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Nutritional value of commercial and traditional lettuce (*Lactuca sativa* L.) and wild relatives: Vitamin C and anthocyanin content

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ABSTRACT

Lettuce is the most consumed leafy vegetable though the most popular varieties have a low nutritional value. Our objective was to accurately quantify vitamin C and anthocyanins in wild relatives, and commercial and traditional varieties.

Wild species and traditional varieties contained more total ascorbic acid (TAA) than commercial varieties (21% and 8%, respectively). In contrast, commercial varieties had significantly higher content of anthocyanins than traditional varieties and wild species (6 and 8 times more, respectively). TAA was significantly higher in green than in red lettuces (18%). TAA was also significantly higher in the leaves of two wild species than in stems. Cyanidin 3-*O*-(6′-*O*-malonylglucoside) was the most abundant anthocyanin (97%), present in most samples.

The rankings of accessions by vitamin C and anthocyanin contents can be useful for consumers worried about the impacts of food on their wellbeing and for breeders aiming to improve lettuce by biofortification with health-promoting compounds.

1. Introduction

Lettuce (*Lactuca sativa* L.) is the most consumed and the most cultivated leafy vegetable worldwide with production continuing to increase yearly. It was estimated that the total area harvested, globally, was more than 1.27 million hectares in 2018, with a total production of approximately 27.3 million tonnes (FAOSTAT, 2018).

L. sativa belongs to Asteraceae family and varieties are classified according to their morphology in 12 types (UPOV, 2019): Batavia, Butterhead, Cos, Frillice, Frisée d'Amérique, Gem, Iceberg, Lollo, Multidivided, Novita, Oakleaf and Stem; and colour: green, semi-red and red. Wild relatives within the genus *Lactuca* also demonstrate enormous variability with more than 100 species (Lebeda et al., 2004). In addition to preserving this rich patrimony, many germplasm banks are carrying out a detailed evaluation of the plant material. The main efforts have been focused on morphological and, more recently, genetic characterisation (Mallor & Díaz, 2016), while the metabolic aspects have barely been taken into account. In the case of lettuce, this metabolic characterisation is very important because the crop is a source of phytonutrients with health-promoting properties, such as phenolic compounds and vitamins (Llorach et al., 2008). However, the most consumed varieties worldwide happen to have a low nutritional value. Differences among the groups under study (commercial varieties, traditional varieties and wild relatives) in the concentration of two compounds with a proven beneficial effect on human health, vitamin C and anthocyanins, will be sought out. In recent years, there has been a growing interest among consumers in the impact of food on health. In fact, many of them are willing to pay a higher price for healthier products, with a higher nutritional value or with potential benefits in disease prevention. This could represent an added value for traditional varieties, as well as a very interesting breeding objective.

Vitamin content, especially vitamin C, is one of the most relevant indicators of the nutritional quality of fruits and vegetables. Vitamin C can be found in two interconvertible forms, ascorbic acid (AA), that exhibits antioxidant activity, and dehydroascorbic acid (DHAA), its oxidation product (Lee & Kader, 2000). Vitamin C is an essential micronutrient for humans, required to ensure a normal physiological function, which has to be taken as part of the diet because humans have

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lost the capacity to synthesize it (Carr & Frei, 1999). Its beneficial properties are related to its role in different processes in the human body, such as collagen formation, reduction of cholesterol levels, inorganic iron absorption, and most important of all, enhancement of the immune system due to its antioxidant activity (Carr & Frei, 1999).

Other compounds with strong antioxidant activity are anthocyanins, a class of water-soluble phenolic compounds which until recently were thought to be synthesized exclusively by plants but have also been found in some fungi (Bu et al., 2020). These play an important role in human health by preventing neuronal and cardiovascular diseases, reducing diabetes risk, and exhibiting anti-cancer activity (Yousuf et al., 2016). In lettuce, they are responsible for the red colour of the leaves, which is an important characteristic, not only because it has a major influencing effect on consumer buying behaviour, but also because it reveals the

Table 1
Description of the plant material used in the present study, commercial and traditional lettuce varieties, as well as wild relatives (*Lactuca* spp.).

Accession name	Species	Group	Type[a]	Leaf colour	Lettuce gene pool	Origin	Source[b]	Accession number
'Begoña'	*Lactuca sativa* L.	Commercial variety	Batavia	Green	Primary	Spain	Ramiro Arnedo Semillas S.A.	–
'Dolomiti G12'	*Lactuca sativa* L.	Commercial variety	Gem	Green	Primary	Spain	Ramiro Arnedo Semillas S.A.	–
'Likarix'	*Lactuca sativa* L.	Commercial variety	Frisée d'Amérique	Red	Primary	Netherlands	CGN	CGN24522
'Lollo Rosso'	*Lactuca sativa* L.	Commercial variety	Lollo	Red	Primary	Italy	CGN	CGN09385
'Winter Crop'	*Lactuca sativa* L.	Commercial variety	Butterhead	Green	Primary	Hungary	BGHZ	CGN05853
'Nestorix'	*Lactuca sativa* L.	Commercial variety	Lollo	Red	Primary	Netherlands	CGN	CGN24712
'Red Sails'	*Lactuca sativa* L.	Commercial variety	Lollo	Red	Primary	Germany	CGN	CGN19014
'Revolution'	*Lactuca sativa* L.	Commercial variety	Lollo	Red	Primary	Netherlands	CGN	CGN20714
'Romana Inverna'	*Lactuca sativa* L.	Commercial variety	Cos	Green	Primary	Spain	BGHZ	BGHZ3604
'Romired'	*Lactuca sativa* L.	Commercial variety	Lollo	Red	Primary	Netherlands	CGN	CGN24713
'Lechuga de Beceite'	*Lactuca sativa* L.	Traditional variety	Cos	Green	Primary	Spain	BGHZ	BGHZ2006
'Lechuga de Bureta'	*Lactuca sativa* L.	Traditional variety	Cos	Semi-red	Primary	Spain	BGHZ	BGHZ4927
'Lechuga de Ensalada'	*Lactuca sativa* L.	Traditional variety	Cos	Green	Primary	Spain	BGHZ	BGHZ2031
'Lechuga de Híjar'	*Lactuca sativa* L.	Traditional variety	Cos	Green	Primary	Spain	BGHZ	BGHZ0529
'Lechuga de Subías'	*Lactuca sativa* L.	Traditional variety	Cos	Green	Primary	Spain	BGHZ	BGHZ1852
'Lechuga del Pirineo'	*Lactuca sativa* L.	Traditional variety	Cos	Green	Primary	Spain	BGHZ	BGHZ2229
'Lechuga del Valle de Tena'	*Lactuca sativa* L.	Traditional variety	Butterhead	Green	Primary	Spain	BGHZ	BGHZ1850
'Lechuga Romana Zaragozana'	*Lactuca sativa* L.	Traditional variety	Cos	Green	Primary	Spain	BGHZ	BGHZ4306
'Lengua de Buey'	*Lactuca sativa* L.	Traditional variety	Cos	Green	Primary	Spain	BGHZ	BGHZ2004
'Morada de Belchite'	*Lactuca sativa* L.	Traditional variety	Cos	Semi-red	Primary	Spain	BGHZ	BGHZ0527
'Morada de Bernués'	*Lactuca sativa* L.	Traditional variety	Batavia	Semi-red	Primary	Spain	BGHZ	BGHZ2097
'Morada de Sorripas'	*Lactuca sativa* L.	Traditional variety	Cos	Semi-red	Primary	Spain	BGHZ	BGHZ2026
'Oreja de Mulo'	*Lactuca sativa* L.	Traditional variety	Cos	Green	Primary	Spain	BGHZ	BGHZ0532
Lactuca biennis	*Lactuca biennis* (Moench) Fernald	Wild crop relative	–	Dark green	Terciary	North America	BGHZ	BGHZ4761
Lactuca dentata	*Lactuca dentata* Makino	Wild crop relative	–	Green	Terciary	East Asia	BGHZ	BGHZ4345
Lactuca dregeana	*Lactuca dregeana* DC.	Wild crop relative	–	Dark green (red stems)	Secondary	South Africa	BGHZ	BGHZ3670
Lactuca floridana	*Lactuca floridana* L. Gaertn.	Wild crop relative	–	Dark green	Terciary	North America	BGHZ	BGHZ5323
Lactuca homblei	*Lactuca homblei* De Wild	Wild crop relative	–	Green (red nerves)	Terciary	Central and South Africa	BGHZ	BGHZ5322
Lactuca squarrosa	*Lactuca squarrosa* (Thunb.) Miq.	Wild crop relative	–	Dark green (red stems)	Terciary	Southeast Asia	BGHZ	BGHZ5124
Lactuca virosa	*Lactuca virosa* L.	Wild crop relative	–	Green (red nerves)	Secondary[c] or terciary[d]	East and North Africa	BGHZ	BGHZ4051

[a]According to UPOV (2019).
[b]BGHZ: Vegetable Germplasm Bank of Zaragoza (Spain); CGN: Centre for Genetic Resources (Wageningen, Netherlands).
[c]According to Koopman et al. (1998) and Koopman (1999).
[d]According to Zohary (1991).

presence of beneficial compounds to health.

Both, vitamin C and anthocyanins, not only have proven beneficial effects on human health but they also help the plants to cope with stresses (biotic and abiotic) mainly thanks to their antioxidant properties, and, in the case of some anthocyanins, protecting the leaves from high radiation without compromising photosynthesis, what ultimately renders higher yields.

On one hand, it is evident that wild *Lactuca* spp. resources have a potential interest in breeding programmes (Lebeda et al., 2014). However, the efforts have been mainly directed to incorporate resistance to pests and diseases, and few studies are focused on enhancing the nutritional value of the crop (van Treuren et al., 2018). Furthermore, only a few wild species have been used in lettuce breeding programmes, even if the diversity within the wild *Lactuca* spp. is worthy of consideration. On the other hand, studies such as those carried out by Llorach et al. (2008) and by Mulabagal et al. (2010), in which anthocyanins and vitamin C were quantified in some green and red varieties, are of great interest. However, they are only focused on commercial varieties. In contrast, the main objective of this study is the analysis and quantification of the nutritional value, specifically, the vitamin C and anthocyanin contents, not only of commercial varieties, but also traditional varieties and lettuce wild relatives.

2. Materials and methods

2.1. Reagent and standards

Acetic acid (≥99% purity), hydrochloric acid (37% purity), *meta*-phosphoric acid (MPA, 33.5–36.5% purity) and sulfuric acid (95–98% purity) were purchased from Sigma-Aldrich (Madrid, Spain). 1,4-Dithiothreitol (DTT, ≥98% purity) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris, ≥99.9% purity) were provided by Roche (Madrid, Spain). Ethylenediaminetetraacetic acid disodium salt (EDTA, 99–101% purity) was obtained from Pancreac (Barcelona, Spain). The solvents for chromatography acetonitrile (99.9% purity) and methanol (≥99.9% purity) were acquired from ChemLab (Zedelgem, Belgium), and formic acid (98–100% purity) from Supelco-Sigma-Aldrich (Madrid, Spain). The standard L-Ascorbic acid (≥99.9% purity) was purchased from Sigma-Aldrich, while the standards Kuromanin chloride (cyanidin 3-*O*-glucoside chloride) (≥96% purity), Peonidin 3-*O*-glucoside chloride (≥95% purity), Oenin chloride (malvidin 3-*O*-glucoside chloride) (≥95% purity) and Myrtillin chloride (delphinidin 3-*O*-glucoside chloride) (≥95% purity) were provided by Extrasynthese (Genay, France).

2.2. Plant material

A total of 30 *Lactuca* accessions were included in this study (Table 1, Supplementary Fig. 1): 10 commercial lettuce varieties (4 green and 6 red), 13 traditional lettuce varieties (9 green and 4 semi-red), and 7 wild relative species. Among the lettuce varieties, 6 out of the 12 types defined by UPOV (2019), Buttherhead, Batavia, Frisée d'Amérique, Lollo, Cos and Gem, are represented. One of the wild species, closely related to *L. sativa*, is included in the primary gene pool (*L. dregeana* DC.), and the rest are more distant, forming part of the secondary or tertiary gene pool (PGR lettuce, www.pgrportal.nl/en/Lettuce-genetic-resources-Portal.htm), though this classification can vary depending on the methodology used to classify them (see Lebeda et al., 2004 and references herein included). In winter 2018/19, three plants per accession were grown in pots (30 × 25 cm and 11.7 L volume) with a mix of black and blonde peat (1:1) supplemented with fertilizer in a greenhouse at Agrifood Research and Technology Centre of Aragón (CITA, Zaragoza, Spain) following a completely randomized block design. No supplementary light was supplied so basal levels of phytochemicals (i.e. anthocyanins) could be measured.

After a period ranging from 2 and a half to 3 and a half months (depending on the accession), the plants were harvested. In all commercial and traditional lettuce varieties, as well as in the wild species with a rosette growth form (i.e. *Lactuca dentata* Makino, *Lactuca homblei* De Wild, and *Lactuca virosa* L.), inner and outer leaves were collected to represent the whole plant. In the case of small wild species (i.e. *Lactuca biennis* (Moench) Fernald and *Lactuca floridana* L. Gaertn), the whole plant was collected. Finally, in the two wild species with an early bushy growth (*L. dregeana* DC. and *Lactuca squarrosa* (Thunb.) Miq.), parts of the main stem were also sampled separate from the leaves. All samples were immediately frozen with liquid nitrogen and then kept at −80 °C until use.

2.3. Sample treatment

2.3.1. Extraction of vitamin C

Vitamin C extraction was carried out following the procedure described by Medina-Lozano et al. (2020). Briefly, 5 mL of extraction solution (8% acetic acid (v/v), 1% MPA (w/v) and 1 mM EDTA) were added to 50 mg of the lyophilized and finely powdered sample. The mixture was shaken in a vortex for 5 s and in an orbital shaker for 10 min at 2000 rpm. After being sonicated for 10 min at room temperature and centrifuged at 4000×*g* for 10 min at 4 °C, the supernatant was filtered through a 0.22-μm regenerated cellulose filter (Agilent, CA, United States) and recovered in a 5-mL amber vial. The obtained filtrate (Extract 1, E1) was used to determine vitamin C content in two steps. First, an aliquot of E1 was directly used to quantify AA as described below. Second, to obtain TAA, DHAA was reduced to AA by adding 200 μL of reducing solution (40 mM DTT with 0.5 M Tris pH 9.0) to a 200-μL aliquot of E1. After 30 min of reaction at room temperature in darkness, 200 μL of 0.4 M sulfuric acid were added to stop and stabilize AA in acidic pH (Extract 2, E2).

2.3.2. Extraction of anthocyanins

Anthocyanin extraction was performed in all the accessions, the green-leaf varieties included, according to the method described by Assefa et al. (2019) with slight modifications. Briefly, 5 mL of the extraction solution (methanol:ultrapure water:formic acid 50:44:6 v:v:v) was added to 40 mg of fine powder of the lyophilized sample. The mixture was vortexed for 5 s, shaken in an orbital shaker for 20 min at 2000 rpm and sonicated in an ultrasonic bath for 20 min at room temperature, followed by a centrifugation at 4000×*g* for 10 min at 4 °C. Supernatant was recovered in a 12-mL amber glass tube. A second extraction was carried out repeating the procedure just described. Both supernatants were blended and filtered through a 0.22-μm polytetrafluoroethylene (PTFE) filter (Agilent) to obtain the final extract (Extract 3, E3). The whole protocol was carried out under low intensity light.

2.4. UPLC conditions

All methods were conducted on the liquid chromatographic Acquity UPLC H-Class system equipped with an Acquity UPLC Photodiode Array PDA eλ Detector (Waters, Milford, MA, USA) and controlled by Empower 3 (Waters) software.

2.4.1. Chromatographic determination of vitamin C

Dilutions of E1 and E2 with ultrapure water (1:4 v:v) were prepared in 2-mL amber screw thread vials with caps and used to quantify AA and TAA, respectively, according to the method described by Medina-Lozano et al. (2020). An Acquity UPLC HSS T3 column (150 mm × 2.1 mm × 1.8 μm, Waters) was used and the mobile phases consisted of methanol (A) and ultrapure water pH 2.0 acidified with formic acid (C) with a flow rate of 0.3 mL min^{-1} of 2% A and 98% C in isocratic mode. The samples and column were kept at 5 °C and 30 °C, respectively. The injection volume was 5 μL and the total running time was 3 min. The wavelength of the detector was set at 245 nm. AA and TAA content were quantified using a calibration curve built with standard solutions at concentrations of 0.5, 2.5, 5.0, 10.0 and 25.0 μg mL^{-1} prepared with the stock solution

(100 μg mL^{-1}) of the AA standard using ultrapure water pH 2.0 acidified with formic acid.

2.4.2. Chromatographic determination of anthocyanins

Anthocyanin extract E3 was transferred to a 2-mL amber screw thread vial with a cap. The chromatographic procedure for anthocyanin determination was based in the method described by Fernández-Barbero et al. (2019) with some modifications. The separations were carried out in an Acquity UPLC BEH C18 column (150 mm × 2.1 mm × 1.7 μm, Waters). The mobile phases consisted of methanol (A) and ultrapure water pH 2.0 acidified with formic acid (C) with a flow rate of 0.3 mL min^{-1} in gradient mode of A and C (Supplementary Table 1). The injection volume was 3 μL and the total running time 20 min. The temperature of the samples and the column were adjusted to 10 °C and 30 °C, respectively, and the UV chromatograms were recorded at 520 nm.

Consistent patterns described in previous works in lettuce (Wu & Prior, 2005; Mulabagal et al, 2010; Becker et al., 2014) allowed us to identify two of the anthocyanins, cyanidin 3-*O*-(6′-*O*-malonylglucoside) and cyanidin 3-(6″-acetylglucoside), present in the samples included in this study. For their quantification, a calibration curve from 0.1 to 50 μg mL^{-1} of cyanidin 3-*O*-glucoside chloride was built. For peonidin 3-*O*-glucoside identification and quantification, a calibration curve from 0.01 to 1 μg mL^{-1} of peonidin 3-*O*-glucoside chloride was generated. No other anthocyanins were found.

2.5. Method validation

To achieve chromatographic separations, the methods were validated by determining different analytical parameters as described by Bertolín et al. (2018). First of all, the selectivity was analysed as the possibility of obtaining a signal free of interferences of other compounds for each analyte. The sensitivity was determined as the slope of the calibration curve. The limit of detection (LOD) and the limit of quantification (LOQ) were established as 3 and 10 times the standard deviation of 10 blanks (5 μL of mobile phase), respectively. The linear range was determined by using five increasing concentrations of the corresponding standards and linearity was presented as a coefficient of determination (R^2), considering an appropriate linearity if this coefficient reached at least a value of 0.970.

The repeatability and the intermediate precision were expressed as coefficients of variation (CV, %) and were determined as standard deviation/average × 100, being n = 5 (five samplings of the matrix, samples treatments and chromatographic conditions within the same day, in the case of repeatability, and on different days in the case of intermediate precision). Finally, the recovery (Rec, %) was calculated using 10 aliquots with 50 mg (AA) or 40 mg (anthocyanins) of the same sample, 5 spiked with 2 mg of analyte g^{-1} of dry matter, and 5 non spiked and was calculated as follows:

$$\frac{[\text{Analyte}]_{\text{spiked sample}} - [\text{Analyte}]_{\text{sample}}}{[\text{Analyte}]_{\text{spiked}}} \times 100$$

where n = 5. Furthermore, AA and anthocyanin stability were checked through consecutive injections of the same sample from 0 to 24 h (data not shown).

2.6. Statistical analysis

Summary statistics were calculated with the data coming from three biological replicates for each accession (n = 3). Effects of the group (according to Table 1), leaf colour and tissue on vitamin C content were tested by ANOVA with post hoc Tukey's test in the whole set of accessions ($p < 0.05$), the lettuce varieties and the two branching wild species (Supplementary Fig. 1), respectively. Regarding the total anthocyanin content, the effects of groups and tissues were checked as described above. Data were normalized when required. Pair-wise Spearman's correlation coefficient and significance ($p < 0.05$) was calculated between seven out of the eight traits measured in all the accessions (cyanidin 3-(6″-acetylglucoside) was only found in one sample, Table 2).

Hierarchical Clustering analysis was applied independently to the vitamin C and total anthocyanin data in all the accessions using the Ward's minimum variance to calculate the distance among clusters.

All statistical analyses were performed using JMP v5.1.2 software for Windows (SAS Institute Inc., Cary, NC).

3. Results and discussion

3.1. Method validation

All analytes showed an appropriate selectivity as they presented a signal free of interferences from other components of similar behaviour. The retention times for AA and anthocyanins were short, 1.874 min and 6.728 min, respectively, as well as the total running times, 3 min and 20 min, respectively.

The statistical parameters were optimized. All compounds presented a good linearity in the linear range tested (0.5–25 μg mL^{-1} for AA, 0.1–50 μg mL^{-1} for cyanidin 3-*O*-glucoside chloride, and 0.01–1 μg mL^{-1} for peonidin 3-*O*-glucoside chloride), with the following coefficients of determination: $R^2 = 0.99998$ for AA, $R^2 = 0.99831$ for cyanidin 3-*O*-glucoside chloride, and $R^2 = 0.99126$ for peonidin 3-*O*-glucoside chloride. The LODs were 13.05 μg AA g^{-1} DW (dry weight), 0.51 μg cyanidin 3-*O*-glucoside chloride g^{-1} DW, and 0.25 μg peonidin 3-*O*-glucoside chloride g^{-1} DW, while the LOQs were 45.21 μg AA g^{-1} DW, 1.77 μg cyanidin 3-*O*-glucoside chloride g^{-1} DW, and 0.87 μg peonidin 3-*O*-glucoside chloride g^{-1} DW.

The analytical methods presented good values of repeatability and intermediate precision: 1.75% and 4.22% for AA, 2.17% and 5.76% for cyanidin 3-*O*-glucoside chloride, and 3.19% and 5.91% for peonidin 3-*O*-glucoside chloride, respectively. Finally, the recovery was also good for AA, cyanidin 3-*O*-glucoside chloride, and peonidin 3-*O*-glucoside chloride: 95.6%, 97.2%, and 96.3%, respectively.

Furthermore, AA and TAA stability was analysed as AA is a very labile molecule that oxidizes easily at high temperatures, high pH, under intense light and in oxidizing atmosphere (Lee and Kader, 2000). AA and TAA degradations started 4 h after extraction (data not shown). Hence, it was necessary to quantify them in this time interval. A short total running time was needed to analyse a high number of samples in the 4 h window. In the case of anthocyanins, their stability was tracked over a 24 h period, without observing significant losses in the analyte concentrations. Chromatograms of 3 and 20 min were achieved without interferences between two consecutive samples in the case of AA and anthocyanins, respectively.

3.2. Quantification of vitamin C

The mean TAA content ranged between 153.24 and 291.11 mg 100 g^{-1} DW in leaf tissue (Table 2, Fig. 1a), the poorest accession being a red-leaf commercial variety ('Nestorix'), while the richest was a wild relative (*L. homblei*). In general, the wild species had higher content of both, DHAA and AA, and consequently also of TAA. In traditional lettuces, the average content of these three compounds was also higher than in the commercial varieties. These results are in agreement with those obtained by van Treuren et al. (2018), that also observed the highest content of AA in primitive lettuces and their wild relatives. This supports the idea that wild *Lactuca* species could play an important role in modern lettuce breeding. Until now, lettuce wild relatives have been mainly used to introduce resistance genes in modern varieties but these results make evident their great potential to improve the nutritional value of a vegetable which is relatively poor in macronutrients like vitamin C (USDA, 2016). When all raw data were taken into account, the plant with the highest content in total vitamin C happened to belong to a

Table 2
Average weight, vitamin C and anthocyanin content (n = 3) in commercial and traditional lettuce varieties and some wild relatives (*Lactuca* spp.).

			Vitamin C (mg 100 g^{-1} DW)			Anthocyanins (mg 100 g^{-1} DW)[a]			
Sample	Group	Weight (g)	DHAA	AA	TAA	Peonidin 3-*O*-glucoside	Cyanidin 3-*O*-(6′-*O*-malonylglucoside)	Cyanidin 3-(6″-acetylglucoside)	Total
'Begoña'	Commercial varieties (green)	102.43 ± 31.64	28.55 ± 3.09	255.48 ± 21.82	284.03 ± 20.65	ND	ND	ND	ND
'Dolomiti G12′		115.17 ± 7.77	26.67 ± 9.49	172.67 ± 9.43	199.35 ± 6.99	ND	ND	ND	ND
'Romana Inverna'		238.63 ± 13.74	19.98 ± 1.49	228.51 ± 24.87	248.50 ± 23.57	ND	ND	ND	ND
'Winter Crop'		208.93 ± 47.73	44.97 ± 15.21	118.74 ± 20.63	163.71 ± 20.63	ND	ND	ND	ND
'Likarix'	Commercial varieties (red)	297.97 ± 29.58	14.43 ± 3.60	205.29 ± 8.27	219.71 ± 5.24	3.37 ± 1.74	123.91 ± 20.50	ND	127.28 ± 21.64
'Lollo Rosso'		291.70 ± 17.68	25.36 ± 5.26	147.09 ± 19.69	172.45 ± 19.70	ND	8.95 ± 1.56	ND	8.95 ± 1.56
'Nestorix'		329.13 ± 16.60	22.12 ± 4.50	131.12 ± 37.94	153.24 ± 40.94	2.60 ± 1.44	55.41 ± 15.13	ND	58.01 ± 16.56
'Red Sails'		343.67 ± 34.70	30.51 ± 5.16	167.01 ± 22.06	197.52 ± 27.14	1.78 ± 0.48	59.58 ± 18.46	ND	61.36 ± 18.73
'Revolution'		215.67 ± 28.76	31.16 ± 11.02	157.32 ± 9.83	188.48 ± 9.70	0.95 ± 0.76	37.91 ± 22.20	ND	38.86 ± 22.96
'Romired'		318.23 ± 32.80	45.79 ± 6.53	150.22 ± 23.66	196.02 ± 17.13	2.34 ± 1.02	58.06 ± 15.99	ND	60.40 ± 16.73
'Lechuga de Beceite'	Traditional varieties (green)	208.80 ± 60.60	21.53 ± 8.35	213.46 ± 72.65	234.99 ± 71.88	ND	ND	ND	ND
'Lechuga de Ensalada'		241.33 ± 47.82	45.53 ± 8.90	164.46 ± 34.14	210.00 ± 40.02	ND	ND	ND	ND
'Lechuga de Híjar'		343.57 ± 83.22	22.94 ± 3.77	165.19 ± 52.27	188.13 ± 54.11	ND	ND	ND	ND
'Lechuga de Subías'		226.30 ± 58.00	21.01 ± 10.14	204.97 ± 26.44	225.98 ± 32.86	ND	ND	ND	ND
'Lechuga del Pirineo'		244.17 ± 109.82	25.45 ± 15.73	238.63 ± 84.74	264.08 ± 100.28	ND	ND	ND	ND
'Lechuga del Valle de Tena'		295.63 ± 45.31	47.03 ± 7.87	165.52 ± 26.94	212.54 ± 26.88	ND	ND	ND	ND
'Lechuga Romana Zaragozana'		163.40 ± 45.80	10.43 ± 7.39	270.38 ± 22.74	280.81 ± 30.13	ND	ND	ND	ND
'Lengua de Buey'[b]		188.97 ± 80.02	31.85 ± 7.97	178.05 ± 47.04	209.91 ± 45.99	ND	1.49 ± 2.59	ND	1.49 ± 2.59
'Oreja de Mulo'		219.90 ± 101.99	29.92 ± 15.64	218.75 ± 60.35	248.68 ± 71.16	ND	ND	ND	ND
'Lechuga de Bureta'	Traditional varieties (semi-red)	171.70 ± 69.04	24.40 ± 3.74	232.97 ± 25.70	257.37 ± 22.20	0.44 ± 0.77	22.48 ± 33.96	ND	22.93 ± 34.72
'Morada de Belchite'		298.57 ± 50.93	10.25 ± 7.63	147.88 ± 30.92	158.13 ± 23.99	0.35 ± 0.61	11.99 ± 14.51	ND	12.34 ± 15.11
'Morada de Bernués'		252.77 ± 37.04	14.61 ± 0.84	183.32 ± 18.39	197.93 ± 18.75	ND	0.50 ± 0.30	ND	0.50 ± 0.30
'Morada de Sorripas'		297.60 ± 18.30	24.07 ± 4.61	157.53 ± 18.82	181.60 ± 14.42	ND	11.41 ± 1.72	ND	11.41 ± 1.72
L. biennis	Wild species (leaf)	4.57 ± 1.56	53.09 ± 11.13	164.68 ± 11.56	217.77 ± 9.51	ND	1.05 ± 0.86	ND	1.05 ± 0.86
L. dentata		38.63 ± 17.33	23.30 ± 4.93	199.05 ± 15.61	222.35 ± 14.21	ND	16.38 ± 5.36	ND	16.38 ± 5.36
L. dregeana		221.90 ± 25.34	40.03 ± 14.85	225.79 ± 16.10	265.82 ± 28.90	ND	12.51 ± 6.34	ND	12.51 ± 6.34
L. floridana		1.23 ± 0.29	150.05 ± 5.87	122.87 ± 18.62	272.92 ± 12.75	ND	14.13 ± 1.09	ND	14.13 ± 1.09
L. homblei		116.27 ± 3.00	50.27 ± 5.41	240.84 ± 34.35	291.11 ± 38.26	ND	3.56 ± 0.46	ND	3.56 ± 0.46
L. squarrosa		186.50 ± 68.64	53.40 ± 22.16	233.13 ± 69.95	286.53 ± 48.76	ND	2.26 ± 1.28	ND	2.26 ± 1.28
L. virosa		102.27 ± 29.71	40.91 ± 6.04	188.45 ± 5.91	229.36 ± 4.61	ND	0.98 ± 0.55	ND	0.98 ± 0.55
L. dregeana	Wild species (stem)	–	17.11 ± 3.08	81.26 ± 25.33	98.38 ± 28.23	ND	15.39 ± 4.34	ND	15.39 ± 4.34
L. squarrosa		–	13.23 ± 5.33	34.27 ± 19.30	47.50 ± 24.25	ND	17.53 ± 15.18	1.95 ± 0.28	19.48 ± 15.23

[a]ND: not detected.
[b]Only one out of the three biological repeats contained anthocyanins.

traditional variety ('Lechuga del Pirineo'), which is a romaine-type lettuce with good organoleptic and agronomic characteristics (Carravedo et al., 2011).

In general, the amounts of vitamin C obtained are higher than those reported in other studies carried out in the same varieties (i.e. 'Lollo Rosso' in Llorach et al., 2008) or in wild *Lactuca* species (i.e. *L. dentata* and *L. virosa* in van Treuren et al., 2018). This is most certainly due to the fact that the starting materials employed are lyophilized leaves, whereas most authors have conducted their experiments using fresh leaves, in which all the compounds are expected to be more diluted. In agreement with this, the results are in the same order of magnitude (even if they are generally higher) than those reported by Złotek et al. (2014), who used lyophilized leaves of *L. sativa* L. *var. capitata*, a variety not included in our study.

Except for *L. floridana*, where DHAA was the most abundant form of vitamin C, AA was the main contributor to the total vitamin C content in all accessions (Table 2, Fig. 1a), representing on average 85% of the TAA. This agrees with observations in most vegetables, where AA is also the most abundant form of vitamin C (Lee and Kader, 2000). However, even if AA is also the form showing the highest biological activity (antioxidant), DHAA is easily converted into AA in the human body (Lee & Kader, 2000). So, DHAA can serve as vitamin C reservoir in certain adverse conditions (i.e. oxidative stress, which is a consequence derived from other stresses, like drought). In this sense, genetic resources like lettuce wild relatives are awaking interest among breeders to increase the crop tolerance to an array of abiotic stresses (Hartman et al., 2014).

When contents of DHAA, AA and TAA were compared among the three groups studied, differences in their average values were observed (Table 2, Fig. 2a), which became significant in the case of TAA ($F = 4.438$, $p = 0.022$), and very significant for DHAA ($F = 6.786$, $p = 0.004$). The wild relatives showed up to 21% and 51% more TAA and DHAA, respectively, when compared to the commercial varieties. In the case of the traditional varieties, the average content of DHAA was similar to the amount present in the commercial varieties, whereas the TAA value was 8% higher. Regarding AA, differences could be also observed, even if they did not reach statistical significance. In fact, the groups formed by the lettuce wild relatives and the traditional varieties exhibited 12% and 11% more AA, respectively, than the lettuce commercial varieties. Until now, lettuce breeding has been focused mainly on obtaining crops that are more productive and resistant to biotic stresses, however little attention has been paid to its nutritional quality. This could have led to a phytonutrient "wash" in modern varieties, which has also been observed in other crops, like apple, where the selection of sweeter and less acidic fruit has resulted in a decrease in their content in vitamin C when wild apples are compared to cultivated ones (Fang et al., 2017). This would explain why non-domesticated plants (Crop Wild Relatives, CWR) or those under low selection pressure (traditional varieties), show higher levels, in this case, of vitamin C.

Considering only the cultivated lettuces, both commercial and traditional varieties, the results obtained allowed to conclude that green-leaf varieties contained higher amounts of vitamin C in all its forms (DHAA, AA and TAA) than red-leaf varieties (Fig. 2b), with up to a 15% more in the case of DHAA, and 18% more in AA and TAA content. Specifically, the differences were significant ($F = 5.480$, $p = 0.029$) and very significant ($F = 8.968$, $p = 0.007$) in AA and TAA content, respectively, between green and red varieties. No interaction between group and leaf colour was detected (data not shown). No exhaustive work comparing both types of lettuce in terms of vitamin C content is available in the literature. However, Llorach et al. (2008) analysed four varieties, two green and two red, and found that the two showing both the highest and the lowest vitamin C contents, were the two green varieties.

The two wild species with an early bushy growth (*L. dregeana* DC. and *L. squarrosa*, Supplementary Fig. 1) offered the opportunity to collect samples from two different tissues, leaf and stem, at the moment of harvest. Both species accumulated more DHAA, AA and TAA in the leaves than in the stems (Table 2, Fig. 2c). In all cases, the differences observed were significant ($F = 20.541$ and $p = 0.045$; $F = 52.119$ and $p = 0.019$; $F = 54.755$ and $p = 0.018$ for DHAA, AA and TAA, respectively).

3.3. Quantification of anthocyanins

Without considering the green-leaf varieties, in which in most cases there was a complete absence of anthocyanins, the average values of total anthocyanins ranged between 0.50 and 127.28 mg 100 g^{-1} DW in leaf tissue (Table 2 and Fig. 1b). The accession with the lowest content was a semi-red traditional variety ('Morada de Bernués') and the one with the highest value was a red commercial variety ('Likarix'). Surprisingly, all wild species assayed contained anthocyanins, almost exclusively cyanidin 3-*O*-(6′-*O*-malonylglucoside), even those apparently being green-leaf coloured, like *L. dentata* (Supplementary Fig. 1). This same anthocyanin was also present in the green-leaf traditional variety 'Lengua de Buey' (Table 1, Supplementary Fig. 1), even if it had been previously described as a lettuce with yellow-green leaves and no anthocyanic colour (Carravedo et al., 2011). Trace amounts of anthocyanins in green-leaf varieties have been previously reported (Kleinhenz et al., 2003; Brücková et al., 2016). However, the cyanidin 3-*O*-(6′-*O*-malonylglucoside) was only found in one out of the three plants analysed from this accession ('Lengua de Buey'-2). These differences (presence/absence of a particular anthocyanin) within the same accession, show the enormous heterogeneity and diversity harboured by the traditional varieties. Cyanidin 3-*O*-(6′-*O*-malonylglucoside) is not only the most abundant anthocyanin, as found before (García-Macías et al., 2007), representing the 97% of the total anthocyanin content on average, but also the only one present in most samples (Fig. 1b). Furthermore, it appears across all the groups, commercial and traditional lettuce varieties, as well as all the wild species studied here. Quite the opposite, peonidin 3-*O*-glucoside is only present in cultivated forms (*L. sativa*). Besides, it is always detected simultaneously with cyanidin 3-*O*-(6′-*O*-malonylglucoside), although in much lower amounts. The cyanidin glucosides are transformed into the peonidin glucosides in a single step catalysed by a methyltransferase. That is why they are commonly found together. Interestingly, the cyanidin 3-(6″-acetylglucoside) was present only in one of the wild species (*L. squarrosa*) and, within it, exclusively in the stem and not in the leaf (Table 2, Fig. 1b). This is the first time that tissue-dependent biosynthesis of a specific anthocyanin has been reported in *Lactuca*, though anthocyanin presence in only certain tissues has been described in *Citrus* genus (Fabroni et al., 2016).

Cyanidin-type is also the most abundant among the anthocyanins present in many other crops, like mulberry (Kim & Lee, 2020), purple corn (Peniche-Pavía & Tiessen, 2020), purple wheat (Abdel-Aal et al., 2018), blood orange (Fabroni et al., 2016), with glucose being the main sugar and malonyl the predominant acyl substituent in many cases, lettuce included (Wu and Prior, 2005; Mulabagal et al., 2010; Becker et al., 2014), as observed in this work (Table 2, Fig. 1b). Regarding the other two minor anthocyanins, cyanidin 3-(6″-acetylglucoside), which was only found in *L. squarrosa* stem, and peonidin 3-*O*-glucoside, both have been rarely reported in lettuce before (Wu and Prior, 2005; Baek et al., 2013; Viacava et al., 2017).

In contrast to what was observed for vitamin C, on average, commercial lettuce varieties had higher content of total anthocyanins than traditional lettuce varieties, and the latter, higher than the wild species (Fig. 2d). However, only the differences between the commercial varieties and the other two groups became very significant statistically ($F = 8.452$, $p = 0.003$). So, this ranking shows the inverse order than the one observed for the average vitamin C content. This effect can be attributed to the fact that the red colour has been enhanced in the new red varieties, as pointed out before (Casals Missio et al., 2018), because it makes the product more attractive for consumers and increases its potential market value (not to mention the benefits for human health derived from a higher anthocyanin content). In this study, commercial varieties

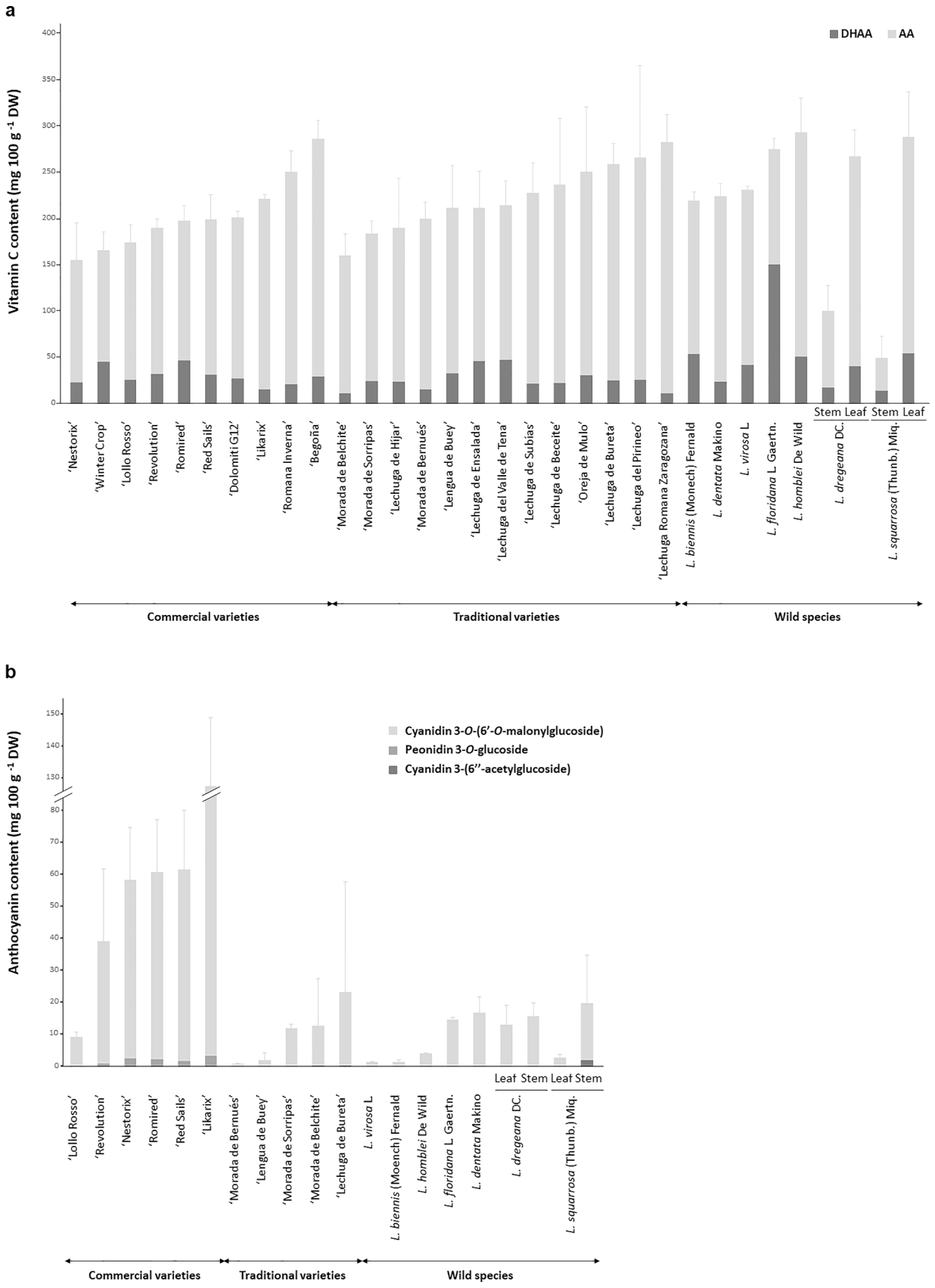

Fig. 1. Total concentration of vitamin C (a) and anthocyanins (b) present in lettuce varieties (commercial and traditional) and some wild relatives. Bars represent the standard deviation of the total (n = 3).

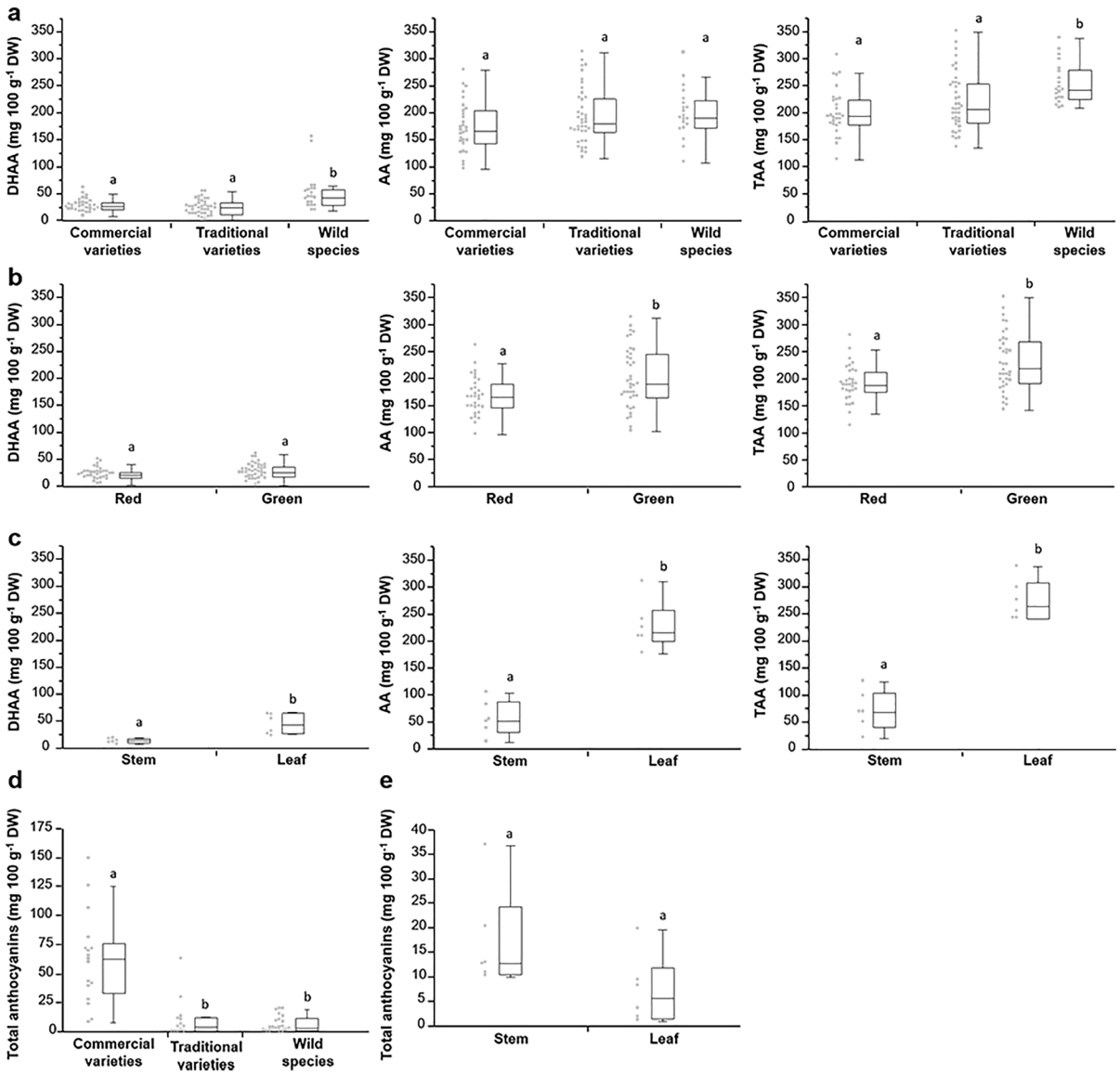

Fig. 2. Box plots of nutrient content: DHAA, AA and TAA in commercial and traditional lettuce varieties and some wild relatives (a), in green and red-leafed lettuce varieties (b), and in stem and leaf of two wild species (see Table 1) (c); total anthocyanins in commercial and traditional lettuce varieties and some wild relatives (d), and in stem and leaf of two wild species (see Table 1) (e). Different letters indicate significant differences ($p < 0.05$) among groups. Note: 'Lengua de Buey'-1, -3 included in the green group and -2 in the red group.

accumulated six and eight times more anthocyanins than traditional varieties and the wild relatives analysed, respectively. However, there are some exceptions, like *L. dentata*, *L. dregeana* and *L. floridana*, in which the mean of the total anthocyanins was higher than in a commercial variety like 'Lollo Rosso' (Table 2), under the particular growing conditions described above. A similar trend was observed in three out of the four semi-red traditional varieties analysed, 'Lechuga de Bureta', 'Morada de Belchite' and 'Morada de Sorripas', which also accumulated more anthocyanins on average than 'Lollo Rosso' (Table 2, Fig. 2b).

A direct comparison of anthocyanin accumulation with previous works is not possible as, in most cases, it has been studied only in one or a few lettuce varieties and under different growing conditions (temperature, illumination, fortification with micronutrients, greenhouse *vs* outdoor cultivation, etc). However, evaluation of lettuce germplasm for anthocyanin content, including cultivated forms (commercial and traditional) as well as wild relatives, has not been carried out before. Even comparing a particular accession, for instance 'Lollo Rosso', the anthocyanin level obtained is both, higher (Ordidge et al., 2010) and lower (García-Macías et al., 2007), than those found under other control conditions in other researches (taking also into account that we referred it to DW unlike in those studies). This is not surprising as anthocyanin synthesis is hugely affected by a number of factors (Chalker-Scott, 1999).

Unlike the vitamin C content in the tissues coming from the wild species *L. dregeana* and *L. squarrosa*, the anthocyanin content was higher in the stem than in the leaf (Table 2), however no significant differences were observed (Fig. 2e). As commented before, the cyanidin 3-(6″-acetylglucoside) was found only in the stem of *L. squarrosa*, but not in the leaves. Furthermore, in this case, this cyanidin glucoside was present in the three biological replicates of *L. squarrosa* tested. Cyanidin 3-(6″-acetylglucoside) has not been identified in any of the cultivated lettuces included in this study, though it has been found previously in some red lettuce varieties (Wu and Prior, 2005; Viacava et al., 2017).

3.4. Pairwise correlations between traits

As expected, AA showed a negative and a positive correlation with DHAA and TAA, respectively, though only the correlation between AA

and TAA was strong and statistically significant (Spearman's rho = 0.868, $p < 0.0001$, Table 3). Those coefficients reflect that AA and DHAA are interconvertible (one appears at the expense of the other) and also that the main contribution to TAA is made by AA. Interestingly, the correlation between total anthocyanins, cyanidin 3-*O*-(6′-*O*-malonylglucoside) and peonidin 3-*O*-glucoside with both, AA and TAA, are all negative. Even if the values of the coefficients were low, in some cases, they were statistically significant (Table 3). Previously, it has been suggested that the biosynthesis of anthocyanins could cause a reduction in other processes of the plant as it is a direct competition for assimilated carbon (García-Macías et al., 2007). This could explain why the red-leaf varieties contained a significantly lower amount of TAA than the green-leaf varieties (Fig. 2b). This also agrees with the inverse ranking observed for average TAA (commercial varieties < traditional varieties < wild relatives) when compared to average total anthocyanins (wild relatives < traditional varieties < commercial varieties). The two main anthocyanins found, cyanidin 3-*O*-(6′-*O*-malonylglucoside) and peonidin 3-*O*-glucoside, show a positive and highly significant correlation (Spearman's rho = 0.717, $p < 0.0001$). This coincides with their simultaneous presence in most of the lettuce varieties studied (8 out of 10, Table 2) and with the peonidin synthesis simply by transferring a methyl group to cyanidin's B ring at 3′ position (Zhang et al, 2014). Both of them were also positively correlated at a highly significant level with the total anthocyanin content, especially the most abundant (cyanidin 3-*O*-(6′-*O*-malonylglucoside), which showed a perfect positive correlation (Spearman's rho = 1, $p < 0.0001$).

Finally, the plant weight showed a negative relationship with the vitamin C content (DHAA, AA and TAA), weak though significant in the case of DHAA ($p = 0.042$) and highly significant for AA and TAA ($p < 0.0001$); whereas it was positive with the two main anthocyanins, as well as with the total anthocyanin content, being highly significant with the peonidin 3-*O*-glucoside ($p < 0.0001$) (Table 3). A negative correlation between biomass and vitamin C content has been previously described in lettuce (Bumgarner et al., 2012) and could be explained by the fact that to synthesize it results costly for the plant cell. The same could be argued for the anthocyanins and, actually, a negative correlation between the biomass and total anthocyanin content has been reported by the same authors (Bumgarner et al., 2012) though, under the experimental conditions of our study, none of the accessions developed a strong anthocyanic colour (Supplementary Fig. 1).

3.5. Nutritional value

The nutritional value of the samples analysed here, expressed as vitamin C and total anthocyanin content, respectively, showed great variation, especially in the case of anthocyanins (Fig. 1). Their hierarchical clustering allowed us to create groups of accessions with similar content of each of the two compounds separately (Fig. 3). Regarding the vitamin C (Fig. 3a), the accessions organised themselves in two main groups (distance = 2.16): high (248–287 mg 100 g^{-1} DW, Cluster I) and medium–low (153–235 mg 100 g^{-1} DW, Cluster II) content. Within Cluster II, subgroups with the lowest (153–198 mg 100 g^{-1} DW, Subcluster IIa), and medium (210–235 mg 100 g^{-1} DW, Subcluster IIb) amount of vitamin C could be distinguished. It is noteworthy that the richest (Cluster I) and the intermediate (Subcluster IIb) groups in vitamin C are mainly formed by traditional varieties and lettuce wild relatives. These results could promote the ongoing initiative to valorise traditional crop varieties and landraces as a source of highly nutritious compounds (Petropoulos et al., 2019). In this sense, Casals Missio et al. (2018) found that lettuce landraces were characterised by higher levels of sugars when compared to modern varieties. In the other extreme, 7 out of the 11 accessions included in the poorest group in terms of vitamin C concentration (Subcluster IIa) are commercial varieties, mainly read-leaf lettuces.

In relation to the total anthocyanins (Fig. 3b), the accessions arranged themselves in two major groups (distance = 2.30): nothing to moderate (0–22.93 mg 100 g^{-1} DW, Cluster I) and high (38.86–127.28 mg 100 g^{-1} DW, Cluster II) amount. In Cluster II, subgroups with nothing or a negligible (0–3.56 mg 100 g^{-1} DW, Subcluster Ia) and medium (11.41–22.93 mg 100 g^{-1} DW, Subcluster Ib) amount of anthocyanins were obtained. Obviously, the poorest group (Subcluster Ia) was mainly integrated by green-leaf lettuces though there was also a semi-red traditional variety and four lettuce wild relatives. The remaining semi-red traditional varieties fall into the medium group (Subcluster Ib) together with the rest of the wild species. The richest group in total anthocyanins (Cluster II) was exclusively formed by red-leaf commercial varieties, with 'Likarix' exceling as it contained one order of magnitude more anthocyanins than the rest. By contrast, 'Lollo Rosso' did not develop a strong red colour (Supplementary Fig. 1) under our specific growing conditions, behaving like the semi-red traditional varieties in relation to the anthocyanin biosynthesis. As commented before, the red-leaf commercial varieties have been bred to accumulate higher concentrations of anthocyanins; this became clear when they were compared to red-leaf landraces performing a colour analysis (Casals Missio et al., 2018).

4. Conclusions

The current market trend reflects the growing interest of consumers in food quality. This justifies making an effort in improving the nutritional value of food, particularly, vegetables. For that, the first logical step should be to evaluate the content of health-promoting compounds in the main crops and their related germplasm. In this sense, wild crop relatives as well as landraces and traditional varieties play a pivotal role, especially the last two as they represent a shortcut in the long path to obtain new biofortified varieties. The characterisation of vitamin C and anthocyanin content of a wide variety of cultivated (commercial and traditional varieties) lettuces and wild forms, has been carried out. Lettuce wild relatives and commercial varieties were the richest groups in vitamin C and anthocyanins, respectively. Conversely, commercial varieties and wild relatives were the poorest groups in vitamin C and anthocyanins, respectively, with the traditional varieties occupying an

Table 3
Spearman's correlation coefficients and significance level ($p < 0.05$) between the traits studied (weight and vitamin C and anthocyanin concentrations) in commercial and traditional lettuce varieties, as well as wild relatives (*Lactuca* spp.).

	DHAA	AA	TAA	Peonidin 3-*O*-glucoside	Cyanidin 3-*O*-(6′-*O*-malonylglucoside)	Total anthocyanins	Weight
DHAA	1.000						
AA	−0.143	1.000					
TAA	0.243*	0.868***	1.000				
Peonidin 3-*O*-glucoside	−0.124	−0.209	−0.271*	1.000			
Cyanidin 3-*O*-(6′-*O*-malonylglucoside)	0.001	−0.217*	−0.180	0.717***	1.000		
Total anthocyanins	0.000	−0.218*	−0.181	0.717***	1.000***	1.000	
Weight	−0.220*	−0.473***	−0.538***	0.400***	0.209	0.210	1.000

***$p < 0.001$; *$p < 0.05$.

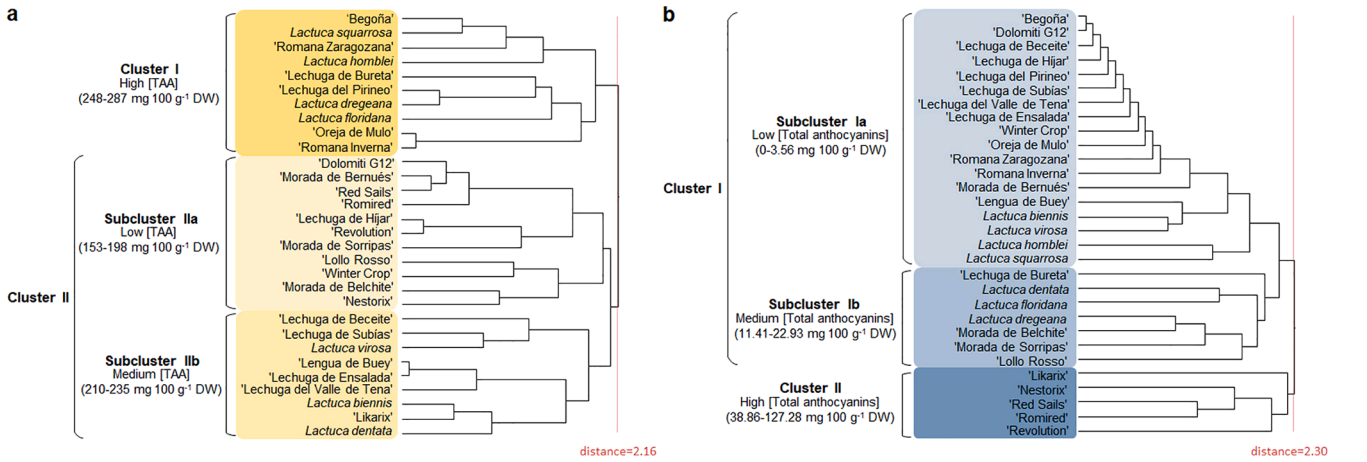

Fig. 3. Hierarchical clustering analysis of the 30 accessions included in this study (10 lettuce commercial varieties, 13 lettuce traditional varieties and 7 wild relatives) attending to their average vitamin C (a) and total anthocyanin (b) content (n = 3).

intermediate position in both cases. Some of the traditional lettuce varieties studied could be considered promising as they are rich in vitamin C and, in some cases, are able to biosynthesize anthocyanins, whose content could be increased by breeding.

The results offer nutritional information on what is the most consumed (but not especially nutritious) leafy vegetable and wild relatives, some of them with potential culinary use. This could be useful for both, consumers and breeders interested in improving the lettuce content of phytochemicals that promote health and reduce the risk of certain diseases.

CRediT authorship contribution statement

Inés Medina-Lozano: Experimentation, Analysis, Writing - review & editing. **Juan Ramón Bertolín:** Experimentation, Data collection, Analysis, Writing - review & editing. **Aurora Díaz:** Funding acquisition, Conceptualisation and design, Supervision, Project administration, Data collection, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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