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Determination of Carotenoids and their Esters in Fruits of Sea Buckthorn (*Hippophae rhamnoides* L.) by HPLC-DAD-APCI-MS

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ABSTRACT:

Introduction – The berries of *Hippophae rhamnoides* Linnaeus have high nutritional and medicinal values and have been used for centuries as food both in Europe and Asia. The oleoresins represent a potential source of carotenoid esters and can be used as food additives, cosmetic ingredients or nutraceuticals.

Objective – The objective of this study was to develop a HPLC-DAD-APCI-MS method, with both positive and negative ionisation modes, for the direct identification of the native carotenoid composition in fruits of *Hippophae rhamnoides*.

Materials and methods – Fruits of *Hippophae rhamnoides*, *cv*. Serbanesti and Victoria, were collected from an experimental field at the Fruit Research Station of Bacau, Romania. Samples were extracted using methanol:ethyl acetate:petroleum ether (1:1:1, v/v/v). The HPLC-DAD-APCI-MS analyses were carried out on a Shimadzu system using a YMC C_{30}-column and a gradient elution.

Results – In total 22 compounds were detected, eight were free carotenoids, nine were xanthophylls monoesters and five were xanthophylls diesters. Differences were observed in the relative percentage composition of the identified components among the two cultivars investigated. Zeaxanthin-C16:0,C16:0 was the most abundant diester. The unsaturated palmitoleic acid was directly detected in its esterified form, in zeaxanthin-C16:0,C16:1, which is reported here for the first time. Although present in small amounts the unsaturated oleic, linoleic, linolenic, hexadecadienoic and hexadecatrienoic acids were detected in their esterified forms as lutein monoesters, the last two having been detected in *Hippophae rhamnoides* for the first time.

Conclusion – A novel (HPLC-DAD-APCI-MS) method was developed for the direct identification of the native carotenoid composition in fruits of *Hippophae rhamnoides*. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: HPLC-DAD-APCI-MS; Carotenoid esters; *Hippophae rhamnoides*

Introduction

The berries of *Hippophae rhamnoides* Linnaeus have high nutritional and medicinal values and have been used for centuries as food both in Europe and Asia (Beveridge *et al*., 1999; Tiitinen *et al*., 2005). Carotenoids have been described as possessing several important functional properties, mainly antioxidant activity (Beutner *et al*., 2001; Caris-Veyrat 2008), as well as prevention of cardiovascular diseases (Arab and Steck, 2000; Rao and Rao, 2007), cancer (Nishino *et al*., 1999), and macular degeneration (Snodderly, 1995; Krinsky and Johnson, 2005) and in some cases, provitamin A activity (Krinsky and Johnson, 2005). Such properties make these compounds ideal for the ever increasing health food industry as well as promoting the consumption of the natural products in which are contained. The chemical structure of carotenoids is usually based in a C_{40} tetraterpenoid moiety with a centrally located, extended conjugated double bond system that acts as the light absorbing chromophore. Taking into account their chemical structure, these compounds can be divided into two different groups: first, hydrocarbon carotenoids, generally termed carotenes; and oxygenated carotenoids, commonly known as xanthophylls. This second group is the most complex one in terms of number of compounds and variations in their structure, and can be found in either its free form or in a more stable fatty acid esterified form in the case of mono- and polyhydroxylated xanthophylls. Thus, in view of the fact that a single carotenoid could be found forming different esters, the already complex natural variability of carotenoids is often increased by the formation of these carotenoid esters. For this reason, to simplify the analysis of these compounds the xanthophylls have most

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frequently been analysed after a saponification step that releases the free xanthophylls, thus losing information about the native composition of the matrix; moreover, during the saponification step, degradation and artefact formation of the carotenoids may occur (Oliver *et al*., 1998). Thus, the study of intact carotenoids (samples without saponification) composition could be useful for increasing knowledge about natural carotenoids and relationships between them, and for establishing authenticity markers among varieties that could potentially be used to prevent adulterations. The characteristic conjugated double bond system of carotenoids produces the main problem associated with this work and with manipulation of carotenoids; i.e. their instability, especially towards light, heat, oxygen and acids. Therefore, a reliable determination of these components in any matrix depends on several precautions being taken at the various steps of analysis, from sampling, to extraction and HPLC analysis (Taungbodhitham *et al*., 1998; Oliver and Palou, 2000; Dias *et al*., 2010; Mertz *et al*., 2010). The correct characterisation of these compounds is necessary to obtain reliable compositional data for realistic and valuable conclusions in nutritional studies. Thus a better approach to carotenoid content is through classifying plant materials depending on a free or esterified xanthophylls profile.

The sea buckthorn oleoresins represent a potential source of carotenoid esters and can be used as food additives, cosmetic ingredients or nutraceuticals. Previous investigations on sea buckthorn berries have shown differences in composition and content of carotenoid, but the exact native carotenoids profile in sea buckthorn remains not fully characterised (Kudritskava *et al*., 1990; Beveridge *et al*., 1999; Bekker and Glushenkova, 2001; Weller and Breithaupt, 2003; Raffo *et al*., 2004; Pintea *et al*., 2005; Parlog *et al*., 2009; Andersson *et al*., 2009).

Sea buckthorn represents a very complex matrix (Tiitinen *et al*., 2005), due to the high oil content – up to 34% in the soft part of the berries. Beside neutral and complex lipids, the high amount of other lipophilic compounds such as tocopherols, tocotrienols and phytosterols (Yang and Kallio, 2001) complicates the analysis of unsaponified extract of sea buckthorn. The fatty acids profile of the carotenoids ester fraction in sea buckthorn berries as determined by GC-MS analysis after transesterification has been reported previously by Pintea *et al*. (2005). Parlog *et al*. (2009) reported the presence of xanthophylls esters fractions in sea buckthorn berries carotenoids extracts determined by HPLC-PDA analysis on a C_{18}-column. Weller and Breithaupt (2003) reported the presence of zeaxanthin dipalmitate and zeaxanthin myristate-palmitate in sea buckthorn berries determined by [LC-(APCI-positive)MS] using a C_{30}-column.

In view of the possible beneficial properties of the carotenoids on human health (Britton *et al*., 2009), our objective was to develop a better HPLC-DAD-APCI-MS method to determine both free carotenoids and carotenoid esters in fruits of *Hippophae rhamnoides* in order to provide information on its specific carotenoids fingerprint.

Experimental

Chemicals

All the reagents and solvents used were of analytical or HPLC grade and were purchased from Sigma-Aldrich (Milan, Italy). Carotenoids standards, namely β-carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin, lutein-di-palmitate and physalein, were purchased from Extrasynthese (Genay, France).

Sample collection

Fully mature fruits of sea buckthorn (*Hippophae rhamnoides* L., ssp. *Carpatica*, *cvs*. Serbanesti and Victoria) were collected from an experimental field at the Fruit Research Station in Bacau, Romania. The cultivars analysed were at the same developmental stage and the fruits were of similar size. Samples were stored at −20° until they were analysed.

Carotenoids extraction

The samples were homogenised using an Ultraturax and aliquots of 20 g were extracted with a mixture of methanol:ethyl acetate:petroleum ether (1:1:1, v/v/v) containing butylated hydroxytoluene (BHT) as antioxidant, according to Pintea *et al*. (2005). The extractions were carried out at room temperature and were made under continuous stirring for 4 h in darkness conditions. The extract was washed successively with water, diethyl ether and NaCl saturated solution. The ether phase was evaporated using a rotavapor at 35 °C. The oleoresin obtained was dissolved in 3 mL of ethyl acetate prior to HPLC analysis.

HPLC-DAD-APCI-MS analysis

The analyses were carried out on an HPLC system (Shimadzu, Milan, Italy) equipped with a CBM-20A controller, two LC-20 AD pumps, a DGU-20A3 degaser, a SIL-20 AC autosampler and a SPD-M20A photo diode array detector. The data were processed with the software Labsolution version 5.10.153 (Shimadzu). For MS analyses a mass spectrometer was used (LCMS-2020, Shimadzu), equipped with an APCI interface, both in positive and negative ionisation modes. Separations were performed on a YMC C_{30}-column (250 × 4.6 mm, 5 μm); the mobile phases consisted of methanol:MTBE:water (83:15:2,v/v/v; eluent A) and methanol:MTBE:water (8:90:2, v/v/v; eluent B), using a gradient program as follows: 0 min 0% B; 20 min 0%B; 160 min 100% B; 161 min 0% B. The flow rate was 0.8 mL/min and the injection volume was 20 μL. The UV-vis spectra were acquired in the range of 250–600 nm, while the chromatograms were extracted at 450 nm (sampling frequency: 15625 Hz; time constant: 0.64 s).

The MS was set up as follows: scan, both APCI positive (+) and negative (−); nebulising gas flow (N_2): 4.0 L/min; event time: 1 s; detector voltage: 0.8 kV; *m/z* range: 350–1200; interface voltage: ±4.5 kV; interface

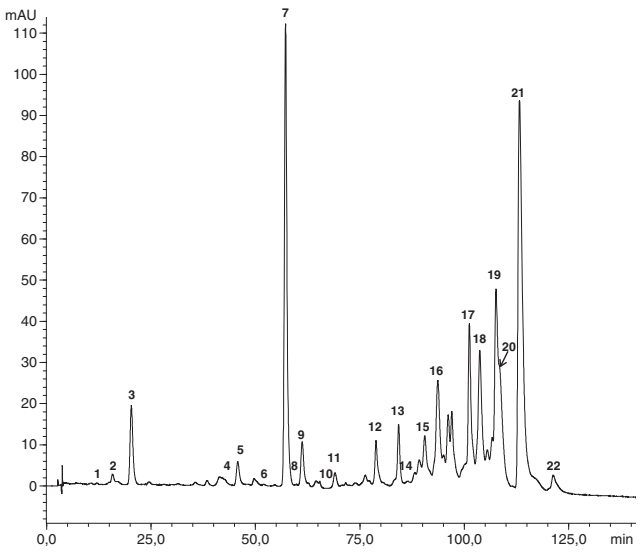

Figure 1. Chromatogram (450 nm) of Sea Buckthorn extract (*cv*. Serbanesti), using a C_{30}-column. For peak identification, see Table 1.

Table 1. UV-vis and MS (APCI (+) and APCI (−), information and identification of the carotenoids found in native sea buckthorn extract

ID	Identification	UV-vis maxima	MS data APCI (+) e (−)
1	Neoxanthin	416, 439, 468	601 (+); 600 (−)
2	Lutein	422, 442, 473	551 (+); 568 (−)
3	Zeaxanthin	427, 450, 476	569 (+); 568 (−)
4	Lutein-C16:3	422, 442, 473	801 (+); 799 (−)
5	n.i.	335, 421, 442, 466	537 (+); 536 (−)
6	Lutein-C18:3	421, 442, 472	829 (+); 827 (−)
7	βcarotene	425, 450, 477	537 (+); 536 (−)
8	Lutein-C16:2	422, 442, 473	803 (+); 801 (−)
9	α-carotene	423, 444, 473	537 (+); 536(−)
10	Lutein-C18:2	421, 442, 472	831 (+); 829 (−)
11	Zeaxanthin-C14:0	427, 450, 476	779 (+); 778 (−)
12	Zeaxanthin − C16:0	427, 450, 476	807 (+); 806 (−)
13	β-cryptoxantin-C14:0	428, 450, 477	763 (+), 535 (+); 762 (−)
14	Lutein-C18:1	421, 442, 473	833 (+); 831 (−)
15	γ-carotene	436, 458, 490	537 (+); 536 (−)
16	β-cryptoxantin-C16:0	428, 450, 477	791 (+), 535 (+); 790 (−)
17	Zeaxanthin-C14:0,C14:0	427, 450, 476	989 (+), 761 (+), 533 (+); 988 (−), 760 (−)
18	Zeaxanthin-C16:0,C16:1	427, 450, 476	1043 (+), 789 (+), 787(+), 533 (+); 1042 (−)
19	Zeaxanthin-C14:0,C16:0	427, 450, 476	1017 (+), 789 (+), 761 (+), 533 (+); 1016 (−), 788 (−), 760 (−)
20	Lutein-C16:0,C16:0	422, 442, 473	1045 (+), 789 (+), 533 (+); 1044 (−)
21	Zeaxanthin-C16:0,C16:0	427, 450, 476	1045 (+), 789 (+), 533 (+); 1044 (−)
22	Lycopene	442, 473, 503	537 (+); 536 (−)

n.i., not identified.

Table 2. Percentage composition of the identified compounds in two sea buckthorn cultivars investigated

ID	Compound	% *cv.* Serbanesti	% *cv.* Victoria
1	Neoxanthin	0.01 ± 0.01	0.08 ± 0.02
2	Lutein	0.27 ± 0.02	0.23 ± 0.03
3	Zeaxanthin	2.65 ± 0.36	3.21 ± 0.32
4	Lutein-C16:3	0.42 ± 0.03	0.62 ± 0.06
5	n.i.	0.88 ± 0.03	2.48 ± 0.43
6	Lutein-C18:3	0.24 ± 0.01	0.07 ± 0.01
7	βcarotene	14.68 ± 0.98	29.06 ± 2.04
8	Lutein-C16:2	0.03 ± 0.01	n.d.
9	α-carotene	1.97 ± 0.08	3.18 ± 0.42
10	Lutein-C18:2	0.2 ± 0.02	0.44 ± 0.02
11	Zeaxanthin-C14:0	0.56 ± 0.03	0.31 ± 0.01
12	Zeaxanthin-C16:0	1.54 ± 0.07	0.6 ± 0.02
13	β-cryptoxantin-C14:0	2.09 ± 0.46	0.9 ± 0.03
14	Lutein-C18:1	0.17 ± 0.01	0.23 ± 0.02
15	γ-carotene	2.39 ± 0.48	3.99 ± 0.32
16	β-cryptoxantin-C16:0	5.73 ± 0.64	3.18 ± 0.28
17	Zeaxanthin-C14:0,C14:0	6.95 ± 0.78	3.66 ± 0.26
18	Zeaxanthin-C16:0,C16:1	7.26 ± 0.62	4.68 ± 0.54
19	Zeaxanthin-C14:0,C16:0	9.13 ± 0.82	6.84 ± 0.62
20	Lutein-C16:0,C16:0	4.57 ± 0.74	3.75 ± 0.38
21	Zeaxanthin-C16:0,C16:0	21.27 ± 1.86	18.53 ± 1.08
22	Lycopene	1.22 ± 0.07	2.08 ± 0.22

Mean values of three determinations, ± SD.

temperature: 350 °C; CDL voltage: 0 V; CDL temperature: 300 °C; heat block: 300 °C; Q-array: 0.0 V; RF: 90 V; sampling: 2 Hz. Samples were analysed in triplicate.

Results and Discussion

Traditionally, the study of the carotenoids esters has been performed by laborious using time-consuming procedures, including the isolation of the ester fraction by column chromatography, saponification and methylation of the fatty acids prior to their analysis by GC.

Here we report a HPLC-DAD-APCI-MS method developed for the direct identification of the native carotenoid composition in fruits of *Hippophae rhamnoides*, which implies a faster analysis and simpler procedure. Figure 1 shows the HPLC chromatogram of the carotenoid composition in sea buckthorn fruit (*cv*. Serbanesti) extract without saponification, and the relative peak identification is shown in Table 1, together with the UV-vis and MS spectra information for the identified compounds. The HPLC qualitative profiles of the two investigated cultivars (*cv*. Serbanesti and *cv*. Victoria) were similar. In total 22 compounds were detected, eight were free carotenoids, nine were xanthophyll monoesters and five were xanthophyll diesters. Compounds were identified by comparison with available standards, by their UV-vis spectra and by their MS spectra recorded in both positive and negative APCI ionisation modes.

As far as detection is concerned, the combination of PDA and MS is mandatory if unambiguous structure assignment is desired, since it helps whenever similarity of the molecules results in overlapping UV spectra. Additionally, the possibility of rapid switchover between negative and positive ionisation mode in the APCI probe allowed us to collect more qualitative information about a sample in a single run, with quasi-molecular ion species dominating the MS spectrum in one case, or abundant fragmentation in the other. Because carotenoids contain an extended polyene chain that can stabilise either a negative or a positive charge, the complex ionisation process in the APCI of carotenoids produces molecular ions and/or protonated or deprotonated molecules depending upon the mobile phase conditions and fragment ions (van Breemen *et al*. 1996). Differences were observed in the relative percentage composition of the identified components in the two cultivars investigated, as shown in Table 2, where mean values ± SD are reported. Among the free carotenoids, β-carotene and zeaxanthin were the most abundant. Zeaxanthin was also the major esterified xanthophyll present, both as monoesters (2.1% *cv*. Serbanesti and 0.91% *cv*. Victoria) and diesters (44.61% *cv*. Serbanesti and 33.71% *cv*. Victoria); in particular, zeaxanthin-C16:0,C16:0 (dipalmitate) was the most abundant diester, and zeaxanthin-C14:0,C16:0 (myristate-palmitate) and zeaxanthin-C14:0,C14:0 (dimyristate) were also well represented in both cultivars. In Fig. 2 is shown the mass spectra of zeaxanthin-C16:0,C16:0 both in

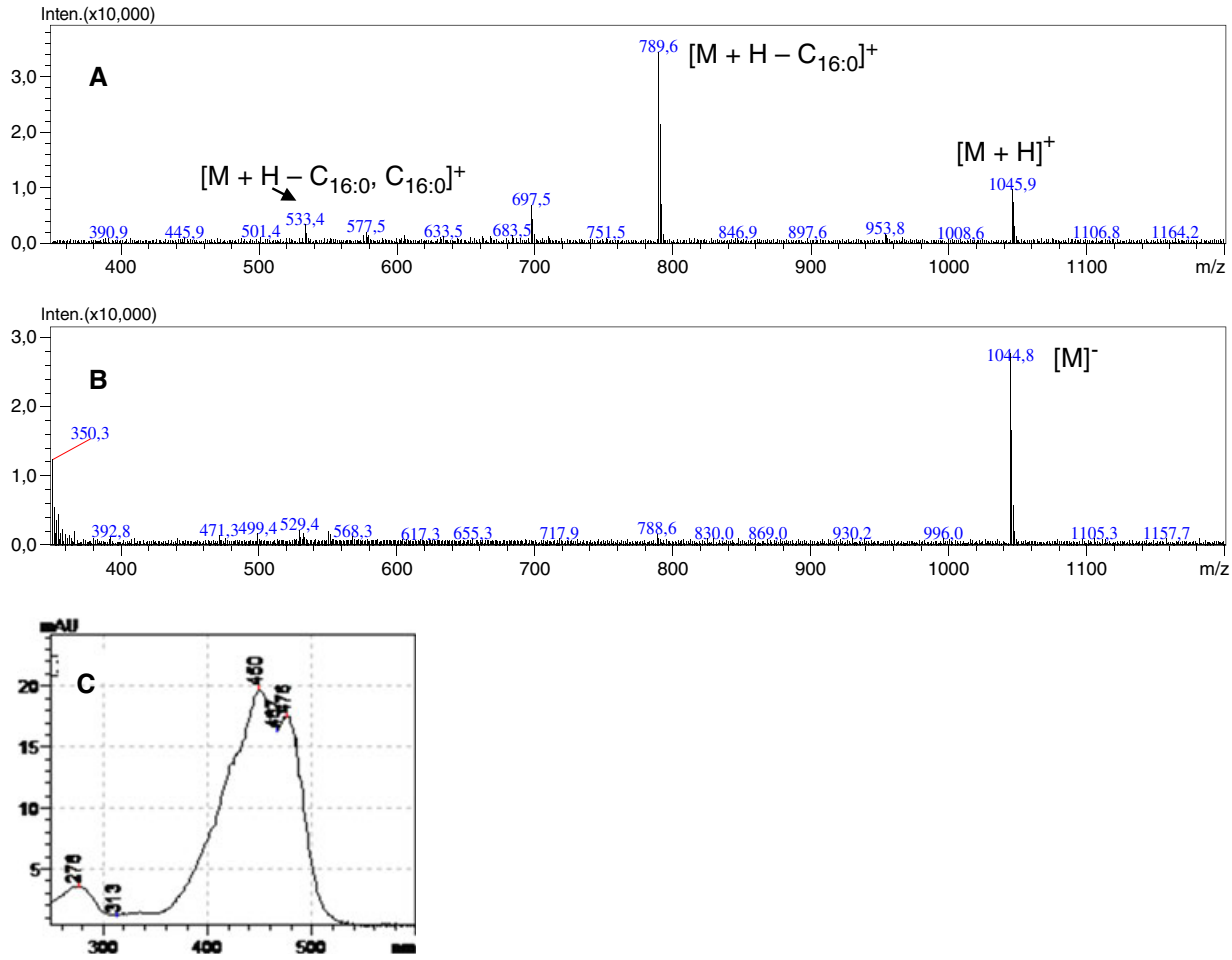

Figure 2. Mass spectra of zeaxanthin-C16:0,C16:0. Peak number **21** in Table 1. (A) APCI positive (+); (B) APCI negative (−); (C) UV-vis spectra for the zeaxanthin ester.

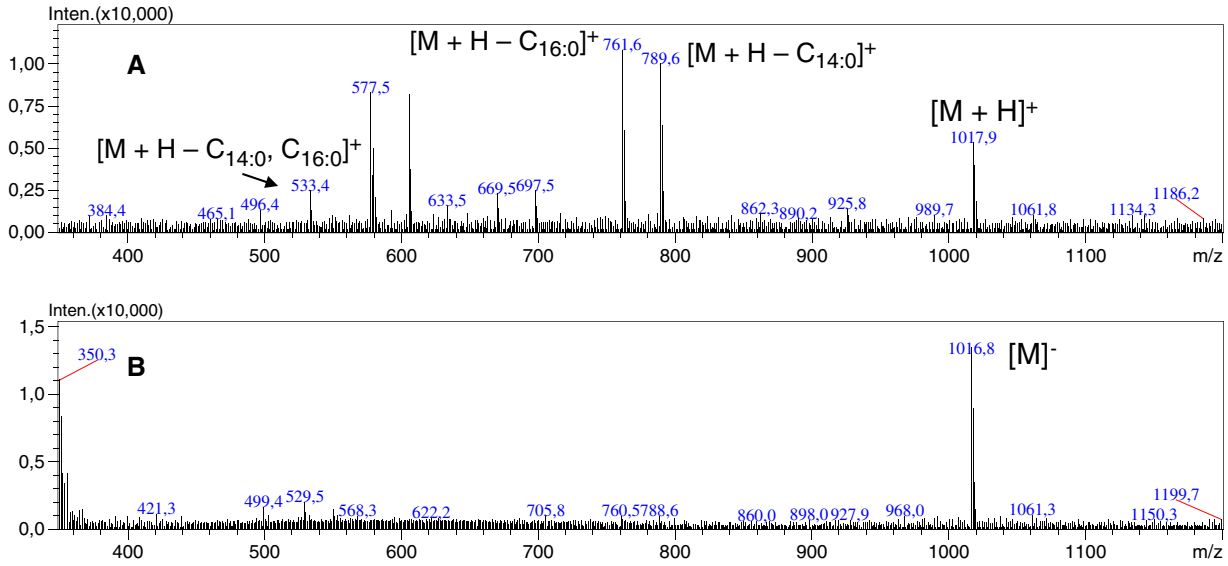

Figure 3. Mass spectra of zeaxanthin-C14:0,C16:0. Peak number **19** in Table 1. (A) APCI positive(+); (B) APCI negative (−).

APCI positive (A) and APCI negative (B) ionisation modes, together with the UV-vis spectra for the zeaxanthin ester, (C); the esters do not change the UV-vis properties of the relative xanthophylls chromophore. In the positive mode (A) the protonated molecular ion $[M+H]^+$ at *m/z* 1045 and the fragment ions at *m/z* 789 and *m/z* 533, corresponding respectively to the loss of one and two molecules of palmitic acid, are clearly observed, and in the negative ionisation mode (B) the quasimolecular $[M]^{-\cdot}$ ion at *m/z* 1044 represents the main peak in the mass spectrum. Similarly in Fig. 3 is shown the mass spectra of zeaxanthin-C14:0,C16:0 both in APCI positive (A) and APCI negative (B) ionisation modes. In the positive mode (A) the protonated molecular ion $[M+H]^+$ at *m/z* 1017 and the fragment ions at *m/z* 789, *m/z* 761 and *m/z* 533 corresponding respectively to the loss of one molecule of myristic acid, one molecule of palmitic acid and both fatty acids are clearly observed, and in the negative ionization mode (B) the quasimolecular $[M]^{-\cdot}$ ion at *m/z* 1016 represents the main peak in the mass spectrum. Interestingly, the unsaturated palmitoleic acid was directly detected in its esterified form, in zeaxanthin-C16:0,C16:1 (7.26 % *cv*. Serbanesti and 4.68 % *cv*. Victoria), which is reported here for the first time. Figure 4 shows the mass spectra of zeaxanthin-C16:0,C16:1 both in APCI positive (A) and APCI negative (B) ionisation modes. In the positive mode (A) the protonated molecular ion $[M+H]^+$ at *m/z* 1043 and the fragment ions at *m/z* 789, *m/z* 787 and *m/z* 533 corresponding respectively to the loss of one molecule of palmitoleic acid, one molecule of palmitic acid and both fatty acids are clearly observed, and in the negative ionisation mode (B) the quasimolecular $[M]^{-\cdot}$ ion at *m/z* 1042 represents the main peak in the mass spectrum. The soft part of sea buckthorn berries is one of the richest sources of palmitoleic acid. Palmitoleic acid was identified only as a zeaxanthin diester, representing the third ester in the total carotenoids extract of both cultivars. Palmitoleic acid is also considered a bioactive molecule. Recently it was identified as a 'lipokine', serving as a lipid signal that mediates communications

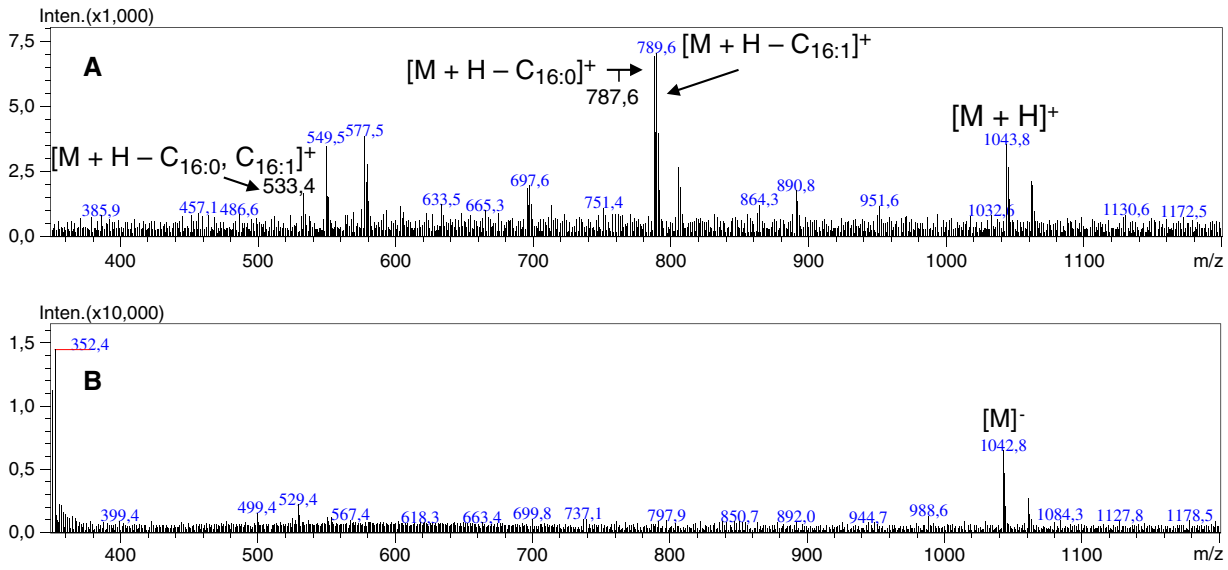

Figure 4. Mass spectra of zeaxanthin-C16:0,C16:1. Peak number **18** in Table 1. (A) APCI positive(+); (B) APCI negative (−).

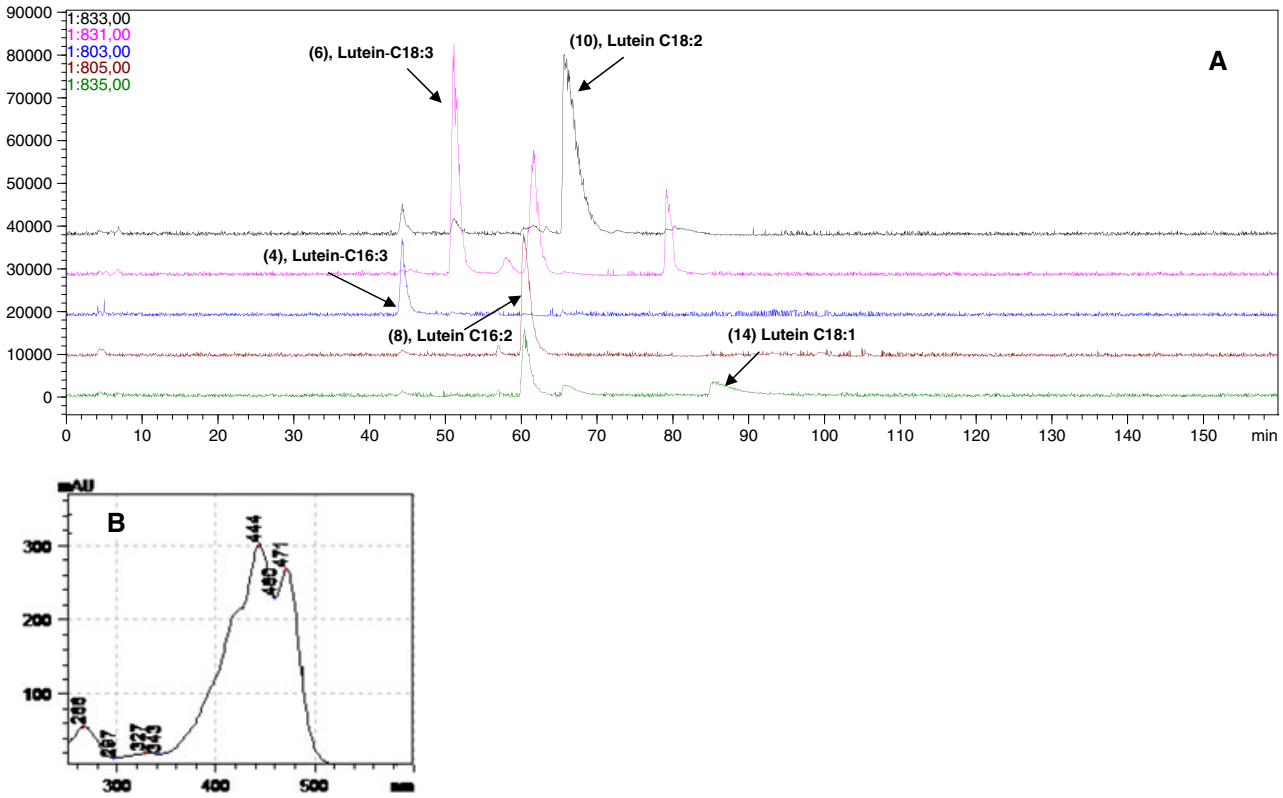

Figure 5. (A) Extracted ion chromatograms (EIC) of sea buckthorn extract, using a C_{30}-column, for ions of peaks **4**, **6**, **8**, **10** and **14**. For peak identification, see Table 1. (B) UV-vis spectra of a lutein ester.

between adipose and other tissues. Palmitoleic acid strongly stimulates muscle insulin action and suppresses hepatosteatosys in mice (Cao *et al.*, 2008). Moreover, although present in small amounts, the unsaturated oleic, linoleic, linolenic, hexadecadienoic and hexadecatrienoic acids were detected in their esterified forms as lutein monoesters, the last two having been detected in *Hippophae rhamnoides* for the first time. Figure 5 shows the MS chromatograms of the extracted ions (EIC) corresponding respectively to the lutein monoesters of the unsaturated oleic, linoleic, linolenic, hexadecadienoic and hexadecatrienoic acids, which were also detected in both the positive ionisation mode, as protonated molecular ions, and in the negative ionisation mode, as deprotonated molecular ions (A), together with the UV-vis spectra for a lutein ester (B). It is known that xanthophylls are preferentially esterified with saturated fatty acids (Weller and Breithaupt, 2003). Here we report that zeaxanthin and lutein also can be esterified with mono- and polyunsaturated fatty acids. These results are in agreement with the report by Pintea *et al.* (2005) on the presence of the unsaturated palmitoleic, oleic, linoleic and linolenic acids in the total lipid extract of sea buckthorn fruits analysed by GC-MS. In conclusion, a HPLC-DAD-APCI-MS method has been developed to directly separate the free carotenoids and their esters in fruits of *Hippophae rhamnoides* by employing a C_{30}-column and a gradient mobile phase. This method may be applied to analyse free carotenoids and carotenoid esters in both food and nutraceuticals.

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