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Tocopherols, carotenoids and phenolics changes during Andean lupin (Lupinus mutabilis Sweet) seeds processing

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Abstract:	Andean lupin (L. mutabilis) seeds, appreciated for their high protein and unsaturated lipid concentrations, must undergo a drastic water debittering process to eliminate endogenous alkaloids. Aim of this research was to investigate the effects of debittering and further technological treatments such as extrusion and spray-drying (with two different coating agents, gum Arabic and maltodextrin) on the antioxidants of three Andean lupin ecotypes. Tocopherols (mainly γ-tocopherol) and free phenolics (flavonoids, phenylethanoids, phenolic acids) were abundant in bitter seeds, while carotenoids were scarce. After debittering, tocopherols slightly increased, xanthophyll carotenoids and bound flavonoids were unchanged but free phenolics and bound phenolic acids decreased (76% and 50%, respectively). Extrusion did not modify tocopherols and phenolics but marginally reduced (7%) xanthophyll carotenoids. Tocopherols, carotenoids and phenolics dropped after spray-drying (24-27%, 32-36% and 53-57%, respectively), without differences between coating agents. Nevertheless, the total tocopherol and phenolic contents were still abundant even after all processing treatments.
Response to Reviewers:	

Dear Editor

please find enclosed our manuscript “Tocopherols, carotenoids and phenolics changes during Andean lupin (*Lupinus mutabilis* Sweet) processing**”** which we wish to submit for consideration as a research article in Journal of Food Composition and Analysis.

Andean lupin (*Lupinus mutabilis* Sweet), a crop that has attracted worldwide interest in recent years, is rich in proteins and lipids but, because of its high alkaloid content, needs a debittering treatment (boiling and repeated washings) before consumption (like the European lupins). Current knowledge about the influence of processing treatments on tocols, carotenoids and phenolics in lupins is limited, hindering the development of new functional foods. Therefore, our research studied the effect of debittering and extrusion of lupin flour, and of spray-drying of lupin drink with two different wall materials (gum Arabic and maltodextrin), on carotenoids, tocols and free and bound phenolics (flavonoids, phenylethanoids, phenolic acids) of three varieties of Andean lupin from Peru. All the compounds were determined by HPLC.
Previous studies report that lipophilic compounds (tocols and carotenoids) were still present in different lupin species after the debittering process. However, the studies focused on the removal of hydrophilic quinolizidine alkaloids seldom give peripheral information about the hydrophilic phenolic compounds (flavonoids, phenolic acid and phenylethanoids).
Our paper presents novel, original research that has not been previously published and that is not under consideration elsewhere. We hope that our article will be suitable for Journal of Food Composition and Analysis.

Looking forward to further word in due time, I remain

yours sincerely

Lorenzo Estivi

Dear Editor,

as you suggested we changed the numerical data to 3 significant figures and added brief methods for moisture, tocols, carotenoids and phenolics.

Best regards,
Lorenzo Estivi

- Debittering, extrusion, and spray-drying effects on lupin antioxidants were tested
- Free phenolics and bound phenolic acids decreased after debittering
- Extrusion did not reduce tocopherols and phenolics but slightly decreased carotenoids
- Tocopherols, carotenoids and phenolics dropped after spray-drying
- Tocopherols and phenolics were still abundant after all treatments

Tocopherols, carotenoids and phenolics changes during Andean lupin (*Lupinus mutabilis* Sweet) seeds processing

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ABSTRACT

Andean lupin (*L. mutabilis*) seeds, appreciated for their high protein and unsaturated lipid concentrations, must undergo a drastic water debittering process to eliminate endogenous alkaloids. Aim of this research was to investigate the effects of debittering and further technological treatments such as extrusion and spray-drying (with two different coating agents, gum Arabic and maltodextrin) on the antioxidants of three Andean lupin ecotypes. Tocopherols (mainly γ-tocopherol) and free phenolics (flavonoids, phenylethanoids, phenolic acids) were abundant in bitter seeds, while carotenoids were scarce. After debittering, tocopherols and bound flavonoids slightly increased, xanthophyll carotenoids were unchanged but free phenolics and bound phenolic acids decreased (76.2% and 50.1%, respectively). Extrusion did not modify tocopherols and phenolics but marginally reduced (14.5%) xanthophyll carotenoids. Tocopherols, carotenoids and phenolics dropped after spray-drying (34.2%, 39.3% and 48.4%, respectively), without differences between coating agents. Nevertheless, the total tocopherol and phenolic contents were still abundant even after all processing treatments.

Keywords. Debittering, extrusion, flavonoids, gum Arabic, maltodextrin, phenolic acids, spray-drying.

1. Introduction

Andean lupin (*L. mutabilis* Sweet) seeds are appreciated for their high content in proteins (Gulisano et al., 2019) and in lipids rich in mono- and poly-unsaturated fatty acids (Carvajal-Larenas et al., 2016; Gulisano et al., 2019). Their carbohydrates are mainly oligosaccharides and cell wall storage polysaccharides (Trugo et al., 2003); furthermore, they contain several bioactive compounds such as tocopherols and carotenoids (Briceño Berru et al., 2021) and phenolic compounds (Czubinski et al., 2021), which exert antioxidant activity (Córdova-Ramos et al., 2020b; Villacrés et al., 2020) with positive effects on human health (Liu et al, 2018).

Unfortunately, lupins generally contain bitter and/or toxic water-soluble alkaloids, particularly abundant in *L. mutabilis* (Caligari et al., 2000), which must be removed by a multi-step debittering process that involves boiling and then soaking the seeds in running water for several days (Musco et al., 2017). Debittering inactivates many enzymes and washes away several water-soluble compounds, such as some minerals and starch, thus modifying the original composition and nutritional value of the beans (Córdova- Ramos et al., 2020a; Villacrés et al., 2020). Other processing treatments may further change several chemical and digestibility characteristics (Córdova- Ramos et al., 2020a), possibly enhancing the nutritional value of foods. For example, flour extrusion leads to protein denaturation, improves the functional properties of lupin dietary fibre (Zhong et al., 2019a) and, depending on the operating conditions, may release bound polyphenols from cell walls (Zhong et al., 2021), while spray-drying reduces the degradation of bioactive compounds and enhances solubility, stability and flow properties (Sosnik and Seremeta 2015). Czubinski et al., (2021) reported that the composition of *Lupinus mutabilis* was significantly different from that of the European lupins, thus any changes due to processing may not be properly predicted from

other species. Few studies addressed the bioactive compounds modifications in Andean lupin seeds and flour due to food manufacturing. Córdova- Ramos et al. (2020b) and Villacrés et al. (2020) described a reduction of total phenolic content and antioxidant activity after aqueous debittering, but nothing is currently available on variations in the composition of hydrophilic (phenolics) and lipophilic (tocols and carotenoids) antioxidants after further treatments.

This dearth of information clearly hinders the development of new functional foods from Andean lupins. Therefore, aim of the research was to analyse the content and the composition of tocols, carotenoids and phenols in seeds of three different Andean lupin ecotypes, and to monitor their changes as a consequence of debittering, extrusion, as well as spray-drying with two different coating agents (gum Arabic and maltodextrin).

2. Materials and methods

2.1. Materials

Three *Lupinus mutabilis* genotypes originating from different regions of Peru (Altagracia, from Ancash, Andenes, from Cusco, and Yunguyo, from Puno) were kindly supplied by the *Programa de Leguminosas de Grano y Oleaginosas* of the Universidad Nacional Agraria La Molina, Lima, Peru.

2.2. Processing methods

2.2.1 Debittering

To remove the alkaloids, debittering was carried out according to Córdova- Ramos et al. (2020a). The lupin beans were hydrated for 12 h at room temperature with a 1:6 (w/v) seeds:water ratio; boiled for 1 h (hydrated seeds:water 1:3 w/v), changing water after 30 min;

soaked in water (cooked seeds:water 1:3 w/v) at room temperature for 5 days, substituting the water every day; dried at 50 °C in a SW-10S dryer (Xinhang, Henan, China) for 18 hours; and stored under dark at room temperature until milling. The complete elimination of the bitter alkaloids was assessed by sensorial analysis of the grains.

2.2.2. Milling

The bitter and debittered lupin grains were ground separately with a Grindomix GM 200 knife mill (Retsch GmbH, Germany) at 6000 RPM for 35 s; each whole meal flour was sieved through a 2.0 mm mesh, packed in high-density polyethylene bags with hermetic closure and stored at 4 °C until the analysis.

2.2.3 Extrusion

The extrusion was performed on debittered flour hydrated to 35%, at a pressure of 20 MPa using a DSE32 laboratory extruder (Jinan Dingrun Machinery Co, Jinan City, China). The temperatures in the different sections of the extruder were set to 95, 120, 140 and 130 °C, as in Lampart-Szczapa et al. (2006). The extrusion pellets were milled for 35 s at 6000 RPM with a Grindomix GM 200 knife mill (Retsch GmbH, Germany). The extruded whole meal flours were packed in high-density polyethylene bags with hermetic closure and stored at 4 °C until further analysis.

2.2.4 Spray-drying

Debittered lupin beans were hydrated (1:6 w/v seeds:water) for 12 h at room temperature, peeled, mixed with cold boiled water (1:4 w/v ratio), ground for 15 min with an Oster BLSTBC4129-053 blender (Sunbeam, Boca Raton, FL, USA) and filtered through a thin-

mesh cloth, to remove coarse material. The lupin drink was fed to a SD-Basic spray-dryer (LabPlant, Filey, North Yorkshire, United Kingdom), with the addition (6% w/w) of a coating agent (gum arabic or maltodextrin; Frutarom SAC, Lima, Peru). The working conditions were: inlet temperature 170 °C, outlet temperature 80-90 °C, 400-600 kPa and feeding speed 12.5 mL/min. The spray-dried lupin powder was stored at 4 ºC in airtight dark glass jars until analysis.

2.3. Analyses

The moisture of the beans and of the flours was determined in triplicate following the gravimetric method 925.10 (AOAC, 2000).

2.3.1. Tocols and carotenoids

The tocols and carotenoids extracts were obtained by saponification as outlined by Hidalgo and Brandolini (2010). Briefly, 2 g of sample were saponified under nitrogen for 45 min at 70 °C, with 5 mL of ethanolic pyrogallol (60 g/L), 2 mL of ethanol (95%), 2 mL of sodium chloride (10 g/L) and 2 mL of potassium hydroxide (600 g/L). After the saponification, the samples were cooled in ice bath and 15 mL of sodium chloride (10 g/L) were added. The suspension was extracted twice with 15 mL hexane:ethyl acetate (9:1 v/v). The organic layer was collected and evaporated under vacuum and nitrogen drying; the residue was dissolved in 2 mL hexane:isopropyl alcohol (99:1 v/v) and filtered through a 0.22 μm PTFE membrane. Tocols quantification was performed by NP-HPLC as detailed in Rodríguez et al. (2021), using: Alltima SI column, 250 x 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); Alltima SI guard column 7.5 x 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); mobile phase, hexane:ethyl acetate:acetic acid (97.3:1.8:0.9, v/v/v); flow rate, 1.6 mL/min;

pump L-2130 Elite LaChrom (VWR, Hitachi, Japan); fluorimetric detector Jasco 821 FP Intelligent Spectrofluorometer (Japan), at excitation-emission wavelengths of 290 nm and 330 nm, respectively, connected to a Hitachi D-7500 integrator (Merck, Darmstadt, Germany). The α-tocopherol (0.40-110 mg/L; Fluka BioChemika, Buchs, Switzerland), β-tocopherol (0.38-72.2 mg/L; Supelco, Bellefonte, PA, USA), γ-tocopherol (0.20-23.2 mg/L; Supelco, Bellefonte, PA, USA), and δ-tocopherol (0.05-9.35 mg/L; Supelco, Bellefonte, PA, USA) standard curves were constructed. Total tocopherols were computed as the sum of the different homologues.

Carotenoids quantification was performed by NP-HPLC as described in Brandolini et al. (2015), using: column Alltima Si column, 250 x 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); Alltima SI guard column 7.5 x 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); column oven at 20 °C L-2300 Elite LaChrom (VWR, Hitachi, Japan); mobile phase, hexane:isopropyl alcohol (5%); flow rate, 1.5 mL/min; pump L-2130 Elite LaChrom (VWR, Hitachi, Japan). The carotenoids were detected at 445 nm by Diode Array Detector L2450 Elite LaChrom (Merck, Hitachi, Japan) in the range 200-650 nm. The HPLC system was controlled by the software EZChrom Client/Server versione 3.1.7. For peak quantification, lutein (0.3-3.0 mg/L; Fluka, St. Louis, MO, USA), and zeaxanthin (0.05-1.03 mg/L; Extrasynthese, Genay, France) calibration curves were built. The total carotenoids were computed as the sum of the different compounds.

The results are reported as mg/kg dry matter (DM). All the analyses were performed in triple.

2.3.2. Phenolics

Soluble free and insoluble bound phenolics were extracted as described by Nakov et al. (2020) and by Yilmaz et al. (2015), respectively. Briefly, exactly 1.0 g of sample was

extracted three times with 15 mL of 80% methanol. After centrifugation, the pooled supernatants were evaporated under vacuum and nitrogen flux, resuspended in 2 mL 80% methanol solution, and filtered with a 0.45 μm PTFE membrane for free phenolic analysis. For insoluble bound phenolics, the sediment was digested with 15 mL of 4M NaOH under nitrogen for 4 h at room temperature, brought to pH 1.5-2 with 6M HCL and extracted twice with 20 mL of diethyl ether/ethyl acetate (1:1, v/v). The extracts were clarified with sodium sulphate, evaporated as previously outlined, resuspended in 2 mL of methanol-water (1:1 v/v) and filtered. The samples were analysed by RP-HPLC following Hidalgo et al. (2019) using a column Adamas® C18-AQ 5 μm 4.6 mm × 250 mm and a precolumn C18 5 μm 4.6 mm × 10 mm (Sepachrom SRL, Rho, Italy) thermostated at 30 °C; L-2130 pump, L-2300 column oven and L2450 Diode Array Detector (Elite LaChrom, Hitachi, Tokyo, Japan). Gradient elution was performed using acetonitrile (A) and 1 % (v/v) formic acid in water (B) mobile phases at 1.0 mL/min flow rate, following the gradient profile: 0-10 min from 10% to 25% A, 10-20 min linear rise up to 60% A, and 20-30 min linear rise up to 70% A, followed by 10 min reverse to 10% A, with 5 min of equilibration time. The identity of the compound was confirmed by congruence of retention times and UV/Vis spectra with those of pure authentic standards. Thirty-three standards were injected; unidentified peaks were quantified using the calibration curve of the compound with similar absorption spectrum (Supplementary Fig. 1) and named as "phenolic derivative". For phenolics quantification, the calibration curves of the identified phenolics were constructed using Sigma-Aldrich (St. Louis, MO, USA) standards recorded at 280 nm for catechin (13.9-99.2 mg/L), genistein (27.5-110 mg/L), naringenin (2.25-9.00 mg/L), tyrosol (3.93-98.2 mg/L), *p*-hydroxybenzoic acid (1.05-70.4 mg/L), *m*-hydroxybenzoic acid (4.68-117 mg/L), 2,4-hydroxybenzoic acid (1.06-10.6 mg/L), cinnamic acid (4.05-19.1 mg/L), *p*-coumaric acid (0.80-3.50 mg/L), salicylic acid (1.05-26.2

mg/L), syringic acid (1.03-10.9 mg/L), and vanillic (1.04-26.1 mg/L), at 320 nm for apigenin (1.00-10.0 mg/L), and ferulic acid (2.61-10.4 mg/L), and at 360 nm for diosmin (5.24-105 mg/L). The calibration curves were linear in the concentration intervals assessed with the respectively following detection limits: 1.86 mg/L, 1.52 mg/L, 0.15 mg/L, 1.40 mg/L, 0.19 mg/L, 2.09 mg/L, 0.10 mg/L, 0.19 mg/L, 0.04 mg/L, 0.53 mg/L, 0.24 mg/L, 0.30 mg/L, 0.06 mg/L, 0.04 mg/L, and 0.41 mg/L.

All analyses were performed thrice; the results are expressed as mg/kg DM.

2.4. Statistical analysis

A two-way analysis of variance (ANOVA) was performed to assess the effect of treatments and lupin ecotypes. When significant differences were found ($p \leq 0.05$), Fisher's lowest significant difference (LSD) at 95% significance was computed. Additionally, to assess the effect of the treatments on each ecotype, a one-way ANOVA was carried out, followed by LSD test. Before the ANOVAs, the data normal distribution was verified and, when necessary, the data underwent logarithmic transformation (free apigenin derivative, free genistein derivative, free tyrosol, total phenylethanoids), inverse transformation (free naringenin derivative, free tyrosol derivative, free *p*-hydroxybenzoic acid, free total flavonoids, free total phenols) or square root (free diosmin, free cinnamic acid derivative, bound syringic acid derivative, bound genistein, free total phenolic acids). All the analyses were performed using the STATGRAPHICS® Centurion statistical programme. Mean and standard error were computed using the software Excel (Microsoft® Office Excel 2007).

3. Results and discussion

3.1. Tocols

All four tocopherol homologues were detected in the whole meals of the three different *L. mutabilis* tested, while tocotrienols were not observed. The most abundant homologue was γ-tocopherol (Supplementary Table 1), with a content ranging from 224 to 228 mg/kg DM in the bitter beans (> 98.0% of total tocopherols), followed by δ-tocopherol (1.66-2.81 mg/kg DM), β-tocopherol (0.93-1.60 mg/kg DM) and α-tocopherol (0.51-0.61 mg/kg DM). The total tocopherols content after the different treatments is shown in Fig. 1A. In the bitter beans the total tocopherol content was 230 mg/kg DM for Altagracia, 231 mg/kg DM for Andenes and 228 mg/kg DM for Yunguyo. These results are in the range (172-250 mg/kg DM) reported by Briceño Berru et al. (2021) for 33 *L. mutabilis* ecotypes and are greater than the amount (103 mg/kg DM of γ-tocopherol) observed by Boschin and Arnoldi (2011) in a wild *L. mutabilis* accession from Ecuador. They are also superior to the values reported for *L. albus* (63.2-153 mg/kg DM; Annicchiarico et al., 2014; Boschin and Arnoldi 2011; Lampart-Szczapa et al., 2003), *L. luteus* (14.5-22.7 mg/kg DM; Fernández-Marín et al., 2014) and *L. angustifolius* (73.5-95.4 mg/kg DM; Boschin and Arnoldi 2011).

The ANOVA (Supplementary Table 2) highlighted that the variation for tocopherol content was mainly influenced by the technological treatment applied, although the genotypic effect was predominant for δ-tocopherol and very important for γ-tocopherol and total tocopherols. The treatment x genotype interaction was significant only for γ-tocopherol and total tocopherols content.

Debittering led to an increase in tocopherols content (Fig. 1A, Supplementary Table 1), because of the rise in lipids (as well as in proteins) caused by water leaching of soluble carbohydrates and minerals (Córdova- Ramos et al., 2020a); the tocopherols surge was 22.7% in Altagracia, 33.4% in Andenes and 61.4% in Yunguyo. These values are coherent with the

range of increase (16.7%-70.8%) inferred from the data of 33 Andean lupins reported by Briceño Berru et al. (2021). The extrusion did not significantly change the tocopherols content compared to the debittered samples, while the spray-dried flours exhibited a 35.2% (maltodextrin) or 32.4% (gum Arabic) drop compared to the debittered flours. This decrease can be attributed to the dilution effect of the coating agents, or maybe also to the loss of lupin fragments and coarse material, strained during the preparation of the liquid used for spray-drying. Additionally, low humidity and high temperature (170 °C) trigger thermal damage (Córdova- Ramos et al., 2020a) and may have led to a partial degradation.

3.2. Carotenoids

Fig. 1B shows the total carotenoid content of the three Andean lupin ecotypes after different treatments. The carotenoids detected (Supplementary Table 1) were lutein (1.06-1.39 mg/kg DM) and zeaxanthin (0.09-0.11 mg/kg DM); total carotenoid content was 1.17 mg/kg DM for Altagracia, 1.29 mg/kg DM for Andenes and 1.48 mg/kg DM for Yunguyo, which are values similar to those described by Briceño Berru et al. (2021) in 33 *L. mutabilis* ecotypes (0.69-2.89 mg/kg DM), but lower than those detected by Fernández-Marín et al. (2014) in a wild accession (7.50 mg/kg DM) and a domesticated accession (4.10 mg/kg DM) of *L. luteus*.

The ANOVA (Supplementary Table 2) indicated that the variation was mainly due to genotype and treatment, while their interaction had only minor effects. After debittering, the carotenoid content was almost unchanged. Probably the above-mentioned lipids increase was counterbalanced by a certain degradation of the carotenoids and by possible changes in the lupin matrix such as the solubilisation of arabinogalactans (Cipriani et al., 2009) that might form supramolecular complexes of different solubility with the carotenoids (Apanasenko et

al., 2015). A decline was observed after extrusion, possibly because these compounds are more heat-sensitive than the tocopherols (Hidalgo and Brandolini, 2010; Hidalgo et al., 2010). This susceptibility was evident also after spray-drying because the average carotenoids content dropped from 1.31 mg/kg DM (debittered) to 0.78-0.84 mg/kg DM (spray-dried with maltodextrin and with gum Arabic, respectively).

3.3. Phenolics

The Supplementary Fig. 2 depicts two chromatograms of free (A) and bound (B) extracts. Not all the peaks could be identified by comparison with the spectrum and retention time of the phenolic standards, therefore, as indicated in the Materials and Methods, they were quantified following the calibration curve of the standard with similar spectrum and defined as derived from said standard, as suggested by Dueñas et al. (2009). The coding of the chromatogram peaks is reported in Supplementary Table 3.

Following this approach, in the free and the bound fractions were detected seven and three apigenin derivative peaks, four and one catechin derivative, fifteen and five genistein derivative, and thirteen and one naringenin derivatives, respectively (Supplementary Table 3). Additionally, in the free fraction extracts four diosmin derivative peaks, two cinnamic acid derivative, one 2,4 hydroxybenzoic acid derivative, two vanillic acid derivative and a one tyrosol derivative were identified, while in the bound fractions were recorded two *m*-hydroxybenzoic acid derivative peaks, two *p*-hydroxybenzoic acid derivative and two syringic acid derivative. Therefore, we decided to group the derivatives of each compound, as depicted in Fig. 2 and 3 (free forms), and Fig. 4 and 5 (bound forms).

3.3.1. Free phenolics

3.3.1.1. Flavonoids

The ANOVA (Supplementary Table 2) showed that all the flavonoid contents were modified mainly by the treatment, although the genotype was always significant except for catechin derivatives and genistein; their interaction was always significant except for catechin derivatives.

Fig. 2 shows the flavonoids in the free fraction of the three *L. mutabilis* cultivars after different treatments. In the bitter seeds these phenolic compounds were, in descending order, genistein derivatives (1280-1375 mg/kg DM), catechin derivatives (926-986-mg/kg DM), diosmin derivatives (159-329 mg/kg DM), apigenin derivatives (154-200 mg/kg DM) and naringenin derivatives (108-138-mg/kg DM); genistein (11.9-12.8 mg/kg DM) was scarce. Although not quantified, in *L. angustifolius* Dueñas et al. (2009) identified the presence of numerous soluble flavonoids, including apigenin derivatives, diosmin derivatives, and genistein and its derivatives. The genistein content was higher than that (2.37 mg/kg DM) reported by Multari et al. (2016) in a commercial lupin variety, but lower than those (22.3-62.6 mg/kg DM) reported by Zhong et al. (2019b) in the seed coats of six *L. angustifolius* accessions and by Gálvez Ranilla et al. (2009) in the cotyledons of six *L. mutabilis* accessions (57.0-70.0 mg/kg DM). The derivative apigenin contents (107-133 mg/kg DM) were comparable to those (130-160 mg/kg DM) reported for heteroside apigenin, an apigenin derivative, by Magalhaes et al. (2016) in three *L. albus*. The total flavonoid content in the bitter beans was 2871 mg/kg DM in Andenes, 2810 mg/kg DM in Altagracia and 2739 mg/kg DM in Yunguyo.

A drastic reduction of flavonoids was observed after debittering: on average the apigenin derivatives decreased from 173 to 18.0 mg/kg DM, the diosmin derivatives from 236 to 68.0

mg/kg and the naringenin derivatives from 118 to 6.89 mg/kgs, while the catechin derivatives disappeared (i.e. were below the detection limit); on the other hand, the genistein derivatives were barely halved, while the genistein increased considerably (from 12.4 to 112 mg/kg DM). A similar genistein behaviour was observed by Dueñas et al. (2009) in germinated lupins and was linked to the protracted debittering hydration. Nevertheless, even after boiling and repeated washings a high total flavonoid concentration was still present, remained stable during the extrusion and decreased after spray-drying, possibly because of the already mentioned diluting effect of the carriers.

The extrusion generally did not modify flavonoid content, but genistein showed a variable trend. Spray-drying led to a further decrease in diosmin derivative, genistein and genistein derivatives, while apigenin derivative and naringenin derivative contents were like those of the extruded samples. No differences were recorded between the two carriers.

3.3.1.2. Phenylethanoids and phenolic acids

The ANOVA (Supplementary Table 2) showed that all the factors influenced the phenylethanoids and phenolic acids content; treatment had the predominant effect, except for *2,4*-hydroxybenzoic acid, where genotype was most important.

The phenylethanoids and phenolic acids in the free extract are presented in Fig. 3. The most abundant phenylethanoids in the bitter lupin samples were tyrosol (398-1044 mg/kg DM) and its derivative (96.1-138 mg/kg DM). Similarly, Multari et al. (2016) found tyrosol in a lupin free fraction, albeit at significantly lower levels (15.3 mg/kg DM). The observed phenolic acids were vanillic acid derivatives (24.4-44.7 mg/kg DM), cinnamic acid derivatives (6.40-30.4 mg/kg DM), *p*-hydroxybenzoic acid (1.99-12.4 mg/kg DM) and *2,4*-hydroxybenzoic acid derivatives (4.04-4.52 mg/kg DM). The *p*-hydroxybenzoic acid contents

were lower than those reported by Siger et al. (2011) for *L. angustifolius* (42.7-43.7 mg/kg DM) and *L. albus* (22.8-27.8 mg/kg DM), but higher than those of *L. luteus* (0.48-0.68 mg/kg DM). They were also higher than the values (0.89 mg/kg DM) described by Multari et al. (2016) in commercial lupin flours. The protocatechuic acid observed by Siger et al. (2011) was not detected in our samples. The different cultivars showed a total phenolic acid content of 71.3 mg/kg (Andenes), 68.0 mg/kg DM (Altagracia) and 64.4 mg/kg DM (Yunguyo).

Debittering drastically reduced the phenylethyl content, as tyrosol and its derivatives suffered a 96.3% drop. In the extruded samples the tyrosol increased from 9.86 to 80.7 mg/kg DM and the derivative did not vary significantly, while in the spray-dried samples they decreased again. The phenolic acids generally decreased after debittering, except the *2,4*-hydroxybenzoic derivative that increased from 4.28 to 10.7 mg/kg DM. Overall their loss was inferior to that of the flavonoids: even after repeated washings, some free phenolic compounds were still detected, suggesting a certain stability or a release from conjugated forms, not evaluated in this research. The extrusion generally did not cause significant changes, while the spray-drying led to a sharp decrease, probably for the already-mentioned effects of filtration and dilution.

3.3.2. Bound phenolics

3.3.2.1. Flavonoids

The ANOVA (Supplementary Table 2) highlighted that the main influence on flavonoids content was exerted by the treatment; genotype and treatment x genotype interaction were generally significant but of minor importance.

Fig. 4 shows the flavonoid content of the bound extracts in the three cultivars analysed after different treatments. In the bitter samples, genistein derivatives (39.9-59.1 mg/kg DM),

catechin derivatives (19.4-31.2 mg/kg DM), apigenin derivatives (10.2-13.1 mg/kg DM) and naringenin derivatives (7.82-9.58 mg/kgs) were found, while genistein was not detected. The apigenin derivatives content was lower than those (24.2-83.1 mg/kg DM) reported by Zhong et al. (2019b) in *L. angustifolius* teguments. On average, the total flavonoid content in the bound fraction was much lower (94.4 mg/kg DM) than in the free fraction (2807 mg/kg DM).

Debittering decreased only marginally the content in catechin and naringenin derivatives and increased slightly those of apigenin, genistein and genistein derivatives. The extrusion led to a rise in genistein (30.5%), genistein derivatives (52.4%) and naringenin derivatives (54.6%), while the other compounds did not change much. The spray-drying induced a sharp flavonoid decrease, inasmuch that catechin, genistein and genistein derivatives became no longer detectable, probably because of teguments removal before spray-drying. This interpretation is upheld by literature reports that identify genistein and apigenin-7-O-glucoside in lupin teguments (Zhong et al, 2019b). No significant differences between the two carriers were observed.

3.3.2.2. Phenolic acids

The ANOVA (Supplementary Table 2) demonstrated that the phenolic acids content was mainly modified by the treatment and, secondly, by the genotype; *p*-coumaric acid, nevertheless, was influenced more by the genotype. The interactions, when significant, had always minor relevance. Fig. 5 depicts the phenolic acids detected in the bound extracts; on average, their content was greater than that in the free extract, regardless of the treatment applied. In the bitter beans were observed, in decreasing order, *p*-hydroxybenzoic acid derivatives (54.9-70.4 mg/kg DM), syringic acid derivatives (19.3-31.2 mg/kg DM), vanillic acid (8.61-20.1 mg/kg DM), *m*-hydroxybenzoic acid derivatives (3.08-9.64 mg/kg DM), *p*-

hydroxybenzoic acid (4.80-6.86 mg/kg DM), *m*-hydroxybenzoic acid (3.71-5.24 mg/kg DM), cinnamic acid (2.56-3.43 mg/kg DM), ferulic acid (1.66-2.16 mg/kg DM), salicylic acid derivative (0.66-0.71 mg/kg DM) and *p*-coumaric acid (0.08-0.16 mg/kg DM.). The content of this last compound was similar to that (0.11-0.18 mg/kg DM) reported by Siger et al. (2011) for *L. albus*, while those of vanillic and cinnamic acids were slightly higher, and those of ferulic acid and *p*-hydroxybenzoic acid slightly lower than those (9.09, 2.14, 3.34 and 10.2 mg/kg DM, respectively) observed in *L. albus* by Multari et al. (2016).

The debittering caused a sharp reduction in the content of syringic acid derivatives and *p*-hydroxybenzoic acid derivatives as well as in *p*-hydroxybenzoic acid and vanillic acid, while induced a slight increase in the content of *m*-hydroxybenzoic acid and cinnamic acid; the other phenolic acids showed limited variation. The extrusion provoked a slight increase of all the phenolic acids, except syringic acid derivatives and *p*-coumaric acid. The spray-drying, on the other hand, triggered a sharp reduction in phenolic acids; no significant differences between coating agents were observed.

Clear differences between flavonoids and phenolic acids behaviour are visible (Fig. 6). After debittering the total free flavonoids contents diminished while the bound ones increased; after spray-drying both decreased, the bound compounds more drastically. The phenolic acids, on the other hand, behaved more evenly: the debittered samples on average contained 50.1% less than the bitter ones, while the reduction was 85.6% in the free and 73.0% in the bound spray-dried products.

The free flavonoids in the final products (debittered, extruded or spray-dried flours) represented the bulk of free extracts (73.0%-96.1%) and of total phenolics (70.9%-91.0%). In the bound fraction, instead, the phenolic acids were more abundant (30.6%-73.5%). No

relevant differences between cultivars were recorded, except for a higher phenylethanoid content in Altagracia.

4. Conclusions

Lupinus mutabilis bitter seeds contain reduced amounts of carotenoids and good concentrations of tocopherols (mainly γ-tocopherol) and phenolics (on average, the free fraction was 94.4% of total phenols). After debittering, the tocopherols content increased slightly while the carotenoids content was largely unchanged and the phenolics concentration markedly decreased (72.9%). The extrusion did not modify tocopherols and phenolics but slightly reduced carotenoids concentration (14.5%). The spray-drying drastically cut tocopherols, carotenoids and phenolics (34.2%, 39.3% and 48.4%, respectively), without differences between coating agents. Nevertheless, even after spray-drying the total tocopherol and free phenolics content was still abundant. The high antioxidant content of the flour of these lupins suggests their possible high availability during the digestion. Further studies to confirm this hypothesis are needed.

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Declarations of interest: none

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Figure captions

Fig. 1. Total tocopherols (A) and total carotenoids (B) content of the three Andean lupin ecotypes after different treatments. SD MD, spray dried with maltodextrin as coating agent; SD GA, spray dried with gum Arabic as coating agent.

Fig. 2. Flavonoids and flavonoids derivatives content in the free extracts of three Andean lupin ecotypes after different treatments. Der, derivative; SD MD, spray dried with maltodextrin as coating agent; SD GA, spray dried with gum Arabic as coating agent.

Fig. 3. Phenylethanoid and phenylethanoid derivatives (left), phenolic acids and phenolic acids derivatives (right) content in the free extracts of three Andean lupin ecotypes after different treatments. Der, derivative; SD MD, spray dried with maltodextrin as coating agent; SD GA, spray dried with gum Arabic as coating agent.

Fig. 4. Flavonoid and flavonoids derivatives content in the bound extracts of three Andean lupin ecotypes after different treatments. Der, derivative; SD MD, spray dried with maltodextrin as coating agent; SD GA, spray dried with gum Arabic as coating agent.

Fig. 5. Phenolic acids and phenolic acids derivatives content in the bound extracts of three Andean lupin ecotypes after different treatments. Der, derivative; SD MD, spray dried with maltodextrin as coating agent; SD GA, spray dried with gum Arabic as coating agent.

Fig. 6. Total free and total bound phenolics content of three Andean lupin ecotypes after different treatments. SD MD, spray dried with maltodextrin as coating agent; SD GA, spray dried with gum Arabic as coating agent.

Figure 1

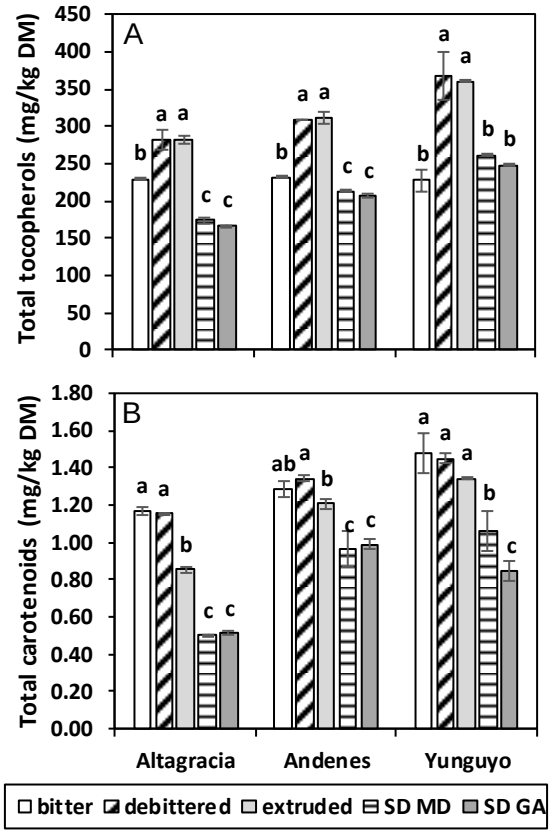

Figure 2

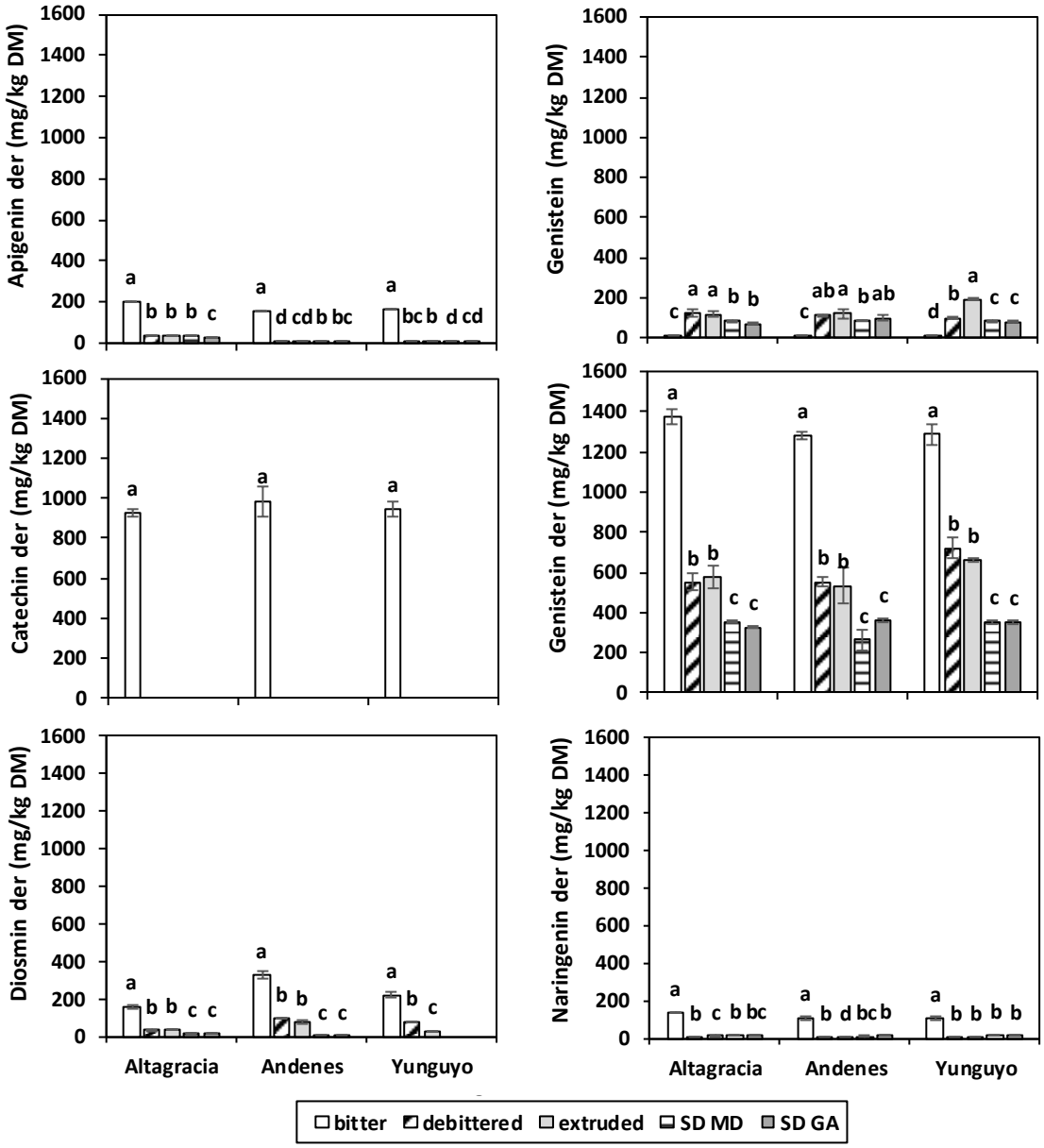

Figure 3

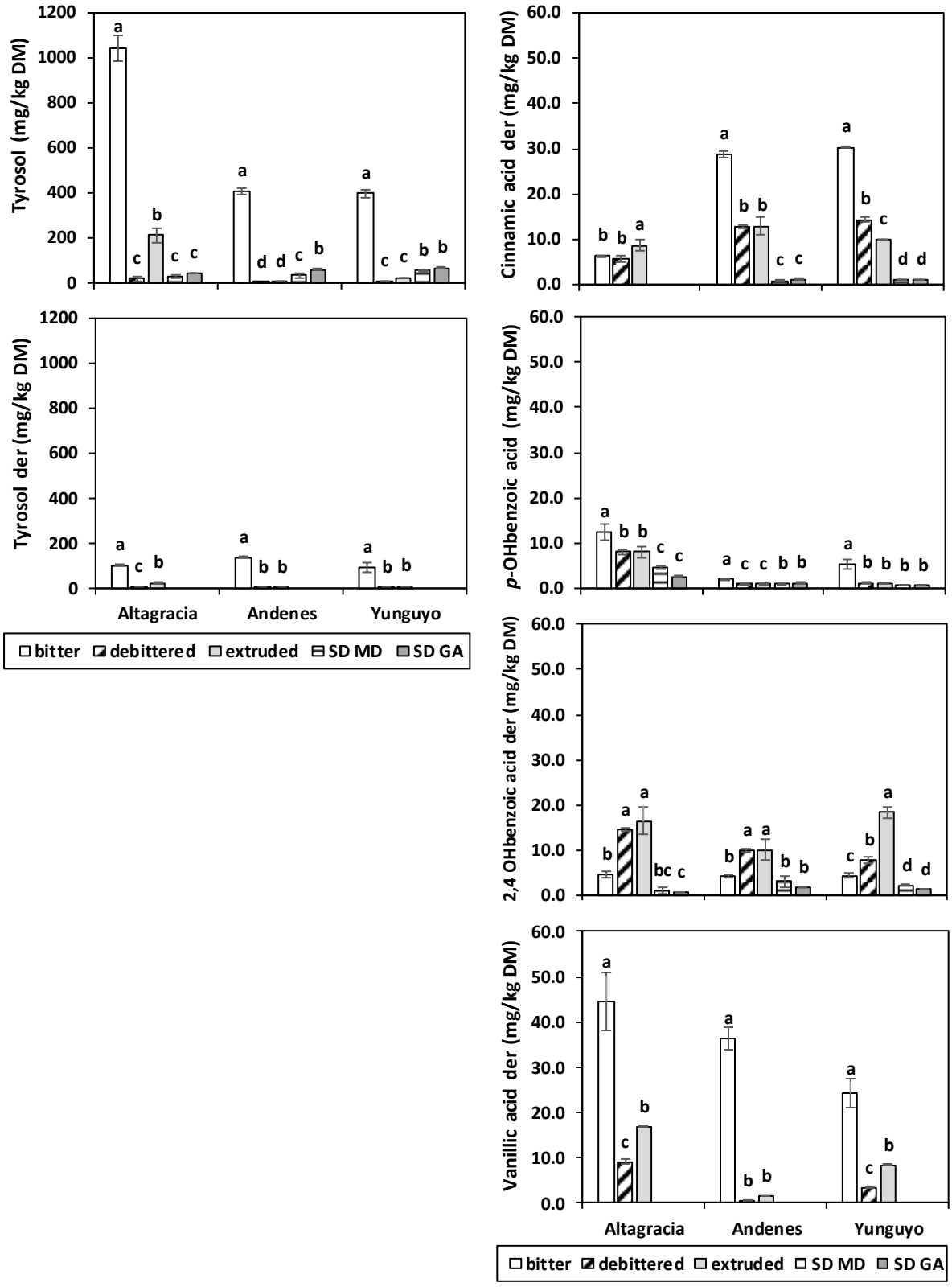

Figure 4

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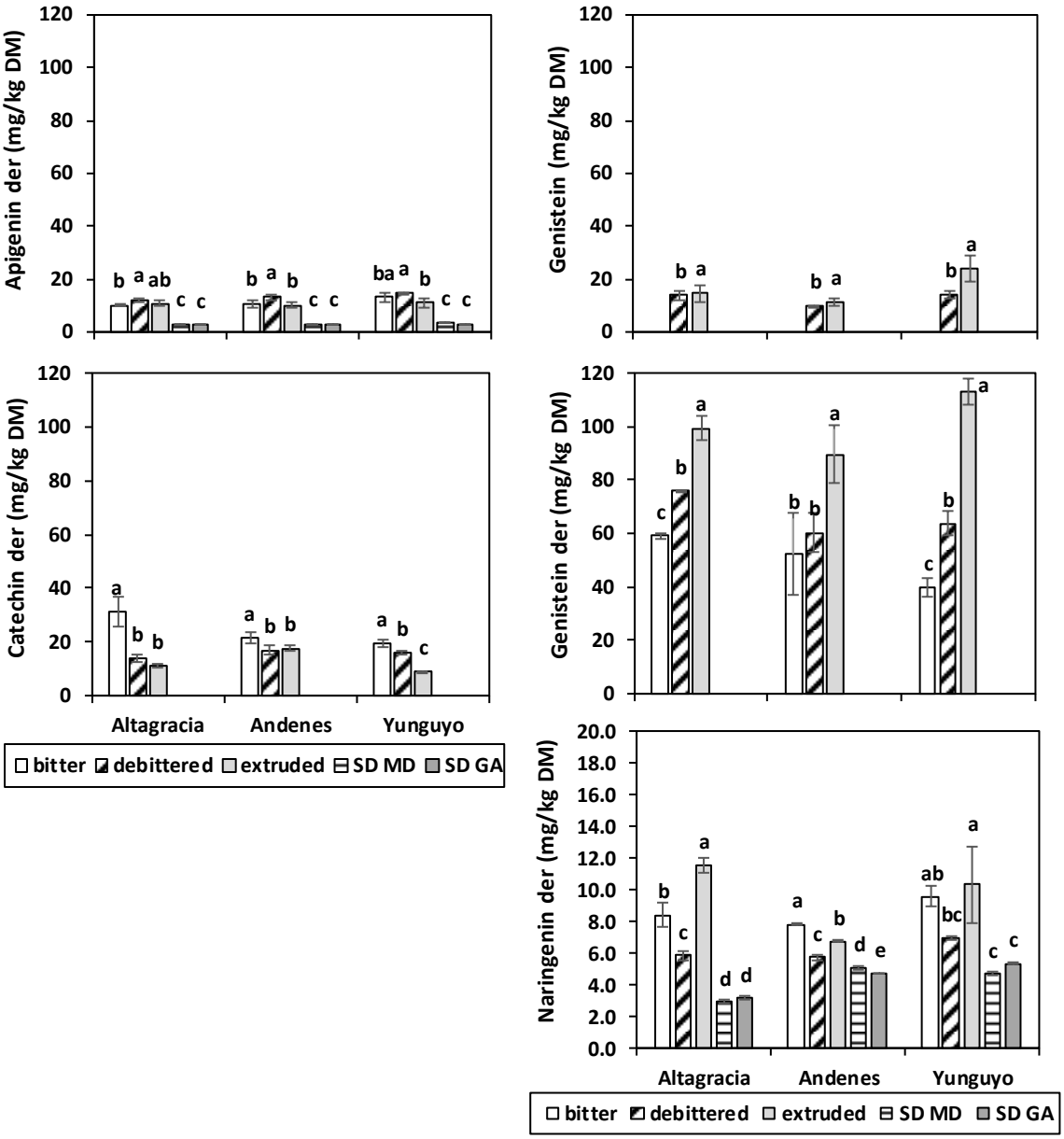

Figure 5

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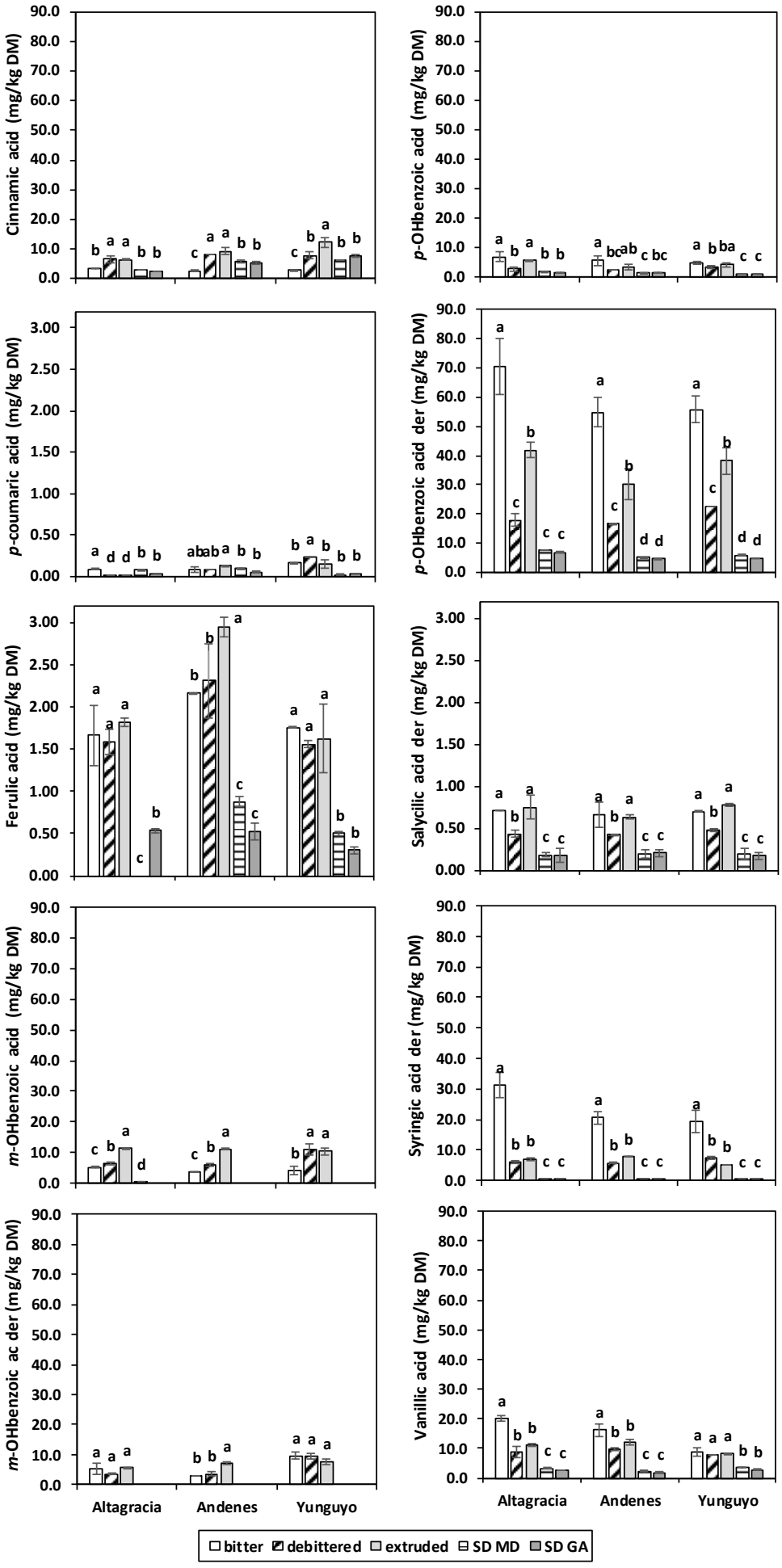
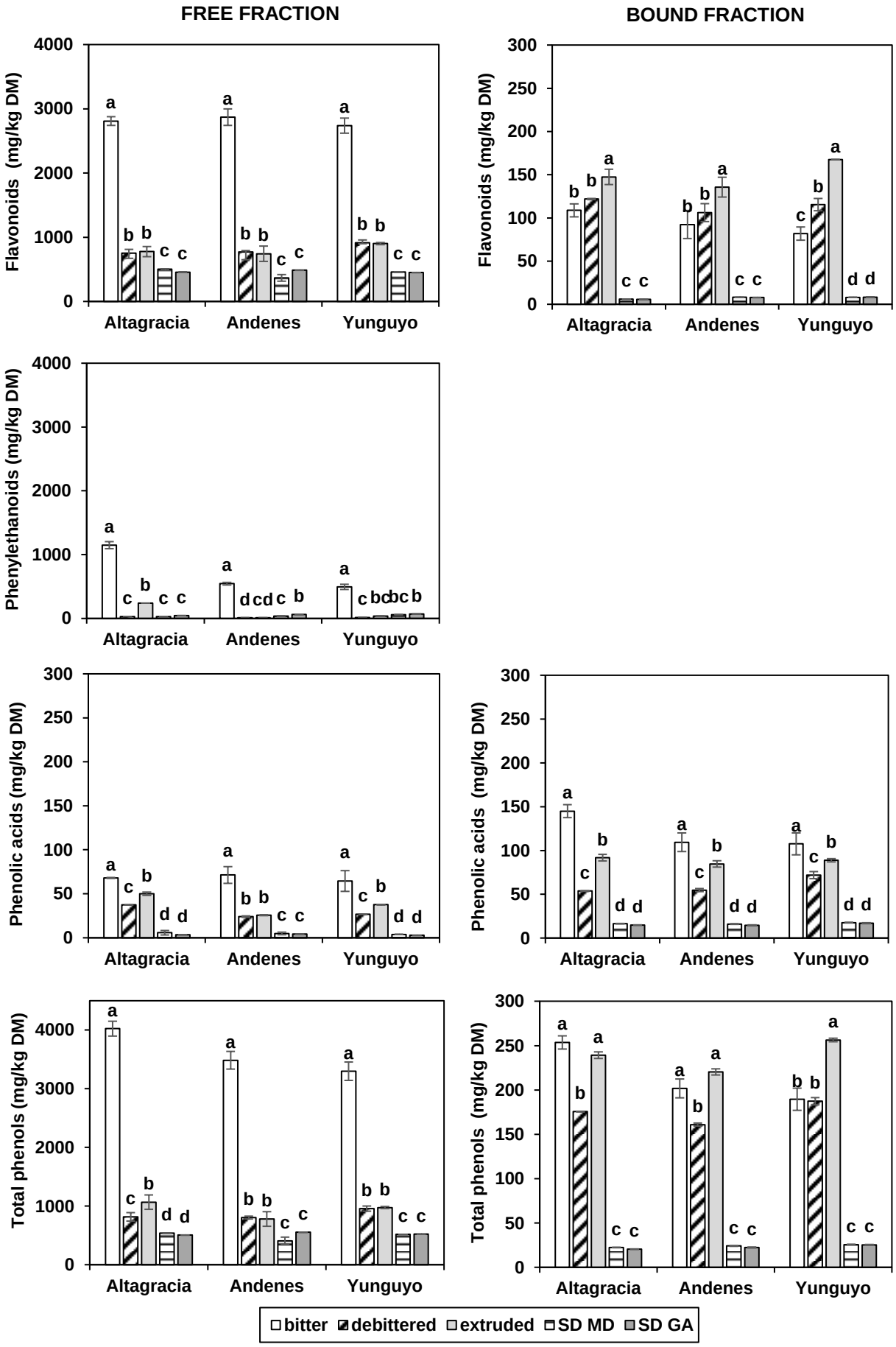


Figure 6



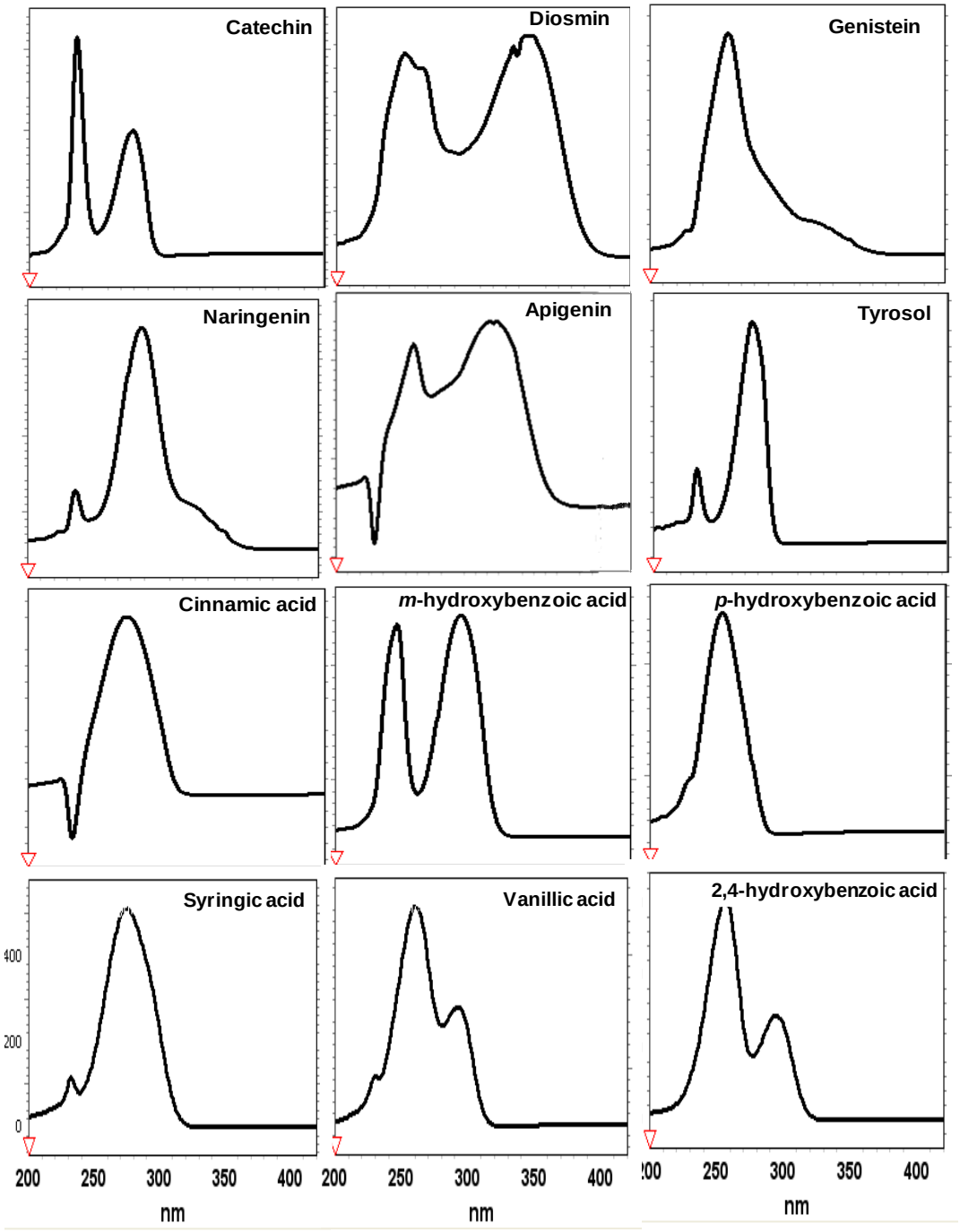
Disclosure of interest

The authors report no conflict of interest

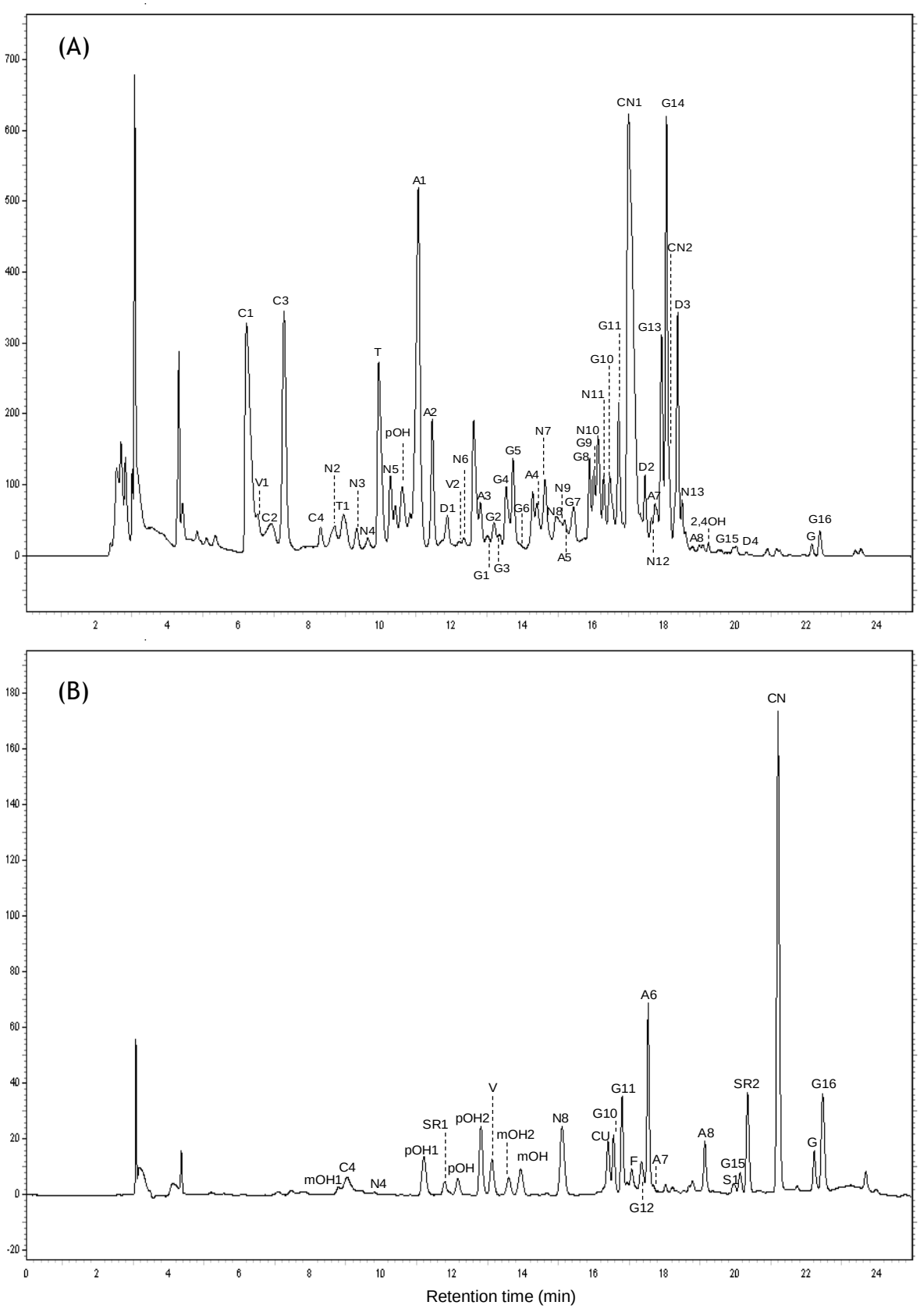
CRediT authorship contribution statement

Andrea Brandolini: Data curation, Formal analysis, Methodology, Writing original draft, Writing-review & editing. **Patricia Glorio-Paulet**: Data curation, Writing-review & editing. **Lorenzo Estivi**: Investigation, Methodology, Writing original draft, Writing-review & editing. **Nicola Locatelli:** Investigation**,** Formal analysis, Data curation. **Javier S. Córdova-Ramos**: Investigation**,** Visualization, Writing review. **Alyssa Hidalgo**: Conceptualization, Supervision, Methodology, Formal analysis, Data curation, Investigation, Writing original draft, Writing review & editing.

Supplementary Fig. 1. Absorbance spectra of the phenolics taken as reference for the derivative compounds



Supplementary Fig. 2. HPLC chromatograms of the free (A) and bound (B) phenols in one *Lupinus mutabilis* ecotype.



Supplementary Table 1. Tocols and carotenoids content (mean ± standard deviation; mg/kg DM) of seeds from three Andean lupin ecotypes after different treatments

	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Lutein	Zeaxanthin
Bitter						
Altagracia	0.51 ± 0.07	1.60 ± 0.50	225 ± 1.49	2.81 ± 0.23	1.06 ± 0.01	0.11 ± 0.01
Andenes	0.55 ± 0.04	0.93 ± 0.10	228 ± 1.11	1.66 ± 0.18	1.20 ± 0.06	0.09 ± 0.02
Yunguyo	0.61 ± 0.00	1.17 ± 0.16	224 ± 14.6	2.23 ± 0.03	1.39 ± 0.12	0.09 ± 0.01
Debittered						
Altagracia	0.63 ± 0.09	2.72 ± 0.08	275 ± 12.6	3.02 ± 0.22	1.04 ± 0.01	0.11 ± 0.01
Andenes	0.66 ± 0.03	1.55 ± 0.22	304 ± 0.30	2.37 ± 0.12	1.23 ± 0.01	0.12 ± 0.01
Yunguyo	0.72 ± 0.02	1.95 ± 1.03	363 ± 33.4	2.55 ± 0.05	1.33 ± 0.03	0.12 ± 0.01
Extruded						
Altagracia	0.64 ± 0.04	3.35 ± 0.12	276 ± 5.71	2.95 ± 0.07	0.76 ± 0.01	0.10 ± 0.02
Andenes	0.68 ± 0.02	2.03 ± 0.02	306 ± 8.24	2.73 ± 0.20	1.11 ± 0.02	0.10 ± 0.01
Yunguyo	0.73 ± 0.01	2.38 ± 0.19	355 ± 1.36	2.29 ± 0.03	1.24 ± 0.01	0.11 ± 0.01
Spray-dried (MD)						
Altagracia	0.55 ± 0.01	1.50 ± 0.06	169 ± 2.63	2.27 ± 0.10	0.44 ± 0.01	0.05 ± 0.01
Andenes	0.54 ± 0.03	1.64 ± 0.04	209 ± 0.98	1.86 ± 0.34	0.90 ± 0.07	0.06 ± 0.03
Yunguyo	0.56 ± 0.03	1.89 ± 0.62	257 ± 2.08	2.17 ± 0.26	1.01 ± 0.09	0.04 ± 0.01
Spray-dried (GA)						
Altagracia	0.51 ± 0.02	1.25 ± 0.17	163 ± 1.23	2.20 ± 0.26	0.45 ± 0.01	0.06 ± 0.01
Andenes	0.51 ± 0.03	1.50 ± 0.07	202 ± 2.82	1.97 ± 0.24	0.92 ± 0.01	0.07 ± 0.01
Yunguyo	0.54 ± 0.12	1.73 ± 0.42	244 ± 0.41	2.16 ± 0.68	0.80 ± 0.06	0.05 ± 0.01

Supplementary Table 2. Two-way ANOVA of tocol, carotenoid and phenol content of three Andean lupin ecotypes seeds after different treatments.

	Treatment (T)	Genotype (G)	TxG	Error
d.f.	**4**	**2**	**8**	**15**
TOCOLS				
α-tocopherol	0.03***	0.01*	0.00	0.00
β-tocopherol	1.69***	0.77*	0.32	0.14
γ-tocopherol	18274***	11276***	759***	107
δ-tocopherol	0.52***	0.74***	0.13	0.07
Total tocols	18810***	11117***	768***	104
CAROTENOIDS				
Lutein	0.31***	0.45***	0.02***	0.00
Zeaxanthin	0.01***	0.00	0.00	0.00
Total carotenoids	0.39***	0.44***	0.02***	0.00
PHENOLS				
Free flavonoids				
Apigenin der.	1.47***	0.92***	0.05***	0.00
Catechin der.	1087800***	371	371	506
Diosmin der.	185***	11.4***	8.80***	0.07
Genistein	14029***	310	985***	85.1
Genistein der.	0.36***	0.01***	0.01*	0.00
Naringenin der.	0.02***	0.01***	0.002***	0.00
Total	0.000004***	0.00000008*	0.00000008**	0.00
Free phenyletanoids				
Tyrosol	2.86***	0.73***	0.28***	0.01
Tyrosol der.	0.04***	0.01***	0.01***	0.00
Total	2.34***	0.40***	0.20***	0.01
Free phenolic acids				
Cinnamic acid der.	17.4***	5.97***	0.62***	0.01
pOH-benzoic acid	0.55***	2.44***	0.27***	0.01
2,4OH-benzoic acid der.	211***	7.40*	14.0***	1.30
Vanillic acid der.	1652***	246***	58.3**	6.50
Total	44.2***	1.08***	0.50***	0.04
Bound flavonoids				
Apigenin der.	144***	4.40*	1.00	0.90
Catechin der.	212***	31.7*	46.9**	4.80
Genistein	61.0***	0.50	1.80***	0.20
Genistein der.	11460***	105	125*	32.2
Naringenin der.	34.9***	4.90**	3.80***	0.50
Total	25120***	172	211**	49.3
Bound phenolic acids				
p-OHbenzoic	22.3***	2.40*	0.80	0.50
p-OHbenzoic der.	3249***	110**	34.5	13.2
m-OHbenzoic	140***	2.20*	3.70***	0.50
m-OHbenzoic der.	3249***	110**	34.5	13.2

Salicilic acid der.	0.40***	0.00	0.00	0.00
Syringic acid der.	18.0***	0.24**	0.21***	0.03
Vanillic acid der.	165.1***	23.5***	13.7***	0.80
Cinnamic acid der.	36.7***	22.3***	3.80***	0.50
p-Coumaric acid	0.01***	0.01***	0.01***	0.00
Ferulic acid	4.02***	1.32***	0.15**	0.04
Total	12513***	180**	235***	25.3
Total free phenols	0.000003***	0.0000001***	0.00000006**	0.00
Total bound phenols	65419***	692*	661**	115

Supplementary Table 3. Phenolic compounds and phenolic compounds derivatives, retention times (min) and codes in HPLC chromatograms of free and bound extracts from three Andean lupins seeds.

	Retention time	Free	Bound
Apigenin derivatives	11.2	A1	
	11.6	A2	
	13.0	A3	
	14.5	A4	
	15.3	A5	
	17.8		A6
	17.9	A7	A7
	19.0	A8	A8
Catechin derivatives	6.30	C1	
	7.00	C2	
	7.30	C3	
	8.40	C4	C4
Diosmin derivaties	12.0	D1	
	17.5	D2	
	18.4	D3	
	19.7	D4	
Genistein	22.2	G	G
Genistein derivatives	13.1	G1	
	13.3	G2	
	13.4	G3	
	13.7	G4	
	13.8	G5	
	13.9	G6	
	15.5	G7	
	16.0	G8	
	16.1	G9	
	16.6	G10	G10
	16.8	G11	G11
	17.3		G12
	18.0	G13	
	18.1	G14	
	20.1	G15	G15
	22.4	G16	G16
Naringenin derivatives	8.20	N1	
	8.90	N2	
	9.40	N3	
	9.80	N4	
	10.4	N5	
	12.4	N6	
	14.7	N7	
	15.1	N8	N8
	15.2	N9	
	16.2	N10	
	16.4	N11	
	17.7	N12	
	18.5	N13	

Cinnamic acid	21.2		CN
Cinnamic acid derivatives	17.2	CN1	
	18.2	CN2	
***p*-comaric acid**	16.4		CU
Ferulic acid	17.1		F
***m*-OHbenzoic acid**	14.0		mOH
***m*-OHbenzoic acid derivatives**	8.90		mOH1
	13.6		mOH2
***p*-OHbenzoic acid**	12.0	pOH	pOH
***p*-OHbenzoic acid derivatives**	11.2		pOH1
	12.8		pOH2
2,4 OHbenzoic acid derivative	19.1	2,4OH	
Salycilic acid derivative	20.0		S1
Syringic acid derivatives	11.8		SR1
	20.0		SR2
Vanillic acid	13.1		V
Vanillic acid derivatives	6.50	V1	
	12.3	V2	
Tyrosol	10.1	T	
Tyrosol derivative	9.10	T1	