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Identification and Quantitative Analysis of Carotenoids and Their Esters from Sarsaparilla (*Smilax aspera* L.) Berries

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Supporting Information

ABSTRACT: The carotenoid composition of sarsaparilla (*Smilax aspera* L.) berries has been analyzed for the first time. Lycopene was found to be the main carotenoid (242.44 μg/g fresh wt) in the pulp, followed by β-carotene (65.76 μg/g fresh wt) and β-cryptoxanthin (42.14 μg/g fresh wt; including the free and esterified forms). Other minor carotenoids were lycophyll (13.70 μg/g fresh wt), zeaxanthin (8.56 μg/g fresh wt; including the free and esterified forms), lutein (0.94 μg/g fresh wt), and antheraxanthin (0.58 μg/g fresh wt). β-Cryptoxanthin and zeaxanthin were present in free and esterified forms. β-Cryptoxanthin was mainly esterified with saturated fatty acids (capric, lauric, myristic, palmitic, and stearic), although a low amount of β-cryptoxanthin oleate was also detected. In the case of zeaxanthin, only a monoester with myristic acid (zeaxanthin monomyristate) was identified. The diverse carotenoid profile, some with provitamin A activity, together with the relatively high content, up to 375 μg/g fresh wt, makes sarsaparilla berries a potential source of carotenoids for the food, animal feed, and pharmaceutical industries.

KEYWORDS: *Smilax aspera L., sarsaparilla, HPLC-DAD-MS, APcI, carotenoids, xanthophyll esters, fatty acids*

INTRODUCTION

The sarsaparilla (*Smilax aspera* L.) is an evergreen perennial climbing plant of the Liliaceae family and typical of the Mediterranean basin. The plant grows and climbs from a rhizome and forms many extended branches, up to 15 m in length, and numerous leaves around shrubs and trees. The stems are semiwoody with abundant prickles. The shiny leaves are generally heart-shaped with a few, tiny and translucent prickles along the margin (see the Supporting Information). Flowers are associated as branched clusters, producing juicy spherical berries (7−9 mm across) as fruits, which are initially green and turn red through ripening, sometimes becoming black when fully ripe.

The popularity of this plant is due to the ancient medicinal uses of the rhizomes, having depurative, diaphoretic, diuretic, stimulant, and tonic properties,[1,2] so that sarsaparilla has been frequently used as an ingredient in soft drinks. These therapeutic actions are mainly attributed to the high content in steroidal saponins.[3] In addition, these healthy properties also might be due to the presence of phenolic compounds, such as *trans*-resveratrol.[4] Recently, the anthocyanins, also phenolic compounds with health-promoting properties, were analyzed and characterized as the main pigments of black sarsaparilla berries;[5] however, several studies in our laboratory have demonstrated that carotenoids are also involved in the color of the ripe berries, especially the red ones.

Carotenoids are an important group of natural pigments responsible for the coloration of most fruits and vegetables, being found in almost all parts of plants: fruits, flowers, roots, leaves, and seeds.[6] In general, plant carotenoids are C_{40} isoprenoids (tetraterpenoids) having a polyene skeleton consisting of a long conjugated double-bond system, which constitutes the chromophore responsible for the color that these pigments confer to most fruits and vegetables, having an important role in attracting animals to act as pollinators and seed dispersion vehicles, including in this process the consumption of food by humans.[6−8] The number of naturally occurring carotenoids is about 750 and continues to rise.[9] Carotenoids can only be synthesized de novo by plants, certain bacteria, and fungi. In contrast, animals are unable to synthesize carotenoids, so they need to take them from the diet. Carotenoids are essential components of the photosynthetic apparatus and are involved in the light-harvesting process, as well as in the photoprotection mechanisms of plants.[8] Xanthophylls are frequently esterified with fatty acids during the ripening of the fruits and stored inside the chromoplasts when the chloroplasts are degraded.[10,11]

The relationship between the ingestion of carotenoids and their health-promoting properties has been widely studied. When carotenoids are ingested and absorbed, they show important biological activities: antioxidant, inhibition of carcinogenesis, enhancement of the immune response and cell protection against reactive oxygen species (ROS) and free radicals, and a reduction in the risk of developing cardiovascular and other degenerative diseases. In addition, some carotenoids (β-carotene, α-carotene, β-cryptoxanthin, etc.) have provitamin A activity. Several epidemiological studies have shown an inverse correlation between the progression of age-related macular degeneration (AMD) and cataracts and the high intake of lutein- and zeaxanthin-rich vegetables, both pigments being present in high concentration at the macula in the retina of humans and primates.[12]

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The increasing interest in the role of dietary carotenoids in human health has boosted the number of studies searching for new natural sources. Therefore, the aim of the present work was to identify and quantitate for the first time the carotenoid profile in *S. aspera* L. berries to evaluate their use as an alternative natural source of carotenoids for the food, animal feed, and pharmaceutical industries.

MATERIALS AND METHODS

Plant Material and Sample Preparation. *S. aspera* L. berries were collected during the autumn (October 2009) in a typical Mediterranean forest located at the Sierra Norte de Sevilla Natural Park (Seville, Spain; 37° 37′ 44.3424″, 6° 24′ 22.4316″). Samples were kept refrigerated during transportation to the laboratory.

Chemicals and Reagents. HPLC grade methanol, acetone, toluene, and heptane were supplied by BDH Prolabo (VWR International Eurolab, SL, Barcelona, Spain). Diethyl ether, containing 7 ppm BHT, was purchased from Scharlau (Scharlab, SL, Barcelona, Spain). HPLC grade deionized water was produced with a Milli-Q 50 system (Millipore Iberica SA, Madrid, Spain). Heptadecanoic acid, butylated hydroxytoluene (BHT), 2,2-dimethoxypropane, and fatty acid methyl ester (FAME) standard mixtures were purchased from Sigma-Aldrich Química, SA (Madrid, Spain). The rest of the reagents were all of analytical grade. For TLC, plates of silica gel 60 GF_{254} (20 × 20 cm plates, thickness = 0.7 mm) supplied by Merck (Darmstadt, Germany) were used.

Extraction of Carotenoids. About 50 sarsaparilla (*S. aspera*) berries were washed and cut and the seeds removed. One gram was extracted with acetone (containing 0.1% BHT) by using a model T-25 Ultra-Turrax homogenizer. The extraction procedure was repeated three or four times until complete extraction of color. The extracts were pooled in a separation funnel, and 50 mL of diethyl ether (containing 6 ppm BHT) was added and shaken vigorously; subsequently, 100 mL of NaCl solution (10% w/v) was added, and the separated aqueous phase (lower phase) was discarded. The upper phase, containing the carotenoid pigments, was washed three additional times with 10% NaCl and finally with 2% (w/v) Na_2SO_4 solution for removing the water contained in the organic fraction. The extract was filtered through an anhydrous Na_2SO_4 bed and subsequently concentrated under vacuum in a rotary evaporator at 30 °C. The dry extract was dissolved in 1.5 mL of acetone and stored at −30 °C until analysis. HPLC analysis was carried out on the same day of the preparation of the extracts. Samples were centrifuged at 13200*g* for 5 min at 4 °C prior to the chromatographic analysis. Analyses were carried out in quadruplicate. All operations were carried out under dimmed light to prevent isomerization and photodegradation of carotenoids.

If saponification was required for hydrolysis of carotenoid esters, a microscale procedure, based on the method of Mínguez-Mosquera and Hornero-Méndez,[13] was used. In this case, 700 μL of the direct extract was dried under nitrogen current, and then 2 mL of diethyl ether and 1 mL of 10% (w/v) KOH/methanol were added and left to react for 40 min at room temperature under a nitrogen atmosphere and with periodic shaking. The organic phase was washed several times with distilled water until the washings were neutral, washed with 2% (w/v) Na_2SO_4 solution, and filtered through an anhydrous Na_2SO_4 bed. The solvent was evaporated to dryness, and the dry extract was dissolved with 1 mL of acetone for subsequent chromatographic analysis (HPLC-DAD or HPLC-DAD-MS).

Pigment Identification. The identification of carotenoids have been carried out following routine procedures, which consisted of a combination of different experiments: separation and isolation of pigments by TLC and cochromatography (TLC and HPLC) with pure standards, analysis of the UV−vis and mass spectra and comparison with the literature values,[9,14−16] and microscale chemical test for 5,6-epoxide groups by controlled treatment with diluted HCl in ethanol following the analysis of the changes in the UV−vis spectrum and the chromatographic mobility. Authentic pigment samples of carotenoids were isolated and purified by means of TLC from natural sources: β-carotene (β,β-carotene), β-cryptoxanthin (β,β-caroten-3-ol), and zeaxanthin (β,β-carotene-3,3′-diol) were obtained from red pepper (*Capsicum annuum* L.); lutein (β,ε-carotene-3,3′-diol) was obtained from mint leaves (*Mentha arvensis*); lycopene (ψ,ψ-carotene) and rubixanthin (β,ψ-caroten-3-ol) were obtained from rose hips (*Rosa rubiginosa*); and lycophyll (ψ,ψ-carotene-16,16′-diol) was obtaind from woody nightshade fruit (*Solanum dulcamara*).[13,14,17] Antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol) was synthesized by the epoxidation of zeaxanthin with 3-chloroperoxybenzoic acid according to the method of Barua and Olson.[18] The identification of *cis* isomers was based on the presence and relative intensity (%A_B/A_{II}) of the *cis* peak at about 330−340 nm in UV−vis spectrum, a reduction in the fine structure, a small hypsochromic shift in λ_{max} with respect to the *all-trans* counterpart, and the chromatographic behavior in the C18 HPLC column (the *cis* isomers show slightly longer retention times than the *all-trans* isomer).[15]

The identification of the fatty acid moiety and the structural assignment of the carotenoid esters were mainly based on the fragmentation pattern obtained with the liquid chromatography−mass spectrometry (LC-MS (APCI+)) conditions described below. The mass spectrum of a xanthophyll acyl ester under APCI (positive mode) is characterized for presenting fragments corresponding to the neutral loss of the fatty acid moiety, allowing the identification of the esterification nature.[19−21]

Quantitative HPLC Analysis of Carotenoids. Quantitative analysis of carotenoids was carried out by HPLC according to the method of Mínguez-Mosquera and Hornero-Méndez[13] with minor modifications. The HPLC system consisted of a Waters 2695 Alliance chromatograph fitted with a Waters 2998 photodiode array detector (DAD) and controlled with Empower2 software (Waters Cromatografía, SA, Barcelona, Spain). A reversed-phase C18 column (200 mm × 4.6 mm i.d., 3 μm, Mediterranea SEA18; Teknokroma, Barcelona, Spain), fitted with a guard column of the same material (10 mm × 4.6 mm), was used. Separation was achieved by a binary gradient elution using an initial composition of 75% acetone and 25% deionized water, which was increased linearly to 95% acetone in 10 min, then held for 7 min, raised to 100% in 3 min, and maintained constant for 10 min. Initial conditions were reached in 5 min. The temperature of the column was kept at 25 °C, and the sample compartment was refrigerated at 15 °C. An injection volume of 10 μL and a flow rate of 1 mL/min were used. Detection was performed at 450 nm, and the online spectra were acquired in the 330−700 nm wavelength range with a resolution of 1.2 nm.

Pigments were quantitated in the direct extracts by using calibration curves (eight concentration levels) prepared with standard stock solutions for each carotenoid in the concentration range of 5−100 μg/mL. Calibration curves were constructed by plotting the peak area at 450 nm versus the pigment concentration. Antheraxanthin and lycophyll were quantified by using the calibration curve of zeaxanthin and lycopene, respectively. The concentrations of xanthophyll esters (zeaxanthin and β-cryptoxanthin esters) were estimated by using the corresponding calibration curve for the free pigments, because the esterification of xanthophylls with fatty acids does not modify the chromophore properties.[15] The *cis* isomers were quantitated by using the calibration curve of the *all-trans* isomer. Concentration values were calculated as micrograms of pigment per gram of fresh sample weight (μg/g fresh wt).

Provitamin A carotenoids content was calculated in two unit systems to allow better comparison with data from the literature: retinol equivalents (RE), in which 1 RE is equivalent to 6 μg of β-carotene or 12 μg of *cis* isomers of β-carotene or any other carotenoid containing one unsubstituted β-ring,[22] and as the more recently defined units retinol activity equivalents (RAEs), proposed by the U.S. Institute of Medicine,[23] so that 1 RAE corresponds to 12 μg of β-carotene or 24 μg of carotenoids containing one unsubstituted β-ring.

Liquid Chromatography−Mass Spectrometry (LC-MS (APCI +)). LC-MS was performed with the same chromatographic system (HPLC-DAD) described above, by coupling a Micromass ZMD4000 mass spectrometer equipped with a single-quadrupole analyzer (Micromass Ltd., Manchester, U.K.) and an atmospheric pressure chemical ionization (APCI) probe to the outlet of the DAD. The system

was controlled with MassLynx 3.2 software (Micromass Ltd.). The mass spectrometer condition parameters were as follows: positive ion mode (APCI+); source temperature, 150 °C; probe temperature, 400 °C; corona voltage, 3.7 kV; high-voltage lens, 0.5 kV; and cone voltage, 30 V. Nitrogen was used as the desolvation and cone gas at 300 and 50 L/h, respectively. Mass spectra were acquired within the m/z 300−1200 range. The chromatographic conditions were the same as described for quantitative analysis of carotenoids.

Analysis of Fatty Acids by Gas Chromatography. For the analysis of total fatty acids profile of sarsaparilla berries, fatty acid methyl esters (FAMEs) were prepared. Extraction and gas chromatographic analysis of FAMEs were carried out according to the method of Garcés and Mancha,[24] which allowed the digestion of the plant tissue, extraction of lipids, and transmethylation in one step. Plant material (0.5 g of lyophilized berries) was placed in a glass tube with Teflon caps together with 100 μL of heptadecanoic acid (C17:0) as internal standard (stock solution 20.0 mg/mL) and placed in 18 mL test tubes with Teflon caps. After the addition of 1.65 mL of methanol/toluene/2,2-dimethoxypropane/H_2SO_4 (39:20:5:2, by vol) and 0.85 mL of heptane (containing 0.05% BHT), the mixture was flushed with nitrogen and heated at 80 °C during 2 h. After cooling to room temperature, 0.5 mL of the upper organic phase, corresponding to heptane, was collected for GC analysis. FAMEs were separated on a Supelcowax 10 fused silica capillary column (30 m length; 0.32 mm i.d.; 0.25 μm film thickness) (Sigma-Aldrich Química, S.A., Madrid, Spain) by using an Agilent Technologies 7890A gas chromatograph (Agilent Technologies España, S.L., Madrid, Spain) fitted with a flame ionization detection (FID), a split/splitless injector, and a 7683B series automatic liquid sampler. Helium was used as carrier gas with a constant linear flow of 1.75 mL/min. The injector and detector temperatures were 250 and 260 °C, respectively. The oven temperature program started at 145 °C, increasing with a ramp of 15 °C/min to 230 °C with a 10 min hold. The injection volume was 1 μL at a split ratio of 1:50. Fatty acids were identified by comparison with known standards. Quantitative determination was performed using the area of the internal standard. Analyses were carried out in triplicate.

Statistical Aalysis. Basic statistics, mean and standard deviation (SD), were calculated for the results with Statistica 6.0 software (Statsoft, 2001).

RESULTS AND DISCUSSION

Characterization of the Carotenoid Profile of Sarsaparilla Berries. The carotenoid pigments occurring in sarsaparilla (*S. aspera* L.) berries have been characterized and identified according to their chemical, chromatographic, and spectroscopic characteristics (UV−vis and mass spectroscopic). Figure 1A shows the reversed phase HPLC chromatogram corresponding to the direct carotenoid extract obtained from fully ripe sarsaparilla (*S. aspera*) berries. When the extract was submitted to saponification with 10% KOH/MeOH, the disappearance of several peaks in the resulting chromatogram (Figure 1B) revealed the presence of xanthophyll acyl esters in the direct extract. As established in a previous work,[11] three different xanthophyll fractions can be distinguished with respect to the degree of esterification under the chromatographic conditions used in the present work (chromatographic separation with C18 reverse phase), namely, free, partially, and totally esterified xanthophylls. The partially esterified forms (monoesters for a dihydroxy xanthophyll) have shorter retention times than β-carotene, and the totally esterified forms (monoester for a monohydroxy xanthophyll and diester for a dihydroxy xanthophyll) are more retained than β-carotene due to their lower polarity. Table 1 summarizes the identification for each chromatographic peak, and Figure 2 shows the corresponding chemical structures. In total 16 carotenoids were detected: 9 free carotenoids and 7 xanthophyll esters.

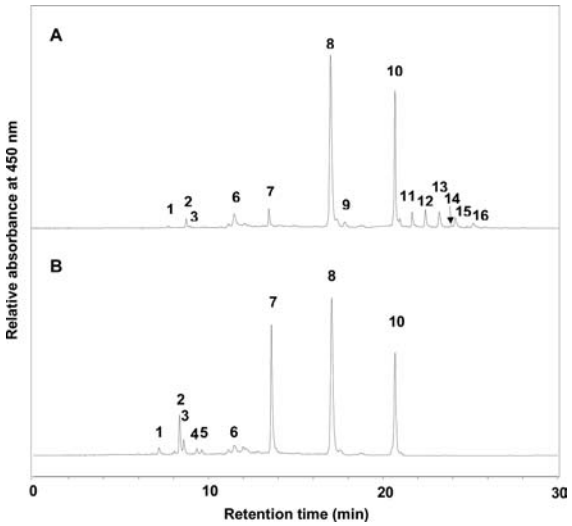

Figure 1. C18 reversed-phase HPLC chromatogram obtained from direct (A) and saponified carotenoid (B) extracts from sarsaparilla (*S. aspera* L.) berries. Peaks: 1, *all-trans*-antheraxanthin; 2, *all-trans*-zeaxanthin; 3, *all-trans*-lutein; 4, *cis* isomer of zeaxanthin; 5, *cis* isomer of lutein; 6, lycophyll; 7, *all-trans*-β-cryptoxanthin; 8, *all-trans*-lycopene; 9, zeaxanthin monomyristate; 10, *all-trans*-β-carotene; 11, *all-trans*-β-cryptoxanthin caprate; 12, *all-trans*-β-cryptoxanthin laurate; 13, *all-trans*-β-cryptoxanthin myristate; 14, *all-trans*-β-cryptoxanthin oleate; 15, *all-trans*-β-cryptoxanthin palmitate; 16, *all-trans*-β-cryptoxanthin stearate. Detection wavelength was 450 nm.

Peak 1 (Rt = 8.33 min) was assigned as antheraxanthin. The UV−vis spectra, with λ_{max} at 427, 450, 479 nm in the mobile phase (%III/II = 57), were in agreement with a chromophore consisting of nine conjugated double bonds and at least one β-ring. In addition, when the direct extract was treated with diluted HCl (epoxide test), the resulting HPLC chromatogram (data not shown) revealed the disappearance of antheraxanthin and the formation of C8-epimers of mutatoxanthin (5,8-epoxy-5,8-dihydro-β,β-carotene-3,3′-diol), the 5,8-epoxide derivative of antheraxanthin generated by the acid-catalyzed rearrangement of the 5,6-epoxy group. The UV−vis spectrum of mutatoxanthin epimers showed λ_{max} at 408, 430, and 456 nm, corresponding to a hypsochromic shift of about 20 nm, which is in agreement with the presence of one 5,6-epoxy group.[25] The LC/MS (APCI+) spectrum was characterized by the presence of a protonated molecule $[M + H]^+$ at m/z 585, which was consistent with the formula $C_{40}H_{56}O_3$ (MW = 584.4229). Fragments at m/z 567 $[M + H - 18]^+$ and 549 $[M + H - 18 - 18]^+$ confirmed the presence of two hydroxyl groups, and a fragment at m/z 505 $[M + H - 80]^+$ was in agreement with the presence of one 5,6-epoxy group.[14] Finally, peak 1 coeluted with an antheraxanthin standard prepared by the epoxidation of zeaxanthin with 3-chloroperoxybenzoic acid according to the method of Barua and Olson.[18]

all-trans-Zeaxanthin was the identity for peak 2 (Rt = 9.35 min). The UV−vis spectra, with λ_{max} at 428, 455, and 481 nm, with a low spectroscopic fine structure (%III/II = 18), suggested a chromophore with nine conjugated double bonds and two β-rings.[15] As discussed later, similar UV−vis properties were found for β-carotene and β-cryptoxanthin, as they share the same chromophore; however, their assignment was discarded for this peak on the basis of the chromatographic behavior. The protonated molecule $[M + H]^+$ detected at m/z 569, which appeared to be the most abundant fragment, was consistent with

Table 1. Chromatographic, UV−Vis, and Mass (APCI+) Spectroscopy Properties of the Carotenoid Pigments Identified in Sarsaparilla (*S. aspera* L.) Berries

							HPLC/APCI(+) MS fragmentation pattern *m/z*	
peak[a]	carotenoid	Rt (min)	λ_{max} (nm)	λ_{max} (nm) according to bibliography in acetone[b]	%III/II	epoxide test	$[M + H]^+$	characteristic fragments
1	*all-trans*-antheraxanthin	8.33	427, 450, 479	425, 449, 478[c]	66	+	585	567 ($[M + H - 18]^+$, 549 ($[M + H - 18 - 18]^+$, 505 ($[M + H - 80]^+$
2	*all-trans*-zeaxanthin	9.35	428, 455, 481	430, 452, 479	38	−	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
3	*all-trans*-lutein	9.56	423, 450, 476	424, 445, 474[c]	73	−	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
4	*cis*-zeaxanthin	10.21	324, 428, 450, 476		6	−	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
5	*cis*-lutein	10.44	337, 421, 444, 470		45	−	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
6	lycophyll	12.01	434, 460, 492	446, 474, 506	65	−	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
7	*all-trans*-β-cryptoxanthin	14.17	430, 454, 480	428, 454, 480	18	−	553	535 $[M + H - 18]^+$
8	*all-trans*-lycopene	17.90	448, 474, 504	448, 474, 505	66	−	537	
9	*trans*-zeaxanthin monomyristate (C14:0)[d]	18.74	430, 454, 481	430, 452, 479	38	−	779	761 $[M + H - 18]^+$, 551 $[M + H - 228]^+$, 533 $[M + H - 228 - 18]^+$
10	*all-trans*-β-carotene	20.68	427, 454, 479	429, 452, 478	16	−	537	
11	*all-trans*-β-cryptoxanthin caprate (C10:0)[d]	21.65	428, 454, 479	428, 454, 480	19	−	707	535 $[M + H - 172]^+$
12	*all-trans*-β-cryptoxanthin laurate (C12:0)[d]	22.42	428, 454, 479	428, 454, 480	24	−	735	535 $[M + H - 200]^+$
13	*all-trans*-β-cryptoxanthin myristate (C14:0)[d]	23.24	428, 452, 479	428, 454, 480	23	−	763	535 $[M + H - 228]^+$
14	*all-trans*-β-cryptoxanthin oleate (C18:1)[d]	23.84	428, 452, 479	428, 454, 480	22	−	817	535 $[M + H - 282]^+$
15	*all-trans*-β-cryptoxanthin palmitate (C16:0)[d]	24.17	426, 452, 479	428, 454, 480	25		791	535 $[M + H - 256]^+$
16	*all-trans*-β-cryptoxanthin stearate (C18:0)[d]	25.21	428, 452, 480	428, 454, 480	21	−	819	535 $[M + H - 284]^+$

[a]Peak numbers are according to Figure 1. [b]Britton, Liaanen-Jensen, and Pfander, 2004. [c]Measured in ethanol. [d]Present only in the direct extract.

the $C_{40}H_{56}O_2$ (MW = 568.4280) formula. The occurrence of fragments at *m/z* 551 $[M + H - 18]^+$ and 533 $[M + H - 18 - 18]^+$ confirmed the presence of two hydroxyl groups. The definitive identity of peak 2 was eventually established after coelution with an authentic sample of zeaxanthin.

Coelution with an authentic lutein sample suggested the assignation of peak 3 (Rt = 9.56 min) as *all-trans*-lutein. This peak showed a UV−vis spectrum similar to the one for zeaxanthin but with a slight hypsochromic shift, with λ_{max} at 423, 450, and 476 nm and a marked spectroscopic fine structure (%III/II = 62), suggesting in this case a chromophore with nine conjugated double bonds and presumably one β-ring and one ε-ring. The unambiguous structural determination of this compound was deduced from the LC/MS (APCI+) spectrum. The mass spectrum of lutein shows a characteristic fragmentation pattern, which has already been used to unequivocally distinguish it from other xanthophylls, such as zeaxanthin.[26,27] Under APCI (positive mode) conditions, the mass spectrum of lutein is characterized by the presence of a protonated molecule ($[M + H]^+$) at *m/z* 569 with low abundance (15−20%) and an ion at *m/z* 551, being the most abundant fragment (100%), corresponding to the neutral loss of one molecule of water ($[M + H - 18]^+$) from the hydroxy group at position 3′ of the ε-ring. An additional neutral loss of a second water molecule from the hydroxy group at position 3 of the β-end ring produces a less abundant fragment (1−3%) at *m/z* 533 ($[M + H - 18 - 18]^+$), which is the lutein backbone. In contrast, for zeaxanthin the most abundant ion correspond to the protonated molecule at *m/z* 569 ($[M + H]^+$), followed by the fragment at *m/z* 551 (10−15%) and by the weak fragment at *m/z* 533 (2−5%). The reason for these differences has been explained on the basis that the cation formed by cleavage at the ε-ring is allylic with respect to the double bond and, therefore, is more stable than the secondary cation generated from the respective cleavage at the β-ring. This characteristic MS fragmentation pattern has recently been used to unequivocally identify the regioisomeric forms of lutein monoesters and diesters in tritordeum (×*Tritordeum* Ascherson et Graebner), a novel cereal.[20] Peaks 4 and 5 (Rt = 10.21 and 10.44 min) were tentatively assigned as geometric isomers (*cis* isomers) of zeaxanthin and lutein, respectively, on the basis of their UV−vis spectra with lower %III/II and slightly shorter λ_{max} when compared with *all-trans* compounds, together with the observation of a "*cis* peak" at 324 and 327 nm. The characteristics of the mass spectra were in accordance with the above-mentioned fragmentation profile for lutein and zeaxanthin allowing the structural assignment of both compounds and taking into account the abundance ratio for ($[M + H]^+$) at *m/z* 569 and ($[M + H - 18]^+$) at *m/z* 551.

The UV−vis spectrum for peak 6 (Rt = 12.01 min) presented λ_{max} at 434, 460, and 492 nm (%III/II = 65), which were in agreement with the presence in the chromophore of ten conjugated double bonds and one β-ring.[15] In principle, these properties together with the chromatographic elution in the zone of the hydroxylated xanthophylls suggested its identification as

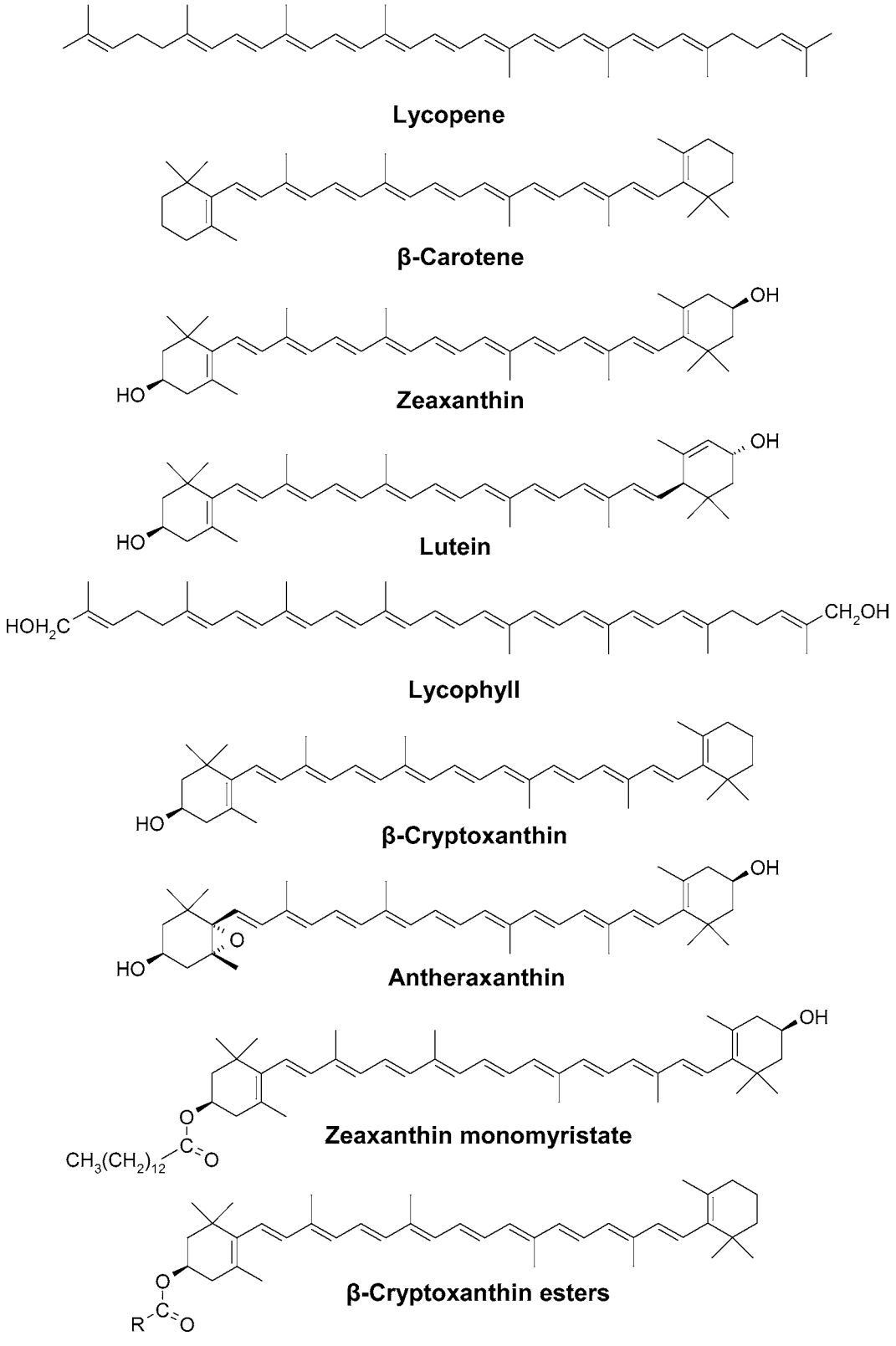

Figure 2. Chemical structures of the carotenoid pigments identified in sarsaparilla (*S. aspera* L.) berries. For *β*-cryptoxanthin esters, R = $CH_3(CH_2)_8$–, caprate; $CH_3(CH_2)_{10}$–, laurate; $CH_3(CH_2)_{12}$–, myristate; $CH_3(CH_2)_{14}$–, palmitate; $CH_3(CH_2)_{16}$–, stearate; $CH_3(CH_2)_7CH{=}CH(CH_2)_7$–, oleate.

rubixanthin. However, peak 6 did not coelute with a rubixanthin standard isolated from rose hips. The mass spectrum showed a protonated molecule $[M + H]^+$ at m/z 569 ($C_{40}H_{56}O_2$), and the presence of fragments at m/z 551 $[M + H - 18]^+$ and m/z 533 $[M + H - 18 - 18]^+$ revealed the existence of two hydroxyl groups. After comparison of these data with data given in the literature,[9] peak 6 (Rt = 12.01 min) was tentatively identified as lycophyll, a dihydroxy derivative of lycopene, which has been found in low concentrations in tomato fruit (*Solanum lycopersicum*) and in larger amounts in woody nightshade fruit

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(*Solanum dulcamara*).[14] The coelution of peak 6 with an standard of lycophyll isolated from *S. dulcamara* berries confirmed the identification.

Peak 7 (Rt = 14.17 min) was initially identified as *β*-cryptoxanthin by chromatographic coelution with a standard. The UV−vis spectrum showed λ_{max} at 430, 454, and 480 nm (% III/II = 18), suggesting a chromophore consisted of nine conjugated double bonds and two *β*-rings. Mass spectra were characterized by a protonated molecule $[M + H]^+$ at *m/z* 553 ($C_{40}H_{56}O$) and a fragment at *m/z* 535 $[M + H - 18]^+$, which is in agreement with the presence of one hydroxy group.

all-trans-Lycopene (peak 8; Rt = 17.90 min) and *all-trans-β*-carotene (peak 10; Rt = 20.68 min) were easily identified by comparing the chromatographic and spectroscopic properties with the standard samples and the literature data. The mass spectrum for both pigments showed the expected protonated molecule $[M + H]^+$ at *m/z* 537, which is in agreement with the $C_{40}H_{56}$ formula, and the typical fragment $[M + H - 92]^+$ at *m/z* 445 corresponding to the in-chain loss of toluene (92 mass units). In addition, a fragment at *m/z* 468 $[M + H - 69]^+$ in the mass spectrum of lycopene was in agreement with the loss of the *ψ*-end groups from both ends of the molecule. The UV−vis spectrum of *β*-carotene presented λ_{max} at 427, 454, and 479 nm and low fine structure (%III/II = 16), which is accordance with a chromophore with nine conjugated double bonds and two *β*-rings. In contrast, the UV−vis spectrum of lycopene was characterized by λ_{max} at longer wavelengths (448, 474, and 504 nm) and a defined fine structure (%III/II = 66), which suggested the presence of 11 conjugated double bonds in the central polyene structure.

As mentioned before, several chromatographic peaks (peaks 9 and 11−16) disappeared upon saponification of the direct extract, indicating their acylated nature. It was also observed, by comparison of the direct and saponified extracts, that the area of the free *β*-cryptoxanthin peak, and zeaxanthin in a minor extension, was increased after saponification, suggesting that most of the xanthophyll esters were mainly acylated forms of *β*-cryptoxanthin followed by a zeaxanthin. The identification of the fatty acid moiety and the structural assignment of the carotenoid esters were carried out by LC-DAD-MS(APCI+) analysis. Because the esterification of xanthophylls with fatty acids does not alter the chromophore properties,[15] the UV−vis spectrum of the acylated form was identical to the spectrum of the free carotenoid as reported in Table 1. The mass spectrum of peak 9 was characterized for presenting a protonated molecule $[M + H]^+$ at *m/z* 779, together with two main fragments at *m/z* 761 and 551, which are derived from the neutral loss of water $[M + H - 18]^+$ from the position 3 or 3′ at the *β*-end ring, and the neutral loss of a myristic acid moiety (C14:0, M_w = 228.21) from the other *β*-end ring $[M + H - \text{myristic}]^+$, respectively. In addition, a fragment at *m/z* 533 was observed, corresponding to the backbone of the carotenoid after the neutral loss of one water molecule and one fatty acid moiety $[M + H - 18 - \text{myristic}]^+$. The UV−vis spectrum presented λ_{max} at 430, 454, and 481 nm, in accordance with the chromophore of zeaxanthin. Therefore, the structure of this compound was assigned as zeaxanthin monomyristate.

In an analogous way, peaks 11−16 were identified as *β*-cryptoxanthin esters according to their mass spectrometric properties, which were characterized by a protonated molecule $[M + H]^+$ (the *m/z* value varies according to the acyl moiety) together with another main fragment at *m/z* 535, which is derived from the neutral loss of the acyl moiety from the *β*-end ring $[M + H - \text{acyl}]^+$, corresponding to the carotenoid backbone. The UV−vis spectra of these peaks presented λ_{max} at 422, 452, and 479 nm, and the absence of the "*cis* peak" was consistent with the chromophore of *all-trans-β*-cryptoxanthin. Figure 3 shows

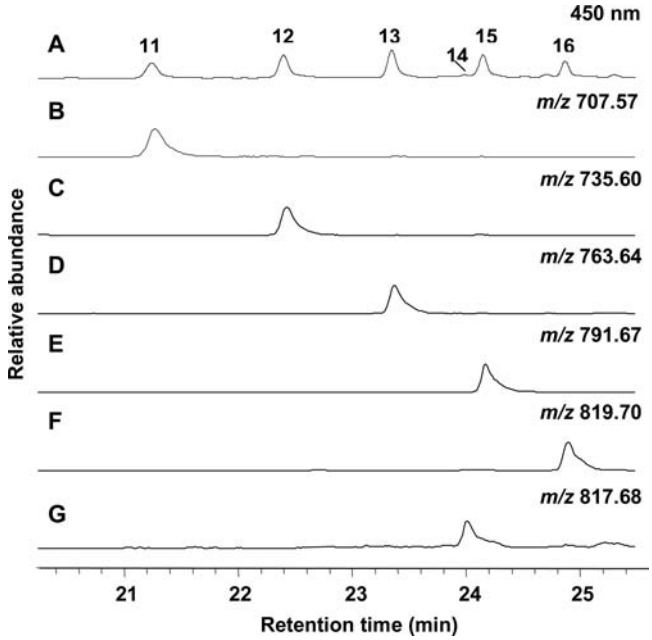

Figure 3. LC-DAD-MS(APCI+) analysis of a direct carotenoid extract from sarsaparilla (*S. aspera* L.) berries. Chromatogram A corresponds to the UV−vis detection at 450 nm (peak identities as in Figure 1 and Table 1), and traces B−G show selected extracted ion chromatograms at specific masses for the detection of the protonated molecular ions for each *β*-cryptoxanthin ester: B, *all-trans-β*-cryptoxanthin caprate (*m/z* 707.57); C, *all-trans-β*-cryptoxanthin laurate (*m/z* 735.60); D, *all-trans-β*-cryptoxanthin myristate (*m/z* 763.64); E, *all-trans-β*-cryptoxanthin palmitate (*m/z* 791.67); F, *all-trans-β*-cryptoxanthin stearate (*m/z* 819.70); G, *all-trans-β*-cryptoxanthin oleate (*m/z* 817.68).

the LC-DAD-MS(APCI+) chromatograms corresponding to the extracted ion at selected masses for the protonated molecule $[M + H^+]$ for each *β*-cryptoxanthin acyl ester, together with the UV−vis chromatogram at 450 nm, demonstrating the unambiguous identification of each peak. As summarized in Table 1, peaks 11−16 were identified as *β*-cryptoxanthin caprate, *β*-cryptoxanthin laurate, *β*-cryptoxanthin myristate, *β*-cryptoxanthin oleate, *β*-cryptoxanthin palmitate, and *β*-cryptoxanthin stearate, respectively.

Quantitative Analysis of Carotenoids. Table 2 summarizes the quantitative analysis of carotenoids from ripe sarsaparilla berries. The total carotenoid content was about 374.12 ± 31.90 *μ*g/g fresh wt, lycopene being the major pigment (242.44 ± 31.90 *μ*g/g fresh wt) accounting for up to 65% of the total carotenoid content and, therefore, the main one responsible for the red color of the ripe berries. The lycopene content in sarsaparilla berries was higher than those reported for many tomato and red-fleshed watermelon cultivars,[28−30] which were in most cases <75 *μ*g/g fresh wt. This fact is interesting because several studies have demonstrated that lycopene is an important antioxidant which protects against the risk of developing certain cancers, atherosclerosis, and cardiovascular disease.[31,32] Other major pigments were *all-trans-β*-carotene (65.76 ± 2.57 *μ*g/g fresh wt) and *all-trans-β*-cryptoxanthin (42.14 ± 1.41 *μ*g/g fresh wt; including the free and esterified forms), both with provitamin

Table 2. Carotenoid Composition in Sarsaparilla (*S. aspera* L.) Berries

carotenoid	concentration (μg/g fresh wt)[a]
all-trans-antheraxanthin	0.58 ± 0.21
all-trans-zeaxanthin	4.35 ± 0.78
all-trans-lutein	0.68 ± 0.04
cis-zeaxanthin	0.23 ± 0.06
cis-lutein	0.26 ± 0.09
lycophyll	13.70 ± 2.11
all-trans-β-cryptoxanthin	7.42 ± 0.44
all-trans-lycopene	242.44 ± 31.69
all-trans-zeaxanthin monomyristate (C14:0)	3.98 ± 0.37
all-trans-β-carotene	65.76 ± 2.57
all-trans-β-cryptoxanthin caprate (C10:0)	7.34 ± 0.67
all-trans-β-cryptoxanthin laurate (C12:0)	8.88 ± 0.74
all-trans-β-cryptoxanthin myristate (C14:0)	8.86 ± 0.83
all-trans-β-cryptoxanthin oleate (C18:1)	0.97 ± 0.02
all-trans-β-cryptoxanthin palmitate (C16:0)	5.26 ± 0.11
all-*trans*-β-cryptoxanthin stearate (C18:0)	3.41 ± 0.32
total β-cryptoxanthin (free + esters)	42.14 ± 1.41
total zeaxanthin (free + esters)	8.56 ± 0.86
total carotenoid	374.12 ± 31.90
provitamin A (RE[b]/100 g)	1447.17 ± 43.10
provitamin A (RAE[c]/100 g)	723.58 ± 21.41

[a]Data are the mean ± standard deviation (n = 4). [b]Retinol equivalents/100 g fresh wt. [c]Retinol activity equivalents/100 g fresh wt.

A activity. When compared with the data reported by Breithaupt and Bamedi[33] for an extensive screening of carotenoid esters in vegetables and fruits, the concentrations of β-cryptoxanthin esters in sarsaparilla were higher than those found in papaya, a fruit characterized for its high β-cryptoxanthin content. For instance, β-cryptoxanthin laurate was similar in sarsaparilla and papaya, 8.88 and 8.92 μg/g fresh wt, respectively; however, the concentrations of β-cryptoxanthin myristate and palmitate were higher in sarsaparilla (8.86 and 5.26 μg/g fresh wt, respectively) than in papaya (1.03 and 0.86 μg/g fresh wt, respectively). Recently, Fu et al.[34] have demonstrated that the esterification of β-cryptoxanthin with saturated fatty acids (lauric, myristic, and palmitic) did not modify the antioxidant activity but increased its stability to heat degradation. Therefore, further research should be carried to investigate the bioaccessibility and bioavailability of the β-cryptoxanthin esters to determine their real contribution to the β-cryptoxanthin incorporated from the diet. Provitamin A accounted for up to 723.58 ± 21.41 RAE/100 g fresh wt (1447.17 ± 43.10 RE/100 g fresh wt), so that sarsaparilla berries can be considered a good source of provitamin A, with similar or even higher values than some Amazonian fruits recently studied.[35] In the present study, it was assumed that β-cryptoxanthin esters have the same provitamin A activity as free β-cryptoxanthin, although this should be further investigated. Other carotenoids were found at lower level, namely, lycophyll (13.70 μg/g fresh wt), zeaxanthin (8.56 μg/g fresh wt; including the free and esterified forms), lutein (0.94 μg/g fresh wt), and antheraxanthin (0.58 μg/g fresh wt).

Xanthophyll esters represented 10% of total carotenoid content, and it was mainly composed of β-cryptoxanthin esters (34.72 ± 1.20 μg/g fresh wt) and a small content of zeaxanthin monomyristate (3.98 ± 0.37 μg/g fresh wt). As shown in Table 2, the β-cryptoxanthin esters fraction was mostly constituted by esters with saturated fatty acid (capric, lauric, myristic, palmitic, and stearic) and only one minor form (0.97 ± 0.02 μg/g fresh wt) was esterified with oleic acid, an unsaturated fatty acid. Within the β-cryptoxanthin esters fraction, acylated forms with capric, lauric, and myristic acids were the major ones, ranging from 7.34 to 8.88 μg/g fresh wt, whereas esters with longer fatty acids, palmitic and stearic acids, were in lower concentrations, 5.26 ± 0.11 and 3.41 ± 0.32 μg/g fresh wt, respectively. Gas chromatography analysis of the fatty acids composition (Table 3)

Table 3. Fatty Acid Composition of Total Lipids from Sarsaparilla (*S. aspera* L.) Berries

fatty acid	composition (%)[a]
capric (C10:0)	11.0 ± 0.4
lauric (C12:0)	24.0 ± 0.6
myristic (C14:0)	2.8 ± 0.0
myristoleic (C14:1)	2.1 ± 0.1
palmitic (C16:0)	15.6 ± 0.1
palmitoleic (C16:1)	0.2 ± 0.0
stearic (C18:0)	10.1 ± 0.1
oleic (C18:1)	1.8 ± 0.0
linoleic (C18:2)	22.7 ± 0.1
linolenic (C18:3)	8.8 ± 0.0
arachidic (C20:0)	0.4 ± 0.0
behenic (C22:0)	0.5 ± 0.1

[a]Data are the mean ± standard deviation (n = 3).

of the sarsaparilla berries revealed that saturated and unsaturated fatty acids accounted for to 63.9 and 36.1%, respectively. The main members of the unsaturated fatty acids were linoleic acid (22.7%) and linolenic acid (8.8%), whereas capric acid (11.1%), lauric acid (24.0%), palmitic acid (15.6%), and stearic acid (10.1%) were the major components of the saturated group. Surprisingly, although myristic acid was in low relative concentration (2.7%), β-cryptoxanthin myristate was among the major esters. On the contrary, unsaturated fatty acids such linoleic and linolenic acids were not involved in the xanthophyll esterification despite being the main members of the total fatty acid profile. The lack of correlation between the fatty acid profile of the total lipid fraction of sarsaparilla and the fatty acid involved in the esterification of the xanthophylls, mainly β-cryptoxanthin esters, suggests that the enzymes responsible for the formation of xanthophyll esters (xanthophyll acyl transferases, XAT) are highly selective regarding the fatty acid, which is in agreement with other previous studies.[11,19,36]

In summary, the diverse carotenoid profile, some of them with provitamin A activity, together with the relatively high content, makes it appropriate to propose sarsaparilla berries as a potential natural source of carotenoids for the food, animal feed, and pharmaceutical industries.

■ ASSOCIATED CONTENT

Supporting Information

Berries, flowers, and European geographical distribution of sarsaparilla (*Smilax aspera* L.). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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