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Analytical Methods

Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole

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Abstract

Salad vegetables could be relevant as dietary sources of natural antioxidants. A better knowledge of their composition can be useful for understanding their potential bioavailability and biological activities. The antioxidant compounds, polyphenols and vitamin C, have been determined in five varieties of lettuce (iceberg, romaine, continental, red oak leaf, lollo rosso) and one variety of escarole (frissé). The polyphenol study by HPLC-DAD-MS/MS ESI allowed the identification of two compounds previously not reported in lettuce; quercetin and luteolin rhamnosyl-hexosides. Qualitative and quantitative differences were observed between the polyphenol profiles. Caffeic acid derivatives were the main phenolics in green varieties, while flavonols were detected in higher quantities in red varieties and escarole, and anthocyanins were only present in red-leafed varieties. The highest total phenolic content was observed in red-leafed varieties while the highest level of vitamin C was detected in the continental variety. The red varieties showed the highest antioxidant activity by all the methods assayed.

Keywords: Lettuce varieties; Hydroxycinnamic acids; Flavones; Chemometric; Hierarchical cluster analysis; Principal component analysis; DPPH; ABTS; FRAP

1. Introduction

Diets rich in fruits and vegetables are associated with a lower risk of cancer and cardiovascular diseases (Hooper & Cassidy, 2006). These beneficial effects are believed to be due to vitamins and phytochemicals such as ascorbic acid, carotenoids, polyphenols and fibre that may protect key biological constituents such as lipoproteins, membranes, and DNA (Szeto, Kwok, & Benzie, 2004). In fact, recent studies have shown the health effects of lettuce in preventing cardiovascular diseases in rats and humans (Nicolle et al., 2004a; Serafini et al., 2002).

Lettuce and escarole, belonging to the *Asteraceae* family, are the most popular vegetables in salads which are consumed in increasing amounts due to their perception as being "healthier" foods (Dupont, Mondi, Willamson, & Price, 2000). The healthy properties are attributed to a large supply of antioxidant compounds mainly vitamin C and polyphenols as well as the fibre content (Nicolle et al., 2004a; Serafini et al., 2002) Polyphenols (flavonols and anthocyanins) have been described to have greater antioxidant activity than vitamins C and E (Rice-Evans, Miller, & Paganga, 1997). Previous studies demonstrate the importance of the chemical nature of these conjugates and suggest that the degree of hydroxylation determines the antioxidant activity (Rice-Evans et al., 1997) and bioavailability (Hollman & Arts, 2000). In addition, antioxidant compounds are susceptible to variation among varieties, growing

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practices, processing and storage conditions on the biologically active compounds (Baur, Klaiber, Koblo, & Carle, 2004; Dupont et al., 2000; Lee & Kader, 2000).

The aim of the present work was a qualitative and quantitative study of antioxidant compounds, polyphenols and vitamin C, in six varieties of the most popular salads. The antioxidant activity was measured by three complementary spectrophotometric assays (ABTS, DPPH and FRAP). The results obtained will be a useful tool in further epidemiological studies, evaluating polyphenol dietary intake and the nutritional recommendations, as well as in the selection of salad ingredients with high antioxidant content.

2. Materials and methods

2.1. Plant material

Five varieties of lettuce (*Lactuca sativa* L.), three green varieties (iceberg, romaine and continental) and two red varieties (red oak leaf and lollo rosso) and one escarole variety (*Cichorium endivia* var. *crispa*) "frissé" were studied. Lettuces and escarole were harvested in Torre Pacheco (Murcia, Spain) and transported within 40 min under refrigerated conditions to the CEBAS laboratory (Murcia, Spain) where the samples were processed. Fresh samples were used to evaluate the vitamin C content while freeze-dried material was used for the polyphenol and antioxidant activity study.

2.2. Identification and quantification of phenolic compound

Freeze-dried material milled to a fine powder was extracted for the identification and quantification of phenolic compounds as described by Ferreres, Gil, Castañer, and Tomás-Barberán (1997), with some modifications. Plant material (0.4 g freeze dried) of each sample was extracted twice with 8 ml of a mixture of MeOH/water/formic acid (25/24/3) (v:v:v) and filtered through a 0.45 μm polyethersulphone filter (Millex HV13, Millipore, Bedford, MA). The extracts (20 μl) were analyzed using an HPLC system equipped with a pump Model L-6200 (Merck Hitachi) and Shimadzu SPD-M6A photodiode array UV–VIS detector. Separations were achieved on a LiChroChart C18 column (250 × 4 mm; 5 μm particle size; Merck, Darmstadt, Germany). The mobile phases were water with 5% formic acid (A) and methanol (B) with a solvent flow rate of 1 ml min^{-1}, in a gradient program starting with 5% B in A, reaching 40% B at 25 min, and then remaining isocratic for 5 min. The UV chromatograms were recorded at 330 and 520 nm. The results presented are the mean of three experiments with the standard error. The identification of phenolic compounds was carried out according to their UV spectra, retention times and HPLC-DAD-MS/MS ESI (Ferreres et al., 1997). Caffeic acid derivatives were quantified by comparison with an external standard of chlorogenic acid (5-*O*-caffeoylquinic acid), flavonoids as rutin (quercetin 3-rhamnosyl-(1-6)-glucoside) and anthocyanins as cyanidin-3-rutinoside (cyanidin 3-rhamnosyl-(1-6)-glucoside).

HPLC-DAD-MS/MS ESI. Extracts from the red lettuce variety "lollo rosso" were used for the HPLC-DAD-MS/MS ESI study as this cultivar showed to the most complex phenolic profile and the chromatographic analysis had been previously reported (Ferreres et al., 1997). The HPLC system was equipped with a DAD and mass detector in series (Agilent 1100 Series LC/MSD Trap). It consisted of an Agilent G1312A HPLC binary pump, an Agilent G1313A autosampler, an Agilent G1322A degasser and an Agilent G1315B Photo-Diode Array Detector controlled by Agilent software v. A.08.03 (Agilent Technologies, Waldbronn, Germany). The Mass Detector was an Agilent G2445A Ion-Trap Mass Spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with an Electrospray Ionisation (ESI) system and controlled by Agilent Software v. 4.1. Nitrogen was used as nebulizing gas at a pressure of 65 psi and the flow was adjusted at 11 l min^{-1}. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass spectra of the phenolic compounds were measured from m/z 100 up to m/z 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with a voltage ramping to 0.3 up to 2 V. MS data were acquired in the negative ionisation mode and MS/MS data in the automatic mode.

2.3. Extraction and analysis of vitamin C

Ascorbic acid (AA) and dehydroascorbic acid (DHA) contents were determined as described by Zapata and Dufour (1992) with some modifications. A 15 g fresh weight (f.w.) sample was homogenized in an Ultra-Turrax (T25, Janke & Kunkel, Germany) for 30 s on an ice bath with 15 ml of extraction medium (0.1 M citric acid, 0.05% w/v ethylenediaminetetraacetic acid disodium salt, 5% v/v methanol, and 4 mM NaF). The homogenate was filtered through cheesecloth and the pH adjusted to 2.2–2.4 by addition of 3 N HCl. The extract (1 ml) was centrifuged at 3600*g* for 3 min and the supernatant was recovered, filtered through a C18 Sep-Pak cartridge (Waters, Milford, MA) and then through a 0.45 μm polyethersulphone filter (Millex HV13, Millipore, Bedford, MA). HPLC analysis of vitamin C (AA and DHA) was achieved after derivatisation of DHA into the fluorophore 3-(1,2-dihydroxyethyl)furo[4-b]quinoxaline-1-one (DFQ), with 1,2-phenylenediamine dihydrochloride. Samples (20 μl) were analysed with a Merck-Hitachi (Tokyo, Japan) liquid chromatograph equipped with a L-4200 UV detector and a L-6000 pump. Separations of DFQ and AA were achieved on a Kromasil 100 C-18 column (250 × 4 mm; 5 μm particle size; Tecnokroma, Barcelona, Spain). The mobile phase was methanol/water (5:95, v/v) containing 5 mM cetrimide and 50 mM potassium dihydrogen phosphate at pH 4.5. The flow rate was 0.9 ml min^{-1}; the detector wavelength was initially set at 348 nm, and after elution of DFQ, was

manually shifted to 261 nm for AA detection. Standard solutions, column conditioning, and derivatisation procedures have been previously described (Gil, Ferreres, & Tomás-Barberán, 1998).

2.4. Antioxidant activity

The antioxidant activity of the six varieties has been evaluated with different assays divided into two basic groups depending of methodology: (i) the free radical scavenging activity was tested by using two assays, DPPH$^{\cdot}$ and ABTS$^{\cdot+}$, with different radicals and solvents (methanol and water solvent) to evaluate the antioxidants present in the extracts with different solvents and (ii) and the ferric reducing ability (FRAP assay). Lettuce and escarole powders (0.4 g freeze dried) were extracted twice with 8 ml of a mixture of MeOH/water/formic acid (25/24/3) (v:v:v) and filtered through a 0.45 μm polyethersulphone filter (Millex HV13, Millipore, Bedford, MA) and homogenates were evaluated in different antioxidant activity assays (Llorach, Tomás-Barberán, & Ferreres, 2004).

DPPH$^{\cdot}$ assay. The free radical scavenging activity using the free radical DPPH$^{\cdot}$ was evaluated by measuring the decrease in absorbance at 515 nm as previously reported (Espín, Soler-Rivas, & Wichers, 2000). The reaction started by adding 20 μl of the corresponding homogenate to the cuvette containing 80 μM (methanol solution) (980 μl) of the free radical (DPPH$^{\cdot}$). Reaction was followed with a UV-1603 Shimadzu spectrophotometer (Tokyo, Japan).

ABTS$^{\cdot+}$ assay. The reaction started by adding 5 μl of the corresponding homogenate to the cuvette containing 32 μM (water solution) (995 μl) of the free radical (ABTS$^{\cdot+}$). The radical was chemically generated with MnO_2 as described by Espín and Wichers (2000). The experiments were always performed on freshly made up solutions. The disappearance of ABTS$^{\cdot+}$ was determined by measuring the decrease in absorbance at 414 nm for 1 h at 25 °C in the above described spectrophotometer.

FRAP assay. The ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain (1996) with some modifications. The freshly made up FRAP solution contained 25 ml of 0.3 M acetate buffer (pH 3.6) plus 2.5 ml of 10 mM TPTZ solution in 40 mM HCl (previously prepared) and 2.5 ml of 20 mM ferric chloride ($FeCl_36H_2O$). This solution was used as blank. FRAP solution (950 μl of warmed, 37 °C) was mixed with 50 μl of the corresponding homogenate. The ferric reducing ability of lettuce and escarole extracts was measured by monitoring the increase of absorbance at 593 nm for 45 min.

All the antioxidant assays were repeated three times. In addition, calibration curves were made for each assay using Trolox as standard. The antioxidant activity (DPPH$^{\cdot}$, ABTS$^{\cdot+}$, FRAP assay) was expressed as Trolox equivalent antioxidant activity (TEAC) following the nomenclature of Rice-Evans and Miller (1994).

2.5. Statistical and multivariate analysis

Statistical ANOVA ($P \leqslant 0.05$) and multivariate analysis were performed using XLSTAT PRO 5.7 (Addinsoft, New York, USA). Statistical ANOVA were carried out to compare the polyphenols, vitamin C and antioxidant capacity values. The multivariate analyses were the Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) using the euclidean distance as distance measurement and Ward's as aggregation method. Such multivariate analyses were carried out to evaluate the influence of polyphenols, vitamin C and antioxidant activity in the classification and differentiation of lettuce and escarole samples.

3. Results and discussion

3.1. Characterization and quantification of phenolic compounds

The analysis of lettuce by HPLC-DAD revealed the presence of phenolic acids as the main phenolic compounds in green lettuce. In addition, anthocyanins (these only in red lettuces), flavones, and flavonols were also identified (Fig. 1).

3.1.1. Identification of phenolic compounds

Recent studies have pointed out the discrepancies on the occurrence and relative abundance of several hydroxycinnamic acid derivatives in lettuce (Baur et al., 2004; Caldwell, 2003). The phenolic composition of lettuce has been re-studied by HPLC-DAD-MS/MS ESI and a missassignation of lettuce hydroxycinnamic acids has been detected in a previous report (Ferreres et al., 1997). The structural study of the caffeic acid derivatives has been carried out following recent HPLC-MS studies including their MS^n fragmentation, and relative abundance of the different ions produced (Clifford, Johnston, Knight, & Kuhnert, 2003; Fang, Yu, & Prior, 2002). It can be concluded that lettuce compounds totally coincide with those reported by Baur et al. (2004). Table 1 shows the MS data of hydroxycinnamic acid derivatives. These analyses show the presence of *O*-caffeoylmalic acid (**CMA**), compound **3**, not reported in our previous study, and di-*O*-caffeoyltartaric acid (chicoric acid, **DCTA**), compound **5**, as characteristic compounds in these vegetables and the main compounds in the green lettuce and escarole varieties studied. In addition, the MS fragmentation of compound **6**, and the relative abundance of its ion fragments coincide with those of compound **5**, as indicated by Baur et al. (2004), and should coincide with *meso*-di-*O*-caffeoyltartaric acid (**mDCTA**). The other identified acids are: *O*-caffeoyltartaric acid (compound **1**) (caftaric acid, **CTA**), 5-*O*-caffeoylquinic acid (chlorogenic acid, **5-CQA**) (compound **2**) and 3,5-di-*O*-caffeoylquinic acid (isochlorogenic acid, **3,5-DCQA**) (compound **7**).

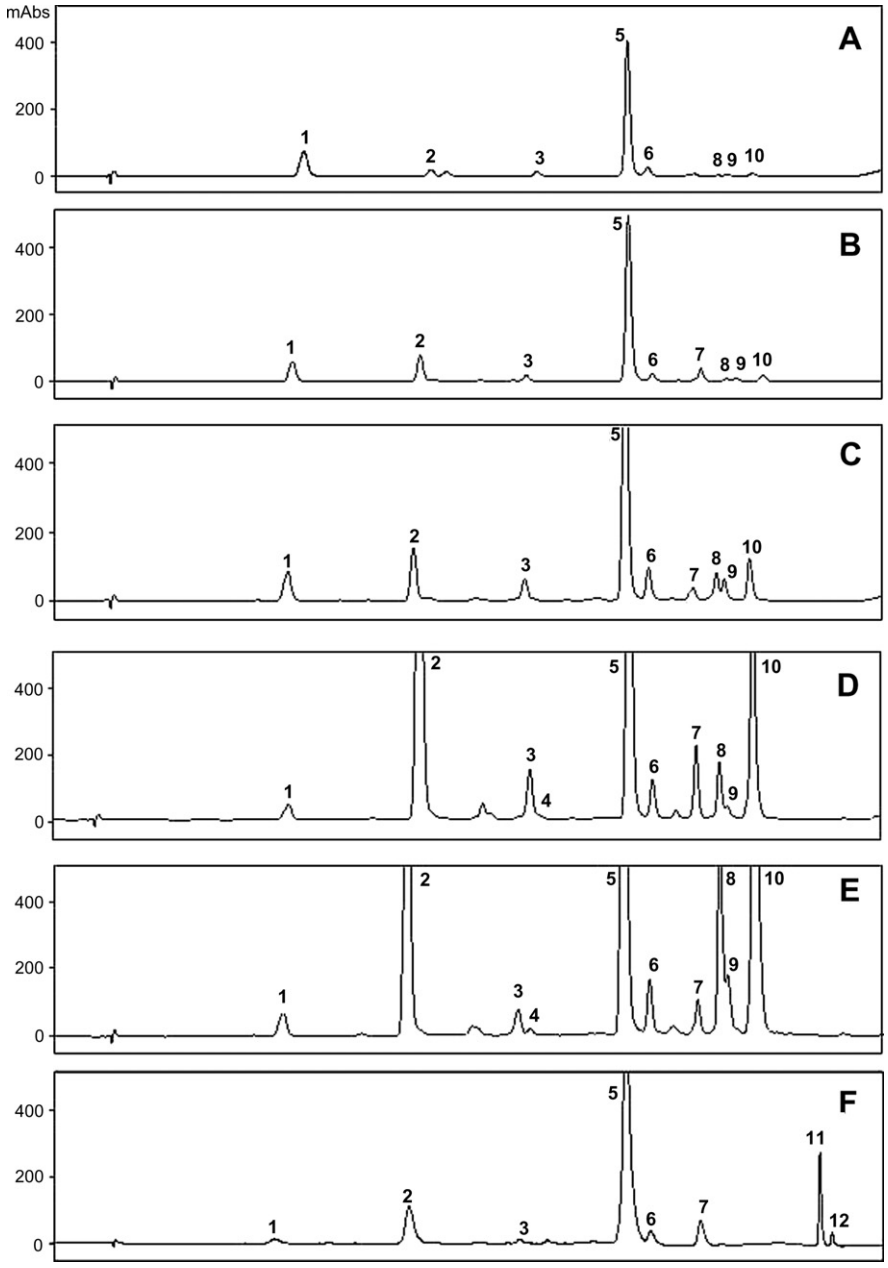

Fig. 1. HPLC profiles (330 nm) of lettuce varieties and escarole. (A) iceberg, (B) romaine, (C) continental, (D) red oak leaf, (E) lollo rosso, (F) escarole. Peak identifications: (**1**) caffeoyltartaric acid; (**2**) chlorogenic acid; (**3**) caffeoylmalic acid; (**4**) quercetin-7-*O*-glucuronide-3-*O*-(6″-*O*-malonyl)-glucoside (**4a**) + quercetin 7-*O*-glucoside-3-*O*-(6″-*O*-malonyl)-glucoside **(4b)**; (**5**) chicoric acid; (**6**) *meso*-di-*O*-caffeoyltartaric acid; (**7**) isochlorogenic acid; (**8**) quercetin-3-*O*-glucuronide (**8a**) + quercetin-3-*O*-glucoside (**8b**) + quercetin-3-*O*-rutinoside (**8c**); (**9**) Luteolin-7-glucuronide (**9a**) + Luteolin-7-glucoside **(9b)** + Luteolin-7-rutinoside (**9c**); (**10**) quercetin-3-*O*-(6″-*O*-malonyl)-glucoside; (**11**) kaempferol-3-*O*-glucuronide; (**12**) kaempferol-3-*O*-(6″-*O*-malonyl)-glucoside.

Lettuce flavonoids have been previously studied (Dupont et al., 2000; Ferreres et al., 1997), showing that quercetin-3-*O*-(6″-*O*-malonyl)-glucoside (compound **10**), is the main phenolic compounds with other quercetin and luteolin derivatives also detected in smaller amounts. In the present study we have identified by HPLC-DAD-MS/MS ESI, some new compounds that had not been previously described in lettuce (Table 1).

In red lettuce, a peak in trace amounts was observed (peak **4**) (Fig. 1D and E) in which two compounds co-eluted with deprotonated molecular ions at *m*/*z* 725 (**4a**) and 711 (**4b**). They showed MS fragmentations with common characteristics (Table 1, Fig. 2). Both flavonoids showed for –MS2[M–H]$^-$ a loss of 44 m.u. from the deprotonated molecular ion indicating a decarboxylation from the di-carboxylic acid linked to the flavonoid glycoside. In the fragmentation –MS3[(M–H) → (M–H–44)]$^-$ losses of 176 (glucuronic) and 162 (hexose) were observed, respectively to yield ions at *m*/*z* 505 (the base peak). This type of fragmentation, in which the loss of a sugar gives the most abundant base peak different from the base peak of the aglycone, indicates that there is a glycosylation in more than one phe-

Table 1
MS″ data of hydroxycinnamic acids and summary of major product ions of flavonoid in lettuce varieties and escarole

Compounds[a]		[M–H]$^-$ *m/z*	–MS2[M–H]$^-$ *m/z* (%)								
			−162	−180	A[b]	B[b]					
1	CTA	311	149(100)[c]		179(86)	135(10)					
2	5-CQA	353	191(100)[c]		179(5)						
3	CMA	295	133(76)[c]		179(100)	135(10)					
5	DCTA	473	311(100)	293(55)							
6	mDCTA	473	311(100)	293(53)							
							–MS3[353]$^-$ *m/z*(%)				
7	3,5-DCQA	515	353(100)		179(3)		191(100)	179(25)			
			–MS2[M–H]$^-$ *m/z* (%)		–MS3[(M–H) → (M–H–44)]$^-$ *m/z* (%)				–MS4[→→505]$^-$ *m/z* (%)		
			–44 (–CO_2)	–44 –176	–42[d]	–176/162	–42 –162	Aglycone-2H[e]/H	–42[d]	Aglycone-2H[e]/H	
Flavonoid-malonylglycosides											
4a	Q-3MG-7Gc	725	681(100)	505(55)		505(100)		300(28)	463(30)	300(100)	
4b	Q-3MG -7G	711	667(100)			505(100)	463(37)	300(49)		300(100)	
10	Q-3MG	549	504(100)		462(33)			300(100)			
12	K-3MG	533	489(100)		447(2)			285(100)			
			Aglycone-2H[e]/H								
Flavonoid glycosides											
8a	Q-3Gc	477	300(100)								
8b	Q-3G	463	300(100)								
8c	Q-3R	609	300(100)								
9a	L-7Gc	461	285(100)								
9b	L-7G	447	285(100)								
9c	L-7R	593	285(100)								
11	K-3Gc	461	285(100)								

[a] CTA: caffeoyltartaric acid; 5-CQA: 5-*O*-caffeoylquinic acid (chlorogenic acid); CMA: caffeoylmalic acid; DCTA: dicaffeoyltartaric acid (chicoric acid); mDCTA: *meso*-dicaffeoyltartaric acid; 3,5-DCQA: 3,5 dicaffeoylquinic acid (isochlorogenic acid); Q-3MG-7Gc: Quercetin-3-MalonylGlucoside-7-Glucuronide; Q-3MG-7G: Quercetin-3-MalonylGlucoside-7-Glucoside; Q-3MG: Quercetin-3-MalonylGlucoside; K-3-MG: Kaempferol-3-MalonylGlucoside; Q-3Gc: Quercetin-3-Glucuronide; Q-3G: Quercetin-3-Glucoside; Q-3R: Quercetin-3-Rutinoside; L-7Gc: Luteolin-7-Glucuronide; L-7G: Luteolin-7-Glucoside; L-7R: Luteolin-7-Rutinoside; K-3Gc: Kaempferol-3-Glucuronide.

[b] Caffeic acid derived fragments.

[c] Ions from loss of caffeoyl acid moiety (162): 149 [Tartaric-H]$^-$; 191 [Quinic-H]$^-$; 133 [Malic-H]$^-$.

[d] Decarboxilated malonyl moiety.

[e] Fragments from homolytic cleavage of the glycosidic bond (Hvattum & Ekeberg, 2003).

nolic hydroxyl (Ferreres, Llorach, & Gil-Izquierdo, 2004; Llorach, Gil-Izquierdo, Ferreres, & Tomás-Barberán, 2003) and in our case indicates the presence of glucuronic acid and glucose in position 7 of compounds **4a** and **4b**, respectively. The loss of glucose carrying the rest of the decarboxylated malonyl residue from the ion at *m/z* 505 (–MS4[505]$^-$) gives rise in both cases to the aglycone (quercetin). Therefore, these compounds were characterized as quercetin-7-*O*-glucuronide-3-*O*-(6″-*O*-malonyl)-glucoside (**4a**) and quercetin-7-*O*-glucoside-3-*O*-(6″-*O*-malonyl)-glucoside (**4b**). The occurrence of the glucuronidated derivative had already been reported recently (Llorach et al., 2004).

Peaks **8** and **9** (Fig. 1) revealed that both quercetin and luteolin derivatives coeluted, and they were identified by MS (Table 1) as quercetin-3-*O*-glucuronide (**8a**), quercetin-3-*O*-glucoside (**8b**), quercetin-3-*O*-rutinoside (**8c**), luteolin-7-*O*-glucuronide (**9a**), luteolin-7-*O*-glucoside (**9b**) and luteolin-7-*O*-rutinoside (**9c**). All these compounds are not always detected in all cultivars, and are found in trace amounts with the exception of red lettuce cultivars. Therefore, the rutinosyl derivatives are the compounds detected less frequently and in lower proportion, and it is probably the reason for not being reported earlier in lettuce. According to this, luteolin derivates have been scarcely identified in lettuce (Dupont et al., 2000) and the only luteolin derivative previously characterized was the glucuronide.

In addition, kaempferol-3-*O*-glucuronide (**11**) and kaempferol-3-*O*-(6″-*O*-malonyl)glucoside (**12**) has been characterized in escarole (Table 1). It is in agreement with previous work where these kaempherol derivatives had been previously identified in different varieties of *Cichorium endivia* (Dupont et al., 2000). In red lettuce varieties, cyanidin-3-*O*-(6″-*O*-malonyl)-glucoside (+MS: 535 [M]$^+$; +MS2[M]$^+$: 287 [Cyanidin]) was also identified.

3.1.2. Quantification of phenolic compounds

The phenolic contents of these salad vegetables were quantified by HPLC-DAD and significant differences in the content of polyphenols between green varieties, red varieties and escarole were detected (Table 2). The main compounds in green lettuces were phenolic acids with a percentage of total phenols between 70% and 94% in

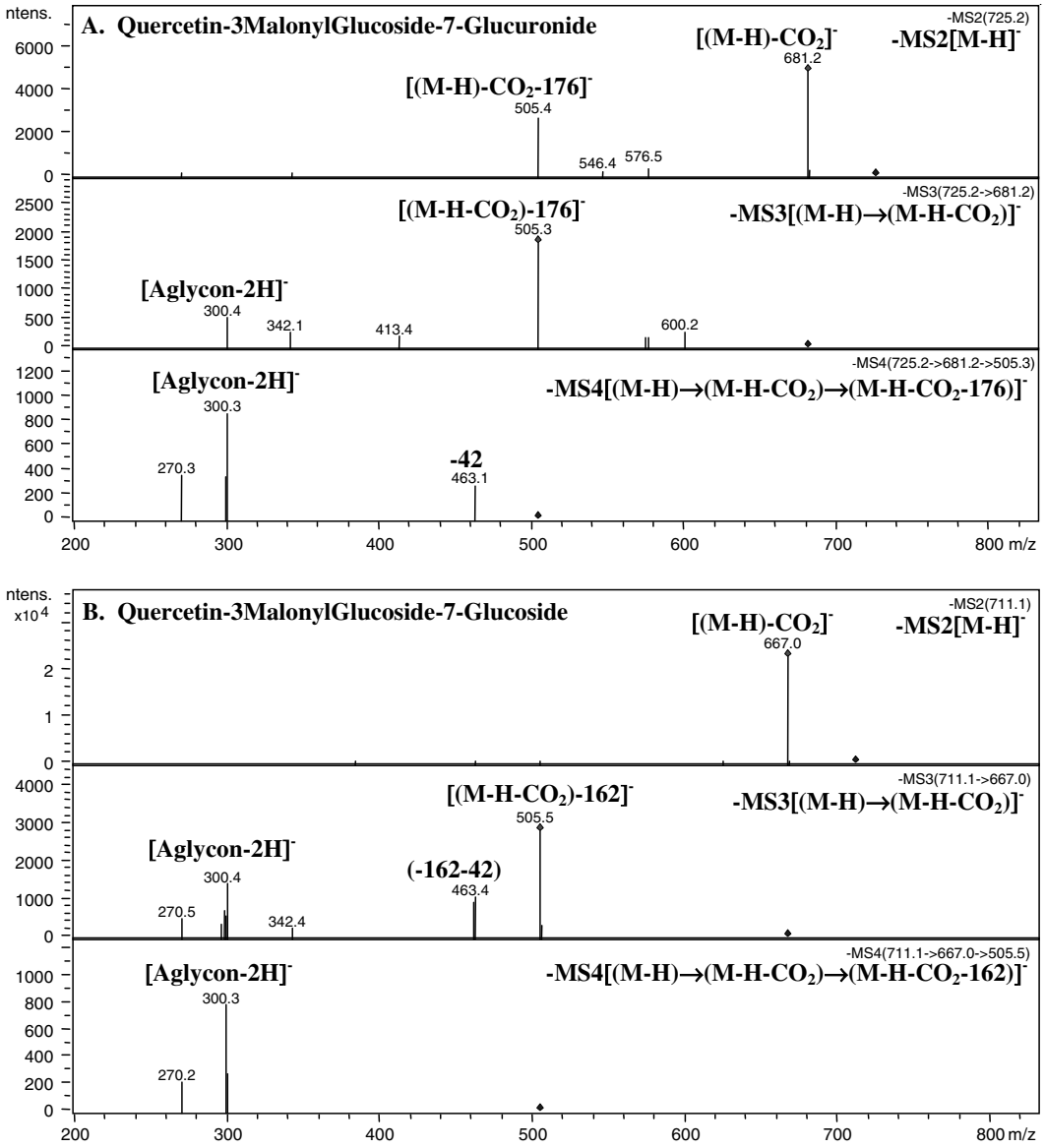

Fig. 2. MS/MS of Quercetin-7-*O*-glucuronide-3-*O*-(6″-malonyl)-glucoside (A) and Quercetin-7-*O*-glucoside-3-*O*-(6″-malonyl)-glucoside (B).

romaine and iceberg varieties, respectively, whereas in red lettuces this percentage decreased to 35% and 45% in lollo rosso and red oak leaf varieties, respectively. Concerning other phenolic compounds, flavonols were the major compounds followed by anthocyanins (identified only in red lettuce varieties) and flavones (Table 2).

The total phenolics content of *Lactuca* and *Cichorium* varieties varied from 18 to 571 mg per 100 g, in iceberg and lollo rosso, respectively. In relation to caffeic acid derivatives, the studied of *Lactuca* and *Cichorium* varieties showed a content ranging from 17 to 203 mg per 100 g, the flavonols content ranged from 1 to 285 mg per 100 g, and flavones content ranged from 0.3 to 38 in iceberg and lollo rosso, respectively (Table 2). Flavones were no detected in escarole samples and anthocyanins were only present in red lettuce varieties, with a content of 26 and 46 mg per 100 g in red oak leaf and lollo rosso, respectively (Table 2). Thus, *Lactuca* and *Cichorium* showed notable levels of phenolic compounds into the range that other vegetables have been considered important sources of polyphenols, such as, apples, tea, broccoli and onions (Hollman & Arts, 2000; Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). These data are important because the polyphenols act as natural antioxidants and may be these compounds are part of the reason for the protective effect against degenerative diseases when this type of food is a significant part of the diet (Dupont et al., 2000). However, different agronomic or environmental conditions and tissue type could affect the phenolic content observed in vegetables (Dupont et al., 2000; Manach et al., 2004; Nicolle et al., 2004b; Tomás-Barberán, Ferreres, & Gil, 2000).

The phenolic content of plants depends both qualitatively and quantitatively on their genetic information (variety). In general, red -leafed vegetables showed a higher

Table 2
Polyphenols, vitamin C and antioxidant activity of lettuce varieties and escarole

Samples	Polyphenols[a,c]					AA[a,c]	DHA[a,c]	Vitamin C[a,c]	Antioxidant activity[b,c]		
	Caffeic acid derivatives	Flavones	Flavonols	Anthocyanins	Total phenolics				DPPH	ABTS	FRAP
Iceberg	17.1 ± 0.5^{f}	0.3 ± 0.1^{e}	0.8 ± 0.1^{e}	N.D.	18.2 ± 0.6^{f}	2.2 ± 0.4^{d}	2.0 ± 0.3^{d}	4.2 ± 0.7^{f}	68.6 ± 5.9^{d}	61.3 ± 5.4^{d}	98.2 ± 11.0^{d}
Romaine	44.8 ± 2.4^{e}	$2.3 \pm 0.3^{b,c}$	$16.6 \pm 1.1^{d,e}$	N.D.	63.5 ± 3.5^{e}	1.5 ± 0.0^{e}	1.3 ± 0.0^{e}	2.8 ± 0.1^{e}	99.7 ± 16.4^{d}	89.5 ± 7.2^{d}	114.3 ± 3.8^{d}
Continental	102.1 ± 4.4^{d}	3.2 ± 0.2^{b}	20.2 ± 0.7^{d}	N.D.	125.5 ± 5.0^{d}	12.3 ± 0.0^{a}	7.2 ± 0.8^{a}	19.5 ± 0.8^{a}	244.1 ± 15.8^{c}	193.2 ± 11.9^{c}	323.4 ± 32.8^{c}
Red oak leaf	146.1 ± 2.6^{b}	35.8 ± 0.7^{a}	114.4 ± 2.1^{c}	25.9 ± 0.7^{b}	322.1 ± 6.1^{b}	8.2 ± 0.6^{b}	6.3 ± 0.5^{a}	14.6 ± 1.0^{b}	585.2 ± 95.1^{b}	412.1 ± 63.2^{b}	665.9 ± 22.3^{b}
Lollo rosso	203.0 ± 11.9^{a}	38.0 ± 2.2^{a}	284.6 ± 16.3^{a}	45.6 ± 2.7^{a}	571.2 ± 33.1^{a}	6.9 ± 0.5^{c}	4.9 ± 0.4^{b}	11.7 ± 0.8^{c}	775.3 ± 85.8^{a}	647.8 ± 16.3^{a}	814.4 ± 38.2^{a}
Escarole	127.2 ± 7.4^{c}	N.D.	132.0 ± 8.7^{b}	N.D.	259.1 ± 15.6^{c}	7.0 ± 0.7^{c}	3.2 ± 0.4^{c}	10.2 ± 1.1^{d}	100.9 ± 3.2^{d}	93.4 ± 9.5^{d}	132.6 ± 29.1^{d}

[a] Values are mg 100 g^{-1} fresh weight ± standard deviation ($n = 3$).
[b] Values are mg TEAC 100 g^{-1} fresh weight ± standard deviation ($n = 3$).
[c] Values in the same column with the same superscript letters are not significantly different ($P \leqslant 0.05$).

content in diverse aglycones and caffeic acid derivatives than green lettuce varieties and escarole (Table 2). This is in agreement with previous work showing that coloured varieties of vegetables (red onion, red cabbage, and red pepper) are especially rich in phenolic compounds (Nicolle et al., 2004b; Stratil, Klejdus, & Kubán, 2006). Therefore, the variability in the compositions and quantities of compounds in different foods indicate the importance of eating a variety of food sources in particular, coloured foods, in every meal.

3.2. Vitamin C

The content of AA, DHA and vitamin C (AA+DHA) of lettuce varieties and escarole are show in Table 2. Vitamin C levels ranged between 2.8 (romaine) and 19.5 (continental) mg 100 g^{-1} f.w. The highest content of vitamin C was observed in a green lettuce, the continental variety, followed by red lettuce varieties. The vitamin C content of continental is higher than those previously reported for other lettuce and escarole cultivars that range between 3 and 15 mg/100 f.w. vitamin C (Bahorun, Luximon-Ramma, Crozier, & Aruoma, 2004; Nicolle et al., 2004a; Szeto et al., 2004).

Regarding both AA and DHA content, significant differences were observed in the samples (Table 2). Lettuces have a high content of DHA, thus overall proportion of AA was 57% while DHA proportion was 43% in these lettuce varieties. AA is the principal biologically active form but L-dehydroascorbic acid (DHA), an oxidative product, can be easily converted into AA in the human body. So it is important to measure both forms, AA and DHA, for vitamin C activity (Lee & Kader, 2000). However, although dehydroascorbic acid is considered like vitamin C, it does not have any antioxidant activity (Nicolle et al., 2004b).

In terms of the relative dietary contributions of a given food, it is not only the nutrient concentrations, but also the level of consumption of the food that are important. Therefore, significant differences were observed between varieties (Table 2). Nevertheless, the vitamin C content not only depends of varieties, other preharvest and postharvest factors can influencing in the vitamin C content (Lee & Kader, 2000). Thus, the ascorbic acid levels could increase in response to high light intensities (Nicolle et al., 2004b). However the selection of the genotype with the highest vitamin C content for a given commodity is a very important factor (Lee & Kader, 2000).In this context, the recommend intake of vitamin C reported by F.A.O. (FAO, 2001) is between 25 and 70 mg per day, thus although the vitamin C content of lettuce is modest a serving of these lettuce varieties could provide around of 26% of this daily intake.

3.3. Antioxidant activity

Three in vitro assays (DPPH, ABTS and FRAP) were used as complementary methods to evaluate the potential

antioxidant activity. Significant differences were observed between the different varieties in the three assays. The antioxidant activity values determined by DPPH, ABTS and FRAP assays for individual varieties decreased in the order; lollo rosso > red oak leaf > continental, while the lowest values were for escarole, romaine and iceberg (Table 2).

The DPPH· scavenging activity of red varieties of lettuce was significantly higher than those of green lettuce and escarole with a range from 1 to 11 fold difference between varieties. Lollo rosso showed the highest antioxidant activity value (775 mg TEAC 100 g^{-1} f.w.), while iceberg, romaine and escarole showed the lowest values (69, 100 and 101 mg TEAC 100 g^{-1} f.w., respectively) (Table 2). The ABTS· scavenging activity of red varieties of lettuce was significantly higher than those of green lettuce and escarole, as in the DPPH assay, showing a range between 1.6 and 10.5 fold difference between varieties. In this assay, once again, lollo rosso showed the highest antioxidant activity (648 mg TEAC 100 g^{-1} f.w.), while iceberg, romaine and escarole showed the lowest values (61, 89 and 93 mg TEAC 100 g^{-1} f.w., respectively) (Table 2). The ferric reducing antioxidant power (FRAP) showed the same trend observed in the previous DPPH and ABTS assays. Thus, red lettuce varieties showed significantly higher activities than green lettuce and escarole with a range from 2 to 8 fold difference between varieties. In this assay, once more, lollo rosso showed the highest antioxidant activity value (814 mg TEAC 100 g^{-1} f.w.), while iceberg, romaine and escarole showed minor values (98, 114 and 133 mg TEAC 100 g^{-1} f.w., respectively) (Table 2). These results agree with previous work reporting the highest antioxidant power in red lettuce vs. green lettuce (Liu et al., 2007; Nicolle et al., 2004b). The high antioxidant activity of red lettuce could be due to the strong antioxidant activity of anthocyanins (Rice-Evans et al., 1997). Moreover, the darker green and red varieties had higher vitamin C and phenols content as well as antioxidant activity. However, escarole, romaine and iceberg showed different total phenolic contents, nevertheless, the antioxidant activity was not significantly different (Table 2). Thus, there are other active compounds in these varieties that contribute to its antioxidant activity, such as carotenoids and vitamin E.

3.4. Chemometric analysis

A multiparametric approach with both hierarchical cluster analysis (HCA) and principal component analysis (PCA) was carried out in order to evaluate the influence of tested parameters in the classification and differentiation of lettuce and escarole samples. A data matrix contained the data of Table 2 was subjected to a HCA of samples, taking the euclidean distance as metric and Ward's method as the agglomeration rule. The dendrogram obtained is shown in Fig. 3A. Two main clusters can be observed, one for the red lettuce and other one for the green lettuce and escarole. This separation could be expected and

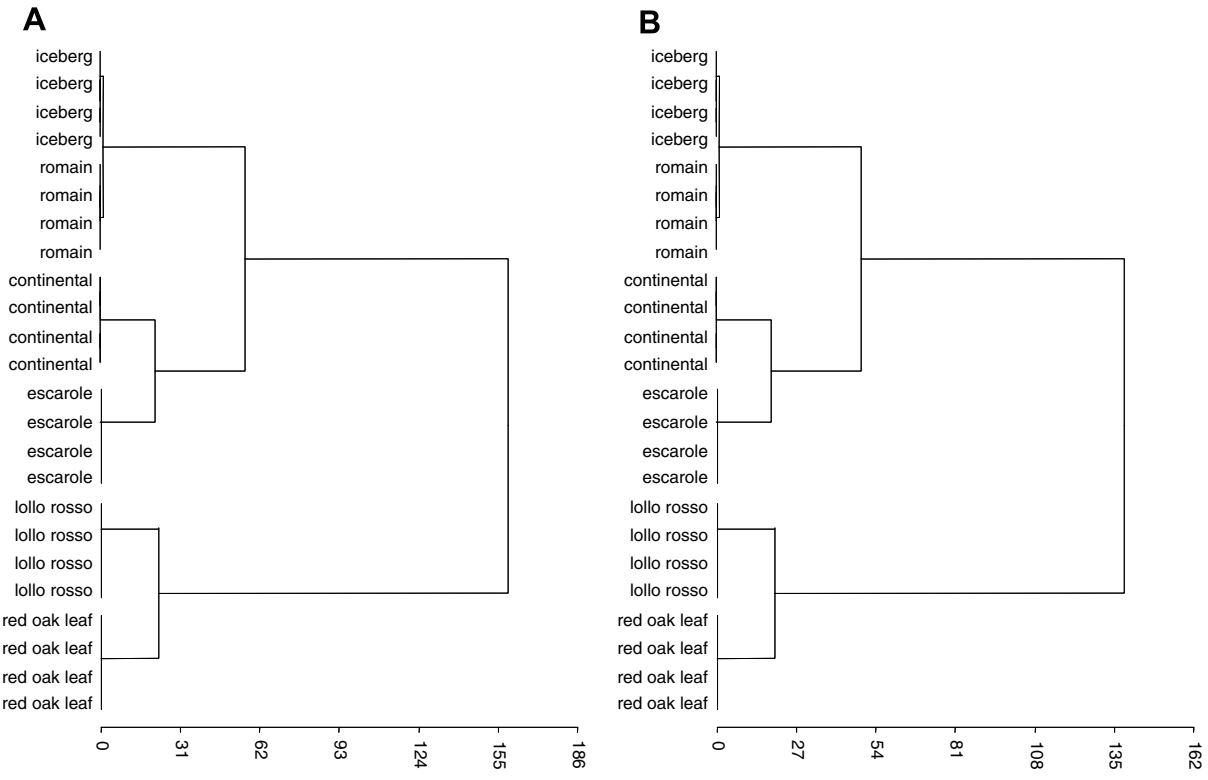

Fig. 3. Dendogram obtained after hierarchical cluster analysis on polyphenols, vitamin C and antioxidant activity of lettuce and escarole samples (A). Dendogram obtained after hierarchical cluster analysis without anthocyanins content (B).

explained by the presence of anthocyanins in the red lettuce varieties. Nevertheless, Fig. 3B shows the same separation pattern even if the anthocyanin content was removed for the analysis. Continental and escarole were clearly separated from iceberg and romaine. These last varieties are very near and showed small difference between them.

In the PCA, a non supervised multivariate analysis was used in order to discriminate between lettuce and escarole samples. A total of 93.38% of difference was explained by relation between principal component **1** vs. principal component **2** (PC1 vs. PC2). Principal component **1** was responsible for 72.39% while component **2** was responsible for 20.99% of the difference. In fact, PC1 allowed differentiation between green (including escarole) and red lettuces (Fig. 4A). Regarding the distribution of the evaluated parameters, the phenolic composition and antioxidant capacity were responsible for the separation in the PC1 while vitamin C (as well as both forms AA and DHA) content was mainly linked to the PC2 separation.

The results show that plants of *Asteraceae* family are relevant food providing interesting antioxidant compounds in enough amounts to the human diet, especially

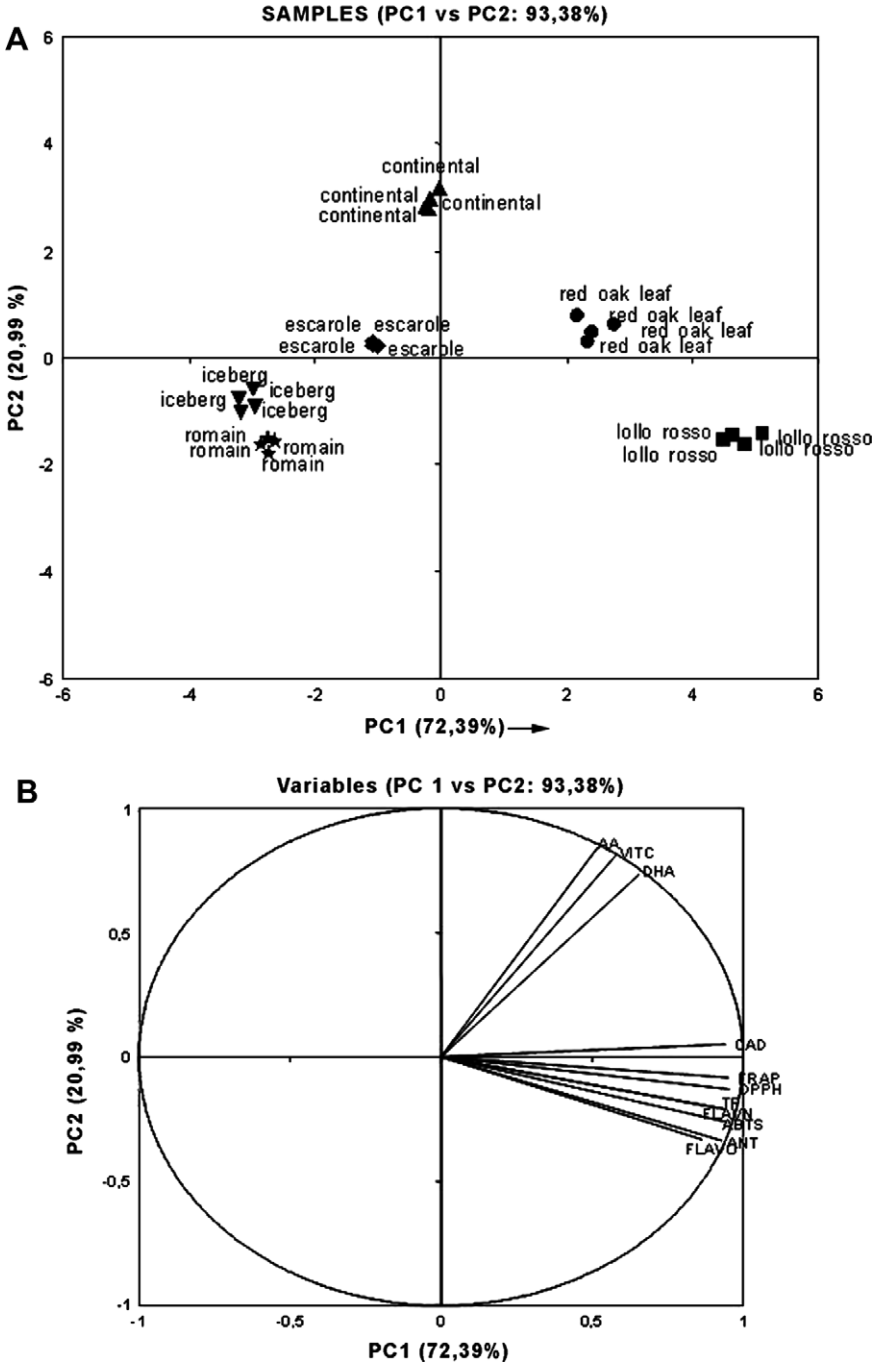

Fig. 4. PCA of lettuce and escarole samples (A). PCA of variables (B).

the anthocyanin-rich samples (the red varieties) which contain not only a large quantity of polyphenols but also an important diversity. Red lettuce varieties showed the highest content of phenolic compounds and had the strongest antioxidant activities than other varieties as green lettuce and escarole within the same growing conditions. However, the green continental lettuce had the highest content of vitamin C. In this study, the genotype has been shown to be an important factor affecting the polyphenol profile and the content vitamin C as well as the antioxidant activity. Thus, green dark varieties showed a high content of antioxidant compounds and escarole showed different flavonoids in the polyphenol profile. The variability in the compositions and quantities of compounds and antioxidants in different foods indicate the importance of eating a variety of food sources in particular, coloured foods, in every meal.

4. Conclusions

The study of polyphenol profiles by HPLC-DAD-MS/MS ESI allowed the identification of two compounds, quercetin and luteolin rhamnosyl-hexosides, not previously reported in lettuce. Differences were observed between the polyphenol profiles among green and red lettuce and escarole. Caffeic acid derivatives were the main polyphenols in green varieties, while flavonols were observed in higher quantities in red varieties and escarole, and anthocyanins were only present in red-leafed varieties. Moreover, lettuce and escarole showed differences in the flavonol composition, as quercetin derivatives were only observed in lettuce samples while kaempferol derivatives were only detected in escarole samples. As a general rule, red-leafed vegetables showed a higher content in both flavonol and caffeic acid derivatives than green lettuce varieties and escarole. Concerning vitamin C, red-leafed salads showed higher content than green-leafed salad vegetables with the exception of the continental variety which showed the highest level. Red varieties showed the highest antioxidant activity for all in vitro methods assayed. This study could help further research concerning the bioavailability of these flavonoids for epidemiological or clinical intervention studies. These red-leafed varieties have considerable potential as healthy leafy salads due to their bioactive phytochemicals.

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