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Molecularization of Bitter Off-Taste Compounds in Pea-Protein Isolates (*Pisum sativum* L.)

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Running Title: Bitter tastants in Pea Protein Isolates

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ABSTRACT

Activity-guided fractionations, combined with taste dilution analyses (TDA), were performed to locate the key compounds contributing to the bitter off-taste of pea-protein isolates (*Pisum sativum* L.). Purification of the compounds perceived with the highest sensory impact, followed by 1D/2D-NMR, (LC-)MS/MS, LC-TOF-MS and MS^E experiments, led to the identification of 14 lipids and lipid oxidation products, namely, 9,10,13-trihydroxyoctadec-12-enoic acid, 9,12,13-trihydroxy-octadec-10-enoic acid, 9,10,11-trihydroxyoctadec-12-enoic, 11,12,13-trihydroxy-octadec-9-enoic acid, (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid, (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, (9*E*,11*E*)-13-hydroxy-octadeca-9,11-dienoic acid, 1-linoleoyl glycerol, *α*-linolenic acid, 2-hydroxypalmitic acid, 2-hydroxyoleic acid, linoleic acid, (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid and, octacosa-6,9,19,22-tetraen. Herein, we present the isolation, structure determination, and sensory activity of these molecules. Depending on their structure, the isolated compounds showed human bitter recognition thresholds between 0.06 and 0.99 mmol/L in water.

Keywords*:* pea, *Pisum sativum*, bitter taste, sensomics, taste dilution analysis, lipids, fatty acids, oxylipins.

INTRODUCTION

Since the world population, and consequently the global food requirements, are steadily rising, proteins have been identified as a future limiting nutrient.[1,2] Therefore, the development of newer and more sustainable protein sources is becoming more and more important to cover this demand. In the food-processing industry proteins – or rather protein isolates – are often used as food ingredients due to their techno-functional properties. They are utilized, for example, as emulsifiers, foaming agents, and gel formers and they are important constituents in the wide field of sports and fitness nutrition.[3–7] For these applications, animal-based protein sources such as casein, whey, or egg are commonly the preferred choices over plant-protein sources such as pea (*Pisum sativum* L.). A major disadvantage of using plant-based proteins is the frequent exhibition of a high bitter off-flavor.[8–10] This bitterness can strongly limit the usage of pea-protein isolates despite being economically and ecologically attractive alternatives to their animal-based analogs.[11,12] Furthermore, a growing interest in vegan and vegetarian products can be observed over the past couple of years, which makes these plant-based proteins appealing to a wide range of consumers.[13,14] To deal with this rising interest in new protein sources, it is necessary to clarify the reasons for their bitter off-taste impressions to get access to these novel protein sources.

In recent years, it has been proposed that a wide variety of secondary plant metabolites could be the reason for the bitter taste of pea-protein isolates by potentially sticking to the protein through non-covalent interactions. Although it is not possible to influence the intrinsic taste of intact proteins without hydrolysation steps, bitter tasting secondary metabolites can possibly be removed by technological and/or

breeding strategies. In the past, several phytochemicals, especially saponins such as soyasaponin I and DDMP saponin, have often been associated with a bitter-astringent off-taste in commercial pea-protein isolates.[15–18] Previous studies could also demonstrate a bitter activity of several amino acids and peptides, which may be due to hydrolysis during processing.[19–23] Bitter lipid oxidation products that are formed either through enzymatic pathways or by autoxidation have been considered as well.[24–28]Taking all these references into account, we realize that no systematic studies on the key bitter molecules of pea-protein isolates have been reported so far.

Taste dilution analysis (TDA), a sensory-guided method for food analysis, has been successfully applied to characterize taste compounds. Many key taste and off-taste compounds in hazelnuts[29], asparagus[30–32], carrots[33], lineseed oil[34], oat[35], coffee,[36] and recently, rapeseed protein isolates[37] could be identified by means of the activity-guided *sensomics* approach.[38] The aim of the present investigation was, therefore, to identify the key compounds contributing to the bitter off-taste of pea-protein isolates, to determine their human recognition thresholds, and evaluate their sensory contributions by means of concentration/activity considerations.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: acetonitrile (ACN), methanol (MeOH) (J.T. Baker, Deventer, The Netherlands); acetone, ethyl acetate, *n*-pentane (BDH Prolabo, Briare, France); dichlormethane (DCM), ethanol (EtOH) (VWR Chemicals, Fontenay-sous-Bois, France); isopropanol (Honeywell, Seelze, Germany); formic acid, H_2SO_4 (Merck, Darmstadt, Germany); caproic acid, $CDCl_3$, CrO_3, (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, leucine enkephalin,

malonic acid, MeOD, Me_2S, NaCl, Na_2SO_4, pyridine-d5, sebacic acid, soyasaponin I, L-tyrosine (Sigma-Aldrich, Steinheim, Germany), (10*E*,12*E*)-9-hydroxy-octadeca-10,12-dienoic acid, (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid, (9*Z*, 11*E*)-13-oxooctadeca-9,11-dienoic acid (Larodan AB, Solna, Schweden). The ACN used for HPLC-MS/MS analysis was LC-MS grade (Honeywell, Seelze, Germany), acetone, ethyl acetate, and *n*-pentane were distilled before use; and all the other solvents were HPLC grade. The water for chromatography was purified using an Advantage A 10 water System (Millipore, Molsheim, France). The pH of bottled water used for sensory analysis (Evian, low mineralization: 405 mg/L) was adjusted to 5.5 using formic acid. A pea-protein isolate with a protein content of ~75% was purchased by the Emsland Group GmbH (Emlichheim, Germany).

Sequential Solvent Extraction. 340 g of the pea-protein isolate were extracted three times with a mixture of MeOH and H_2O (1+1, v+v; 1400 mL) by stirring for 30 min at room temperature, followed by filtration using a Büchner funnel (Rotilabo®, 185 mm, type 111A, Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The filtrates were combined, freed from solvent in vacuum at 40 °C and freeze-dried to give the MeOH/H_2O extractables (fraction I). The residue was extracted three times with MeOH (1400 mL; fraction II), followed by extraction with MeOH/acetone (6+4, v/v; 1400 mL; fraction III), ethyl acetate (1400 mL; fraction IV) and *n*-pentane (1400 mL; fraction V). The corresponding fractions I – V were lyophilized twice (to remove trace amounts of solvents) and kept at –20°C until being used for comparative taste profile analysis (**Table 1**).

Separation of Fraction I by Medium Pressure Liquid Chromatography (MPLC). For MPLC chromatography, fraction I was dissolved in 0.1% formic acid in H_2O (v/v; 3 g/100 mL) with ultrasonification (15 min). 10 mL of this solution were

126 separated on a self-packed polypropylene cartridge (150 x 40 mm i.d., Büchi) filled
127 with LiChroprep RP-18 (25 – 40 µm) material (Merck, Darmstadt, Germany).
128 Chromatography was run at a flow rate of 40 mL/min, using 0.1% formic acid in H_2O
129 (v/v) as solvent A and MeOH as solvent B. Thirteen fractions (I-1 to I-13) were
130 collected by monitoring the effluent using a Sedex LT-ELSD detector Model 80
131 (Sedere, Alfortville, France) at Gain 8 using the following gradient: 0 min, 0% B;
132 7 min, 0% B; 8 min, 10% B; 13 min, 10% B; 14 min, 20% B; 19 min, 20% B, 20 min,
133 30% B; 25 min, 30% B; 26 min, 40% B; 31 min, 40% B; 32 min, 50% B; 37 min,
134 50% B; 38 min, 60% B; 43 min, 60% B; 44 min, 70% B; 49 min, 70% B; 50 min,
135 80% B; 55 min, 80% B; 56 min, 90% B; 61 min, 90% B; 62 min, 100% B; 92 min,
136 100% B. All 13 fractions were collected in a fraction collector, freed from solvent
137 (vacuum, 40 °C), lyophilized twice, and then kept at -20°C until being used for sensory
138 and chemical analyses.

139 **Identification of Soyasaponin I (1) in Fraction I-10.** An aliquot (20 mg) of
140 fraction I-10 (t_R = 56 – 61 min) was dissolved in of H_2O/ACN (20 mL, 6+4, v+v) with
141 ultrasonification (15 min). After carrying out membrane filtration, the sample (250 µL)
142 was injected into a Synergi 4u Hydro-RP column (250 x 21.1 mm, 5 µm, 80 Å,
143 Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same
144 type at a flow rate of 21 mL/min and using solvents consisting of 0.1% formic acid in
145 H_2O (v/v) as solvent A and ACN as solvent B. The effluent was monitored using a
146 Sedex LT-ELSD detector Model 85 (Sedere, Alfortville, France) at Gain 7. For
147 separation, the following gradient was used: 0 min, 50% B; 6 min, 70% B; 7 min,
148 100% B; 10 min, 100% B; 12 min, 50% B; 17 min, 50% B. Fraction I-10-1
149 (t_R = 56 – 61 min), containing the purified soyasaponin I (**1**), was collected in multiple
150 HPLC runs, combined, freed from the solvent (vacuum, 40 °C), and then freeze-dried

twice. The obtained residues were used for structural analysis, quantification methods and taste threshold determination. 1/2D NMR, TOF-MS, and MS/MS experiments, and a comparison of the observed retention time with that of a commercial reference verified the structure of soyasaponin I (**1**).

soyasaponin I, **1** (**Figure 1**): LC-TOF-MS: *m/z* 943.5271 (measured), *m/z* 943.5266 (calcd. for $[C_{48}H_{79}O_{18}]^+$), *m/z* 941.5110; *m/z* detektiert: 941.5110 (calcd. for $[C_{48}H_{77}O_{18}]^-$). MS/MS (DP = 20 V, CE = 30 V): 405 [Aglycon-OH-$2H_2O$]$^+$ (4), 424 [Aglycon-OH-H_2O]$^+$ (64), 442 [Aglycon-OH]$^+$ (100), 460 [M-GlcA-Gal-Rha+H]$^+$ (14), 600 [M-GlcA-Gal-$2H_2O$+H]$^+$ (24), 618 [M-GlcA-Gal-H_2O+H]$^+$ (11), 636 [M-GlcA-Gal+H]$^+$ (6), 798 [M-Rha+H]$^+$ (16), 944 [M+H]$^+$ (10). ^{1}H-NMR (500 MHz, pyridine-d5) *δ*/ppm 0.71 [s, 3H, H-C(25)], 0.85 [m, 1H, H-C(1-*α*)], 0.88 [m, 1H, H-C(5)], 0.96 [s, 3H, H-C(26)], 1.00 [s, 3H, H-C(29)], 1.13 [m, 1H, H-C(19-*α*)], 1.24 [s, 3H, H-C(28)], 1.28 [m, 1H, H-C(6-*β*)], 1.30 [s, 3H, H-C(30)], 1.31 [s, 3H, H-C(27)], 1.36 [m, 1H, H-C(7-*β*)], 1.37 [m, 1H, H-C(1-*β*)], 1.45 [s, 3H, H-C(23)], 1.54 [m, 1H, H-C(7-*α*)], 1.57 [m, 1H, H-C(6-*α*)], 1.62 [m, 1H, H-C(9)], 1.65 [m, 1H, H-C(21-*α*)], 1.77 [m, 1H, H-C(21-β)], 1.79 [m, 1H, H-C(11-α)], 1.80 [d, 3H, *J* = 6.9 Hz, H-C(6‘‘‘)], 1.81 – 1.91 [m, 2H, H-C(15), H-C(16)], 1.86 [m, 1H, H-C(11-*β*)], 1.87 [m, 1H, H-C(2-*β*)], 1.91 [m, 1H, H-C(19-*β*)], 2.16 – 2.22 [m, 2H, H-C(15), H-C(16)], 2.21 [m, 1H, H-C(2-*α*)], 2.41 [d, 1H, *J* = 12.4 Hz, H-C(18)], 3.27 [d, 2H, *J* = 11.4 Hz, H-C(24)], 3.41 [dd, 1H, *J* = 4.1 Hz/11.4 Hz, H-C(3)], 3.75 [dd, 1H, *J* = 3.2 Hz/6.3 Hz, H-C(22)], 3.95 [ddd, 1H, *J* = 1.6 Hz/5,7 Hz/6.5 Hz, H-C(5‘‘)], 4.13 [dd, 1H, *J* = 3.7 Hz/9.8 Hz, H-C(3‘‘)], 4.29 [d, 1H, *J* = 11.4 Hz, H-C(24)], 4.34 [dd, 1H, *J* = 5.7 Hz/11.2 Hz, H-C(6‘‘)], 4.36 [t, 1H, *J* = 9.3 Hz, H-C(4‘‘‘)], 4.42 [dd, 1H, *J* = 1.6 Hz/3.4 Hz, H-C(4‘‘)], 4.44 [dd, 1H, *J* = 6.5Hz/11.3 Hz, H-C(6‘‘)], 4.49 [dd, 1H, *J* = 7.5 Hz/10.6 Hz, H-C(4‘)], 4.58 [dd, 1H, *J* = 7.5 Hz/9.3 Hz, H-C(2‘‘)], 4.63 [m, 1H, H-C(2‘)], 4.66 [m, 2H, H-C(3‘),

H-C(5‘)], 4.70 [dd, 1H, *J* = 3.7 Hz/9.2 Hz, H-C(3‘‘)], 4.83 [dd, 1H, *J* = 2.0 Hz/3.7 Hz, H-C(2‘‘)], 5.00 [d, 1H, *J* = 8.1 Hz, H-C(1‘)], 5.02 [dq, 1H, *J* = 6.6 Hz/9.3 Hz, H-C(5‘‘)], 5.31 [t, 1H, *J* = 4.0 Hz, H-C(12)], 5.83 [d, 1H, *J* = 7.5 Hz, H-C(1‘‘)], 6.31 [d, 1H, *J* = 1.4 Hz, H-C(1‘‘‘)]. ^{13}C-NMR (500 MHz, pyridine-d5) *δ*/ppm 16.18 [C(25)], 17.27 [C(26)], 18.47 [C(6)], 19.43 [C(6’’’)], 21.56 [C(30)], 23.38 [C(23)], 24.31 [C(11)], 26.01 [C(27)], 26.97 [C(15)], 27.06 [C(2)], 27.07 [C(16)], 28.96 [C(28)], 31.26 [C(20)], 33.70 [C(7)], 34.36 [C(29)], 36.91 [C(10)], 38.33 [C(17)], 39.11 [C(1)], 40.35 [C(8)], 42.24 [C(21)], 42.77 [C(14)], 44.27 [C(4)], 45.59 [C(18)], 47.26 [C(19)], 48.11 [C(9)], 56.46 [C(5)], 62.01 [C(6’’)], 64.19 [C(24)], 68.85 [C(5’’’)], 71.57 [C(4’’)], 72.86 [C(2’’’)], 73.28 [C(3’’’)], 74.82 [C(4’’’)], 76.01 [C(22)], 76.78 [C(5’’)], 77.08 [C(2’), C(5’)], 77.11 [C(3’’)], 78.04 [C(2’)], 78.27 [C(2’’)], 78.94 [C(2’)], 79.27 [C(4’)], 91.56 [C(3)], 102.11 [C(1’’’)], 102.95 [C(1’’)], 105.80 [C(1’)], 122.76 [C(12)], 146.03 [C(13)], 172.96 [C(6’)].

Separation of Fraction I-11 by Preparative High Performance Liquid Chromatography (HPLC). Fraction I-11 (t_R = 62 – 72 min), which exhibited the highest bitter impact, was dissolved in H_2O/ACN (6+4, v+v; 340 mg in 6 mL) with ultrasonification (15 min). After membrane filtration the sample (300 µL) was injected into a Synergi 4u Hydro-RP column (250 x 21.1 mm, 5 µm, 80 Å, Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same type. Separation was performed at a flow rate of 21 mL/min and using 0.1% formic acid in H_2O (v/v) as solvent A and acetonitrile as solvent B. The effluent was monitored using a Sedex LT-ELSD detector Model 85 (Sedere, Alfortville, France) at Gain 8. For chromatography, the following gradient was used: 0 min, 40% B; 2 min, 40% B; 5 min, 50% B; 25 min, 100% B; 28 min, 100% B; 33 min, 40% B; 40 min, 40% B. The effluent was separated to give 16 fractions (I-11-1 to I-11-16) and freed from solvent

(vacuum, 40 °C). The residues were dissolved in water, lyophilized twice, and then kept at -20 °C until further use.

Identification of the Key Bitter Compounds in Fraction I-11-8. Fraction I-11-8 was dissolved in ACN/isopropanol (3+2, v+v; 10 mg/mL) and, after membrane filtration, separated by semi-preparative HPLC using a Nucleodur C18 Pyramid column (250 x 10 mm, 5 µm, 110 Å, Macherey-Nagel, Düren, Germany) equipped with a guard column of the same type with a binary gradient using 0.1% formic acid in H_2O as solvent A and ACN/isopropanol/formic acid (600+400+1, v+v+v) as solvent B (flow rate 4.7 mL/min): 0 min, 30% B; 2 min, 35% B; 6 min, 100% B; 11 min, 100% B; 14 min, 30% B; 17 min, 30% B. A Sedex LT-ELSD detector Model 85 (Sedere, Alfortville, France) was used at Gain 5. Fractions I-11-8-1 (t_R = 8.0 min) and I-11-8-2 (t_R = 8.3 min), containing the bitter target compounds, were collected in multiple HPLC runs, combined, separated from the solvent (vacuum, 40 °C), and then lyophilized twice. The residues obtained were used for taste dilution analysis (TDA) and structure identification experiments. LC-TOF-MS and 1/2D NMR analyses revealed that I-11-8-1 and I-11-8-2 were isomeric mixtures of trihydroxyoctadecenoic acids (THOAs), namely, 9,10,13-trihydroxyoctadec-11-enoic acid (**2**), 9,12,13-trihydroxyoctadec-10-enoic acid (**3**), 9,10,11-trihydroxyoctadec-12-enoic acid (**4**) and 11,12,13-trihydroxyoctadec-9-enoic acid (**5**).

9,10,13-trihydroxyoctadec-11-enoic acid, **2** (**Figure 1**): LC-TOF-MS: *m/z* 329,2350 (measured), *m/z* 329,2328 (calcd. for $[C_{18}H_{33}O_5]^-$); MS^E (ESI^-, Ramp Transfer Collision Energy = 20 - 40 eV) *m/z* (%): 99.08 (11), 127.11 (12), 139.11 (61), 157.12 (5), 171.10 (100), 193.08 (54), 329.23 (91), 351.21 (37); ^{1}H-NMR (500 MHz, MeOD) *δ*/ppm 0.93 [t, *J* = 6.5 Hz, 3H, H-C(18)], 1.31 – 1.41 [m, 14H, H-C(4), H-C(5), H-C(6), H-C(7), H-C(15), H-C(16), H-C(17)], 1.49 – 1.57 [m, 4H, H-C(8), H-(C14)],

1.64 [m, 2H, H-C(3)], 2.37 [m, 2H, H-C(2)], 3.44 [m, 1H, H-C(13)], 3.94 [m, 1H, H-C(9)], 4.07 [m, 1H, H-C(10)], 5.71 [m, 1H, H-C(11)], 5.73 [m, 1H, H-C(12)]; ^{13}C-NMR (500 MHz, MeOD) *δ*/ppm 13.03 [C(18)], 22.33 [C(17)], 24.86 [C(3)], 28.61 – 29.62 [C(4), C(5), C(6), C(7), C(15)], 31.72 [C(16)], 32.86 [C(14)], 33.78 [C(2)], 37.09 [C(8)], 71.74 [C(10)], 74.40 [C(13)], 75.34 [C(9)], 129.63 [C(12)], 135.20 [C(11)], 175.13 [C(1)].

9,12,13-trihydroxyoctadec-10-enoic acid, **3** (**Figure 1**): LC-TOF-MS: *m/z* 329.2327 (measured), *m/z* 329.2328 (calcd. for $[C_{18}H_{33}O_5]^-$); MS^E (ESI^-, Ramp Transfer Collision Energy = 20 - 40 eV) *m/z* (%) 99.08 (11), 211.13 (84), 229.14 (23), 233.12 (81), 329.23 (100), 351.21 (84); ^{1}H-NMR (500 MHz, MeOD) *δ*/ppm 0,93 [t, *J* = 6.5 Hz, 3H, H-C(18)], 1.31 – 1.41 [m, 14H, H-C(4), H-C(5), H-C(6), H-C(7), H-C(15), H-C(16), H-C(17)], 1.49 – 1.57 [m, 4H, H-C(8), H-(C14)], 1.64 [m, 2H, H-C(3)], 2.37 [m, 2H, H-C(2)], 3.44 [m, 1H, H-C(9)], 3.94 [m, 1H, H-C(13)], 4.07 [m, 1H, H-C(12)], 5.71 [m, 1H, H-C(11)], 5.73 [m, 1H, H-C(10)]; ^{13}C-NMR (500 MHz, MeOD) *δ*/ppm 13.03 [C(18)], 22.33 [C(17)], 24.86 [C(3)], 28.61 – 29.62 [C(4), C(5), C(6), C(7), C(15)], 31.72 [C(16)], 32.86 [C(8)], 33.78 [C(2)], 37.09 [C(14)], 71.74 [C(12)], 74.40 [C(9)], 75.34 [C(13)], 129.63 [C(10)], 135.20 [C(11)], 175.13 [C(1)].

9,10,11-trihydroxyoctadec-12-enoic acid, **4** (**Figure 1**): LC-TOF-MS: *m/z* 329.2333 (measured), *m/z* 329.2328 (calcd. for $[C_{18}H_{33}O_5]^-$); MS^E (ESI^-, Ramp Transfer Collision Energy = 20 - 40 eV) *m/z* (%) 127.11 (23), 139.11 (14), 155.11 (13), 171.10 (68), 193.08 (54), 201.11 (61), 223.09 (32), 329.23 (100), 351,21 (91); ^{1}H-NMR (500 MHz, MeOD) *δ*/ppm 0.93 [t, *J* = 7.7 Hz, 3H, H-C(18)], 1.25 – 1.39 [m, 14H, H-C(4), H-C(5), H-C(6), H-C(7), H-C(15), H-C(16), H-C(17)], 1.56 [m, 2H, H-C(8)], 1.66 [m, 2H, H-C(3)], 2.08 [m, 2H, H-C(14)], 2.38 [m, 2H, H-C(2)], 3.24 [m, 1H, H-C(10)], 3.59 [m, 1H, H-C(9)], 4.55 [m, 1H, H-C(11)], 5.43 [t, *J* = 9.8 Hz ,1H,

H-C(12)], 5.76 [m, 1H, H-C(13)]; ^{13}C-NMR (500 MHz, MeOD) *δ*/ppm 13.03 [C(18)], 22.53 [C(17)], 24.39 [C(3)], 28.88 – 29.40 [C(4), C(5), C(6), C(7), C(15)], 31.77 [C(16)], 32.37 [C(14)], 33.61 [C(8)], 34.37 [C(2)], 68.35 [C(11)], 71.24 [C(9)], 76.16 [C(10)], 128.91 [C(12)], 133.24 [C(13)], 175.15 [C(1)].

11,12,13-trihydroxyoctadec-9-enoic acid, **5** (**Figure 1**): LC-TOF-MS: *m/z* 329.2330 (measured), *m/z* 329.2328 (calcd. for $[C_{18}H_{33}O_5]^-$); MS^E (ESI^-, Ramp Transfer Collision Energy = 20-40 eV) *m/z* (%) 99.08 (17); 112.99 (7); 129.09 (12); 153.13 (7); 199.13 (26); 211.13 (8); 221.12 (36); 329.23 (44); 351.21 (100); ^{1}H-NMR (500 MHz, MeOD) *δ*/ppm 0.93 [t, *J* = 7,7 Hz, 3H, H-C(18)], 1.25 – 1.39 [m, 14H, H-C(4), H-C(5), H-C(6), H-C(7), H-C(15), H-C(16), H-C(17)], 1.56 [m, 2H, H-C(14)], 1.66 [m, 2H, H-C(3)], 2.08 [m, 2H, H-C(8)], 2.38 [m, 2H, H-C(2)], 3.24 [m, 1H, H-C(12)], 3.59 [m, 1H, H-C(13)], 4.55 [m, 1H, H-C(11)], 5.43 [t, *J* = 9,8 Hz ,1H, H-C(10)], 5.76 [m, 1H, H-C(9)]; ^{13}C-NMR (500 MHz, MeOD) *δ*/ppm 13.03 [C(18)], 22.53 [C(17)], 24.39 [C(3)], 28.88 – 29.40 [C(4), C(5), C(6), C(7), C(15)], 31.77 [C(16)], 32.37 [C(8)], 33.61 [C(14)], 34.37 [C(2)], 68.35 [C(11)], 71.24 [C(13)], 76.16 [C(12)], 128.91 [C(10)], 133.24 [C(9)], 175.15 [C(1)].

Identification of the Key Bitter Compounds in Fraction I-11-14. LC-MS experiments demonstrated only quantitative differences between the bitter active fractions I-11-8, I-11-14 and I-11-15 (data not shown). Therefore, the key bitter tastants were isolated from fractions I-11-8 and I-11-15.

Identification of the Key Bitter Compounds in Fraction I-11-15. Fraction I-11-15, evaluated with the highest bitter impact, was dissolved in ACN/isopropanol (3+2, v+v; 6 mg/mL) and, after membrane filtration, it was injected into a semi-preparative Luna® C5 column (250 x 10 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same type operated

at a flow rate of 4.7 mL/min and using solvents consisting of 0.1% formic acid in H_2O (solvent A) and ACN/isopropanol/formic acid (600+400+1, v+v+v) (solvent B). Chromatography was achieved by applying the following gradient: 0 min, 74% B; 15 min, 74% B; 19 min, 100% B; 26 min, 100% B; 29 min, 74% B; 33 min, 74% B. The effluent was detected using a Sedex LT-ELSD detector Model 85 (Sedere, Alfortville, France) at Gain 7. The individually collected fractions, namely, I-11-15-1 to I-11-15-6, were freed from solvent in vacuum at 40 °C and freeze-dried twice. The obtained residues were then used for structural analysis and taste dilution analysis. Based on the MS/MS, TOF-MS and 1/2D NMR results, the structures of the taste compounds I-11-15-1 to I-11-15-6 could be determined as (10*E*,12*E*)-9-hydroxy-octadeca-10,12-dienoic acid (**6**, t_R = 9.7 min), (9*Z*,11*E*)-13-hydroxy-octadeca-9,11-dienoic acid (**7**, t_R = 9.7 min), (9*E*,11*E*)-13-hydroxy-octadeca-9,11-dienoic acid (**8**, t_R = 9.7 min), 1-linoleoyl glycerol (**9**, t_R = 10.6 min), *α*-linolenic acid (**10**, t_R = 11.1 min), 2-hydroxypalmitic acid (**11**, t_R = 12.0 min), 2-hydroxyoleic acid (**12**, t_R = 13.1 min) and linoleic acid (**13**, t_R = 14.2 min). The isomers of hydroxy-octadecadienoic acid (**6 – 8**) were identified by LC-TOF-MS using the corresponding commercial references.

(10E,12E)-9-hydroxyoctadeca-10,12-dienoic acid, **6** (**Figure 1**): LC-TOF-MS: *m/z* 295.2267 (measured), *m/z* 295.2273 (calcd. for $[C_{18}H_{31}O_3]^-$); MS^E (ESI^-, Ramp Transfer Collision Energy = 20-40 eV) *m/z* (%) 112.98 (11), 171.10 (32), 277.22 (52), 295.23 (100), 317.21 (20); ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*/ppm 0.81 [t, *J* = 6.8 Hz, 3H, H-C(18)], 1.23 [m, 14H, H-C(4), H-C(5), H-C(6), H-C(7), H-C(15), H-C(16), H-C(17)],1.52 [m, 2H, H-C(8)], 1.56 [m, 2H, H-C(3)], 1.98 [m, 2H, H-C(14)], 2.27 [t, *J* = 7.4 Hz, 2H, H-C(2)], 3.44 [m, 1H, H-C(9)], 5.31 [m,1H, H-C(10)], 5.62 [m, 1H, H-C(13)], 5.98 [m, 1H, H-C(12)], 6.06 [m, 1H, H-C(11)]; ^{13}C-NMR (500 MHz, $CDCl_3$)

300 *δ*/ppm 14.07 [C(18)], 22.53 [C(17)], 24.69 [C(3)], 27.21 [C(14)], 28.88 – 29.38 [C(4),
301 C(5), C(6), C(7), C(15)], 31.45 [C(16)], 33.69 [C(2)], 35.68 [C(8)], 82.36 [C(9)], 129.40
302 [C(12)], 131.39 [C(10)], 132.86 [C(11)], 135.33 [C(13)], 177.78 [C(1)].

303 *(9Z,11E)-13-hydroxyoctadeca-9,11-dienoic acid,* **7** (**Figure 1**): LC-TOF-MS:
304 *m/z* 295.2269 (measured), *m/z* 295.2273 (calcd. for $[C_{18}H_{31}O_3]^-$); MS^E (ESI^-, Ramp
305 Transfer Collision Energy = 20-40 eV) *m/z* (%) 195.14 (9), 277.22 (9), 295.23 (100),
306 317.21 (26); ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*/ppm 0.81 [t, *J* = 6,8 Hz, 3H, H-C(18)], 1.23
307 [m, 12H, H-C(4), H-C(5), H-C(6), H-C(7), H-C(16), H-C(17)], 1.52 [m, 2H, H-C(14)],
308 1.56 [m, 2H, H-C(3)], 1.98 [m, 2H, H-C(7)], 2.27 [t, *J* = 7.4 Hz, 2H, H-C(2)], 3.44 [m,
309 1H, H-C(13)], 5.31 [m,1H, H-C(12)], 5.62 [m, 1H, H-C(9)], 5.98 [m, 1H, H-C(10)], 6.06
310 [m, 1H, H-C(11)]; ^{13}C-NMR (500 MHz, $CDCl_3$) *δ*/ppm 14.07 [C(18)], 22.53 [C(17)],
311 24.69 [C(3)], 35.68 [C(14)], 28.88 – 29.38 [C(4), C(5), C(6), C(7), C(15)], 31.45
312 [C(16)], 33.69 [C(2)], 27.21 [C(8)], 82.36 [C(13)], 129.40 [C(10)], 131.39 [C(12)],
313 132.86 [C(11)], 135.33 [C(9)], 177.78 [C(1)].

314 *(9E,11E)-13-hydroxyoctadeca-9,11-dienoic acid*, **8** (**Figure 1**): LC-TOF-MS:
315 *m/z* 295.2270 (measured), *m/z* 295.2273 (calcd. for $[C_{18}H_{31}O_3]^-$); MS^E (ESI^-, Ramp
316 Transfer Collision Energy = 20-40 eV) *m/z* (%) 112.98 (11), 195.14 (34), 277.22 (35),
317 295.23 (100), 317.21 (35); ^{1}H-NMR (500 MHz, $CDCl_3$) δ/ppm 0.81 [t, *J* = 6,8 Hz, 3H,
318 H-C(18)], 1.23 [m, 14H, H-C(4), H-C(5), H-C(6), H-C(7), H-C(15), H-C(16), H-C(17)],
319 1.52 [m, 2H, H-C(8)], 1.56 [m, 2H, H-C(3)], 1.98 [m, 2H, H-C(14)], 2.27 [t, *J* = 7.4 Hz,
320 2H, H-C(2)], 3.44 [m, 1H, H-C(9)], 5.31 [m,1H, H-C(10)], 5.62 [m, 1H, H-C(13)], 5.98
321 [m, 1H, H-C(12)], 6.06 [m, 1H, H-C(11)]; ^{13}C-NMR (500 MHz, $CDCl_3$) *δ*/ppm 14.07
322 [C(18)], 22.53 [C(17)], 24.69 [C(3)], 35.68 [C(14)], 28.88 – 29.38 [C(4), C(5), C(6),
323 C(7), C(15)], 31.45 [C(16)], 33.69 [C(2)], 27.21 [C(8)], 82.36 [C(13)], 129.40 [C(10)],
324 131.39 [C(12)], 132.86 [C(11)], 135.33 [C(9)], 177.78 [C(1)].

1-linoleoyl glycerol, **9** (**Figure 1**): LC-TOF-MS: *m/z* 377.2707 (measured), *m/z* 377.2668 (calcd. for $[C_{21}H_{38}O_4Na]^+$); MS/MS (DP = 60 V, CE ramp = 1 – 23 V): *m/z* (%) 137 (13), 245 $[M\text{-glycerol-}H_2O]^+$ (25), 263 $[M\text{-glycerol}]^+$ (100), 337 $[M\text{-}H_2O]^+$ (44), 355 $[M+H]^+$ (31); ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*/ppm 0.82 [t, *J* = 5.7 Hz, 3H, H-C(18)], 1.20 – 1.34 [m, 14H, H-C(4), H-C(5), H-C(6), H-C(7), H-C(15), H-C(16), H-C(17)], 1.56 [m, 2H, H-C(3)], 1.99 [m, 4H, H-C(8), H-C(14)], 2.28 [t, *J* = 7,4 Hz, 2H, H-C(2)], 2.70 [dt, *J* = 6.5 Hz/17.5 Hz, 2H, H-C(11)], 3.60 [m, 2H, H-C(3‘)], 3.86 [m, 2H, H-C(2‘)],4.08 [dd, *J* = 4.5 Hz/11.4 Hz, 1H, H_α-C(2‘)], 4.14 [dd, *J* = 4.5 Hz/11.4 Hz, 1H, H_β-C(2‘)], 5.27 [m, 2H, H-C(10), H-C(12)], 5.30 [m, 2H, H-C(9), H-C(13)]; ^{13}C-NMR (500 MHz, $CDCl_3$) *δ*/ppm 14.07 [C(18)], 22.58 [C(17)], 24.90 [C(3)], 25.62 [C(11)], 27.21 [C(8), C(14)], 29.04 – 29.59 [C(4), C(5), C(6), C(7), C(15)], 31.53 [C(16)], 34.14 [C(2)], 63.34 [C(3‘)], 65.19 [C(1‘)], 70.28 [C(2‘)], 127.89/128.08 [C(10)/C(12)], 130.01/130.25 [C(9)/C(13)], 174.29 [C(1)].

α-linolenic acid, **10** (**Figure 1**): LC-TOF-MS: *m/z* 277.2154 (measured), *m/z* 277.2168 (calcd. for $[C_{18}H_{29}O_2]^-$); MS/MS (DP = -80 V, CE Ramp = (-52) V – (-40) V) *m/z* (%): 97 (16), 99 (8), 113 (47), 141 $[M\text{-}C_{10}H_{17}]^-$ (19), 167 $[M\text{-}C_8H_{15}]^-$ (6), 181 $[M\text{-}C_7H_{13}]^-$ (5), 207 $[M\text{-}_5H_{11}]^-$ (5), 234 (5), 247 $[M\text{-}C_2H_7]^-$ (4), 277 $[M\text{-}H]^-$ (19); ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*/ppm 0.91 (t, *J* = 6.7 Hz, 3H, H-C(18)], 1.25 [m, 8H, H-C(4), H-C(5), H-C(6), H-C(7)], 1.56 [m, 2H, *J* = 7.2 Hz, H-C(3)], 1.98 [dt, 2H, *J* = 7.0/15.1 Hz, H-C(17)], 2.28 [t, *J* = 7.5 Hz, 2H, H-C(2)], 2.74 [t, *J* = 6.3 Hz, 4H, H-C(11), H-C(14)], 5.30 [m, 6H, H-C(9), H-C(10), H-C(12), H-C(13), H-C(15), H-C(16)]; ^{13}C-NMR (400 MHz, $CDCl_3$) *δ*/ppm 14.21 [C(18)], 20.55 [C(17)], 24.57 [C(3)], 25.62 [C(11), C(14)], 27,20 [C(8)], 29.08 – 29.73 [C(4), C(5), C(6), C(7)], 127.11 [C(15)], 33.76 [C(2)], 127.75 [C(13)], 128.24/128.29 [C(12), C(10)], 130.24 [C(9)], 131.96 [C(16)], 178.44 [C(1)].

2-hydroxypalmitic acid, **11** (**Figure 1**): LC-TOF-MS: *m/z* 271.2266 (measured), *m/z* 271.2273 (calcd. for $[C_{16}H_{31}O_3]^-$); MS/MS (DP = -60 V, CE ramp = (43) V – (-28) V): *m/z* (%) 225 $[M\text{-}CO_2\text{-}H_2]^-$ (100), 253 $[M\text{-}H_2O]^-$ (3), 271 $[M\text{-}H]^-$ (24); ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*/ppm 0.81 [t, *J* = 6.8 Hz, 3H, H-C(16)], 1.20 [m, 22H, H-C(5), H-C(6), H-C(7), H-C(8), H-C(9), H-C(10), H-C(11), H-C(12), H-C(13), H-C(14), H-C(15)], 1.57 [m, 2H, H-C(4)], 1.64 [m, 2H, H-C(3)], 4.19 [dd, *J* = 3.9/7.5 Hz, 1H, H-C(2)]; ^{13}C-NMR (500 MHz, $CDCl_3$) *δ*/ppm 14.28 [C(16)], 22.69 [C(15)], 24.77 [C(4)], 29.29 – 29.69 [C(5), C(6), C(7), C(8), C(9), C(10), C(11), C(12), C(13)], 31.93 [C(14)], 34.24 [C(3)], 70.23 [C(2)], 177.96 [C(1)].

2-hydroxyoleic acid, **12** (**Figure 1**): LC-TOF-MS: *m/z* 297.2434 (measured), *m/z* 297.2430 (calcd. for $[C_{18}H_{33}O_3]^-$); MS/MS (DP = -40 V, CE ramp = (43) V – (-28) V): *m/z* (%) 251 $[M\text{-}CO_2\text{-}H_2]^-$ (100), 279 $[M\text{-}H_2O]^-$ (6), 297 $[M\text{-}H]^-$ (53); ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*/ppm 0.81 [t, *J* = 7.15 Hz, 3H, H-C(18)], 1.22 [m, 18H, H-C(5), H-C(6), H-C(7), H-C(12), H-C(13), H-C(14), H-C(15), H-C(16), H-C(17)], 1.56 [m, 2H, H-C(4)], 1.96 [m, 4H, H-C(8), H-C(11)], 2.28 [t, *J* = 7.42 Hz, 2H, H-C(3)], 4.18 [m, 1H, H-C(2)], 5.25/5.31 [m, 2H, H-C(9), H-C(10)]; ^{13}C-NMR (400 MHz, $CDCl_3$) *δ*/ppm 14.11 [C(18)], 22.68 [C(17)], 24.77 [C(4)], 27.23 [C(8), C(11)], 29.03 – 29.77 [C(5), C(6), C(7), C(12), C(13), C(14), C(15)], 31.91 [C(16)], 34.28 [C(3)], 70.22 [C(2)], 129.69/130.04 [C(9), C(10)], 178.12 [C(1)].

linoleic acid, **13** (**Figure 1**): LC-TOF-MS: *m/z* 279.2315 (measured), *m/z* 279.2324 (calcd. for $[C_{18}H_{31}O_2]^-$); MS/MS (DP = -50 V, CE Ramp = (-40 V) – (28 V)) *m/z* (%): 261 $[M\text{-}H_2O]^-$ (2), 279 $[M\text{-}H]^-$ (100); ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*/ppm 0.91 [t, 3H, *J* = 6.8 Hz, H-C(18)], 1.33 – 1.36 [m, 12H, H-C(4), H-C(5), H-C(6), H-C(7), H-C(15), H-C(16), H-C(17)], 1.65 [m, 2H, H-C(3)], 2.07 [m, 4H, H-C(8), H-C(14)], 2.36 [t, 2H, *J* = 7.5 Hz, H-C(2)], 2.79 [t, 2H, *J* = 6.4 Hz, H-C(11)], 5.36 [m, 2H, H-C(10),

H-C(12)], 5.39 [m, 2H, H-C(9), H-C(13)]; ^{13}C-NMR (500 MHz, $CDCl_3$) δ/ppm 14.06 [C(18)], 22.57 [C(17)], 24.71 [C(3)], 25.63 [C(11)], 27.19 [C(8), C(14)], 29.04 – 29.68 [C(4), C(5), C(6), C(7), C(15)], 31.53 [C(16)], 33.88 [C(2)], 127.90/128.05 [C(10), C(12)], 130.03/130.21 [C(9), C(13)], 178.55 [C(1)].

Identification of the Key Bitter Compounds in Fraction I-11-16. After dissolving the fraction in ACN/isopropanol (3+2, v+v; 6 mg/mL) and carrying out membrane filtration, fractionation was performed using a Luna® C5 column (250 x 10 mm, 5 µm, 100 Å, Phenomenex, Aschaffenburg, Germany), equipped with a guard column of the same type, with a flow rate of 4.7 mL/min and using 0.1% formic acid in H_2O as solvent A and ACN/isopropanol/formic acid (600+400+1, v+v+v) as solvent B. A semi-preparative analysis was performed by using the following gradient: 0 min, 74% B; 15 min, 74% B; 19 min, 100% B; 26 min, 100% B; 29 min, 74% B; 33 min, 74% B. The effluent of the fractionation was monitored using a Sedex LT-ELSD detector Model 85 (Sedere, Alfortville, France) at Gain 7 and by UV detection at a wavelength of 230 nm. The fraction was separated into two further fractions, namely, I-11-16-1 (t_R = 6.9 min) and I-11-16-2 (t_R = 13.9 min). The individually collected fractions were freed from solvent in vacuum at 40 °C and lyophilized twice. The obtained residues were then used for the taste dilution analysis (TDA) and for structure-elucidation experiments. Based on TOF-MS and 1/2D NMR results – and the comparison of the retentions times obtained for the samples with those observed for commercially available references – compound I-11-16-1 was identified as (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (**14**). 1/2D NMR experiments suggested that I-11-16-2 is octacosa-6,9,19,22-tetraen (**15**). However, the theoretical *m/z* of 385,38 ($[M–H]^-$) or 387,39 ($[M+H]^+$) could not be detected by means of MS/MS, LC-TOF-MS, or GC-MS to confirm the suggested compound (data

not shown). To verify the structure of the isolated substance, 2 mg aliquot of the sample was ozonolyzed and, after a reductive workup, oxidized to malonic acid, sebacic acid, and caproic acid. The generated (di)carboxylic acids were identified by LC-MS/MS, by comparing the observed retention times to those of their commercial references.

(9Z,11E)-13-oxooctadeca-9,11-dienoic acid, **14** (**Figure 1**): LC-TOF-MS: *m/z* 293.2114 (measured), *m/z* 293.2117 (calcd. for $[C_{18}H_{29}O_3]^-$); MS^E (ESI^-, Ramp Transfer Collision Energy = 20-40 eV) *m/z* (%) 97.06 (5), 99.92 (5), 113.10 (73), 139.11 (10), 183.01 (7), 195.14 (8), 293.21 (100), 315.19 (22); ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*/ppm 0.92 [t, 3H, *J* = 6.8 Hz, H-C(18)], 1.35 [m, 10H, H-C(4), H-C(5), H-C(6), H-C(16), H-C(17)], 1.43 [m, 2H, H-C(7)], 1.66 [m, 2H, H-C(15)], 1.67 [m, 2H, H-C(3)], 2.32 [dt, 2H, *J* = 7.3 Hz, H-C(8)], 2.38 [m, 2H, H-C(2)], 2.57 [t, 2H, *J* = 6.8 Hz, H-C(14)], 5.95 [dt, 1H, *J* = 7.5/11.1 Hz, H-C(9)], 6.15 [t, 1H, *J* = 11.5 Hz, H-C(10)], 6.20 [d, 1H, *J* = 15.4 Hz, H-C(12)], 7.53 [dd, 1H, *J* = 11.5/15.4 Hz, H-C(11)]; ^{13}C-NMR (500 MHz, $CDCl_3$) *δ*/ppm 14.26 [C(18)], 22.59 [C(17)], 24.08/24.59 [C(3), C(15)], 28.24 [C(8)], 28.80 – 29.18 [C(4), C(5), C(6), C(7)], 31.92 [C(16)], 34.34 [C(2)], 41.45 [C(14)], 126.91 [C(10)], 129.45 [C(12)], 137.13 [C(11)], 142.78 [C(9)], 178.88 [C(1)], 202.01 [C(13)].

octacosa-6,9,19,22-tetraen, **15** (**Figure 1**): ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*/ppm 0.93 [t, 6H, *J* = 6,5 Hz, H-C(1), H-C(28)], 1.28 – 1.34 [m, 24H, H-C(2), H-C(3), H-C(4), H-C(12), H-C(13), H-C(14), H-C(15), H-C(16), H-C(17), H-C(25), H-C(26), H-C(27)], 2.08 [m, 8H, H-C(5), H-C(11), H-C(18) H-C(24)], 2.80 [t, 4H, *J* = 6,4 Hz, H-C(8), H-C(21)] 5.34 [m, 4H, H-C(7), H-C(9), H-C(20), H-C(22)], 5.39 [m, 4H, H-C(6), H-C(10), H-C(19), H-C(23)]; ^{13}C-NMR (500 MHz, $CDCl_3$) *δ*/ppm 13.93 [C(1), C(28)], 22.57 [C(2), C(27)], 25.61 [C(8) C(21)], 27.16/27.22 [C(5), C(11), C(18), C(24)],

29.04 – 29.64 [C(4), C(12), C(13), C(14), C(15), C(16), C(17), C(25)], 31.23 [C(3), C(26)], 127.88/128.01 [C(7), C(9), C(20), C(22)], 130.03/130.20 [C(6), C(10), C(19), C(23)].

Sensory Analysis. *Sensory Panel Training and Sample Pretreatment.* Seven female and seven male panelists (23 – 31 years old), who had given their informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, were trained weekly for at least two years to become familiar with the sensory methodologies used and be able to evaluate aqueous references solutions of taste compounds.[31,39,40] Sensory analyses were performed in a sensory panel room at 22–25 °C using nose clips to prevent cross-model interactions with olfactory cues. Prior to the sensory analysis, the isolated fractions and compounds were confirmed to be effectively free of solvent traces.[31]

Taste Profile Analysis. An aliquot (3 g) of the pea protein isolate was suspended in water (50 mL; pH 5.5) and presented to the trained panel. The suspension was stirred the whole time during the test to avoid sedimentation. The panel was asked to evaluate the bitter, sweet, sour, umami, salty, and astringent taste perception on a scale from 0 (not detectable) to 5 (strongly detectable). In addition, an aliquot of fraction I was taken up in bottled water (25 mL, pH 5.5) in natural concentrations and then evaluated by the sensory panelists for bitterness, sweetness, sourness, umami, saltiness, and astringency.

Comparative Taste Profile Analysis. Aliquots of the lyophilized extracts II – V were dissolved in their natural concentrations (corresponding to 3 g of pea-protein isolate) in bottled water. After ultrasonification (15 min), they were presented to the sensory group, which was asked to rate the intensity of the individual taste qualities on a linear

scale from 0 (not detectable) to 5 (strongly detectable) in comparison to fraction I as the control.

Taste Dilution Analysis (TDA). The MPLC fractions I-1 – I -11, isolated from 11 g of fraction I, and the HPLC sub-fractions I-11-1 – I-11-16, isolated from 450 mg of fraction I-11, were dissolved in their natural concentrations in bottled water (20 mL, pH 5.5, 2% EtOH) and then, sequentially diluted 1:1 (v/v) with bottled water (pH 5.5). The serial dilutions of each of these fractions were then presented in order of increasing concentration to the panel, which was asked to evaluate each dilution step starting with the highest dilution level and, then, to mark where there was a first detectable difference between a negative control (bottled water, pH 5.5) and the sample. The dilution at which a taste difference between the sample and the blank could just be detected, was defined as taste dilution (TD) factor.[41]

Human Taste Recognition Thresholds. The threshold concentration, at which the bitter-taste quality of the compound was just detectable, was determined by performing a two-alternative forced-choice test (2-AFC).[42] Therefore, the purified substances were dissolved in bottled water (3% EtOH) at increasing concentrations and followed the procedure as mentioned. The geometric means of the first missed and last correctly identified concentrations were calculated and taken as the individual recognition thresholds. The taste threshold of the sensory group was approximated by averaging the threshold values of each panelist from two independent sessions.

Medium-Pressure Liquid Chromatography (MPLC). The MeOH/H_2O extract (fraction I) was separated on an MPLC apparatus (Büchi, Flawil, Switzerland) consisting of a binary pump module C-605, a control unit C-620, a fraction collector C-660, and a Sedex LT-ELSD detector Model 80 (Sedere, Alfortville, France). Chromatography (40 mL/min) was performed on a 150 x 40 mm polypropylene

474 cartridge filled with 25 – 40 µm LiChroprep RP-18 material (Merck, Darmstadt,
475 Germany). Instrument control and data analysis were performed using the software
476 SepaControl version 1.2.4000 (Büchi).

477 **High-Performance Liquid Chromatography (HPLC).** The HPLC apparatus
478 (Jasco, Groß-Umstadt, Germany) consisted of a binary pump system PU-2080 Plus,
479 an DG-2080-53 degaser and an Rh 7725i type Rheodyne injection valve (Rheodyne,
480 Bensheim, Germany). To monitor the effluent, an MD-2010 Plus diode array detector
481 (Jasco, Groß-Umstadt, Germany) working in the range of 200 – 500 nm and a Sedex
482 LT-ELSD detector Model 85 (Sedere, Alfortville, France) were used. Chromatography
483 measurements were run either on a preparative Synergi 4u Hydro-RP column (250 x
484 21.1 mm, 5 µm, 80Å, Phenomenex, Aschaffenburg, Germany), on a semi-preparative
485 Nucleodur C18 Pyramid column (250 x 10 mm, 5 µm, 110 Å, Macherey-Nagel,
486 Düren, Germany), or on a semi-preparative Luna® C5 column (250 x 10 mm, 5 µm,
487 100 Å, Phenomenex, Aschaffenburg, Germany), all equipped with a guard column of
488 the same type. The Chrompass Chromatography Data System, version 1.9, was used
489 for data acquisition.

490 **UPLC/Time-of-Flight Mass Spectrometry (LC-TOF-MS) and MS^E**
491 **Experiments**. High-resolution mass spectra were obtained by injecting aliquots
492 (2 µL) of all analytes in ACN/H_2O (80+20, v+v) into an Acquity UPLC core system
493 (Waters, Manchester, UK) consisting of a binary solvent manager, a sample manager
494 and a column oven. Chromatography measurements were run on a BEH C18 column
495 (150 x 2 mm, 1.7 µm, 130 Å, Waters, Manchester, UK) at a flow rate of 0.4 mL/min
496 and a temperature of 40 °C with 0.1% formic acid in H_2O (v/v) as solvent A and 0.1%
497 formic acid in ACN (v/v) as solvent B. Depending on the substances different
498 gradients were used. Compound **1** was analyzed using the following gradient: 0 min,

499 50% B; 4 min, 100% B; 4.5 min, 100% B; 4.6 min, 50% B; 5 min, 100% B. To
500 separate the compounds **2 – 5** the following gradient was used: 0 min, 36% B;
501 11 min, 40% B; 13 min, 100% B; 13.5 min, 100% B; 13.6 min, 36% B; 15 min,
502 100% B. The chromatographic analyses for substances **6 – 8** were performed with
503 the gradient: 0 min, 50% B; 11 min, 58% B; 13 min, 100% B; 13.5 min, 100% B;
504 13.6 min, 50% B; 15 min, 50% B. LC-TOF-MS experiments were performed for all the
505 other key bitter substances using the following gradient: 0 min, 70% B; 4 min,
506 100% B; 4.5 min, 100% B; 4.6 min, 70% B; 5 min, 70% B. High-resolution mass
507 spectra were recorded on a Synapt G2-S HDMS (Waters, Manchester, UK) in positive
508 and negative ESI resolution mode using 2.5 kV and -1.7 kV capillary voltage, 50 V
509 sampling cone, 4.0 kV extraction cone, 150 °C source temperature, 450 °C
510 desolvation temperature, 2 and 30 L/h cone gas and 800 L/h desolvation gas. The
511 mass spectrometer was calibrated (*m/z* 50 – 1200) using a solution of sodium formate
512 (0.5 mmol/L) dissolved in isopropanol/H_2O (9+1, v+v). The mass data were
513 autocorrected by infusing (at 10 µL/min) a solution of leucine enkephalin (1 ng/µL,
514 *m/z* 556.2771, $[M+H]^+$ and *m/z* 554.2615, $[M-H]^-$). The data were processed using
515 MassLynx 4.1 SCN 8.51 (Waters, Manchester, UK) and the elemental composition
516 tool.

517 **Tandem Mass Spectrometry (MS/MS).** An API 4000 QTRAP mass spectrometer
518 (Sciex, Darmstadt, Germany) with direct-flow infusion was used to obtain the mass
519 spectra. The samples were directly injected into the system through an injection valve
520 at a flow of 0.25 mL/min (ACN/H_2O, 1+1, v+v). The MS system operated in full-scan
521 mode (positive and negative, ion-spray voltage: 5500 and -4500 V) in the *m/z* range
522 of 50 – 1200: curtain gas, 10 psi; temperature, 350 °C; gas 1, 10 psi; gas 2, 0 psi;
523 CAD, -2 V; EP, 10 V and -10 V; CXP, 10 V and -9 V. Mass spectra were obtained

running a CE ramp in the range of 5 – 120 V and (-5) – (-120) V with the DP being optimized for each compound individually. Data acquisition and analysis were performed using the Analyst software 1.6.2 (Sciex, Darmstadt, Germany).

Nuclear Magnetic Resonance Spectrometry (NMR). 1D and 2D NMR spectra were recorded on a 400 MHz ultrashield Avance III spectrometer with a Broadband Observe BBFOplus probehead and a 500 MHz ultrashield plus Avance III spectrometer with a Tripple Resonance Cryo Probe TCI probehead (Bruker, Rheinstetten, Germany). Using pyridine-d5, $CDCl_3$ and MeOD as solvents, the chemical shifts were quoted in parts per million relative to the solvent signals. Quantitative NMR experiments were performed after calibrating the Bruker Avance III 400 MHz spectrometer with the ERETIC 2 tool using the PULCON methodology, as reported earlier.[43] The data was processed using the software Topspin 3.2 (Bruker, Rheinstetten, Germany) and MestReNova 10.0 (Mestrelab Research, Santiago de Compostela, Spain).

Quantification of Soyasaponin I (1) by UHPLC-MS/MS. *Solvent Extraction for Quantification.* For a triple determination, 3 x 500 mg of the pea-protein isolate were extracted using a mixture of MeOH and H_2O (1+1, v+v; 5 mL) on an Analogue Orbital Shaker 3005 (GFL, Burgwedel, Germany) for 1 h at 300 U/min. After membrane filtration, the extracts were analyzed by LC-MS/MS.

External Calibration Curve and Linear Range. A stock solution of commercial soyasaponin I (**1**) was prepared in MeOH/H_2O (80+20, v+v) and its exact concentration (0.94 mg/mL) was verified by qNMR.[43] This stock solution was diluted 1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10000, 1:20000, 1:50000 and 1:100000 with MeOH/H_2O (80+20, v+v). The dilutions were analyzed by LC-MS/MS using the characteristic MRM transition Q1→Q3 of *m/z*

549 943.43→441.20 as the quantifier. The parameters for soyasaponin I (**1**) were
550 optimized by flow injection (10 µL/min), detecting the fragmentation of the
551 pseudomolecular ion ($[M+H]^+$) into specific product ions by a special tuning process.
552 The instrument settings were optimized to the following parameters: DP, 46 V; EP,
553 10 V; CE, 37 V, and CXP; 50 V. An external calibration curve
554 ($y = 7.1242e5x + 5174.0555$, $R^2 = 0.9903$) was received by plotting the peak area
555 rations against the corresponding concentrations using the software MultiQuant 3.03
556 (Sciex, Darmstadt, Germany).

557 *UHPLC-MS/MS System and Parameters.* For quantification, a QTRAP 6500 mass
558 spectrometer (Sciex, Darmstadt, Germany) was used and operated in the full-scan
559 mode (ion-spray voltage: 5500 V) using the following parameters: curtain gas, 35 psi;
560 temperature, 500 °C; gas 1, 55 psi; gas 2, 65 psi and collision activated dissociation,
561 -2 V. The instrument was controlled using the Analyst 1.6.3 software (Sciex,
562 Darmstadt, Germany). The mass spectrometer was connected to a Nexera UHPLC
563 system (Shimadzu Europa GmbH, Duisburg, Germany) consisting of two LC pump
564 systems 30AD, a DGU-20A5 degassser, a SIL-30AC autosampler, a CTO-30A
565 column oven and a CBM-20A controller.

566 After injecting the samples (1 µL), chromatography was run on a Kinetex® F5
567 column (100 x 2.1 mm, 1.7 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) at a
568 flow rate of 0.5 mL/min using 0.1% formic acid in H_2O (v/v) as solvent A and 0.1%
569 formic acid in ACN (v/v) as solvent B. For separation, the following gradient was used:
570 0 min, 10% B; 0.25 min, 10% B; 3.5 min, 70% B; 3.7 min, 100% B; 4.7 min, 100% B;
571 5 min, 10% B; 6 min, 10% B.

572 **Ozonolysis of Octacosa-6,9,19,22-tetraen (15) and Identification of the**
573 **Generated (Di)carboxylic Acids by LC-MS/MS.** The structure of compound **14** was

574 confirmed by ozonolysis, followed by a reductive workup, oxidation to malonic acid,
575 sebacic acid and caproic acid, and subsequent analysis of the generated
576 (di)carboxylic acids by LC-MS/MS. Ozone was generated by an ozone generator
577 Model 502 (Fischer, Bonn-Bad Godesberg, Germany), operating with an oxygen flow
578 of 200 L/h (purity, 5.0; Westfalen, Germany) and 76 W. Ozone was bubbled through
579 a baked out Schlenk tube containing a stirred solution consisting of 2 mg of isolated
580 octacosa-6,9,19,22-tetraen in DCM (2 mL) until the solution turned blue (-78 °C).
581 After removing the excess of ozone with a stream of nitrogen (indicated by the
582 disappearance of the blue color), Me_2S (0.1 mL) was added and the solution was
583 stirred for 1 h at -78 °C. Afterwards, the reaction mixture was brought to room
584 temperature and stirred overnight. The mixture was then freed from solvent and re-
585 dissolved in 1 mL of acetone, followed by the addition of 0.1 ml of Jones reagent
586 (1.33 g of CrO_3 in 1 mL H_2O). After cooling the solution to 0 °C, 1.2 mL of
587 concentrated H_2SO_4 and 1 mL of H_2O were given into the flask. The mixture was then
588 stirred for 15 min and 0.2 mL of isopropanol were added to the flask. After removing
589 the solvent under vacuum, the residue was transferred quantitatively to a separating
590 funnel with a saturated solution of NaCl in H_2O (10 mL) and extracted three times
591 with ethyl acetate (10 mL). The combined organic extracts were dried with anhydrous
592 Na_2SO_4, filtered, and freed from solvent by vacuum evaporation.

593 The residue was taken up in MeOH (5 mL) and, after membrane filtration, an
594 aliquot was used for LC-MS/MS analysis. Therefore, a QTRAP 6500+ mass
595 spectrometer (Sciex, Darmstadt, Germany) was connected to an ExionLC UHPLC
596 System (Sciex, Darmstadt, Germany) consisting of two LC pump systems ExionLC
597 AD Pump, an ExionLC degasser, an ExionLC AD autosampler, an ExionLC AC
598 column oven, and an ExionLC controller. The system operated in the MRM- (low

mass) mode (ion-spray voltage: -4500 V) 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. Data acquisition and instrumental control were performed using the Analyst 1.6.3 software (Sciex, Darmstadt, Germany).

The declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) were optimized for commercial references of malonic acid, sebacic acid, and caproic acid by flow injection (10 µL/min), detecting the fragmentation of the pseudomolecular ions ($[M-H]^-$) into specific product ions by a special tuning process. The parameters were optimized to the following values: malonic acid (MRM 103.02→58.90): DP, -30 V; EP, -10 V; CE, - 10 V, CXP; -14 V; sebacic acid (MRM 201.06→182.90): DP, -55 V; EP, -10 V; CE, - 10 V, CXP; -18 V; caproic acid (MRM 114.84→115.00): DP, -20 V; EP, -10 V; CE, - 8 V, CXP; -13 V.

After sample injection (10 µL), chromatography was run on a Kinetex® C18 column (100 x 2.1 mm, 1.7 µm, 100 Å, Phenomenex, Aschaffenburg, Germany) with gradient elution at a flow rate of 0.4 mL/min. Using 0.1% formic acid in H_2O (v/v) as solvent A and 0.1% formic acid in ACN (v/v) as solvent B, separation was performed with the following gradient: 0 min, 0% B; 2 min, 0% B; 6 min, 65% B; 6.5 min, 100% B; 8.5 min, 100% B; 9.5 min, 0% B; 11.5 min, 0% B. A comparison of the observed retention times with those of the reference compounds led to the identification of malonic acid, sebacic acid and caproic acid as the generated (di)carboxylic acids released from **15** upon ozonolysis.

RESULTS AND DISCUSSION

In recent years, a wide range of phytochemicals have been considered to cause the bitter off-taste of pea-protein isolates. Especially saponins, such as soyasaponin I (**1**), were often discussed in literature to be main contributors to the bitterness of pea-based products.[15–18] In order to estimate the sensory contribution of soyasaponin I (**1**) to the bitterness of pea protein isolates, it was isolated, identified, and its human bitter taste threshold was determined. After quantification of the saponin, its influence on the overall bitter taste perception could be elucidated by calculating the Dose-over-Threshold factor (DoT).

Isolation and Identification of Soyasaponin I (1). A pea-protein isolate was extracted three times with MeOH/H_2O (fraction I). Fraction I was separated from the solvent and lyophylized twice. The obtained residue was further fractionated by MPLC-ELSD and 13 fractions (I-1 to I-13) were collected. LC-TOF-MS runs of all MPLC fractions indicated the presence of soyasaponin I in fraction I-10 (data not shown). To obtain the target molecule, fraction I-10 was purified by preparative RP-HPLC. 1/2D NMR, MS/MS and LC-TOF-MS data of the isolated soyasaponin I (**1**) were well in line with those reported in literature.[44–50]

Sensory Activity of Soyasaponin I (1). Prior to the determination of a human recognition threshold, the purity of compound **1** as well as the concentration of a stock solution were checked by LC-TOF-MS and qNMR.[43] The bitter threshold concentration of soyasaponin I (**1**) was evaluated by a two-alternative forced choice (2-AFC) test in 3% aqueous EtOH.[42] The test revealed a bitter and astringent taste perception induced by the saponin with recognition thresholds of 1.62 mmol/L and 0.64 mmol/L, respectively.

Quantitation of Soyasaponin I (1) and Dose-Activity Considerations in Pea-Protein Isolates. To gain a first insight into the influence of soyasaponin I (**1**) on the

overall bitter taste of pea-protein isolates, the saponin was quantified by LC-MS/MS using external calibration. Quantitative analysis revealed a concentration of 1.1 mmol/kg for soyasaponin I (**1**) in the pea-protein isolate. To assess the impact of compound **1** in the pea-protein isolate, Dose-over-threshold (DoT)-factors were determined as ratio of the concentration to the taste threshold of the respective taste active substance.[51–55] The DoT-factor for bitterness of soyasaponin I (**1**) was determined to be 0.7, indicating that it has no major impact on the bitter taste of the tested pea-protein isolate. For astringency, the DoT-factor was determined to be 1.8, thus indicating that the concentration of the saponin exceeded its astringent taste threshold be a factor of 1.8.

Sensomics Approach. To isolate and identify the key bitter compounds evoking the bitter off-taste of pea-protein isolates, the sensomics approach was applied. Therefore, a pea-protein isolate was first analyzed by a trained panel by means of a taste profile analysis. The panelists were asked to rate the taste intensities of bitter, sweet, sour, umami, salty, and astringent on a scale from 0 (not detectable) to 5 (strongly detectable) (**Table 1**). The highest scores of 1.4 and 1.3 were observed for the intensities of the bitter- and sweet-taste sensation, followed by astringency judged with a score of 1.1. Sourness (0.7), umami (0.7), and saltiness (0.5) were perceived with lower intensities. To separate the taste active compounds from the insoluble protein residue the pea protein isolate was extracted using different solvents.

Sequential Solvent Extraction of Pea-Protein Isolate. The pea-protein isolate was extracted with MeOH/H_2O (fraction I), followed by MeOH (fraction II), MeOH/acetone (fraction III), ethyl acetate (fraction IV), and *n*-pentane (fraction V) (**Figure 2**). The fractions were first separated from the solvent by using a rotary evaporator, freeze-dried twice, then taken up in sensory water at natural

673 concentrations, and finally evaluated by comparative taste profile analysis (**Table 1**).
674 Fraction I exhibited the highest bitter taste, with an intensity score of 1.5 while
675 fractions II – IV only showed low intensities perceived for bitterness. Due to its bitter
676 impact, further fractionation steps were focused on the identification of the key
677 molecules in fraction I.

678 **Activity-Guided Identification of the Key Bitter Compounds in Fraction I.** To
679 achieve the isolation of individual bitter molecules, fraction I was separated by MPLC-
680 ELSD to yield 13 fractions (I-1 to I-13), which were then freeze-dried, taken up in
681 equal amounts of water, and used for TDA (**Figure 2**). Fraction I-11 was judged with
682 the highest TD-factor for bitterness, namely 32, while the other sub-fractions showed
683 TD-factors of 16 and lower or had a sour taste (**Figure 3 A**).

684 Fraction I-11 was further fractionated by preparative RP18-HPLC-ELSD and
685 16 fractions were collected (I-11-1 to I-11-16). The solvent was separated in vacuum,
686 then, each sub-fraction was dissolved in equal amounts of water and used for TDA
687 at increasing concentrations. (**Figure 2**). Among the 16 fractions, fraction I-11-15 was
688 evaluated with the highest TD-factor (64), followed by sub-fractions I-11-8, I-11-14
689 and I-11-16 with a TD-factor of 32. (**Figure 3 B**). To identify the bitter molecules in
690 these HPLC fractions, the compounds were purified by re-chromatography and the
691 chemical structure was determined by 1/2D NMR, tandem-MS/MS, LC-TOF-MS, and
692 MS^E.

693 LC-TOF-MS (ESI^-) analysis of the purified fractions of I-11-8 showed a total of
694 13 peaks, all with *m/z* 329.23 as the pseudo molecular ion ($[M-H]^-$). The molecular
695 formula of these compounds was calculated to be $C_{18}H_{34}O_5$, fitting with a molecular
696 mass of 330.5 g/mol. The mass spectrum of each peak could be obtained by applying
697 the MS^E technique, which showed four different fragmentation patterns. (**Figure 4**).

As reported earlier, for trihydroxyoctadecenoic acids a cleavage between the vicinal hydroxy groups is likely.[56–58] This behavior could be observed with the MS^E experiments as well. The detection of the fragment ions *m/z* 171 and 157 (or rather 139 after dehydration) indicates the presence of 9,10,13-trihydroxyoctadec-12-enoic acid (**2**).[28,57–59] Furthermore, for 9,12,13-trihydroxyoctadec-10-enoic acid (**3**) the formation of the fragment ions *m/z* 229 (and 211 after dehydration) could be observed.[57,58,60,61] 9,10,11-trihydroxyoctadec-12-enoic (**4**) cleaved between both vicinal OH groups during fragmentation, forming the fragment ions *m/z* 201 and 171.[56] To the best of our knowledge, not much is known about the fragmentation of 11,12,13-trihydroxyoctadec-9-enoic acid. (**5**). Our studies showed the cleavage between the vicinal hydroxy groups, leading to the formation of fragment ions *m/z* 199 and, after the neutral loss of H_2O (-18), *m/z* 211. Interestingly, all THOAs formed $[-2H+Na]^-$ adducts during the ESI^- MS^E ionization process. Additionally, 1/2D NMR experiments could confirm the mixture of compounds **2 – 5**. The steric configuration of the total of 13 isomers had not be determined. Although trihydroxyoctadecenoic acids could be identified earlier in several plants[56,62–68] and plant based products,[69–72] they have been isolated from pea-protein isolates for the first time. The bitter activity of compound **2** and **3** has been previously reported in the literature.[24,25,28,73]

Structure determination of the key compounds present in fraction I-11-15 revealed an isomeric mixture of hydroxyoctadecadienoic acids, namely, (10*E*,12*E*)-9-hydroxy-octadeca-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**). The *E/Z*-configurations of the isomers were determined by comparison of the retention times of the analytes with those of their corresponding commercial references by LC-TOF-MS. Moreover, NMR, LC-TOF-MS and MS/MS experiments allowed the straightforward identification

of 1-linoleoyl glycerol (**9**), *α*-linolenic acid (**10**), 2-hydroxypalmitic acid (**11**), 2-hydroxyoleic acid (**12**), and linoleic acid (**13**) in fraction I-11-15.

Hydroxyoctadecadienoic acids (**6 – 8**) have been isolated from several plants before [68,74–77] and are known to be metabolites in the lipoxygenase pathway formed through the action of 9-/13-LOX and subsequent reduction by a peroxygenase or other peroxidase activity.[78–81] Their bitter activity was reported earlier in literature.[28] In addition, 1-linoleoyl glycerol (**9**) was isolated from the pea-protein isolate. Presumably, this monoglyceride is a degradation product of di- or triacylglycerids, which are highly abundant lipids in peas,[82] whereas the identified polyunsaturated fatty acids, namely, *α*-linolenic acid (**10**) and linoleic acid (**13**), are two of the dominant free fatty acids in peas.[83,84] Human bitter-taste thresholds of **9**, **10** and **13** have been published in earlier studies.[26,28] The 2-hydroxy derivatives **11** and **12** were known to be products from the *α*-oxidation enzyme system in peas (*Pisum sativum* L.) and other higher plants[81,85–88] but could not yet be reported to be bitter tastants.

Isolation of fraction I-11-16 by semi-preparative HPLC, followed by 1/2D NMR spectroscopy, and TOF-MS experiments, as well as by a comparison of the observed retention time with that of a commercial reference revealed (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (**14**). The isolation of oxooctadecadienoic acids from plants has been reported earlier.[76,77,89] It can be assumed that the identified 13-oxooctadeca-9,11-dienoic acid was formed during the LOX pathway. In this enzymatic route, hydroperoxides are formed by 9- and 13-LOXs, followed by dehydration or dehydrogenation of the fatty acid hydroxide.[81,90,91]

A second compound eluting in fraction I-11-16 was purified by re-chromatography and analyzed by 1/2D NMR spectroscopy. At first sight, the ^{13}C- and ^{1}H-NMR spectra demonstrated a total of 14 carbon atoms resonating between 13.93 and 128.01 ppm

and 25 proton signals resonating between 0.93 and 5.93 ppm. A combination of HSQC and DEPT-135 experiments helped us to visualize one methyl group, nine methylene groups and four olefinic groups. The connection of these C-atoms and protons was readily assigned by means of $^{1}H,^{1}H$-COSY-correlations and $^{1}H,^{13}C$-correlations in the HMBC and H2BC spectrum. The methyl proton signal H-C(1)/H-C(28), detected at 0.93 ppm, exhibited a $^{2}J_{H\text{-}C}$ coupling to the alkyl carbon atoms C(2)/C(27) resonating at 22.57 ppm and a $^{3}J_{H\text{-}C}$ coupling to the alkyl carbon C(3)/C(26) atoms resonating at 31.23 ppm (**Figure 5**). In addition, a 1,3-pentadiene system between C(6)/C(10) and C(19)/C(23) could be detected. The observed $^{2}J_{H\text{-}C}$ couplings from H-C(7)/H-C(22) and H-C(9)/H-C(20) at 5.34 ppm to C(8)/C(21) at 25.61 ppm deduced the connection of two double bonds over one methylene group. Additionally, the heteronuclear couplings of H-C(6)/H-C(23) and H-C(10)/H-(19) at 5.39 ppm to C(5)/C(24) and C(11)/C(18) resonating at 27.16/27.22 ppm confirmed the connection of the pentadiene system to the alkyl moieties from C(2) to C(5), from C(11) to C(18) and C(24) to C(27), respectively. Since integration of the proton signals only showed one methyl group but no other functional group could be detected, it could be assumed that the detected carbon atoms and protons are magnetically and chemically equivalent pairs resulting in a symmetric molecule. Based on the NMR experiments the structure of an octacosa-6,9,19,22-tetraen (**15**) could be postulated.

However, no *m/z* indicating the theoretical elemental composition of $C_{28}H_{50}$ could be detected by MS/MS, LC-TOF-MS or GC-MS (data not shown). To confirm the structure of compound **15**, an aliquot was ozonolyzed, worked up reductively, and oxidized to generate the cleavage products malonic acid, sebacic acid, and caproic acid. Afterwards, the reaction mixture was analyzed by LC-MS/MS and the

(di)carboxylic acids malonic acid, sebacic acid, and caproic acid could be identified by comparison with their corresponding commercial standard (**Figure 6**). Taking all these data into account, the target bitter compound was identified as octacosa-6,9,19,22-tetraen (**15**), which – to the best of our knowledge – has not yet been reported.

Sensory Activity of Bitter Compounds To analyze the sensory activity of the previously identified substances, the taste threshold of each compound was determined. Prior to the sensory evaluation, the purity and identity of the isolated compounds were reviewed by LC-TOF-MS and qNMR.[43] To overcome the limited water-solubility of the hydrophobic compounds, the human bitter recognition threshold concentrations were determined in 3% aqueous EtOH by a two-alternative forced choice test procedure (2-AFC).[42] The taste threshold concentrations for compounds **2**, **3**, **6 – 10** and **13** were taken from literature sources in which a similar procedure was used.[26,28,61] The lowest threshold concentrations of 0.06 and 0.07 mmol/L were found for 2-hydroxyoleic acid (**12**) and 1-linoleoyl glycerol (**9**) (**Table 2**). 9,10,13-Trihydroxyoctadec-12-enoic acid (**2**) and different isomeric mixtures of the isolated trihydroxy derivates (**2 – 5**) exhibited bitter thresholds of 0.08 and 0.13 mmol/L, respectively. In comparison to the triols as well as compounds **9** and **12**, 2-hydroxypalmitic acid (**11**), α-linolenic acid (**10**), octacosa-6,9,19,22-tetraen (**15**) and (10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid (**6**) showed thresholds that were approximately two to three times higher (i.e., between 0.22 and 0.35 mmol/L). For compound **6**, the same bitter threshold as its 10*E*,12*Z*-isomer was assumed, following the assumption that the *E*,*Z*- and *E,E*-isomers have a rather similar taste threshold.[28] This trend could also be observed for (9*Z*,11*E*)-13-hydroxy-octadeca-9,11-dienoic acid (**7**) and (9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid

798 (**8**), showing values of 0.97 and 0,79 mmol/L, respectively. The highest
799 concentrations (of 0.93 and 0.99 mmol/L) were determined for linoleic acid (**13**) and
800 (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (**14**).
801
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804
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812 for LC-MS/MS measurements.
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Figure Captions

Figure 1. Chemical structures of soyasaponin I (**1**) and the bitter key molecules isolated from pea protein isolate: 9,10,13-trihydroxyoctadec-12-enoic acid (**2**), 9,12,13-trihydroxyoctadec-10-enoic acid (**3**), 9,10,11-trihydroxyoctadec-12-enoic (**4**), 11,12,13-trihydroxy-octadec-9-enoic 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**), (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid (**14**), and octacosa-6,9,19,22-tetraen (**15**).

Figure 2. Separation scheme used to isolate bitter-tasting compounds from the commercial pea protein.

Figure 3. (**A**) MPLC chromatogram with taste dilution (TD-)factors of fraction I and (**B**) RP-HPLC chromatogram of fraction I-11 with TD-factors of fraction I-11.

Figure 4. MS^E spectra of (**A**) 9,10,13-trihydroxyoctadec-12-enoic acid (**2**), (**B**) 9,12,13-trihydroxyoctadec-10-enoic acid (**3**), (**C**) 9,10,11-trihydroxy-octadec-12-enoic (**4**) and (**D**) 11,12,13-trihydroxyoctadec-9-enoic acid. (**5**).

Figure 5. Structure and HMBC spectrum (500 MHz, $CDCl_3$) of octacosa-6,9,19,22-tetraen (**15**).

Figure 6. (**A**) Ozonolysis of octacosa-6,9,19,22-tetraen (**15**) and (**B**) LC-MS/MS chromatogram of the resulting (di)carboxylic acids: (**a**) malonic acid, (**b**) sebacic acid, and (**c**) caproic acid.

Table 1: Sensory Evaluation of Fractions Isolated from the Pea Protein Isolate.

Taste quality	Intensities for individual taste qualities[a]					
	Pea Protein	Fraction I[b]	Fraction II[b]	Fraction III[b]	Fraction IV[b]	Fraction V[b]
sweet	0.5	0.3	0.5	0.4	0.4	0.5
sour	0.7	0.6	0.5	0.5	0.4	0.4
umami	0.4	0.4	0.4	0.3	0.3	0.3
salty	0.4	0.3	0.3	0.2	0.2	0.2
bitter	1.7	1.5	0.7	0.8	0.7	0.7
astringent	1.4	0.8	0.9	0.9	0.3	0.8

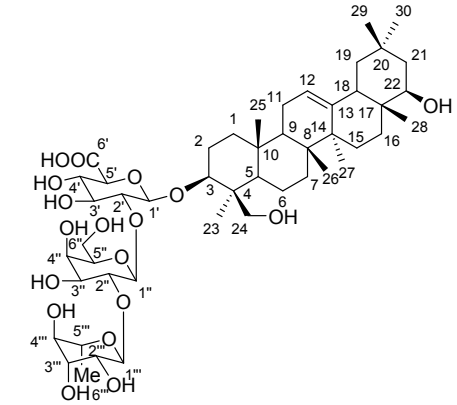
[a]The taste intensity of the individual taste descriptors was rated on by a trained panel on a linear scale from 0 (not detectable) to 5 (strongly detectable).
[b]The panelists were asked to rate aqueous solutions of the natural concentrations of the fractions I – V.

Table 2: Bitter taste threshold concentrations of compounds isolated from pea protein isolate.

Compound No.	Compound	Bitter threshold concentration [mmol/L]
2	9,10,13-trihydroxyoctadec-12-enoic acid	0.08[#]
3	9,12,13-trihydroxyoctadec-10-enoic acid	0.13[+,a]
4	9,10,11-trihydroxyoctadec-12-enoic acid	0.13[b]
5	11,12,13-trihydroxyoctadec-9-enoic acid	0.13[b]
6	(10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid	0.35[#,c]
7	(9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	0.79[#]
8	(9*E*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid	0.97[#]
9	1-linoleoyl glycerol	0.07*
10	*α*-linolenic acid	0.28[#]
11	2-hydroxypalmitic acid	0.22
12	2-hydroxyoleic acid	0.06
13	linoleic acid	0.93[#]
14	(9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid	0.99
15	octacosa-6,9,19,22-tetraen	0.33

[#]taste threshold taken from Lainer et al. (2019).
[+]taste threshold taken from Lainer (2019).
*taste threshold taken from Degenhardt & Hofmann. (2010).
[a]in mixture with 9,10,13-trihydroxyoctadec-12-enoic acid.
[b]in an isomeric mixture of 9,10,13-trihydroxyoctadec-12-enoic acid, 9,12,13-trihydroxyoctadec-10-enoic acid, 9,10,11-trihydroxyoctadec-12-enoic and 11,12,13-trihydroxyoctadec-9-enoic acid.
[c]bitter taste threshold concentration of the 10*E*,12*Z*-isomer.

Gläser et al. (Figure 1)



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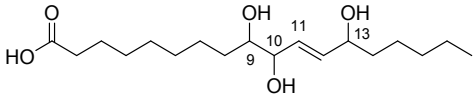

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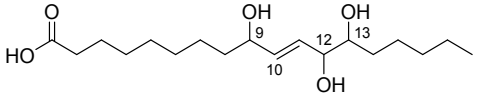

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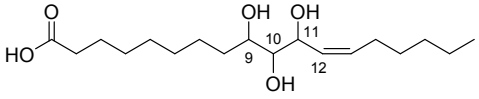

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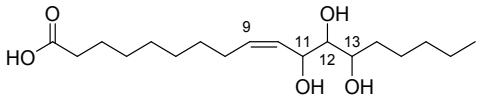

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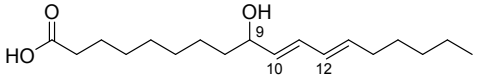

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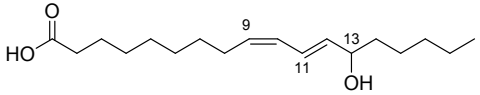

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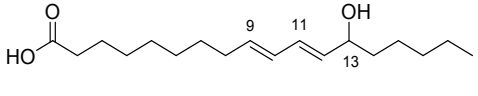

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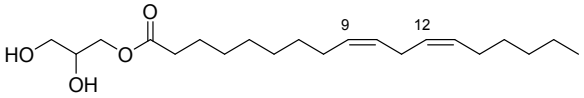

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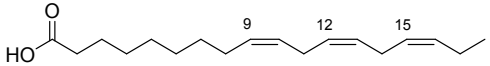

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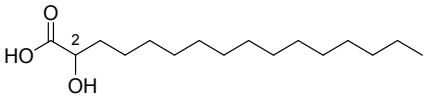

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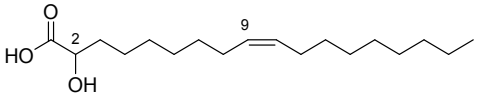

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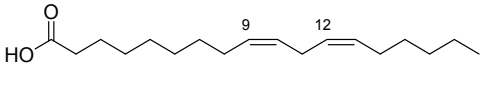

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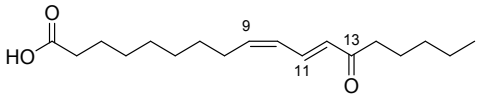

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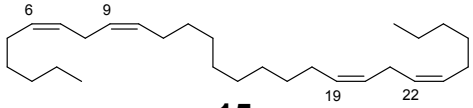

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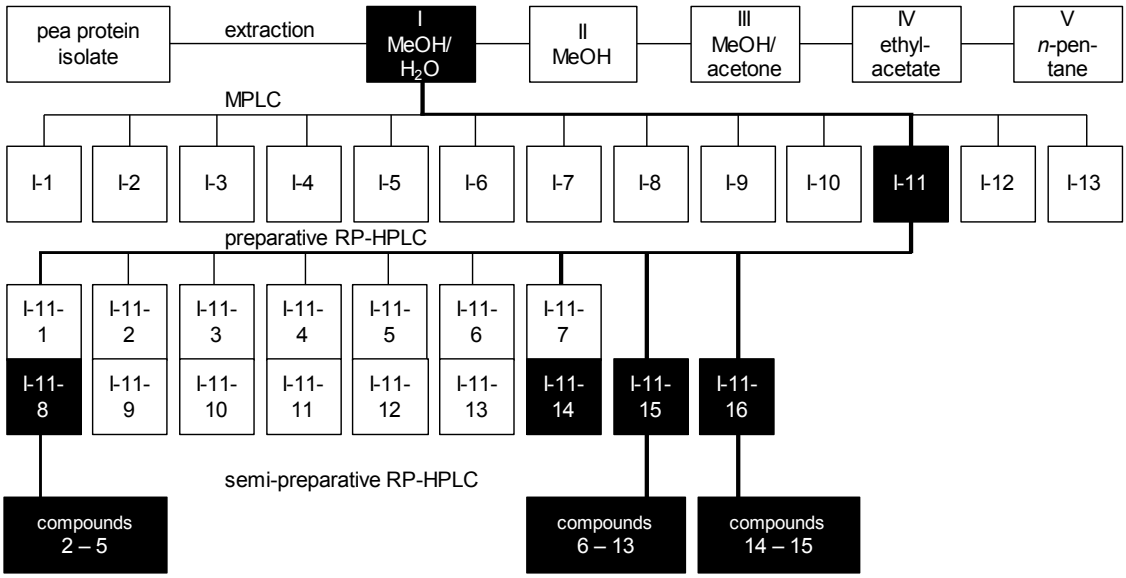
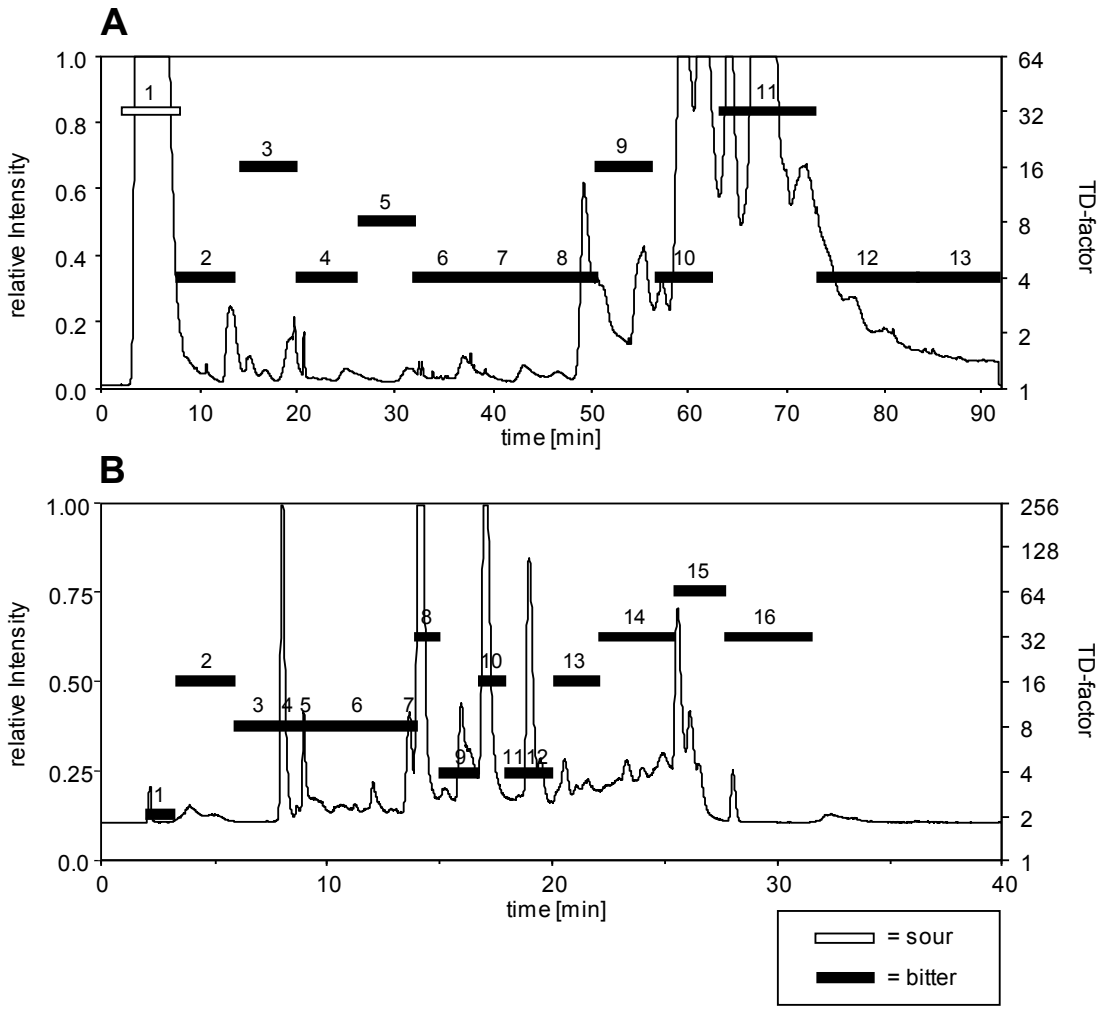
Gläser et al. (Figure 2)



Gläser et al. (Figure 3)



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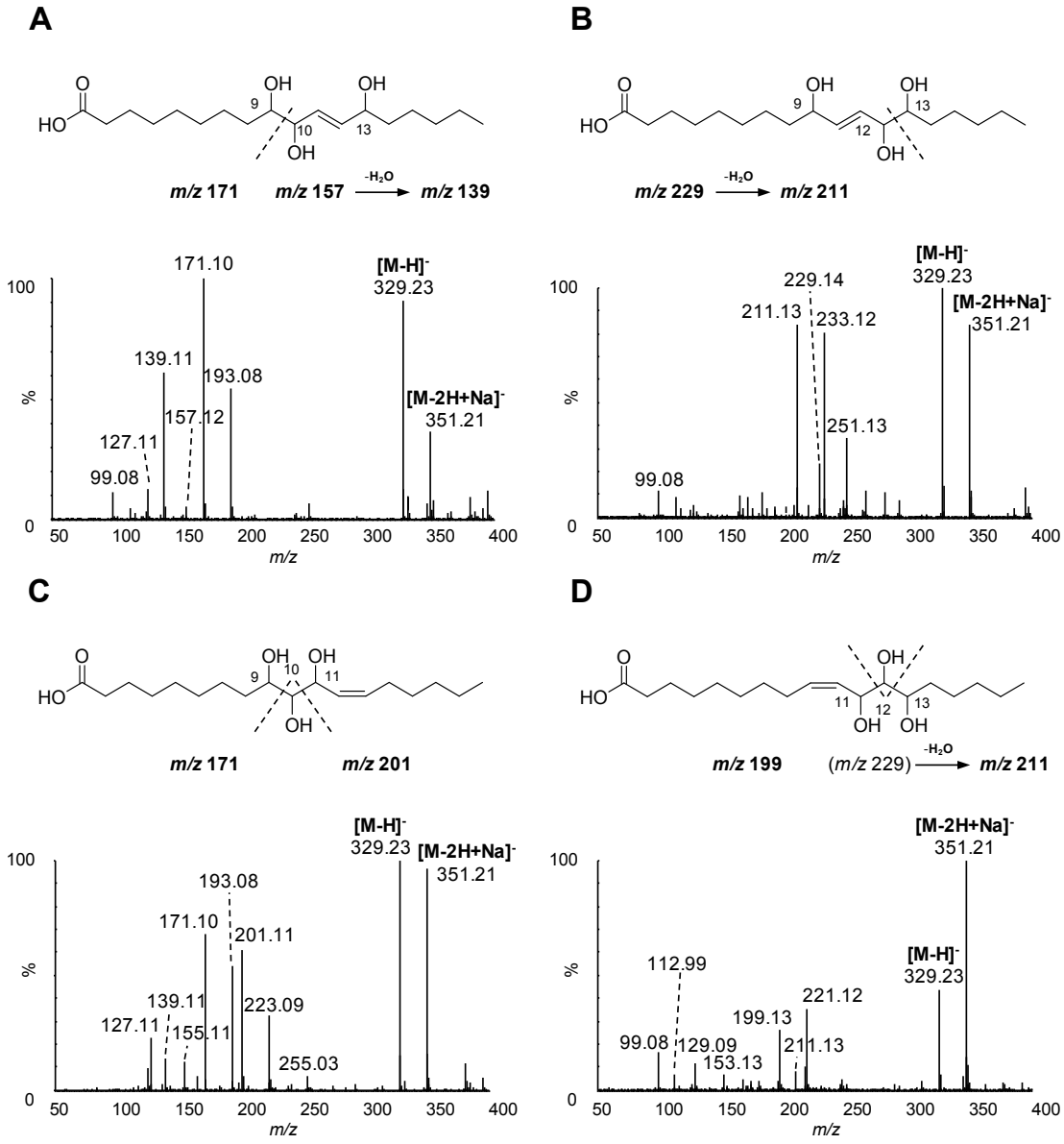

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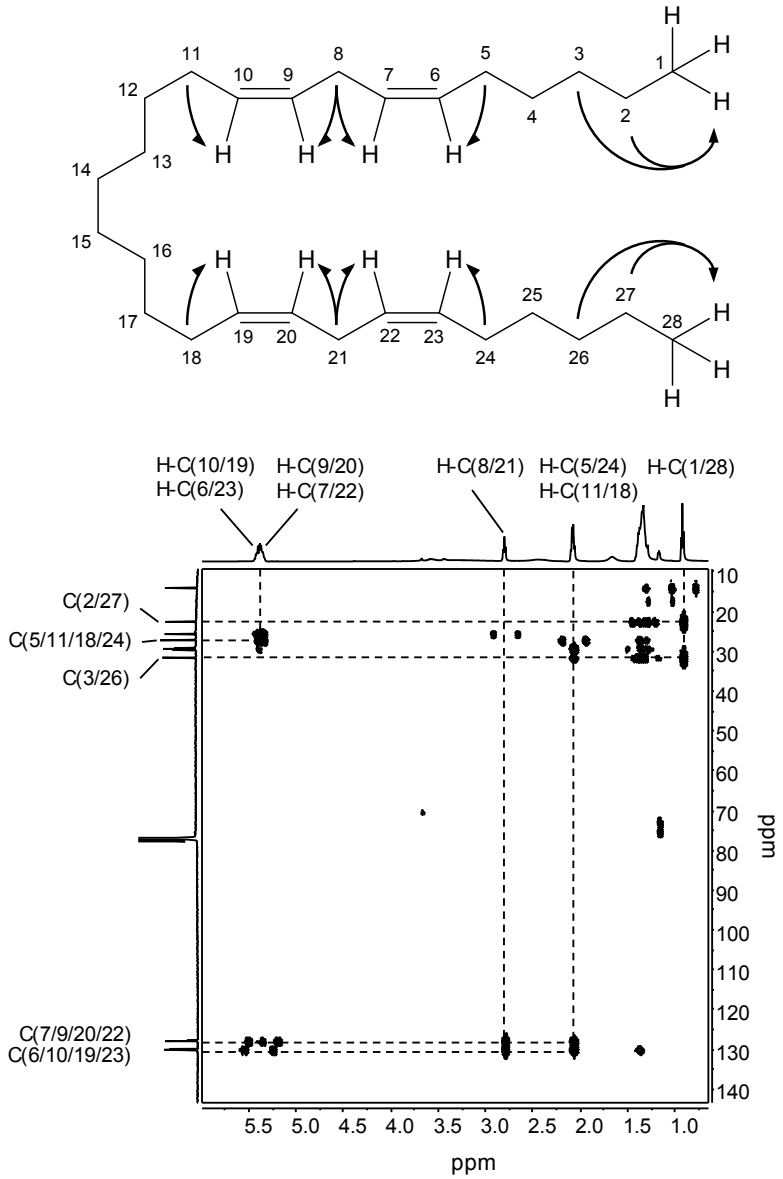
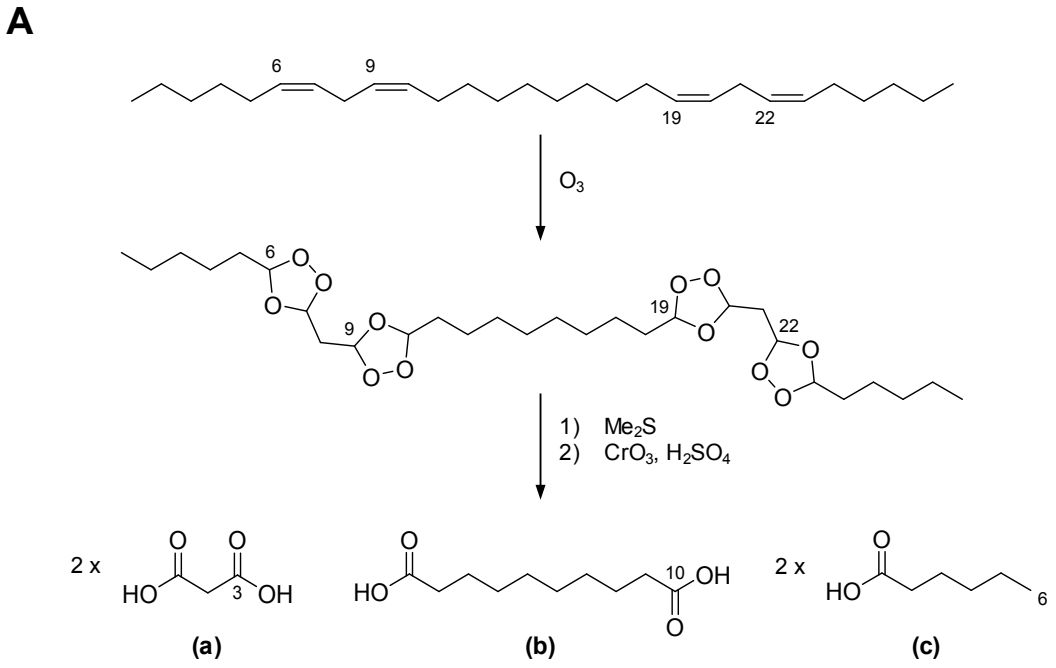


Gläser et al. (Figure 6)



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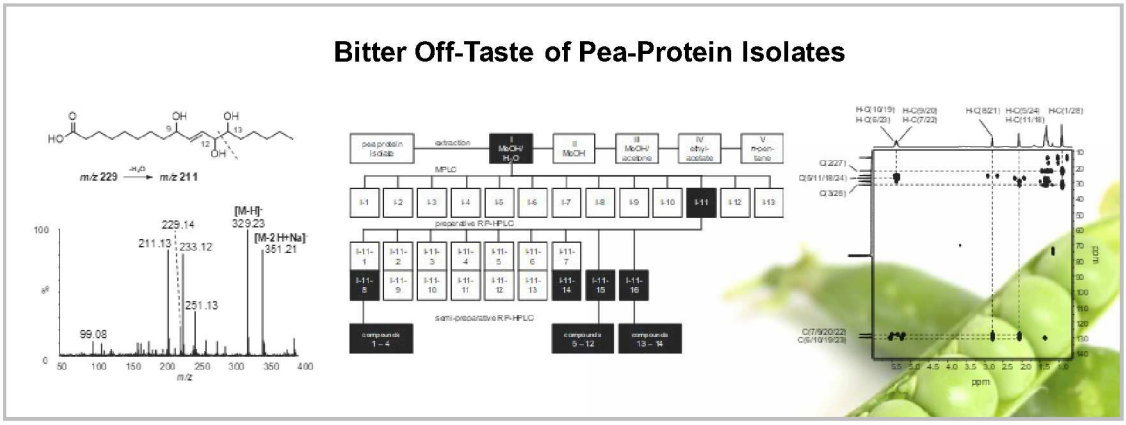
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m/z 211
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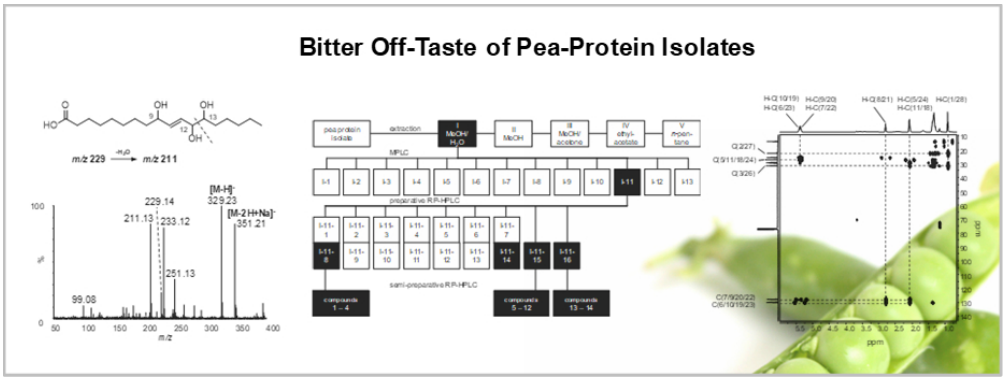
329.23
[M-2 H+Na]-
351.21
229.14
211.13
233.12
251.13
99.08
pea protein isolate
extraction
MPLC
preparative RP-HPLC
semi-preparative RP-HPLC
compounds 1 – 4
compounds 5 – 12
compounds 13 – 14
ppm



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