Identification and quantification of key phytochemicals in peas – Linking compounds with sensory attributes

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Title: Identification and quantification of key phytochemicals in peas - linking compounds with sensory attributes

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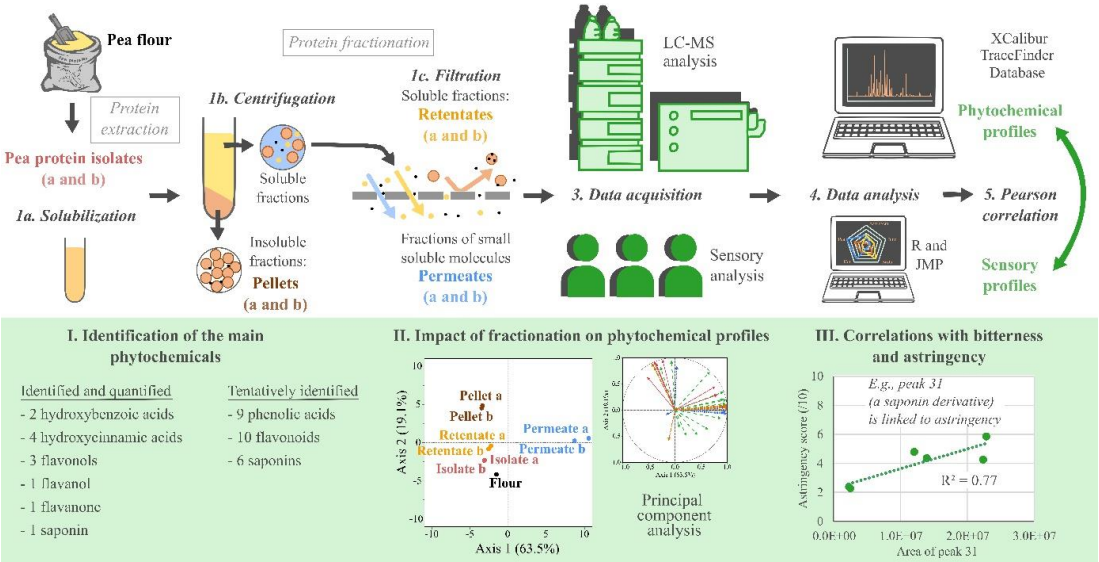
Abstract : Pea protein isolates contain high-quality plant protein. However, they have sensory drawbacks, notably bitterness and astringency, that have limited their use in commercial foods. This study's aim was thus to identify the main phytochemicals in pea-based samples and to examine associations with sensory attributes. The phytochemical profiles of pea flour, pea protein isolates, and pea protein isolate fractions were characterized via UHPLC-DAD-MS. Forty-eight phytochemicals were observed: 6 phenolic acids, 5 flavonoids, and 1 saponin were unambiguously identified and quantified, while another 9 phenolic acids, 10 flavonoids, and 6 saponins were tentatively identified. The impacts of protein extraction and fractionation were studied. These processes appear to have caused some compound degradation. It was found that 29 compounds were correlated with perceived bitterness and/or astringency. Therefore, these results show that certain phytochemicals can lead to negative sensory attributes in pea-protein-based products.

Keywords: Pulse, Flavonoids, Saponins, Bitterness, Astringency, Correlation

Graphical Abstract



Highlights

- 6 phenolic acids, 5 flavonoids, and 1 saponin were identified and quantified
- 9 phenolic acids, 10 flavonoids, and 6 saponins were tentatively identified
- Protein fractionation led to some compound degradation
- 29 compounds were correlated with perceived bitterness and/or astringen

1. Introduction

Over the past several years, the agrifood industry has taken on the challenge of developing replacements for animal proteins. The latter have pronounced environmental impacts and may become scarce in the future. Consequently, industry stakeholders and consumers have begun focusing on plant proteins. For example, pea (*L. Pisum sativum*) protein isolates are increasingly being incorporated into foods because of their functional properties, protein content, environmental sustainability, and low price. However, there are sensory drawbacks to the use of plant proteins, notably when the source is isolate fractions derived from raw plant materials. In particularly, unpleasant olfactory and gustatory sensations may arise, which presents a hurdle for the development of commercial plant-based foods. Research on pea-based foods has largely focused on how volatile aroma compounds lead to perceived beaniness. However, far less studied is why such products are also perceived as bitter and/or astringent.

It has been suggested that a wide variety of phytochemicals could be responsible for the bitter and astringent notes associated with pea protein isolates. Research has particularly highlighted the potential role played by lipids, saponins, and phenolics. Scientists have also explored the contribution of bitter lipid oxidation products formed either through enzymatic pathways or via autoxidation. Using pea protein isolate fractions, recent work identified 14 lipids and lipid oxidation products that are associated with greater perceived bitterness (Gläser *et al.*, 2020). Similarly, saponins have been found to give rise to bitter and astringent notes. Such is namely the case for soyasaponin I and DDMP saponins (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) (Heng *et al.*, 2006; Reim & Rohn, 2015). The work by Gläser *et al.* examined soyasaponin I's influence on the overall bitter and astringent notes associated with pea protein isolates by calculating dose-over-threshold factors (the ratio of compound concentration to taste threshold for each test substance). For bitterness, the factor value was below 1, indicating a limited sensory contribution. For astringency, the factor value was 1.8, indicating a more pronounced sensory contribution. However, to date, there has been no detailed research on the composition, content, or impact of other saponins on the sensory perception of pea-based products.

Such research is similarly scarce for phenolic compounds. That said, several phenolic acids in plant-based materials other than peas have been found to result in bitter and astringent notes. For example, perceived bitterness and astringency appear to arise from caffeic acid and o-caffeoylquinic acid in coffee (Frank *et al.*, 2006) and hydroxybenzoic and hydroxycinnamic acids in wine (Hufnagel & Hofmann, 2008). Several flavonoids seem to have the same effect, such as catechin and gallic acid in red wine (Robichaud & Noble, 1990) and flavan-3-ols and flavonol glycosides in cocoa (Stark *et al.*, 2006). A range of studies have found that the yellow pea contains at least 115 different phenolics (Fahim *et al.*, 2019; Neugart *et al.*, 2015; Stanisavljevic *et al.*, 2015), mainly glycosylated flavonols, although other flavonoids are also present, including flavanols, anthocyanins, and isoflavonoids (Fahim *et al.*, 2019). Many kaempferol and quercetin 3-O-glycosides have also been characterized in the pea (Neugart *et al.*, 2015; Stanisavljevic *et al.*, 2015). It is therefore possible that these compounds also contribute to the perceived bitterness and astringency of pea protein isolates.

This study thus aimed to identify the key phytochemicals (phenolic acids, flavonoids, and saponins) found in pea protein isolates and to explore their links with bitter and astringent sensory attributes. To this end, pea flour, pea protein isolates, and pea protein isolate fractions were utilized. First, the phytochemical profiles of these different sample types were characterized using ultra-high-performance liquid chromatography–diode array detector–tandem mass spectrometry (UHPLC-DAD-MS). Second, the impacts of processing (i.e., extraction and fractionation) on the phytochemical profiles were examined. Third, the contribution of key phytochemical compounds to perceived bitterness and astringency was explored.

2. Materials and methods

2.1. Chemical reagents

Based on past research, 12 phenolic compounds were chosen as standards: kaempferol-3-O-glucoside (CAS 480-10-4, grade: analytical standard), soyasapogenol B (CAS 595-15-3, grade: ≥98% HPLC), caffeic acid (CAS 331-39-5, grade: ≥98.0% HPLC), (+)-catechin (CAS 225937-10-0, grade: ≥98% HPLC), *trans*-ferulic acid (CAS 537-98-4, grade: ≥99.0% HPLC), gallic acid (CAS 149-91-7, grade: 97.5–102.5% titration), naringin (CAS 10236-47-2, grade: ≥95% HPLC), *trans-p*-coumaric acid (CAS

501-98-4, grade: ≥98.0% HPLC), *p*-hydroxybenzoic acid (CAS 99-96-7, grade: ≥99% FG), quercetin-3-O-glucoside (CAS 482-35-9, grade: ≥90% HPLC), quercetin-3-O-rutinoside (CAS 207671-50-9, grade: ≥94% HPLC), and sinapic acid (CAS 530-59-6, grade: ≥98%). All 12 were purchased from Sigma-Aldrich. Other reagents included acetonitrile (grade: Optima™ for HPLC-MS), water (grade: Optima™ for HPLC-MS), formic acid (grade: Optima™ for HPLC-MS), and methanol (grade: Optima™ for HPLC-MS); they were purchased from Thermo Fisher. Prior to performing the UHPLC-DAD-MS analysis, the solutions were run through polytetrafluoroethylene (PTFE) filters (13 mm with a porosity of 0.22 µm; Fisherbrand). Stock solutions of the individual analytes and working solutions were prepared and immediately placed in glass vials stored at -80°C (Vial N9: 1.5 mL, 11.6 x 32 mm; MACHEREY-NAGEL).

2.2. Pea sample types

Pea flour and two commercial pea protein isolates (a and b) (protein content = nitrogen [N] content × 6.25; 83% dry matter [DM] V/V) were obtained from Roquette Frères (Lestrem, France). These two pea protein isolates differed because they were produced under different protein extraction and heat treatment conditions.

Six fractions were obtained from these two isolates as fully described in Cosson *et al.* (2021): pellets a and b; permeates a and b; and retentates a and b. In brief, the isolates were first dispersed in tap water (4% DM content V/V) and kept under conditions of constant agitation (12 h, 3°C). Second, the solutions were centrifuged (Jouan Kr4i and Sorvall Lynx 4000 [Thermo Scientific, Waltham, US]; 6000 g, 10 min, 4 °C). The supernatant was manually separated from the pellet, which was then diluted with tap water (12.35% DM content V/V). Third, the supernatant was filtered with a tangential filtration module (TIA, Bollene, France) equipped with two ST-3B-1812 PES Synder membranes (46-mil spacer, 10-kDa MWCO) and a high-pressure diaphragm pump (Wanner Hydra-Cell G10, Wanner International Ltd, Church Crookham, UK). Fourth, ultrafiltration was used to obtain 10 L of permeate; the retentate was washed with 1 diavolume during diafiltration. During the latter process, the retentate was at 13°C, inlet pressure (P1) was 1.5 bar, outlet retentate pressure (P2) was 1 bar, and mean transmembrane pressure ([P1 + P2]/2) was 1.25 bar. All the fractionation steps were carried out at

4°C, except for membrane filtration, which was carried out at 13°C. Finally, the samples were stored at -20°C in 50 mL glass flasks.

DM content was determined via drying (analysis system: prepASH®219). The nine sample types had the following DM levels: flour—92%; isolates a and b—93.7%; pellets a and b—6.2%; retentates a and b—1.7%; and permeates a and b—0.2%.

2.3. Standard and sample preparation

2.3.1. Standard solutions

For each standard, solutions of 0.1 g/L were prepared in methanol and water (70/30 [V/V]). For quercetin-3-O-glucoside and quercetin-3-O-rutinoside, solubilization was promoted using 15 min of sonication. Then, 0.01 g/L stock solutions were prepared by mixing each standard with methanol and water (70/30 [V/V]). These stock solutions were diluted with methanol to arrive at working solutions ranging in concentrations from 0.05 to 15 ng/µl for p-hydroxybenzoic acid and from 1 to 10 ng/µl for the other standards. The stock and working solutions were run through a 0.22-µm PTFE filter and stored in the dark at -80°C until analysis. Calibration curves were obtained by plotting the peak areas of positive compound mass (see the conditions described in section 2.4.) as a function of standard solution concentration using TraceFinder software (Thermo Scientific, USA). The calibration curves were linear with R^2 values exceeding 0.99. The detection limit (DL) was 0.02 ng/µl, and the quantification limit (QL) was 8.0 ng/µl. There were two exceptions: for soyasapogenol B, DL = 0.07 ng/g and QL = 3 ng/g, and for p-hydroxybenzoic acid, DL = 0.05 ng/g and QL = 30.0 ng/g.

2.3.2. Sample types with higher dry matter content

For the sample types with higher DM content (flour, isolates a and b, pellets a and b), 6.0 g of material was placed in 150-mL glass vials (Schott vials, Dutscher, France) and extracted three times with 30 mL of a methanol and formic acid mixture (99/1 [V/V]). The extraction process lasted 2 h, used a magnetic stirrer, and occurred at room temperature in the dark. The supernatants were then kept at -20°C for 10 min before being separated via centrifugation (4,500 rpm, 20 min, 4°C; Eppendorf 5804R). The supernatant was evaporated down at 40°C using a vacuum concentrator (Jouan Thermo

Electron Corporation, RC 1022); the process took place in darkness. To remove certain precipitates generated during evaporation, centrifugation was performed before the solvent evaporated entirely (4,500 rpm, 20 min, 4°C). The evaporation process was then relaunched using the supernatants and continued until the solvent was gone. The samples were solubilized in 1 mL of a methanol and water mixture (80/20 [V/V]) and then kept at -20°C for 10 min to promote precipitation. At that point, the samples were separated by centrifugation (4,500 rpm, 20 min, 4°C). The supernatants were run through a 0.22-µm PTFE filter and stored at -80°C in darkness until analysis. This series of filtration and centrifugation steps was performed to obtain clear extracts that contained no precipitates. The samples were prepared in triplicate.

2.3.3. Sample types with lower dry matter content

For the sample types with lower DM content (retentates a and b, permeates a and b), a similar preparation process was used. Only the extraction step differed: 6.0 g of material was mixed with 1 mL of a methanol and formic acid mixture (99/1 [V/V]) for 10 min at room temperature in darkness.

2.4. Ultra-high-performance liquid chromatography–diode array detector–tandem mass spectrometry

The samples were subject to UHPLC analysis (Ultimate 3000, Thermo Scientific, USA) using a Hypersil GOLD column (100 mm x 2.1 mm x 1.9 µm, Thermo Scientific). The mobile phase consisted of (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. A gradient program with a flow rate of 0.25 mL/min was used: there was 4 min of 98% (A); a linear gradient from 98% to 70% of (A) over 26 min; a linear gradient from 70% to 2% of (A) over 6 min; and 9 min of 2% (A). Initial gradient pressure was 280 bar. The injection volume was 5 µL, and the injector temperature was 7°C. The oven temperature was 25°C.

The UHPLC system was coupled with a high-resolution mass spectrometer (Q Exactive Orbitrap, Thermo Scientific, USA) equipped with a heated electrospray ionization probe (HESI II, Thermo Scientific, USA). The mass spectrometer was operated in both negative and positive ion modes. The parameters for the ion source were as follows: sheath gas pressure = 2.4×10^5 Pa; auxiliary gas flow rate = 10; sweep gas flow rate = null; spray voltage = 3 kV; capillary temperature = 300°C, S-lens

radio frequency = 50 V; and heater temperature = 300°C. The spectra (MS^1 and MS^2) were acquired using full MS^1 and full MS^1/ddMS^2 across a range from 85 to 1,000 amu at two resolution levels (70,000 and 17,500, respectively). The system was also coupled with a diode array detector covering the full range of acquisition (190–600 nm).

Phytochemicals were identified based on the following features: mass spectra, accurate mass, characteristic fragmentation, UV spectrum, and characteristic retention time. Xcalibur (Thermo Scientific, USA) was used for instrument control, data acquisition, and data analysis. Phytochemical quantification was carried out by comparing the specific reconstructed ion current with the calibration curves using TraceFinder software (Thermo Scientific, USA). To propose hypothetical compound formulas and to identify peaks, Compound Discoverer software (Thermo Scientific, USA) was used alongside several databases (PubChem, Phenol-Explorer, Flavonoid Database, Arita Database, NPASS Database, KNApSAcK Database).

2.5. Sensory analysis

Pea solutions were characterized using static profiling performed by 17 trained panelists (13 women and 4 men; mean age = 23 years old) as fully described in Cosson *et al.* (2021). Briefly, attribute selection was carried out using a check-all-that-apply (CATA) questionnaire. The panelists were trained to evaluate attribute intensity along an unstructured scale (range: 0–10) using external references. The attributes were evaluated in blocks. The first block focused on attributes shaped by olfactory perception in the nose (attribute block 1: pea, broth, nuts, almond, potato, and cereals). The second block focused on attributes shaped by taste perception and mouthfeel (attribute block 2: salty, sugar, bitter, astringent, mouthfeel, and granularity). The third block focused on attributes shaped by olfactory perception in the mouth (attribute block 3: pea, broth, nuts, almond, potato, and cereals). Each solution was evaluated in duplicate by the 17 panelists. In generally, they arrived at repeatable, homogeneous scores, and there was no between-session drift in scoring. Additional details on attribute selection, panelist training, and method characterization can be found elsewhere (Cosson *et al.*, 2021). In this study, only the data for the bitter and astringent attributes perceived in mouth are discussed.

2.6. Statistical analysis

Analyses were performed using R (R Core Team, 2019) and JMP (v. 13.1.0; SAS Institute Inc., Cary, SC, USA). For the inferential analyses, $\alpha = 0.05$ was the threshold for statistical significance. Principal component analysis (PCA; centered reduced variables, Pearson's n) was used on a correlation matrix to visually explore differences in the phytochemical profiles of the nine sample types. The relationships between the phytochemical concentrations and the sensory attribute scores (i.e., bitterness and astringency) were also explored using a Pearson correlation matrix.

3. Results and discussion

3.1. Phytochemical identification

This research characterized the main soluble phytochemicals in pea flour, protein isolates, and isolate fractions. The results of the UHPLC-DAD-MS analysis led to the tentatively identification of 54 substances (Supplementary Figure 1), which are either native seed compounds or reaction products formed during extraction and fractionation. By comparing the retention times and accurate MS^1 and MS^2 data for the samples with those for the standards, 12 compounds were unambiguously identified: 2 hydroxybenzoic acids, 4 hydroxycinnamic acids, 3 flavonols, 2 flavanols, and 1 saponin. In addition, based on the UV–vis, accurate MS^1, and MS^2 data; the web databases; and published research results, 26 compounds were tentatively identified: 9 phenolic acids, 10 flavonoids, 6 terpenoids, and 1 compound from another chemical family. A range of data were collected: retention times, assigned identities, UV–vis absorption levels, molecular formulas, accurate masses, the main MS data, and the web database(s) used in identification (Table 1).

3.1.1. Phenolic acid identification

Gallic acid (peak 1), p-hydroxybenzoic acid (peak 5), caffeic acid (peak 8), *trans*-*p*-coumaric acid (peak 11), *trans*-ferulic acid (peak 12), and sinapic acid (peak 13) were identified by comparing the retention times, the UV spectra, and MS spectra of the samples with those of the standards (in positive and negative ion mode). ESI ionization in the negative ion mode showed better sensitivity. In addition,

nine phenolic acids were tentatively identified; the results obtained in the negative ion mode are discussed below.

Peak 2 had two absorption bands characteristic of phenolic acids, one at 241sh-257 and one at 293 nm. It displayed a major molecular ion at m/z 299.0773. A fragment at m/z 137.0235 was seen in the MS^2 spectra; it may indicate the presence of a hydroxybenzoic moiety and the loss of a hexoside residue (-162). A fragment at m/z 93.0334 was also observed and was related to the loss of a carboxylic acid functional group (-44). Peak 2 was thus tentatively identified as an hydroxybenzoic hexoside. Peak 3 had two absorption bands, one at 256 nm and one at 293 nm. It displayed a major molecular ion at m/z 153.0183. A fragment ion was seen in the MS^2 spectra at m/z 109.0284 and may correspond to the loss of COO. This peak was thus tentatively identified as protocatechuic acid, which has previously been observed in the pea (Klejdus *et al.*, 2008; Singh *et al.*, 2017).

Peak 6 had two absorption bands, one at 271 nm and one at 317 nm. It displayed a major molecular ion at m/z 341.0874. A fragment ion was seen in the MS^2 spectra at m/z 179.0343; it may correspond to [caffeic acid$-H^+$]. The single secondary fragment at m/z 135 may correspond to [caffeic acid$-CO2-H^+$] (Jaiswal *et al.*, 2014). This peak was thus tentatively identified as caffeoyl hexoside, previously observed in pea leaves (Klopsch *et al.*, 2019; Neugart *et al.*, 2015). Peak 7 had an absorption band at 295 nm and a major molecular ion at m/z 325.0928. The fragment ions at m/z 163.0396 and 119.0492 in the MS^2 spectra could correspond to [coumaric acid$-H^+$] and [coumaric acid$-CO2-H^+$] (Iswaldi *et al.*, 2013). The peak was thus tentatively identified as coumaroyl-4-O-hexoside, also previously observed in pea leaves (Klopsch *et al.*, 2019).

Peaks 4, 9, 10, 14, and 15 all displayed even ion mass, the presence of an odd number of nitrogen atoms, and a fragment at m/z 132.0293 that was attributed to [aspartic acid$–H^+$]. They also had a fragment ion resulting from the neutral loss of part of an aspartic acid (116 amu, C4H4O4), which indicates a link between the phenolic acid and the aspartic acid in the form of an amide bond. Fragment ions corresponding to [phenolic acid $–H^+$] were also observed, as described earlier for N-caffeoylaspartic acid (Fayeulle et al, 2019); they apparently arose following the fragmentation of the peptides containing aspartic acid (Waugh et al, 1991; Harrison and Young, 2006).

Peak 4 had an absorption band at 254 nm and a major molecular ion at m/z 252.0512. There were fragment ions at m/z 132.0293, 136.0394, 137.0235, and 93.0334 in the MS^2 spectra that may correspond to [aspartic acid–H^+], [hydroxybenzoyl amide – H^+] (neutral loss of 116 amu), [hydroxybenzoic acid –H^+], and a [phenol –H^+], respectively. Peak 4 was tentatively identified as N-p-hydroxybenzoyl aspartic acid based on the fragment at m/z 136.0394, which confirmed the presence of an amide link, and the fragment at m/z 132.0293, which confirmed the presence of aspartic acid (Clifford et Knight 2004).

Similarly, peaks 9, 10, 14, and 15 displayed even ion mass and a fragment at m/z 132.0293, which was attributed to aspartic acid. Peaks 9 and 10 presented two absorption bands characteristic of hydroxycinnamic acids at 295/293 nm and 310/320 nm, respectively. Peak 9 showed evidence of a major molecular ion at m/z 278.067. The fragment ion at m/z 162.0552 could correspond to coumaroyl amide following the loss of 116 amu. Peak 9 was thus tentatively identified as N-coumaroyl aspartic acid (Oracz *et al.*, 2019).

Peak 10 displayed signs of a major molecular ion at m/z 308.0776. The fragment at m/z 192.0660 could correspond to [feruloyl amide –H^+]. The peak was thus tentatively identified as a N-feruloyl aspartic acid. Peaks 14 and 15 had an absorption band at 250 nm. Peak 14 had a major molecular ion at m/z 250.0720 and a fragment at m/z 134.0601, which may correspond to [phenylacetyl amide –H^+]. Peak 14 was thus tentatively identified as an N-phenylacetyl aspartic acid. Peak 15 had a major molecular ion at m/z 266.0670 and was tentatively identified as a hydroxyphenylacetic acid-aspartic acid conjugate (Waugh et al, 1991; Harrison and Young, 2006).

3.1.2. Flavonoid identification

The standard solutions of kaempferol 3-O-glucoside (peak 20), quercetin-3-O-rutinoside (peak 25), quercetin-3-O-glucoside (peak 26), catechin (peak 28), and naringin (peak 29) were analyzed using the ESI source in positive and negative ion modes. There was greater sensitivity in the negative ion mode for these compounds. In addition, ten other flavonoids were tentatively identified and are discussed below, based on the results obtained in negative ion mode. These compounds displayed two absorption bands characteristic of flavonoids: one within the range of 211–267 nm, corresponding to the phenolic

296 core, and the second within the range of 347–373 nm, corresponding to the conjugated system (Mabry
297 *et al.*, 1970).
298 In the MS^2 spectra, peaks 16 to 22 had fragment ions at m/z 287.0545 (positive ion mode) and either
299 m/z 284.0237 or 285.0405 (negative ion mode), which suggests the presence of a kaempferol moiety.
300 Also in the MS^2 spectra, peaks 16, 17, 19, 21, and 22 had a fragment at m/z 609.1436, corresponding
301 to [kaempferol + 2 hexoses], and peaks 21 and 22 had a fragment at m/z 771.1990, corresponding to
302 [kaempferol + 3 hexoses]. Kaempferol derivatives have been observed in pea seed coats (Duenas *et*
303 *al.*, 2004; Stanisavljevic *et al.*, 2015), pea seeds (Jha *et al.*, 2019), pea leaves (Klopsch *et al.*, 2019;
304 Neugart *et al.*, 2015), and pea shoots (Ferreres *et al.*, 1995; Santos *et al.*, 2014). Peak 16 displayed
305 signs of a major molecular ion at m/z 771.1978 and was tentatively identified as kaempferol
306 sophorotrioside, which has previously been detected in pea shoots using nuclear magnetic resonance
307 (NMR) spectroscopy (Ferreres *et al.*, 1995). Peak 19 had a major molecular ion at m/z 695.1442 and
308 was tentatively identified as kaempferol malonyl di-hexoside. Peak 22 had a major molecular ion at
309 m/z 947.2452 and was tentatively identified as kaempferol feruloyl tri-hexoside. This identification
310 was based on comparisons with data for quercetin feruloyl tri-hexoside, found in pea shoots using
311 NMR spectroscopy (Ferreres *et al.*, 1995), and the results of Goupy *et al.* (2013). Peak 21 displayed
312 signs of a major molecular ion at m/z 977.2558 and was tentatively identified as kaempferol sinapoyl
313 tri-hexoside based on comparisons with data for quercetin sinapoyl tri-hexoside, also found in pea
314 shoots using NMR spectroscopy (Ferreres *et al.*, 1995). Peaks 17 and 18 had major molecular ions at
315 m/z 753.1879 and 593.1510, respectively. They were tentatively identified as kaempferol derivatives.
316 In the MS^2 spectra, peaks 23 through 27 displayed a fragment ion at either m/z 303.0497 (positive ion
317 mode) or m/z 300.0276 (negative ion mode), which could indicate the presence of a quercetin moiety
318 or a flavone of the same mass. Also in the MS^2 spectra, peaks 23 and 24 had a fragment at m/z
319 445.0775, which could correspond to [quercetin + hexose - H2O]. Like kaempferol derivatives,
320 quercetin derivatives have been found in pea seed coats (Duenas *et al.*, 2004; Stanisavljevic *et al.*,
321 2015), pea seeds (Jha *et al.*, 2019), pea leaves (Klopsch *et al.*, 2019; Neugart *et al.*, 2015), and pea
322 shoots (Ferreres *et al.*, 1995; Santos *et al.*, 2014). Peak 23 had a major molecular ion at m/z 787.1926
323 and two absorption bands, at 256-267sh nm and 350 nm, respectively. The peak was tentatively

identified as quercetin tri-hexoside. Peak 24 had a major molecular ion at m/z 625.1401 and two absorption bands, at 256 nm and at 355 nm, respectively. The peak was tentatively identified as quercetin di-hexoside. Peak 27 had a major molecular ion at m/z 301.0352 and an absorption band at 370 nm; it was also associated with a relatively higher retention time (35.95 min). The peak was tentatively identified as quercetin aglycone, which has been observed in pea seed coats (Stanisavljevic *et al.*, 2015) and pea seeds (Jha *et al.*, 2019).

Peak 30 had two absorption bands, at 267 nm and 336 nm, respectively, and a major molecular ion at m/z 431.0981. In the MS^2 spectra, fragment ions occurred at m/z 269.0456, potentially corresponding to an apigenin moiety, and at m/z 164.0448, potentially corresponding to an O-hexoside. The peak was tentatively identified as apigenin-7-O-glucoside, which has been found in pea roots (based on comparison with a standard; Šibul *et al.*, 2016).

3.1.3. Terpenoid identification

The standard solution of soyasapogenol B (peak 37) was analyzed using the ESI source in positive and negative ion modes. There was greater sensitivity in the positive ion mode for soyasapogenol B. The peak had a single, small absorption band whose maximum was at 228 nm and a major ion at m/z 459.3844 in the MS^1 spectra (positive ion mode). There were two fragment ions at m/z 441.3723 and 423.3616 in the MS^2 spectra (positive ion mode).

In addition, seven peaks were tentatively identified as saponins (peaks 31 to 36). Sensitivity was better in the positive ion mode for all seven; each peak displayed only one small absorption band whose maximum occurred between 193 nm and 229 nm, a feature characteristic of saponins (Decroos *et al.*, 2005). The peaks' retention times were also high (between 35.9 and 37.6 min), confirming that they could be extremely apolar compounds. In the MS^2 spectra (positive ion mode), the peaks displayed the same set of fragment ions—located at m/z 85.0291, 141.0183, 365.3195, 423.3616, 581.3833, and 441.3723—which supports the interpretation that they belong to the same compound family. Soyasapogenol B also had fragments at m/z 441.3723 and 423.3616. The fragments at m/z 581.3833 and 423.3616 have been observed for DDMP saponin (Daveby *et al.*, 1998). In addition, saponins have been seen in pea seeds (Curl *et al.*, 1985; Daveby *et al.*, 1998; Heng *et al.*, 2006; Reim & Rohn,

2015), and the masses associated with peaks 31 through 36 correspond to *Pisum sativum* saponins in the KNApSAcK database. Peak 33 specifically had a major molecular ion at m/z 943.5251 in the MS^1 spectra (positive ion mode) and was tentatively identified as saponin B (Heng *et al.*, 2006).

Finally, the last peak, peak 38, did belong to any of the above families. It displayed a major molecular ion at m/z 203.0821 in the negative ion mode and a characteristic absorption band at 279–289sh nm. It was tentatively identified as tryptophan.

3.2. Pea phytochemical profiles and key compounds underlying bitterness and astringency

3.2.1. Pea phytochemical quantification and impacts of extraction and fractionation

This study used six phenolic acids, five flavonoids, and one saponin as standards to characterize key compounds in pea flour, isolates, and fractions. The analysis was based on the UHPLC-DAD-MS results (mass and calibration curves). Table 2 shows the concentrations (± standard deviation) of the 12 standards. Quercetin-3-O-rutinoside and catechin were identified but could not be quantified because they occurred at very low concentrations in all the sample types. Gallic acid, sinapic acid, and quercetin-3-O-glucoside could only be quantified in the flour samples because their concentrations were too low in the other sample types. In general, compound concentrations were higher in the flour samples than in the isolates. The two exceptions were kaempferol-3-O-glucoside and soyasapogenol B, for which the opposite was seen. In the case of p-hydroxybenzoic acid, the difference in concentrations was relatively small. Furthermore, compound concentrations were generally higher in the isolates than in the fractions, except in the case of soyasapogenol B. The latter had higher concentrations in the pellets. It is difficult to interpret these findings because the samples differed in nature; notably different MS rates and extraction protocols were employed.

Concentrations varied between 0 and 2,000 ng/g (i.e., 0.002 mg/g) for the different compounds and sample types. The literature contains few sources of quantitative data on the phytochemicals in pea flour and isolates. Dvorak *et al.* (2011) found that several phenolic acids occurred at varying concentrations (range: 0–0.026 mg/g) in pea samples. Another study found that the total phenolic acid concentrations in peas varied between 12 and 19 mg/g; for total flavonoids, the range was 0–9 mg/g (Nithiyanantham *et al.*, 2012). Research on pea flour found that DDMP saponin occurred at levels of

1.5 mg/g (Reim & Rohn, 2015) and of 0.7–1.90 mg/g (Heng *et al.*, 2006). Also in pea flour, soyasaponin I occurred within a range of 0.82–2.5 mg/g (Curl *et al.*, 1985); in pea protein isolates, levels reached 1.4 mg/g (1.1 mmol/kg; Gläser *et al.*, 2020). However, these values cannot be reliably compared because they were obtained from different raw materials and underwent different extraction and analysis procedures.

In this study, the ratio between peak area and DM content was established to better compare the different pea protein sample types. When characterizing the compounds identified, data were used that described the peak areas in positive mass. The PCA found that the phytochemical profiles of the flour, isolate, and fraction (pellet, retentate, and permeate) solutions were well distributed along axes F1 and F2, which accounted for 82.6% of the variance (Fig. 1). Thus, maps based on the first two axes provided a high-quality representation of the initial multidimensional data. Most of the phytochemical traits were clustered within one quarter of the correlation circles along axis 1 and thus are clearly correlated. Examples can seen in peak 7 (coumaroyl-4-O-hexoside), peak 10 (N-feruoyl aspartic acid derivative), peak 30 (apigenine-7-O-glucoside), and peak 31 (a saponin derivative). In particular, permeates had large areas associated with phenolic acids, such as peak 14 (a N-phenylacetyl aspartic acid derivative), peak 5 (p-hydroxybenzoic acid), and peak 7 (coumaroyl-4-O-hexoside). A smaller number of phytochemicals representing the different families present were orthogonal to this larger group. For example, along axis 2 are peak 1 (gallic acid), peak 27 (quercetin aglycone), and peak 32 (a saponin derivative). Accounting for DM content, the nine sample types varied in their phytochemical profiles. Permeates a and b had the largest overall areas across all the phytochemical peaks. The flour had the largest areas for the flavonoid peaks. Finally, pellets a and b had the largest terpenoid peaks.

Commercial pea protein isolates undergo significant temperature and pH changes during processing. In particular, pea proteins are usually extracted via isoelectric precipitation, during which pH falls to 4–5. To promote flocculation, the raw extract can also be heated to increase protein denaturation (Murat *et al.*, 2013). The resulting protein solutions subsequently undergo drum drying or spray drying, which involves a rise in temperature. Flavonoids and especially phenolic acids are highly unstable and easily degraded due to changes in pH, temperature, light conditions, or enzyme presence. Thus, pulses subject to processing display significantly reduced levels of phenolics (Nithiyanantham *et*

al., 2012), as observed in our results (i.e., the differences between the flour and isolates). However, different factors can result in synergistic or contradictory actions, which might explain why a small set of phytochemicals could present different behaviors. For example, the following can occur during processing: oxidative reactions; compound formation, breakdown, or leaching; losses of solids; and interactions between proteins and other compounds (Nithiyanantham *et al.*, 2012). According to the review by Singh *et al.* (2017), saponins are also highly vulnerable to degradation due to modifications in pH and temperature during processing. Furthermore, the large number of phytochemicals in the permeates may have resulted from water-soluble compounds having leached away.

3.2.2. Key compounds underlying bitterness and astringency

The phytochemical profiles of the six fractions displayed correlations with the bitterness and astringency scores (analyzed and discussed in Cosson *et al.* [2021]). The main results of this prior research are described here, including a summary of the scores for the two isolates (4% DM), the two permeates, the two retentates, and the two pellets (6% DM) as well as the results of a stepwise multiple comparisons procedure (Newman-Keuls test, alpha level = 0.05) (Fig. 2). Bitterness scores were highest for the retentates and isolates and lowest for the permeates. Furthermore, scores were lower for sample types produced from isolate a than from isolate b. The astringency scores showed less pronounced differences: pellet b had the highest score, and the permeates had the lowest scores. Correlations were characterized between the areas of the phytochemical peaks and the attribute scores (Pearson's r; alpha level = 0.05). The same sample types were used in the sensory analysis and the phytochemical analysis. Based on psychophysical curves, the perception of a compound in a product depends on compound concentration (Chambers & Koppel, 2013). The relationship between compound concentration and sensory intensity may be linear (i.e., above a threshold) or non-linear (i.e., below a threshold). Consequently, this work explored both linear and logarithmic correlations. Different degrees of correlation were seen between the phytochemical peak areas and the attribute scores in the linear and logarithmic models (Table 3). The linear correlations with the attribute scores are presented in Supplementary Figure 2. Overall, 11 phenolic acids, 2 flavonoids, 6 saponins, and 10

non-identified compounds were significantly correlated with bitterness and astringency (Table 3, Supplementary Figure 2).

Among the phenolic acids, caffeic acid (peak 8) was positively correlated with bitterness ($R^2 = 0.90$) and astringency ($R^2 = 0.87$). Caffeic acid is known to cause a persistent sensation of intense bitterness in plant products such as coffee (Frank *et al.*, 2006). Caffeoyl hexoside (peak 6) was negatively correlated with both bitterness and astringency. The degradation of caffeic acid derivatives may lead to an increase in the concentration of free caffeic acid. The other phenolic acids—hydroxybenzoic hexoside (peak 2), protocatechuic acid (peak 3), N-p-hydroxybenzoyl aspartic acid (peak 4), p-hydroxybenzoic acid (peak 5), coumaroyl-4-O-hexoside (peak 7), N-phenylacetyl aspartic acid (peak 14), and the aspartic acid derivative (peak 15)—were negatively correlated with astringency. Bitterness was negatively correlated with peaks 4, 5, and 15. Finally, N-coumaroyl aspartic acid (peak 9) and N-feruloyl aspartic acid (peak 10) were negatively correlated with bitterness. A variety of hydroxybenzoic acid ethyl esters and hydroxycinnamic acid ethyl esters have been identified as bitter compounds in wine (Hufnagel & Hofmann, 2008). However, these ethyl esters are less polar than the acids, which could explain their bitterness. A range of N-phenylpropenoyl-L-amino acids have also been identified as key astringent compounds in roasted cocoa (Stark *et al.*, 2006), and several hydroxycinnamic acids act as precursors of off-flavors in fruit (Naim *et al.*, 1992). Thus, the negative correlations above could also be explained by the release of related compounds (not identified here) that are responsible for perceived bitterness and astringency. With regards to the threshold values, various figures have been reported and are matrix dependent. For example, threshold values for chlorogenic, caffeic, and p-coumaric acids were 40–90 mg/L in water; 520–690 mg/L in beer; and 10–32 mg/L in wine (Boulet *et al.*, 2017). All these compound concentrations are much higher than those in the isolates.

Among the flavonoids, a kaempferol derivative (peak 17) was negatively correlated with bitterness and astringency ($R^2 = -0.93$ and $R^2 = -0.94$). Quercetin-3-O-glucoside (peak 26) was positively correlated with astringency ($R^2 = 0.85$). Past research has shown that flavonoids can contribute to both these attributes, although most of this work was focused on polyphenols in red wine (Hufnagel & Hofmann, 2008). From a mechanistic perspective, the structural configuration of flavonoid compounds

plays a role in activating bitterness receptors. (+)-catechin can activate the TAS2R14 and TAS2R39 receptors, while (-)-epicatechin can activate the TAS2R4, TAS2R5, TAS2R14, and TAS2R39 receptors (Roland *et al.*, 2017). Moreover, the molecular size of polyphenol compounds also plays a role in receptor activation dynamics. Larger polymers tend to result in less bitterness and more astringency, whereas smaller polymers tend to result in more bitterness and less astringency (Sun *et al.*, 2007). In addition, the presence of galloyl groups on epicatechin can affect receptor activation, and the aglycone isomers of isoflavones, being more hydrophobic, are more compatible with the receptors than are their glucoside counterparts. Thus, the negative correlations could be explained by the presence of derivatives associated with peak 17 (e.g., more or less glycolyzed compound forms not identified here) that could play a role in perceived bitterness and astringency. The other flavonoids identified in this study were not correlated with either attribute. However, given that flavone threshold values of 0.1–20 mg/L have been reported for red wine (Sáenz-Navajas *et al.*, 2010), it could be that compound concentrations in the isolates were too low.

Finally, six saponins were positively correlated with astringency. Saponins are generally perceived as bitter and astringent (Heng *et al.*, 2006). Here, the saponin concentrations in the pea protein isolates were calculated based on the soyasapogenol B standard and then compared to values in the literature. The disadvantage of this approach was that a single standard was employed for all the saponins instead of a unique standard for each. However, no commercial standards are available for pea saponins given that their purification remains challenging. The mean concentrations (± standard deviation) of the six saponins were calculated, and their correlations with the astringency scores were examined (Fig. 3). Saponin B (peak 33) occurred at the highest concentration (0.05 mg/g). The panelists performing the sensory analysis were therefore exposed to saponin B levels of 20 mg/L, given the compound's relative concentration (4%) in the pea protein isolates. Previous work found that saponin-mediated bitterness could be perceived by panelists at very low concentrations in dry peas, at around 2 mg/L for a saponin mixture (saponin B and DDMP saponin in a 1:4 ratio) and around 8 mg/L for saponin B (Heng *et al.*, 2006). In this study, the concentration of saponin B should have been high enough to be perceived by the panelists and to thus contribute to sensations of bitterness and astringency. In contrast, soyasaponin I appears to have perception thresholds of 1.62 mmol/L (1,528 mg/L) for

bitterness and 0.64 mmol/L (604 mg/L) for astringency (Gläser *et al.*, 2020). Thus, this saponin was not concentrated enough to contribute to bitterness and astringency on its own; it may, however, have exerted an influence through interactions with other compounds.

4. Conclusion

In this study, UHPLC-DAD-MS was used to identify the main phytochemicals present in pea flour, isolates, and fractions. Several key results emerged. First, 48 phytochemicals were observed. Fifteen compounds were tentatively identified as phenolic acids, 15 flavonoids, and 7 saponins. Furthermore, when the MS data were compared with the reference standards data, it was possible to unambiguously identify and quantify 2 hydroxybenzoic acids, 4 hydroxycinnamic acids, 3 flavonols, 2 flavanols, and 1 saponin. Second, based on the peak areas for the compounds, larger amounts of phytochemicals were present in the flour than in the isolates and fractions, suggesting compounds experienced degradation during processing. However, when accounting for DM content, the permeates contained larger amounts of phytochemicals, which could have resulted from the leaching away of water-soluble compounds. Third, the peak areas of the compounds displayed different degrees of correlation with perceived bitterness and astringency. A total of 29 compounds (phenolic acids, flavonoids, and saponins) were correlated with one or both attributes. It is possible that the complex mixture of these compounds (which includes other compounds as well, such as peptides) could influence overall perceptions of bitterness and astringency. Consequently, at this stage, it remains difficult to make concrete recommendations about which phytochemicals could be removed to improve the desirability of commercial pea-protein-based products. That said, one promising strategy could be to explore different phytochemical compositions, such as those resulting from a decrease in oxidative reactions or the leaching of water-soluble compounds.

5. CRediT author statement

Audrey Cosson: Methodology, Investigation, Formal analysis, Writing - Original Draft. Emmanuelle Meudec: Resources, Investigation. Christian Ginies: Investigation. Alice Danel: Resources, Investigation. Pascale Lieben: Resources, Investigation. Nicolas Descamps: Funding acquisition.

Véronique Cheynier: Resources, Supervision, Writing - review & editing. Anne Saint-Eve: Methodology, Writing - review & editing. Isabelle Souchon: Conceptualization, Supervision, Writing - review & editing.

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7. Declaration of interest

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669 **Figure captions**

670

671 **Figure 1**: Principal component analysis (centered reduced variables, Pearson's n) examining the
672 phytochemical profiles of the different sample types: pea flour, isolates, and fractions (pellets,
673 retentates, and permeates). On the right is a loading plot showing the correlational relationships
674 between PCA axes 1 and 2 and the peak areas (accounting for the sample type's dry matter content)
675 for the 54 phytochemicals identified in the study: the phenolic acids are in blue (dotted line), the
676 terpenoids are in red (solid line), the flavonoids are in green (dashed line), and the other compounds
677 are in orange (thick dashed and dotted line). On the left is a PCA plot with the same two axes that
678 shows the relative similarity of the nine sample types.

679

680 **Figure 2**: Bitterness and astringency scores (out of 10) for the different sample types as determined
681 via static profiling by trained panelists (Cosson *et al.*, 2021). Significant differences between groups
682 are indicated by differences in letters (Newman-Keuls test, alpha level = 0.05).

683

684 **Figure 3**: Mean concentrations (± standard deviation; ng/g) of the six saponins that contributed to
685 perceived astringency.

686

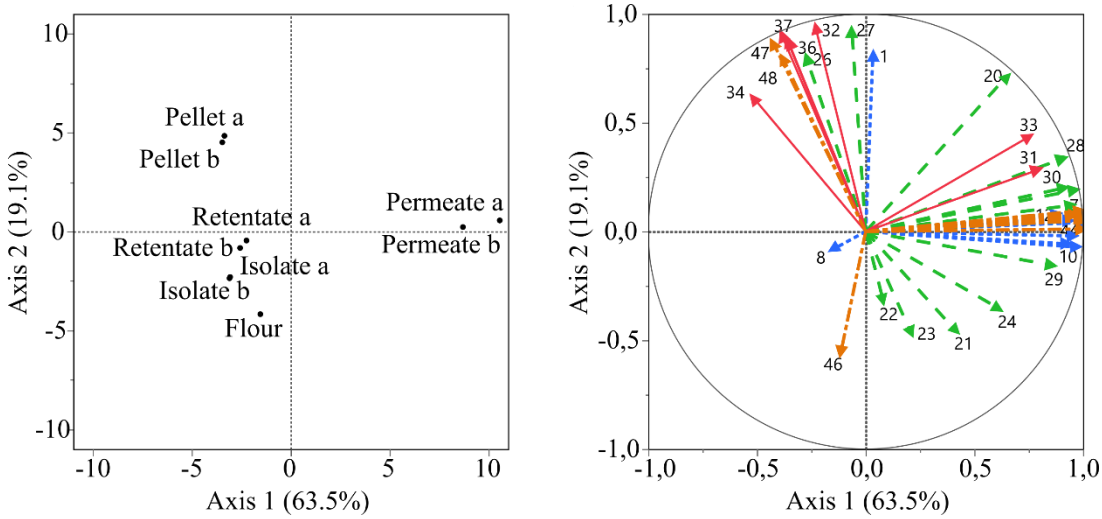


Figure 1: Principal component analysis (centered reduced variables, Pearson's n) examining the phytochemical profiles of the different sample types: pea flour, isolates, and fractions (pellets, retentates, and permeates). On the right is a loading plot showing the correlational relationships between PCA axes 1 and 2 and the peak areas (accounting for the sample type's dry matter content) for the 54 phytochemicals identified in the study: the phenolic acids are in blue (dotted line), the terpenoids are in red (solid line), the flavonoids are in green (dashed line), and the other compounds are in orange (thick dashed and dotted line). On the left is a PCA plot with the same two axes that shows the relative similarity of the nine sample types.

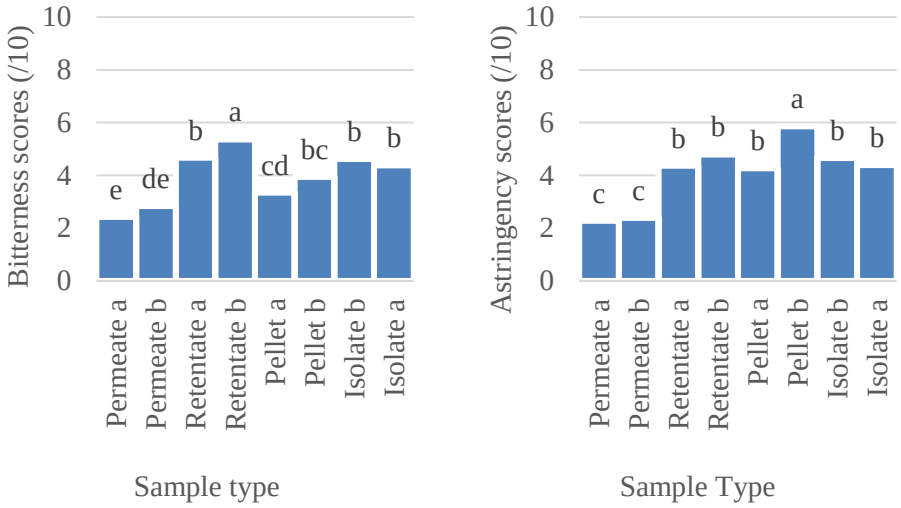


Figure 2: Bitterness and astringency scores (out of 10) for the different sample types as determined via static profiling by trained panelists (Cosson *et al.*, 2021). Significant differences between groups are indicated by differences in letters (Newman-Keuls test, alpha level = 0.05).

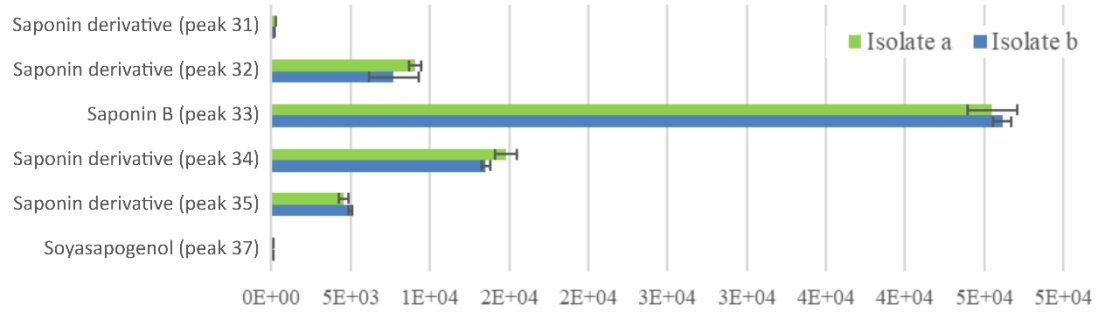
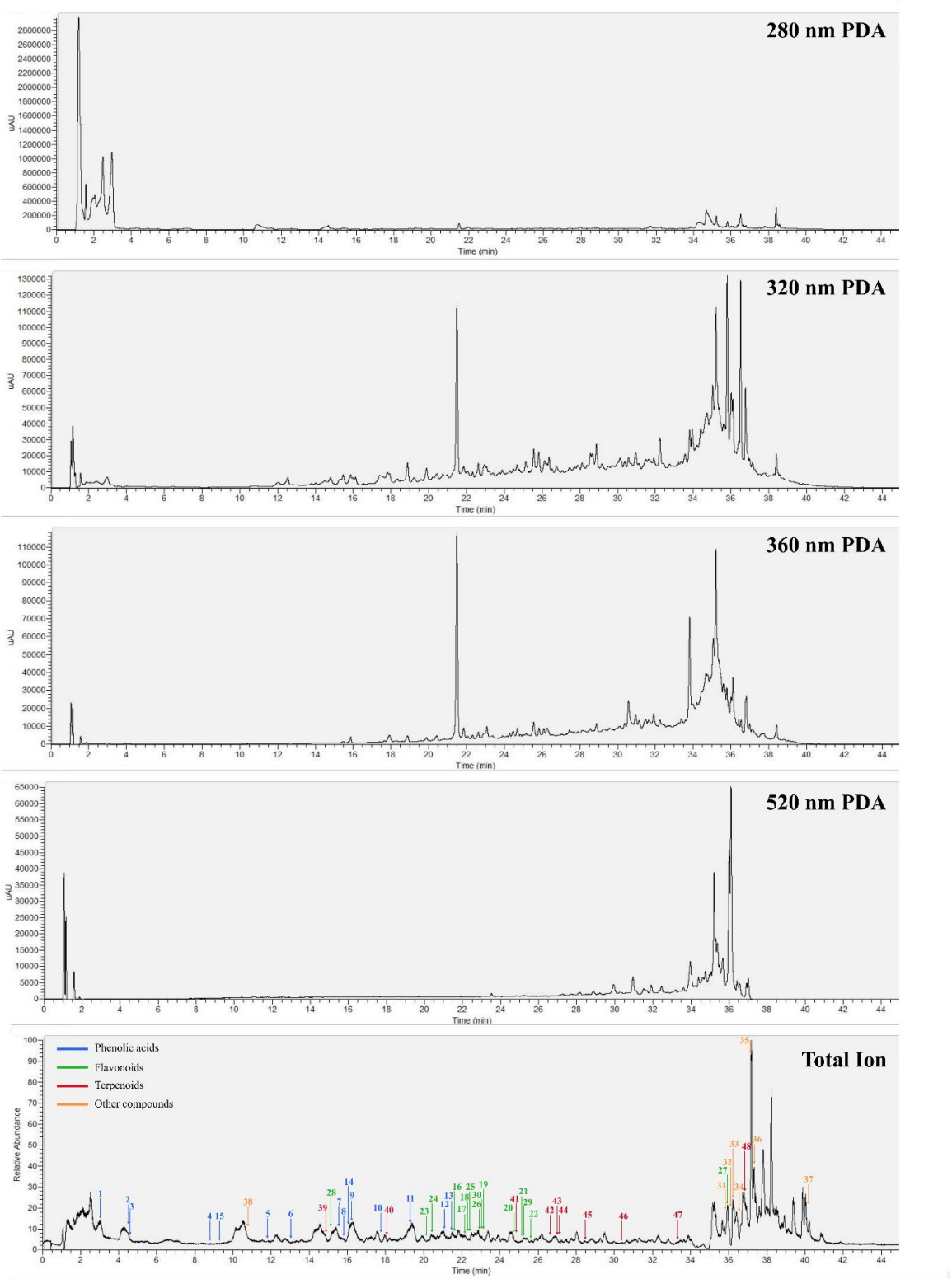


Figure 3: Mean concentrations (± standard deviation; ng/g) of the six saponins that contributed to perceived astringency.

718 **Supplementary Figures**

719 **Figure 1**: Total ion chromatogram and diode array detector (PDA) chromatogram at 280, 320, 360,

720 and 520 nm for isolate a.



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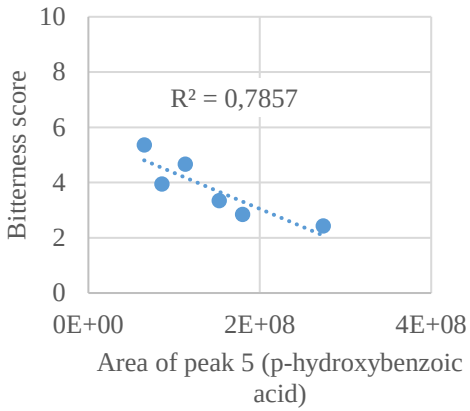
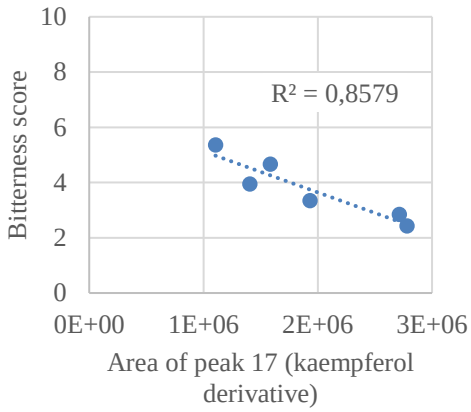
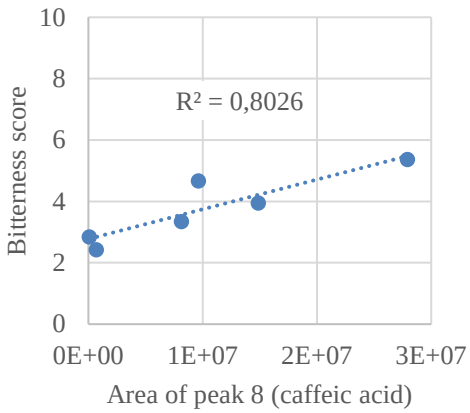
Supplementary Figure 2: Representative positive and negative linear correlations between the phytochemicals (peak areas determined via UHPLC-DAD-MS) and the scores for bitterness or astringency (out of 10); the R^2 values are indicated.







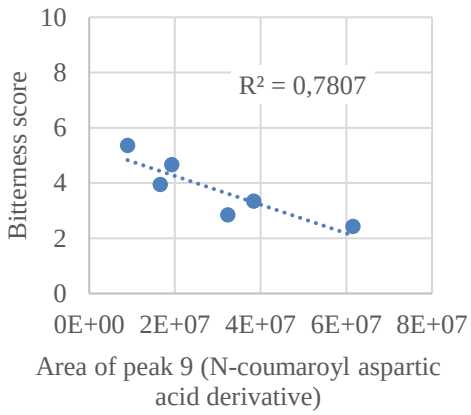


Table captions

Table 1: Phytochemicals identified in the pea flour, isolates, and fractions. Indicated are peak number, RT [min], UV (nm), MS^1 [M+H]+, MS^2 [M+H]+, MS^1 [M-H]-, MS^2 [M-H]-, theoretical molecular mass, theoretical [M+H]+, theoretical [M-H]-, hypothetical chemical formula, tentative of identification, and the database used for identification. The compounds identified using standards are in bold.

Table 2: Concentrations (± standard deviation) of the 12 standards (performed in triplicate) on 10-3 mg/g. The detection limit (DL) was 0.02 ng/µl, and the quantification limit (QL) was 8.0 ng/µl. There were two exceptions: soyasapogenol B, where DL = 0.07 ng/g and QL = 3 ng/g, and p-hydroxybenzoic acid, where DL = 0.05 ng/g and QL = 30.0 ng/g.

Table 3: The coefficients (Pearson's r) for the correlations between the phytochemical compounds (peak areas determined via UHPLC-DAD-MS) and the scores for bitterness and astringency (out of 10) determined using linear and logarithmic models. In bold are the negative correlation coefficients. Only the statistically significant values are indicated (p-value < 0.05).

746 **Table 1**

Peak number	RT [min]	UV (nm)	MS^1 [M+H]$^+$	MS^2 [M+H]$^+$	MS^1 [M-H]$^-$	MS^2 [M-H]$^-$	Hypothetical formula	Expected [M+H]+	Error (ppm)	Expected [M-H] -	Error (ppm)	Hypothetical class	Hypothetical compound	Database	Relative quantification
Phenolic acids															
1	**3.05**	**NA**	**171.0291**	**NA**	**169.0134**	**125.0234**	**C7H6O5**	**171.0288**	**-1.8**	**169.0142**	**5**	**Hydroxybenzoic acid**	**gallic acid**	**Standard**	**NEG**
2	4.43	241sh, 257, 293	301.1021	NA	299.0773	137.0235; 93.0334	C13H16O8	301.0918	-34.2	299.0772	-0.2	Hydroxybenzoic acid	p-hydroxybenzoic hexoside or isomer	Arita	NEG
3	4.45	256; 293	155.0339	NA	153.0183	109.0284	C7H6O4	155.0339	-0.1	153.0193	6.7	Hydroxybenzoic acid	protocatechuic acid	Arita	NEG
4	8.71	254	254.0658	NA	252.0512	136.0394 (p-hydroxybenzoyl amide-H^+); 137.0235 (hydroxybenzoic $-H^+$); 132.0293 (aspartic acid$-H^+$); 93.0334 (phenol moiety)	C11H11NO6	254.0659	0.4	252.0514	0.6	Hydroxybenzoic acid	N-p-hydroxybenzoyl aspartic acid	PubChem	NEG
5	11.7	257	139.039	NA	137.0234	93.0334	C7H6O3	139.039	-0.2	137.0244	7.4	Hydroxybenzoic acid	p-hydroxybenzoic acid	Standard	NEG
6	13	271; 317	343.1023	NA	341.0874	135.0442 (caffeic acid$-CO_2-H^+$); 179.0343 (caffeic acid$-H^+$)	C15H18O9	343.1024	0.2	341.0878	1.2	Hydroxycinnamic acid	caffeoyl hexoside	PubChem	NEG
7	15.47	295	327.1085	NA	325.0928	163.0396 (coumaric acid$-H^+$); 119.0492 (coumaric acid$-CO2-H^+$)	C15H18O8	327.1074	-3.2	325.0929	0.3	Hydroxycinnamic acid	coumaroyl- 4-O-hexoside	KNApSAcK	NEG
8	**15.61**	**320**	**181.0496**	**NA**	**179.0342**	**135.0442**	**C9H8O4**	**181.0495**	**-0.4**	**179.035**	**4.4**	**Hydroxycinnamic acid**	**caffeic acid**	**Standard**	**NEG**
9	16.13	295sh, 310	280.0812	NA	278.067	132.0292 (aspartic acid $-H^+$); 162.0552 (coumaroyl amide $-H^+$); 163.0392 (coumaric acid-H^+)	C13H13NO6	280.0816	1.3	278.067	0	Hydroxycinnamic acid	N-coumaroyl aspartic acid derivative	NA	NEG
10	17.7	293sh, 320	310.0916	NA	308.0776	132.0292 (aspartic acid$-H^+$); 192.0660 (feruloyl amide $-H^+$); 193.0502 (ferulic acid $-H^+$)	C14H15NO7	310.0921	1.7	308.0776	-0.1	Hydroxycinnamic acid	N-feruoyl aspartic acid derivative	NA	NEG
11	**19.34**	**310**	**165.0546**	**NA**	**163.0391**	**119.0491**	**C9H8O3**	**165.0546**	**0.1**	**163.0401**	**5.9**	**Hydroxycinnamic acid**	**trans-p-coumaric acid**	**Standard**	**NEG**
12	**21.08**	**324**	**195.0651**	**NA**	**193.05**	**134.0364; 178.0265; 149.0597**	**C10H10O4**	**195.0652**	**0.4**	**193.0506**	**3.3**	**Hydroxycinnamic acid**	**trans-ferulic acid**	**Standard**	**NEG**
13	**21.44**	**325**	**225.0757**	**NA**	**223.0608**	**208.0372**	**C11H12O5**	**225.0757**	**0.2**	**223.0612**	**1.8**	**Hydroxycinnamic acid**	**sinapic acid**	**Standard**	**NEG**
14	16.02	250	252.0864	NA	250.072	132.0293 (aspartic acid$-H^+$); 135.0442 (phenylacetic acid $-H^+$); 134.0601 (phenylacetyl amide-H^+)	C12H13NO5	252.0866	1	250.0721	0.4	Phenylacetic acid	N-phenylacetyl aspartic acid derivative	NA	NEG
15	9.34	250	268.0814	NA	266.067	132.0293 (aspartic acid$-H^+$)	C12H13NO6	268.0816	0.6	266.067	0	Other phenolic acid	hydroxyphenylacetic acid aspartic acid conjugate	PubChem	NEG
Flavonoids															
16	21.55	266; 347	773.2117	287.0545 (kaempferol)	771.1978	609.1436 (kaempferol + 2 hexoses); 429.0825; 327.0507; 284.0237 (kaempferol);	C33H40O21	773.2135	2.3	771.1989	1.5	Flavonol (kaempferol derivative)	kaempferol sophorotrioside	Arita, KNApSAcK	NEG

						285.0393 (kaempferol); 255.0297; 771.2020									
17	22.13	345	755.201	287.0545 (kaempferol)	753.1879	193.0501; 255.0297; 285.0405 (kaempferol); 609.1436 (kaempferol + 2 hexoses)	C33H38O20	755.2029	2.5	753.1884	0.6	Flavonol (kaempferol derivative)	kaempferol derivative	Arita	NEG
18	22.27	258; 345	595.1647	287.0545 (kaempferol)	593.151	488.1147; 389.2183; 285.0405 (kaempferol); 255.0297	C27H30O15	595.1657	1.8	593.1512	0.3	Flavonol (kaempferol derivative)	kaempferol derivative	Arita	NEG
19	23.1	ND	697.1592	287.0545 (kaempferol)	695.1442	488.1147; 489.1039 (-44-hexose); 477.0941 (kaempferol + hexose); 389.2183; 285.0405 (kaempferol); 255.0297; 609.1436 (kaempferol + 2 hexoses); 447.0902 (-malonylhexose)	C30H32O19	697.1611	2.7	695.1465	3.3	Flavonol (kaempferol derivative)	kaempferol malonyl di-hexoside	Arita	NEG
20	**24.75**	**266; 347**	**449.1071**	**287.0545 (kaempferol)**	**447.0928**	**284.0323; 255.0297**	**C21H20O11**	**449.1078**	**1.6**	**447.0933**	**1.1**	**Flavonol (kaempferol derivative)**	**kaempferol-3-O-glucoside**	**Standard**	**NEG**
21	25.12	269, 346	979.2695	287.0545 (kaempferol)	977.2558	284.0237 (kaempferol); 255.0297; 609.1436 (kaempferol + 2 hexoses); 771.2020 (kaempferol + 3 hexoses)	C44H50O25	979.2714	1.9	977.2568	1.1	Flavonol (kaempferol derivative)	kaempferol sinapoyl triglucoside	Arita, KNApSAcK	NEG
22	25.62	ND	949.2592	287.0545 (kaempferol)	947.2452	771.1990 (kaempferol + 3 hexoses); 609.1436 (kaempferol + 2 hexoses); 429.0825; 327.0507; 284.0237 (kaempferol); 255.0297; 300.0277; 389.2183	C43H48O24	949.2608	1.7	947.2463	1.1	Flavonol (kaempferol derivative)	kaempferol feruloyl tri-hexoside	Arita	NEG
23	20.18	256, 267sh, 350	789.2068	303.0497 (quercetine)	787.1926	300.0276 (quercetine); 445.0775 (quercetine + hexose - H2O); 271.0230; 178.9980	C33H40O22	789.2084	2	787.1938	1.6	Flavonol (quercetin derivative)	quercetin tri-hexoside	KNApSAcK	NEG
24	20.41	256, 355	627.154	303.0496 (quercetine)	625.1401	300.0276 (quercetine); 445.0775 (quercetine + hexose - H2O); 271.0230; 178.9980	C27H30O17	627.1556	2.5	625.141	1.5	Flavonol (quercetin derivative)	quercetin di-hexoside	KNApSAcK	NEG
25	**22.41**	**258; 355**	**611.1589**	**303.0496 (quercetine)**	**609.1456**	**300.0275 (quercetine); 178.9980**	**C27H30O16**	**611.1607**	**2.9**	**609.1461**	**0.8**	**Flavonol (quercetin derivative)**	**quercetin-3-O-rutinoside**	**Standard**	**NEG**
26	**22.94**	**258; 355**	**465.1016**	**303.0496 (quercetine)**	**463.0885**	**300.0274 (quercetine); 271.0230; 178.9980**	**C21H20O12**	**465.1028**	**2.5**	**463.0882**	**-0.6**	**Flavonol (quercetin derivative)**	**quercetin-3-O-glucoside**	**Standard**	**NEG**
27	35.95	370	303.0277	NA	301.0352	NA	C15H10O7	303.0499	73.4	301.0354	0.6	Flavonol (quercetin derivative)	quercetin aglycone	Arita	NEG
28	**15.02**	**279**	**291.086**	**NA**	**289.0714**	**178.998; 271.0612; 151.0389**	**C15H14O6**	**291.0863**	**1**	**289.0718**	**1.4**	**Flavanol**	**catechin**	**Standard**	**NEG**
29	**25.26**	**284**	**581.1872**	**273.0754**	**579.1717**	**271.0612; 151.0389**	**C27H32O14**	**581.1865**	**-1.2**	**579.1719**	**0.4**	**Flavanone**	**naringin**	**Standard**	**NEG**
30	23	267, 336	NA	NA	431.0981	269.0456 (apigenine); 164.0448; 271.0628	C21H20O10	433.1129	NA	431.0984	0.6	Flavone (apigenin derivative)	apigenine-7-O-glucoside	KNApSAcK	NEG
Terpenoids															
31	35.9	193-222	925.5158	85.0291; 141.0183; 365.3195; 423.3616; 581.3833; 441.3723	923.4626	NA	C48H76O17	925.5155	-0.3	923.501	41.6	Saponin	saponin derivative	KNApSAcK	POS
32	36.06	193-222	797.4666	85.0290; 141.0182; 365.3194; 423.3615; 581.3832; 441,3723	795.4531	NA	C42H68O14	797.4682	2	795.4536	0.7	Saponin	saponin derivative	KNApSAcK	POS

33	36.12	195-205	943.5251	85.0293; 141.0181; 365.3193; 423.3614; 581.3831; 441.3723	941.5092	NA	C48H78O18	943.5261	1.1	941.5115	2.5	Saponin	saponin B	PubChem, KNApSAcK	POS
34	36.53	228	941.5086	85.0291; 141.0182; 365.3191; 423.3615; 581.3833; 441.3723	939.4941	NA	C48H76O18	941.5104	2	939.4959	1.9	Saponin	saponin derivative	KNApSAcK	POS
35	37.22	226	971.5193	85.0290; 141.0184; 365.3194; 423.3617; 581.3832	969.5037	NA	C49H78O19	971.521	1.8	969.5065	2.8	Saponin	saponin derivative	KNApSAcK	POS
36	37.6	229	825.4613	85.0288; 141.0180; 365.3192; 423.3615; 581.3831	823.4426	NA	C43H68O15	825.4631	2.2	823.4485	7.2	Saponin	saponin derivative	KNApSAcK	POS
37	**40.2**	**228**	**459.3844**	**441.3723; 423.3618**	**NA**	**NA**	**C30H50O3**	**459.3833**	**-2.5**	**457.3687**	**NA**	**Saponin**	**soyasapogenol B**	**Standard**	**POS**

Others

38	10.79	279-289sh	205.0973	159.0917	203.0821	116.0495; 159.0920	C11H12N2O2	205.0972	-0.7	203.0826	2.5	Amino acid	tryptophan	PubChem	NEG
39	14.9	214; 280; 320sh	457.2171	NA	455.1774	293.1245; 179.0554	NA	NA	NA	NA	NA	NA	NA	NA	NEG
40	18.03	212; 283	389.1794	NA	387.1659	163.1120; 225.1129	NA	389.1806	3.1	387.1661	0.4	NA	NA	NA	NEG
41	24.82	193-218sh	549.2483	NA	547.2394	161.0446; 149.0445; 89.0232	NA	549.2542	10.7	547.2396	0.4	NA	NA	NA	NEG
42	26.6	349	741.222	309.2784; 599.5033; 703.5117	739.207	164.0448; 271.0628	NA	741.2237	2.2	739.2091	2.8	NA	NA	NA	NEG
43	27	280; 340	591.1696	285.0753	589.1566	149.0446; 283.0612; 178.9980	NA	591.1708	2.1	589.1563	-0.5	NA	NA	NA	NEG
44	27.15	193-218sh; 277	705.2587	NA	703.2447	161.0446; 149.0445; 89.0232	NA	705.26	1.9	703.2455	1.1	NA	NA	NA	NEG
45	28.48	250	591.2638	NA	589.2498	161.0446; 149.0445; 89.0232	NA	591.2647	1.6	589.2502	0.6	NA	NA	NA	NEG
46	30.39	258	619.2751	NA	617.2639	NA	NA	NA	NA	NA	NA	NA	NA	NA	NEG
47	33.38	270; 370	271.0598	NA	269.0453	223.0819. 161.0448	NA	271.0601	1.1	269.0455	0.9	NA	NA	NA	NEG
48	36.85	282; 333	313.0711	285.0753; 163.0391; 257.0805	311.0568	267.1967; 134.0463; 271.0612; 283.0627	NA	313.0707	-1.4	311.0561	-2.2	NA	NA	NA	NEG

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750 **Table 2**

Compound	**gallic acid**	**p-hydroxybenzoic acid**	**caffeic acid**	**coumaric acid**	**ferulic acid**	**sinapic acid**	**kaempferol-3-O-glucoside**	**quercetin-3-O-rutinoside**	**quercetin-3-O-glucoside**	**catechin**	**naringin**	**soyasapogenol B**
Peak number	*1*	*2*	*8*	*11*	*12*	*13*	*20*	*25*	*26*	*28*	*29*	*37*
Isolate b	QL	1265.9 ± 6.9	DL	3.0 ± 1.7	3.0 ± 1.7	DL	108.9 ± 1.7	QL	QL	QL	5.6 ± 1.7	91.0 ± 10.0
Isolate a	QL	1682.0 ± 56.8	QL	8.0 ± 1.7	8.4 ± 1.7	DL	105.9 ± 1.7	QL	QL	QL	10.2 ± 1.7	93.9 ± 13.42
Permeate a	QL	170.8 ± 6.4	QL	DL	DL	QL	DL	QL	QL	QL	QL	QL
Permeate b	QL	258.5 ± 1.8	QL	2.0 ± 1.7	DL	QL	DL	QL	QL	QL	QL	QL
Pellet b	QL	72.0 ± 6.8	3.1 ± 2.3	DL	DL	QL	29.0 ± 1.7	QL	QL	QL	QL	175.6 ± 15.4
Pellet a	QL	126.5 ± 4.4	DL	1.7 ± 1.7	2.3 ± 1.7	QL	41.7 ± 1.7	QL	QL	QL	QL	188.6 ± 41.6
Retentate b	QL	54.5 ± 6.2	5.8 ± 2.1	DL	DL	QL	2.3 ± 1.7	QL	QL	QL	QL	9.4 ± 10.0
Retentate a	QL	94.2 ± 2.3	DL	DL	DL	QL	3.0 ± 1.7	QL	QL	QL	QL	15.7 ± 10.0
Flour	16.08 ± 1.08	1999.0 ± 18.4	90.7 ± 2.4	124.0 ± 2.2	151.3 ± 1.8	44.49 ± 1.7	62.9 ± 11.1	QL	14.8 ± 1.7	QL	81.7 ± 3.1	48.4 ± 10.0

751

Table 3

			Linear model - Bitterness		Linear model - Astringency		Logarithmic model - Bitterness		Logarithmic model - Astringency	
Hypothetical family	**Peak number**	**Hypothetical compound**	**p-value**	**R^2**	**p-value**	**R^2**	**p-value**	**R^2**	**p-value**	**R^2**
Phenolic acids	2	hydroxybenzoic hexoside	NA	NA	<0.01	**-0.94**	NA	NA	<0.01	**-0.96**
	3	protocatechuic acid	NA	NA	<0.01	**-0.96**	NA	NA	<0.01	**-0.99**
	4	N-p-hydroxybenzoyl aspartic acid	0.04	**-0.83**	<0.01	**-0.95**	0.03	**-0.85**	<0.01	**-0.95**
	5	p-hydroxybenzoic acid	0.02	**-0.89**	0.03	**-0.87**	0.01	**-0.92**	0.03	**-0.86**
	6	caffeoyl hexoside	0.02	**-0.88**	0.01	**-0.91**	0.02	**-0.89**	0.01	**-0.90**
	7	coumaroyl-4-O-hexoside	NA	NA	<0.01	**-0.96**	NA	NA	<0.01	**-0.98**
	8	caffeic acid	0.02	0.90	NA	NA	NA	NA	0.02	0.87
	9	N-coumaroyl aspartic acid derivative	0.02	**-0.88**	NA	NA	0.01	**-0.94**	NA	NA
	10	N-feruoyl aspartic acid derivative	NA	NA	NA	NA	0.05	**-0.82**	NA	NA
	14	N-phenylacetyl aspartic acid derivative	NA	NA	0.01	**-0.93**	NA	NA	<0.01	**-0.97**
	15	aspartic acid derivative	0.03	**-0.84**	<0.01	**-0.95**	0.02	**-0.87**	<0.01	**-0.95**
Flavonoids	17	kaempferol derivative	0.01	**-0.93**	0.01	**-0.92**	0.01	**-0.94**	0.02	**-0.88**
	26	quercetin-3-O-glucoside	NA	NA	NA	NA	NA	NA	0.03	0.85
Terpenoids	31	saponin derivative	NA	NA	0.02	0.88	NA	NA	0.01	0.92
	32	saponin derivative	NA	NA	NA	NA	NA	NA	0.02	0.88
	33	saponin B	NA	NA	NA	NA	NA	NA	0.02	0.90
	34	saponin derivative	NA	NA	NA	NA	NA	NA	0.01	0.92
	35	saponin derivative	NA	NA	NA	NA	NA	NA	0.04	0.83
	37	soyasapogenol B	NA	NA	NA	NA	NA	NA	0.02	0.88
Others	39	NA	NA	NA	<0.01	**-0.95**	NA	NA	<0.01	**-0.96**
	42	NA	0.02	**-0.90**	0.01	**-0.92**	0.01	**-0.90**	0.01	**-0.90**
	43	NA	NA	NA	NA	NA	0.05	**-0.81**	NA	NA
	40	NA	NA	NA	<0.01	**-0.97**	NA	NA	<0.01	**-0.97**
	41	NA	<0.01	**-0.97**	0.03	**-0.84**	<0.01	-0.98	NA	NA
	44	NA	NA	NA	<0.01	**-0.97**	NA	NA	NA	NA
	45	NA	NA	NA	<0.01	**-0.96**	NA	NA	NA	NA
	46	NA	NA	NA	0.04	0.84	NA	NA	NA	NA
	47	NA	NA	NA	NA	NA	NA	NA	0.02	0.88
	48	NA	NA	NA	NA	NA	NA	NA	0.03	0.86