, **5**−**8**, and **10**−**14** on consecutive days. The interday precision of the quantification method was determined by replicate analysis and expressed by the relative standard deviation: **1** (38%), **2** (27%), **3** (12%), **5** (11%), **6** (38%), **7** (11%), **8** (34%), **10** (9%), **11** (15%), **12** (14%), **13** (10%), and **14** (19%). For the intraday precision, six aliquots of the same pea-protein isolate were analyzed on the same day and the precision (relative standard deviation) was as follows: **1** (31%), **2** (18%), **3** (12%), **5** (12%), **6** (34%), **7** (13%), **8** (24%), **10** (7%), **11** (12%), **12** (17%), **13** (7%), and **14** (23%).

UHPLC−DMS−MS/MS System and Parameters. The MS/MS analysis was performed on a QTrap 6500 + mass spectrometer equipped with a SelexION + DMS cell (Sciex, Darmstadt, Germany) in the negative ionization mode (except for compound **9**). Nitrogen was used as a carrier gas for the modifier isopropanol. Ion mobility parameters, such as the type of chemical modifier and its flow rate, the SV (SV = 500−3500 V), and the DMS temperature (DT = 150, 225, and 300 °C), were optimized to separate the HODEs (**5**−**8**) to the following final conditions: isopropanol as the chemical modifier at the flow rate of 363.6 μL/min (low), an SV of 3500 V, a DMS temperature of 225 °C (medium), a DMS offset of 3 V, and a DMS resolution set to open. The declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) were optimized for commercial references of methanolic solutions of the analytes (**1**−**3** and **5**−**14**) and internal standards (**IS1**−**3**) by flow injection (10 μL/min). The detection of the pseudomolecular ions' ($[M - H]^-$ or $[M - H]^+$) fragmentation into specific product ions was done by a tuning process (Table 1). Compound **9** was analyzed without using the DMS cell in ESI positive ionization.

The mass spectrometer was operated in the MRM mode (ion-spray voltage: 5500 V for ESI positive ionization and −4500 V for ESI negative ionization) using the following parameters: curtain gas, 35 psi; temperature, 450 °C; gas 1, 55 psi; gas 2, 65 psi; and collision activated dissociation, −2 V. The MS/MS system was connected to an ExionLC UHPLC system (Sciex, Darmstadt, Germany) consisting of two ExionLC Binary Gradient pumps, an ExionLC degasser, an ExionLC autosampler AD, an ExionLC column oven AC, and an ExionLC controller.

After injecting the samples (1 μL), chromatography was run on a Kinetex F5 column (100 × 2.1 mm, 1.7 μm, 100 Å, Phenomenex, Aschaffenburg, Germany) with a binary gradient using 5 mM NH_4Ac in H_2O at pH 5.0 as solvent A and 5 mM NH_4Ac in H_2O at pH 5.0/ACN/isopropanol (5 + 55+40, v + v + v) as solvent B (flow rate of 0.35 mL/min): 0 min, 30% B; 4 min, 60% B; 10 min, 71% B; 12 min, 100% B; 14 min, 100% B; 15 min, 30% B; 16 min, 30% B.

The instrument was controlled using the Analyst 1.6.3 software (Sciex, Darmstadt, Germany). Microsoft Excel (Microsoft Office, 2016) and Multiquant (version 3.0.2, Sciex, Darmstadt, Germany) were used for data analysis.

Quantitative NMR Spectrometry (qNMR). qNMR was recorded on a 400 MHz ultrashield Avance III spectrometer with a

https://doi.org/10.1021/acs.jafc.1c02889
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Table 1. Optimized MRM Transitions and Parameters of Analyzed Compounds (1−14) and the Internal Standards (IS1−3)[e]

compound no.	Q1 (Da)	Q3 (Da)	DP (V)[a]	EP (V)[b]	CE (V)[c]	CXP (V)[d]
1	329.1	171.0	−75	−10	−30	−19
2	329.1	211.0	−90	−10	−30	−23
3	329.1	201.0	−95	−10	−30	−23
4	329.1	199.0	−115	−10	−32	−19
5	295.1	171.0	−85	−10	−24	−19
6	295.1	171.0	−85	−10	−22	−19
7	295.1	195.0	−105	−10	−24	−21
8	295.1	195.0	−95	−10	−22	−21
9	355.2	263.1	61	10	11	32
10	277.1	277.1	−90	−10	−8	−33
11	271.1	225.1	−75	−10	−28	−25
12	297.1	251.2	−100	−10	−26	−21
13	279.2	279.1	−160	−10	−12	−33
14	293.1	112.9	−90	−10	−28	−13
IS1	297.1	297.2	−115	−10	−14	−29
IS2	297.1	251.1	−100	−10	−30	−29
IS3	303.2	285.2	41	10	11	14

[a]Declustering potential. [b]Entrance potential. [c]Collision energy. [d]Cell exit potential. [e]Compensation Voltage.

Broadband Observe BBFOplus probe (Bruker, Rheinstetten, Germany). MeOD (600 μL) was used as a solvent, and chemical shifts were quoted in parts per million relative to the solvent signals. The experiments were performed after calibrating the spectrometer with the ERETIC 2 tool using the PULCON methodology, as reported earlier.[38] The data were processed using the software Topspin 3.2 (Bruker, Rheinstetten, Germany).

Statistical Analysis. The quantitative data were visualized as a heatmap and faceted scatterplot using R (Version 4.0.2, R Foundation).[39] Visualization was done using the packages "ggplot2" and "ComplexHeatmap".[40,41]

RESULTS AND DISCUSSION

Bitter lipids and oxylipins have been discovered in various plants and foodstuff, for example, poppy seeds (*Papaver*

Table 2. Optimal CoV Values of the Bitter Compounds from Pea-Protein Isolates (1−14) and the Internal Standards (IS1−3)

compound no.	compound[a]	CoV [V][b]
1	9,10,13-trihydroxyoctadec-11-enoic acid	−3
2	9,12,13-trihydroxyoctadec-10-enoic acid	0
3	9,10,11-trihydroxyoctadec-12-enoic acid	2
4	11,12,13-trihydroxyoctadec-9-enoic acid	1.5
5	(10E,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid	−9
6	(10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid	−16
7	(9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	−8
8	(9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	−16
9	1-linoleoyl glycerol	n.d.
10	α-linolenic acid	−17
11	2-hydroxypalmitic acid	−11
12	2-hydroxyoleic acid	−8
13	linoleic acid	−16
14	(9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid	−13
IS1	[$^{13}C_{18}$]-linoleic acid	−16
IS2	18-hydroxyoleic acid	−10
IS3	1-myristoyl glycerol	n.d.

[a]Chemical structures shown in Figures 1 and 2. [b]Determined under the following conditions: an SV of 3500 V, a DMS temperature of 225 °C, isopropanol as the modifier at the flow rate of 363.6 μL/min (low), and a DMS offset of 3 V. The signal intensities are normalized. n.d. = not determined.

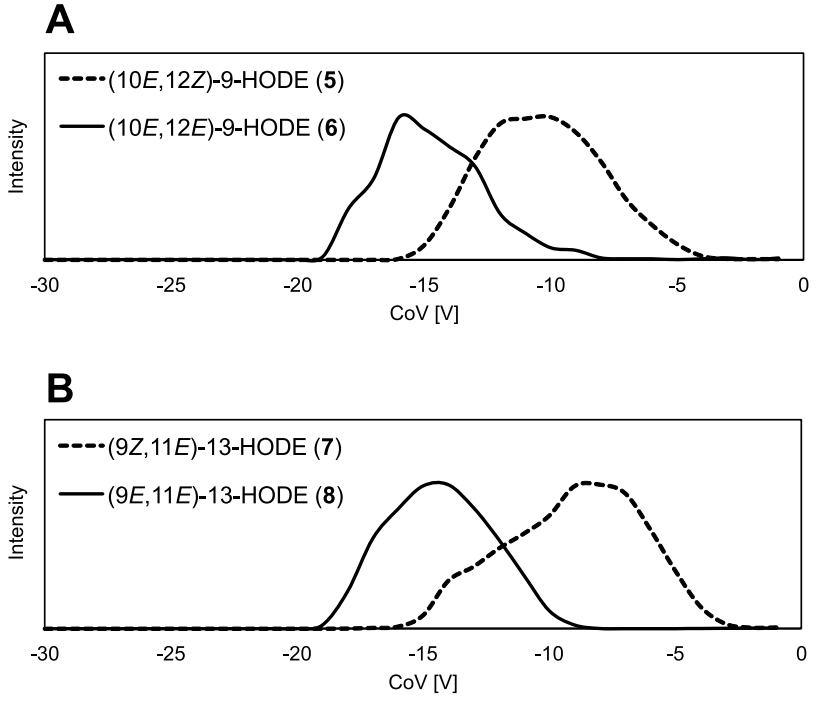

Figure 3. DMS separation of (**A**) 9-HODE isomers **5** and **6** and (**B**) 13-HODE isomers **7** and **8** with the optimized parameters: an SV of 3500 V, a DMS temperature of 225 °C, isopropanol as the modifier at the flow rate of 363.6 μL/min (low), and a DMS offset of 3 V. The signal intensities are normalized.

https://doi.org/10.1021/acs.jafc.1c02889
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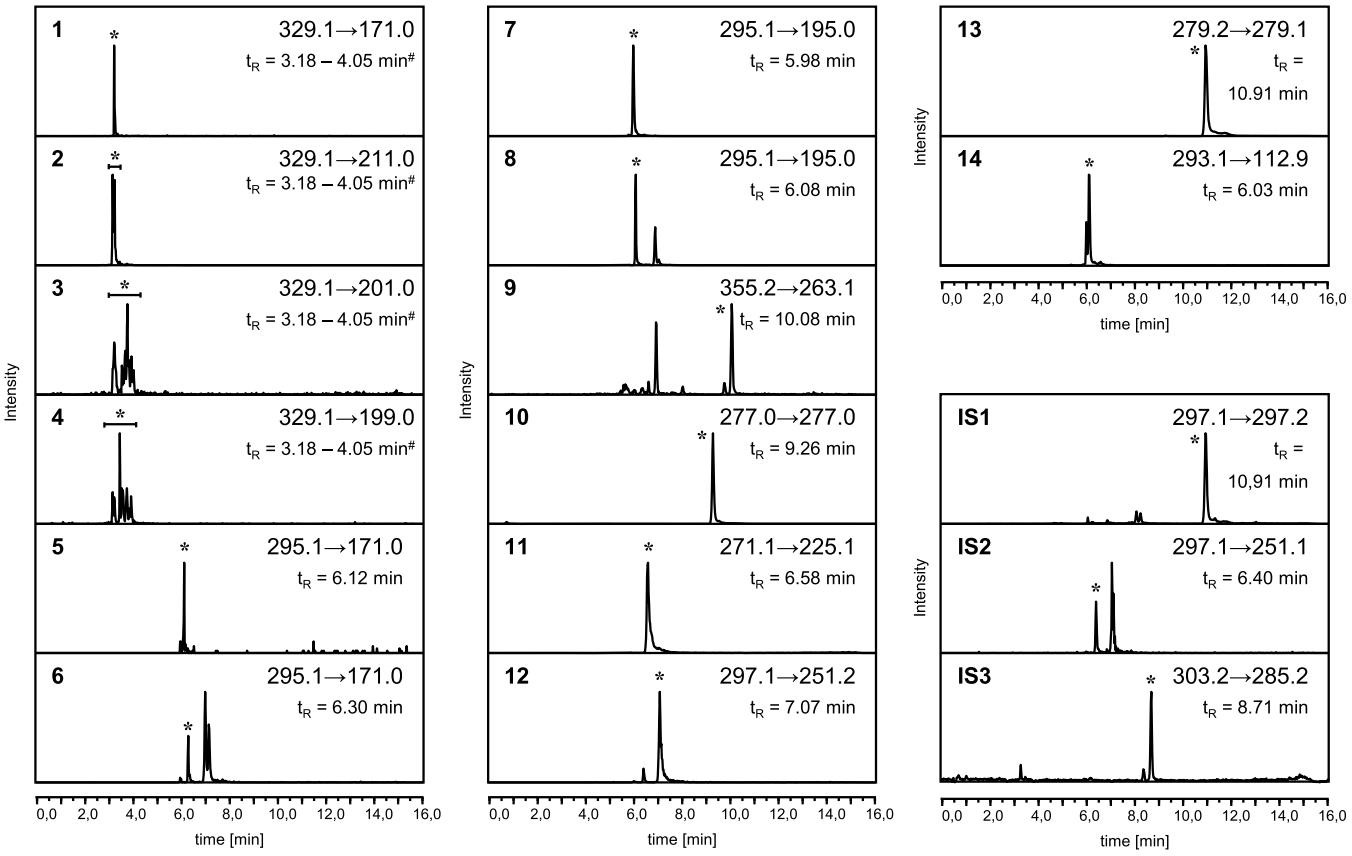

Figure 4. UHPLC−DMS−MS/MS analysis of a pea-protein isolate (isolate 5) showing the mass transitions for the quantification of the bitter compounds 9,10,13-trihydroxyoctadec-11-enoic acid (**1**), 9,12,13-trihydroxyoctadec-10-enoic acid (**2**), 9,10,11-trihydroxyoctadec-12-enoic (**3**), 11,12,13-trihydroxyoctadec-9-enoic acid (**4**), (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**5**), (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid (**6**), (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**7**), (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (**8**), 1-linoleoyl glycerol (**9**), *α*-linolenic acid (**10**), 2-hydroxypalmitic acid (**11**), 2-hydroxyoleic acid (**12**), linoleic acid (**13**), and (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (**14**), as well as the internal standards [$^{13}C_{18}$]-linoleic acid (**IS1**), 18-hydroxyoleic acid (**IS2**), and 1-myristoyl glycerol (**IS3**). The signal intensity of each mass transition is normalized.

somniferum L.),[26] avocados (*Persea americana* Mill),[24] and potato fibers.[27] Furthermore, the bitter lipids **1**−**4** and **6**−**14** (Figure 1) have recently been identified as bitter compounds in pea-protein isolates (*P. sativum*) by activity-guided fractionation.[28] An accurate UHPLC−DMS−MS/MS method was developed to assess the identified compounds' contribution to the overall off-taste of different commercial pea-protein isolates. Further studies showed no major difference in the bitter threshold between the *E,Z*- and *E,E*-isomers of HODEs. Therefore, (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**5**, Figure 1) was also added to the pool of analytes to provide quantitative data for all 9- and 13-HODE isomers.[26]

Method Development for the Quantitative Analysis of Compounds 1−14 in Commercial Pea-Protein Isolates. Reference compounds for substances **1**−**3** and **5**−**14** have been purchased commercially. For accurate quantification, three different commercially available internal standards have been chosen that share structural similarities with the respective analytes to be investigated: [$^{13}C_{18}$]-linoleic acid (**IS1**) for compounds **10** and **13**; 18-hydroxyoleic acid (**IS2**) for compounds **1**−**8**, **11**, **12**, and **14**; and 1-myristoyl glycerol (**IS3**) for compound **9** (Figure 2). The MS/MS parameters were individually tuned for each analyte and internal standard in the ESI negative ionization mode (**1**−**8**, **10**−**14**, and **IS1**−**3**) or ESI positive ionization mode (**9**) to sensitively monitor the pseudomolecular ions' fragmentation into specific product ions. Therefore, the corresponding reference compounds were infused directly into the MS/MS system with a syringe pump. For compound **4**, the ionization parameters were determined manually by injecting an isomeric mixture containing trihydroxyoctadecenoic acids (THOAs) **1**−**4** isolated from a pea-protein isolate as reported earlier.[28] The most abundant mass transition was selected for each of the compounds for quantification, and a second mass transition was chosen for the target molecule's unequivocal identification. Additionally, the DMS technique was implemented to overcome the mass transitions' spectral overlap 295.1 → 171.0 for 9-HODE isomers **5** and **6** and 295.1 → 195.0 for 13-HODE isomers **7** and **8**, respectively. In the past, DMS had already been used to enhance the selectivity for analyzing isobaric and isomeric substances.[30,32,33,42]

Using the DMS technique, a third separation dimension is implemented to the method besides the retention time and mass transition. In the DMS cell, the SV shifts the ion trajectories in the DMS cell and prevents the ions from entering the MS/MS system. This voltage is negated by the CoV—a compound-specific parameter that compensates for the SV-dependent shift of an ion trajectory for specific ions to enter the MS. Several parameters, for example, the temperature or the use and type of a chemical modifier, affect the DMS cell's gas equilibrium.[30,33,35−37] Based on Mittermeier et al. (2020), each parameter's optimization was performed on the

https://doi.org/10.1021/acs.jafc.1c02889
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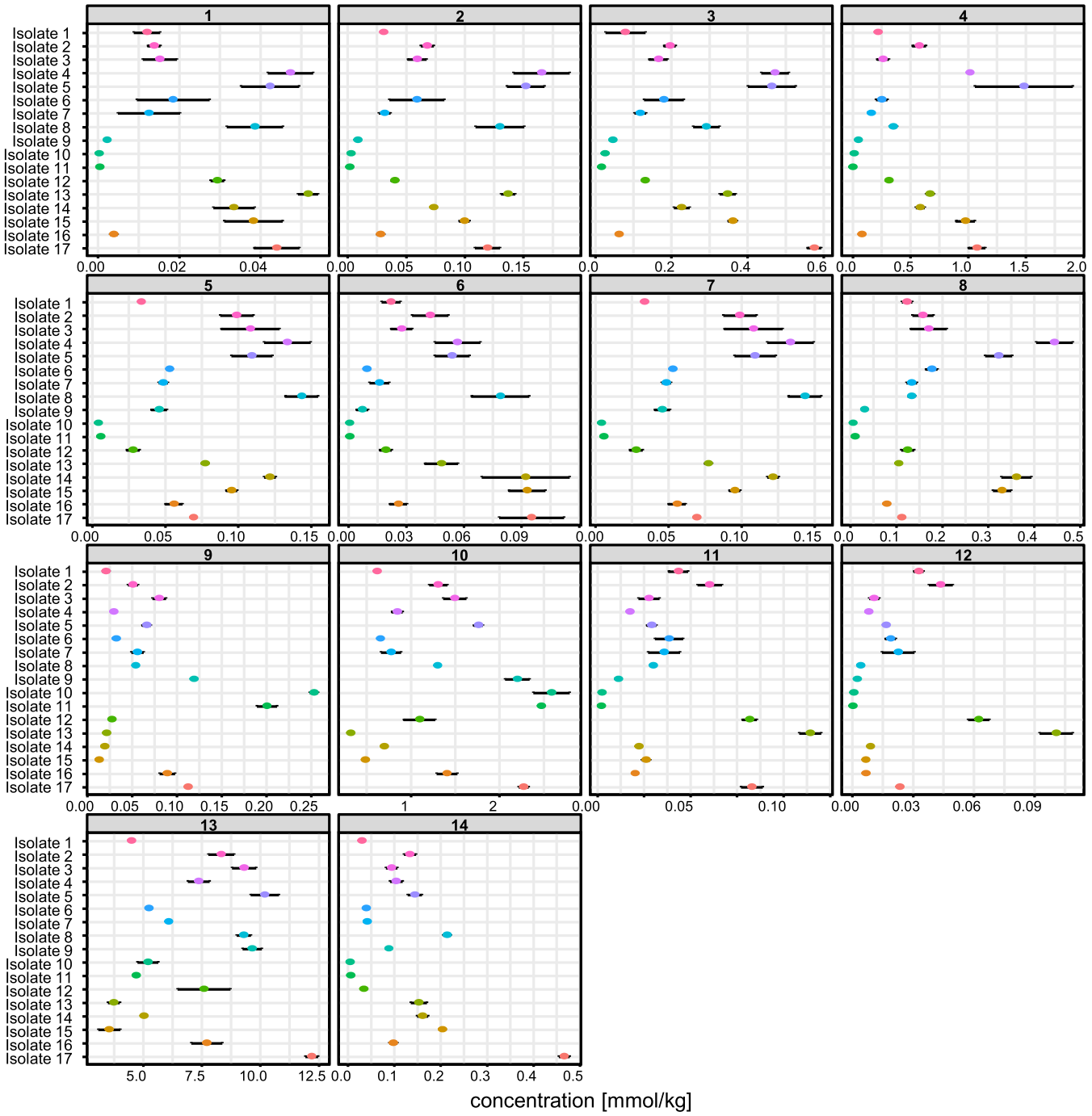

Figure 5. Faceted scatterplot of quantitative data [mmol/kg] for compounds **1**−**14** in 17 pea-protein isolates.

column individually.[30] Accordingly, each HODE isomer (**5**−**8**) was injected separately into the UHPLC−DMS−MS/MS system, and the CoV was ramped from −30 to 15 V for a fixed set of SVs (500, 1000, 1500, 2000, 2500, 3000, and 3500 V). Tested modifiers were isopropanol, MeOH, and ACN at different flow rates and DMS temperatures (150, 225, and 300 °C). The parameters were optimized for the highest possible CoV resolution and signal intensity for the HODE isomers (Figure 3). The final measurements for all the analytes (**1**−**14**) and internal standards (**IS1**−**3**) were performed using the optimized settings shown in Table 2.

The following sample workup was developed to accurately quantify the target molecules by UHPLC−DMS−MS/MS: 500 mg of the pea-protein isolate, 5 mL of MeOH/H_2O (1 + 1, v + v) as well as 25 μL of [$^{13}C_{18}$]-linoleic acid (**IS1**, 1.0 mM in MeOH), 25 μL of 18-hydroxyoleic acid (**IS2**, 1.0 mM in MeOH), and 25 μL of 1-myristoyl glycerol (**IS3**, 1.0 mM in MeOH) were added to a cryogenic tube (10 mL, VWR Chemicals, Fontenay-sous-Bois, France). The tubes were fixed on an Analogue Orbital Shaker 3005 (GFL, Burgwedel, Germany) and shaken for 1 h at 300 U/min. Every protein isolate was worked up in triplicate. After membrane filtration, the extracts were injected into the UHPLC−DMS−MS/MS system (Figure 4).

The aliquots of protein isolate 5 were spiked with three different analytes' concentration levels (**1**−**3** and **5**−**14**), prior

https://doi.org/10.1021/acs.jafc.1c02889
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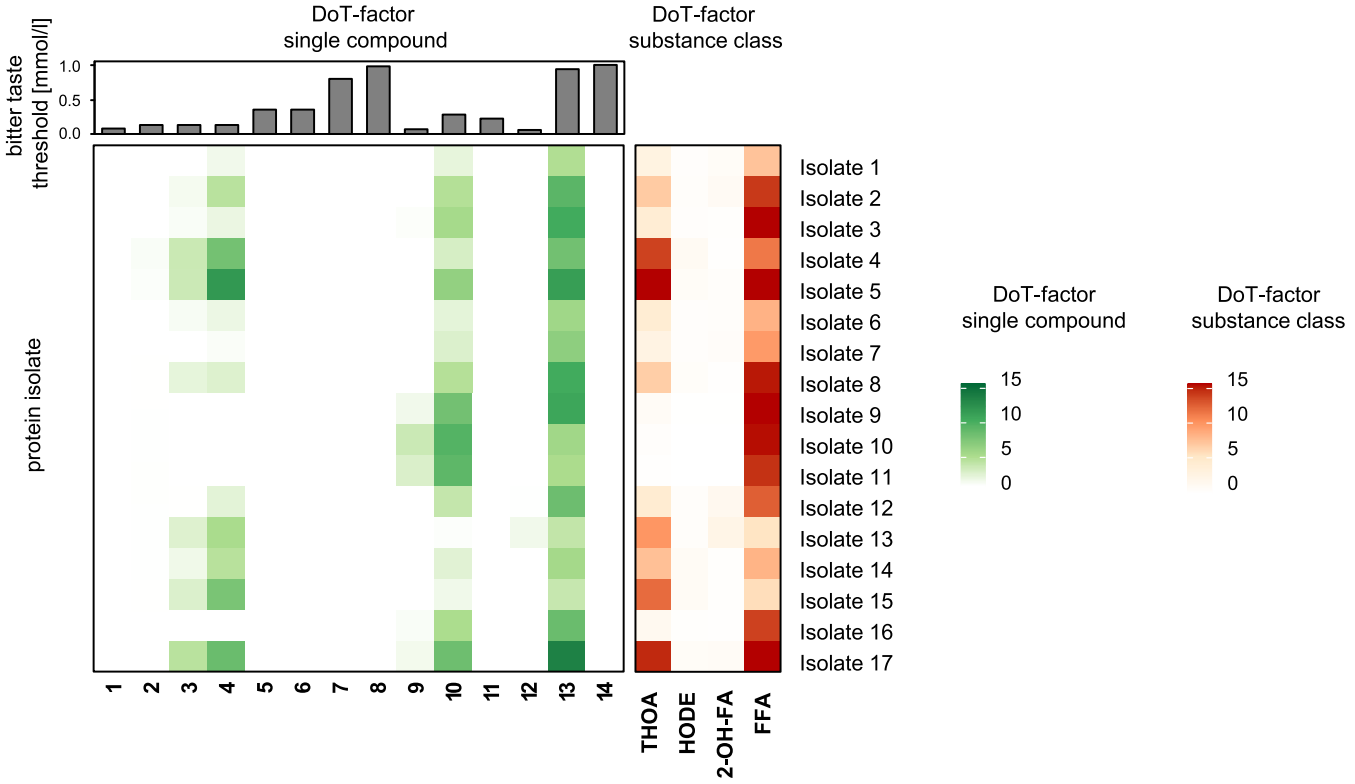

Figure 6. DoT-factors' heatmaps were determined for the single compounds **1**−**14** and summarized as substance classes in 17 pea-protein isolates (THOAs: **1**−**4**; HODEs: **5**−**8**; 2-OH-FAs: **11** and **12**; and FFAs: **10** and **13**).

to the quantification to determine the recovery rates. Comparison of the amounts determined with those found in the blank control pea-protein isolate (isolate 5) revealed recovery rates of 73% (**1**), 93% (**2**), 99% (**3**), 103% (**5**), 70% (**6**), 105% (**7**), 76% (**8**), 89% (**9**), 96% (**10**), 105% (**11**), 70% (**12**), 104% (**13**), and 93% (**14**).

Quantification of bitter compounds **1**−**14** in commercial pea-protein isolates and dose−activity considerations: The UHPLC−DMS−MS/MS method developed was applied to quantify the key bitter molecules in 17 pea-protein isolates. The quantitative data (mmol/kg) are summarized in a faceted scatterplot (Figure 5).

The free fatty acids (FFAs) α-linolenic acid (**10**) and linoleic acid (**13**) were found as the predominant compounds in the tested pea-protein isolates, with concentrations ranging from 0.32 to 2.59 mmol/kg and from 3.55 to 12.19 mmol/kg, respectively. Although there are no comparative quantitative data available for other pea-protein isolates, this trend is in line with the literature in which, for these two compounds, the highest amounts of all tested FFAs were determined in seeds of *P. sativum* L. and lipophilic extracts of different pea samples.[43,44] The tri- and monohydroxy fatty acids THOAs **1**−**4** and HODEs were present in much lower levels between 0.00001 and 1.48 mmol/kg and between 0.004 and 0.44 mmol/kg, respectively. Even lower concentrations for 2-hydroxy derivatives **11** and **12** were determined to range from 0.0002 to 0.11 mmol/kg. The monoglyceride 1-linoleoyl glycerol (**9**) was present in concentrations between 0.01 and 0.25 mmol/kg, and for (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (13OxoDE, **14**), levels ranging from 0.004 to 0.47 mmol/kg were measured.

Dose-over-threshold (DoT) factors were determined as the ratio of the concentration of the taste threshold of a respective tastant[45] to evaluate the bitter taste impact of compounds **1**−**13**. The taste thresholds for calculating the values were taken from the literature.[24,26,28,46] The data were displayed in two heatmaps (Figure 6): one of them for every single compound individually and the other one summarized to the following groups: THOAs (**1**−**4**), HODEs (**5**−**8**), 2-OH-FAs (**11** and **12**), and FFAs (**10** and **13**). The compounds 1-linoleoyl glycerol (**9**) and 13OxoDE (**14**) were not added to any groups.

The DoT-factors' calculation revealed the highest bitter impact for linoleic acid (**13**) in the 17 tested pea-protein isolates, exceeding its bitter recognition threshold by an average factor of 7.6, followed by α-linolenic acid (**10**) showing an average DoT-factor of 4.7. In all the tested isolates, the FFAs showed values over 1, indicating a high bitter impact on this substance class's bitterness. In 6 of the 17 pea-protein isolates, a bitter taste contribution of 1-linoleoyl glycerol (**9**) could be shown, and an average DoT-factor of 1.1 could be determined. The highest bitter impact within the oxidized fatty acids could be shown for the THOAs, especially for 9,10,11-trihydroxyoctadec-12-enoic (**3**) and 11,12,13-trihydroxyoctadec-9-enoic acid (**4**). In 14 pea-protein isolates, values over 1.0 could be determined for the trihydroxy derivatives group, with an average DoT-factor of 6.2. According to the calculated DoT-factors, none of the other tested compounds directly contributes to the pea-protein isolates' bitter taste.

In summary, a suitable analytical method enabling the mapping as well as the simultaneous quantification of bitter-tasting lipids and lipid oxidation products (**1**−**14**) in different pea-protein isolates by means of UHPLC−DMS−MS/MS was developed. For the first time, the DMS technique could be used to simultaneously quantify *E,Z*- and *E,E* isomers of HODEs (HODEs, **5**−**8**). Until now, no quantitative data of the main bitter compounds in pea-protein isolates have been

https://doi.org/10.1021/acs.jafc.1c02889
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reported. By applying this newly developed UHPLC−DMS−MS/MS method to 17 commercial pea-protein isolates, first insights into the quantities of the bitter components could be gained. Based on these data and bitter taste thresholds, DoT factors were determined to evaluate the bitter impact of the individual lipids and lipid oxidation products. The FFAs α-linolenic acid (**10**) and linoleic acid (**13**), as well as the THOAs, especially 9,10,11-trihydroxyoctadec-12-enoic (**3**) and 11,12,13-trihydroxyoctadec-9-enoic acids (**4**), were demonstrated to be major contributors to the isolates' bitterness. Furthermore, the impact of 1-linoleoyl glycerol (**9**) on the bitter taste could be shown for 14 of the 17 tested pea-protein isolates. On the basis of this method, it can be concluded that product-specific taste differences can be objectified and growing programs as well as technological process parameters can be knowledge-based-optimized in future.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This IGF project of the FEI is supported via AiF within the program for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi) based on a resolution of the German Parliament under grant number AiF-18814 N.

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J. Agric. Food Chem. 2021, 69, 8768−8776