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Determination of the geographical origin of valencia orange juice using carotenoid liquid chromatographic profiles

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Abstract

We present a simultaneous liquid chromatographic method for quantitation of the major carotenoid pigment in pure valencia orange juice from two country origins. This technique involves the use of a C_{30} non-endcapped reversed-phase column and a ternary gradient, methanol–methyl *tert*.-butyl ether–water. Identification of carotenoids is achieved using a photodiode array detection at 350, 430 and 486 nm. The spectra of saponified carotenoid pigments from 23 orange juices samples were compared and discussed in the context of results that were published previously. The behaviour of the C_{30} column with time, on the resolution of two carotenoids, zeaxanthin and isolutein is discussed. This technique has been applied to the determination of the geographical origin of carotenoid compounds using eighteen samples of valencia pure orange juice from two countries (Belize and Spain), collected over two consecutive years. Quantitative determination seems to be a convenient method for determination of the origin of orange juice. The total carotenoids considered were higher in valencia juice from Spain (17.0 ± 5.0 mg l^{-1}) than in valencia juice from Belize (4.8 ± 1.0 mg l^{-1}). Some carotenoids play a large role in origin differentiation, such as phytofluene, which is lower from Belize than from Spain (0.5 vs. 1.8%), or ξ-carotene, which is higher in valencia from Spain (4.9%) than in valencia from Belize (1.8%).

Keywords: Fruit juices; Food analysis; Carotenoids

1. Introduction

Carotenoids are one of the main classes of natural pigments that have been investigated extensively, due to their wide distribution in the plant kingdom. Their basic structure is composed of eight isoprene units; the structure of all carotenoids is derived from that of lycopene, a compound that is characteristic of red and pink grapefruit [1], and containing some structural modification [2]. Carotenoids can be divided into two classes: (i) carotenes or hydrocarbon carotenoids, composed of only carbon and hydrogen, such as α- and β-carotene; (ii) xanthophylls or oxygenated carotenoids, which bear the following

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functions on their terminal ring: epoxy, carbonyl, hydroxyl, ester or acid, such as cryptoxanthin [3].

Carotenoids have been extensively studied in citrus fruits, which are a complex source of carotenoids [4] and, in particular, in orange. The correlation between orange juice colour and carotenoid composition has been studied [5,6]. The presence of numerous conjugated double bond explains the intense colour of the main carotenoids, such as zeaxanthin or β-carotene [4,7], compared to α-carotene and lutein, which have one double bond less. Many liquid chromatographic methods have been developed for the identification of carotenoids or for the separation of the *cis–trans* isomers of α- and β-carotene [8]. The chromatographic system most often employed for the separation and quantitation of several citrus carotenoids was the non-aqueous reversed-phase system [9–11], which was used, in particular, for saponified carotenoid extracts. Nevertheless, Philip et al. [12] separated numerous orange juice carotenoid esters and characterized the presence of tangerine and mandarin in valencia and navel orange juices using the cryptoxanthin palmitate–cryptoxanthin laurate ratio and the cryptoxanthin esters–lutein diesters ratio. Perfetti et al. [13] have detected orange juice adulteration using a fingerprint of unsaponified orange juice carotenoids.

Diode array detectors were used commonly for the identification of carotenoids in citrus due to the specific spectral data of many of these pigments [14,15,16]. Some authors [16] have used a gradient mobile phase composed of a mixture of methanol–water–methyl *tert*.-butyl ether (MTBE) for the separation of a saponified carotenoid extract. Separations were achieved on a material that was commercially available, a non-endcapped polymeric C_{30} reversed-phase column. Such a stationary phase has been proposed by Sander and Wise [17] to be adequate for the separation of lutein and zeaxanthin [17].

The purpose of this study was to develop a quantitative method for the determination of carotenoids in orange juices, starting from chromatographic conditions soon developed [16]. The quantitative composition of carotenoid compounds from eighteen pure valencia orange juices made in Spain and Belize and obtained from fruits harvested over two consecutive years was investigated.

2. Experimental

2.1. Materials

Solvents were of HPLC grade. Methanol was from Carlo Erba (Val de Reuil, France), water was from Riedel-de Haën (Seelze, Germany) and MTBE was from Sigma–Aldrich (Steinheim, Germany). The commercial standards used for determination of retention times and spectral identification were purchased from Extrasynthese (Genay, France) and were zeaxanthin (**12**), β-cryptoxanthin (**14**) and β-carotene (**18**). Five pure orange juices were purchased at a local market in Marseilles and 18 authentic pure orange juices (valencia var.) prepared from fruits harvested during the 1996–1997 and 1997–1998 seasons, nine of which were from Spain and nine from Belize, were given to us by the bureau Couecou (15 samples; Biarritz, France) and three were from the Fruival society (Valence, France).

2.2. Chromatographic conditions

Separations were performed on a YMC column (Hampsted, NC, USA) stainless steel column (250× 4.6 mm I.D.) packed with 5 μm silica spheres that were chemically bonded with C_{30} material and non-endcapped. The gradient profile and the mobile phase composition are given in Table 1. A Waters 600 controller pump was used for analyses. Samples were introduced onto the column via an automatic injector (Waters 717) equipped with a sample loop (20 μl). A Waters 996 diode array detector was set at

Table 1
Gradient profile used in the liquid chromatographic separation of carotenoids from pure orange juice

Time[a] (min)	MTBE[b,c] (%, v/v)	Methanol[c] (%, v/v)	Water[c] (%, v/v)
0 to 12	5	90	5 to 0[a]
12 to 25	5 to 11[a]	95 to 89[a]	0
25 to 40	11 to 25[a]	89 to 75[a]	0
40 to 60	25 to 50[a]	75 to 50[a]	0
60 to 62	50 to 5[a]	50 to 90[a]	0 to 5[a]

[a] Linear gradient.
[b] Methyl *tert*.-butyl ether.
[c] Adapted from ref. [16].

350, 430 and 486 nm, chromatographic data and UV–visible spectra were handled by a Millenium driver station. The column was set at ambient temperature (20–25°C), the inlet pressure was 7 MPa and the flow-rate was fixed at 1.0 ml min^{-1}.

2.3. Sample preparation

2.3.1. Standards

All carotenoid standards were diluted in methanol–acetone, (2:1, v/v) to give a final concentration of 25 mg l^{-1} for zeaxanthin, 20 mg l^{-1} for β-cryptoxanthin and 5 mg l^{-1} for β-carotene.

2.3.2. Citrus juice preparation

Samples was prepared as follows [16]: A 50-ml volume of orange juice was precipitated with 1 ml of an aqueous solution of $ZnSO_4 \cdot H_2O$ (300 g l^{-1}) and 1 ml of $K_4[Fe(CN)_6] \cdot 3H_2O$ (150 g l^{-1}) for precipitation of the total carotenoid compounds contained in orange juices. After mixing, the solution was allowed to stand for 10 min before centrifugation, following which, the supernatant was decanted and discarded. The carotenoids contained in the precipitate were extracted two-fold with acetone (Riedel-de Haën; 40 and 20 ml). The mixture of precipitate and acetone was stirred vigorously for 3 min with a glass rod and centrifuged for 5 min. All acetonic layers were placed in a separatory funnel containing 50 ml of light petroleum (boiling range: 40–70°C; Carlo Erba). The organic phase was washed with 50 ml of water. The carotenoid–petroleum phase was dried with 2 g of anhydrous sodium sulfate and centrifuged. After filtration, the remaining carotenoids contained in sodium sulfate were dissolved with approximately 30 ml of light petroleum. All petroleum extracts were concentrated to dryness in a rotary evaporator at 40°C under vacuum. The residue was dissolved in 6 ml of diethyl ether and 6 ml of 10% methanolic KOH. After standing for 12 h in the dark at room temperature, the methanolic KOH layer was extracted with 20+30 ml of diethyl ether (50 ml). An aqueous solution of NaCl (100 ml of a 10%) was added to the separatory funnel and, after shaking, the ether layer was removed and washed with distilled water (50 ml) until free of alkali. The ether layer was dried with sodium sulfate and evaporated to dryness under vacuum. The carotenoids were dissolved in 500 μl of acetone and 1 ml of methanol and placed in sealed amber vials until analysis.

2.4. Determination of carotenoids in pure valencia orange juice

The carotenoids contained in pure valencia orange juice and in commercially available orange juices were identified by comparison of their retention times and UV–visible spectra with values from literature and with those of standards that were commercially available, **12**, **14**, **18**. For each sample solution, the concentrations of carotenoid pigments were determined using the response factor obtained from β-carotene as a standard and were expressed as a percentage of the total peak taken into account for the quantitative study.

3. Results and discussion

The separation of carotenoid pigments contained in pure valencia orange juice, using the chromatographic conditions given in Table 1, is shown in Fig. 1. We used a slowly increasing percentage of MTBE (between 5 and 25%) in methanol over 10 min. We obtained a slight improvement in the carotenoid profile resolution, especially between mutatoxanthin B (**9**) and lutein (**10**) (Fig. 1A). We observed an inversion of the elution order between two compounds [isolutein (**11**) and zeaxanthin (**12**)] compared with the elution order previously described [16]: in our work, isolutein (**11**) eluted before zeaxanthin (**12**) (Fig. 1A). However, after approximately 100 injections, we established coelution on these two compounds (**11** and **12**) under the same chromatographic conditions, thus indicating the evolution of this C_{30} column with time (Fig. 1B). Fig. 2 shows the separation of commercially available carotenoids after stabilisation of the column (after 100 injections).

Peak identification was achieved using UV–visible spectral data found in the literature [4,16,18]. Results are given in Table 2 and spectral profiles are shown in Fig. 3. Absorption maxima of peaks from 23

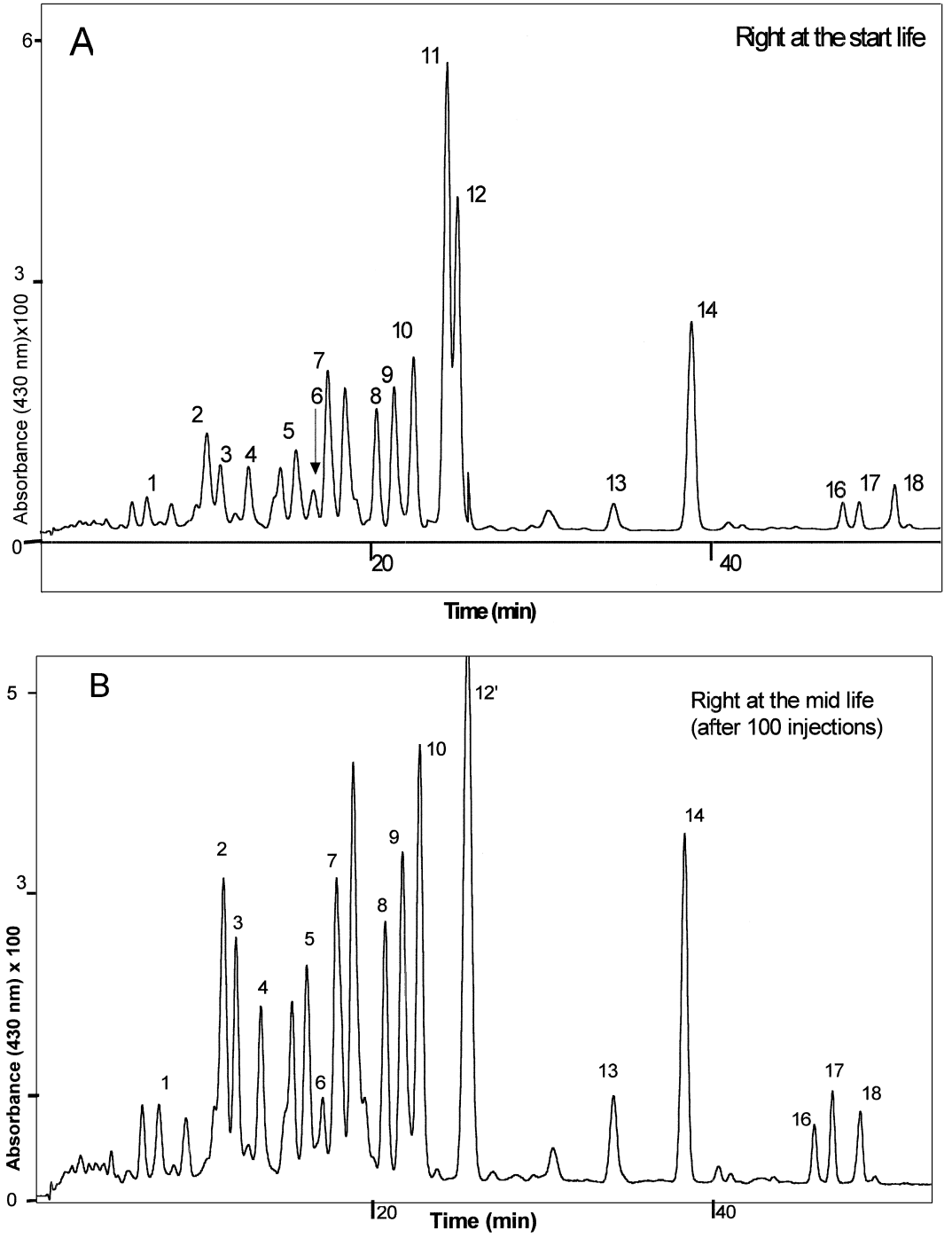

Fig. 1. Chromatographic resolution characteristics during the life of a polymeric C_{30} column [(YMC), 250×4.6 mm I.D., 5 μm non-endcapped], used for saponified carotenoid orange juice. See Table 1 for the conditions of gradient elution and Table 2 for compound identification. Flow-rate, 1 ml min^{-1}; visible detection, 430 nm; amount injected, 20 μl.

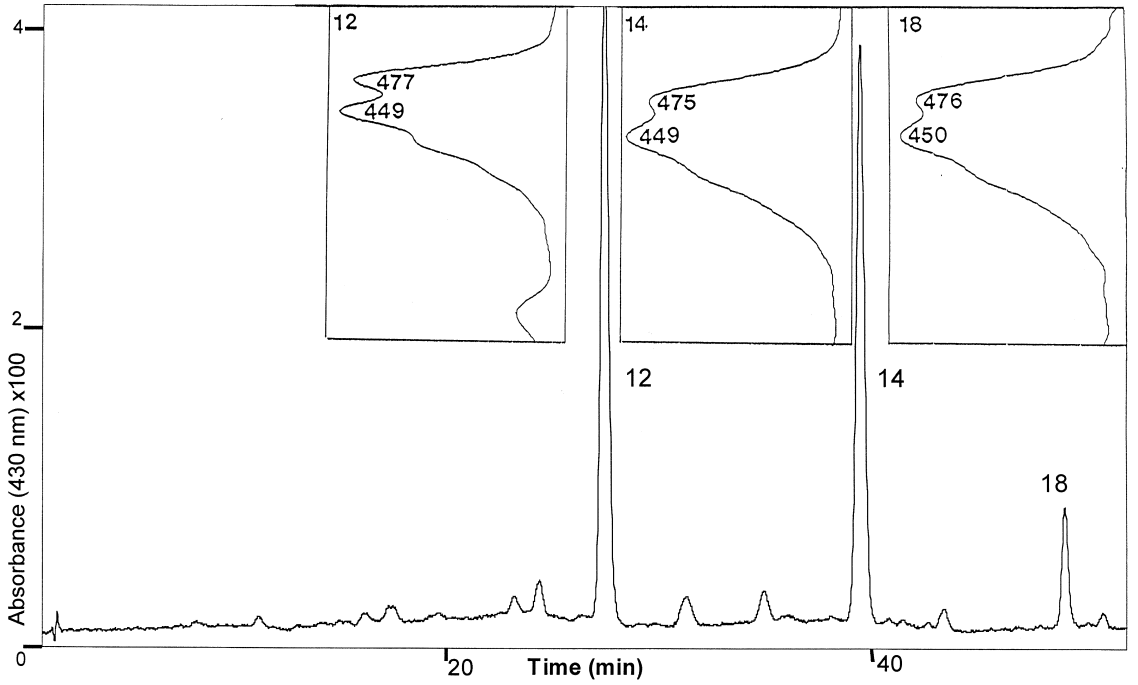

Fig. 2. Separation and spectral characteristics of carotenoid standards. For chromatographic conditions and sample preparation, see Experimental and Table 1. Amount injected, 20 μl of a solution at 25 mg l^{-1} for **12**, 20 mg l^{-1} for **14** and 5 mg l^{-1} for **18**. For compound identification, see Table 2.

orange juice samples were determined and standard deviations are reported in Table 2. Standard deviations are slightly more important using the maxima shoulder measurement, due to the difficulty in measuring the right value on the maxima shoulder compared to the maxima peak (Fig. 3). The standard deviation obtained on the different maxima spectra is good (±0.6 nm at 3.6 nm), the only exception being peak **12′** (isolutein **11**+zeaxanthin **12**), due to the coelution of these compounds.

There were some small differences in the spectral characteristics of carotenoid pigments between our results and those obtained in previous work [16], due partly to the materials and resolution conditions used and in partly due to the percentage of MTBE used, which was slightly different at the time of complete acquisition spectra. For compound **3** (*cis*-antheraxanthin), the first maximum observed is a shoulder (Fig. 3) and for compound **4** (neoxanthin), the third maxima observed was at 467 nm. We noted for compound **4** (neoxanthin) that the absorption maxima given in the literature were 438 and 467 nm, which were determined in ethanol [18]. In Table 2, the α value found in our chromatographic gradient is reported, showing worse resolution between compounds **2** and **3** and compounds **11** and **12**.

The use of a diode array detector allowed for the simultaneous acquisition of complete spectra and therefore allowed us to detect all carotenoids (absorbing at various wavelengths) contained in orange juices, such as phytofluene (330.0, 346.4 and 364.7 nm) and β-carotene (449.9 and 475.8 nm). To avoid the overlapping of peaks for quantitative analysis, we extracted three wavelengths for quantification of some of the carotenoids contained in orange juices: e.g., 350 nm for the quantification of phytofluene (**15**), 430 nm for the quantification of *cis*-violaxanthin (**7**), isolutein+zeaxanthin (**12′**), α-cryptoxanthin (**13**), α-carotene (**16**), ξ-carotene (**17**) and β-carotene (**18**) and 486 nm for the quantitation of lutein (**10**) (better resolution at this wavelength) and β-cryptoxanthin (**14**).

Good repeatabilities, as relative standard deviations, for carotenoid pigments based on relative

Table 2
Comparative chromatographic and spectral characteristics of carotenoids in orange juice

Name	Peak number	Our results[a]						Literature values (nm)		
		α[b]		Maxima spectral absorption[c] (λ), observations (nm)				λ_2	λ_3	Ref.
		Mean[c]	SD[d]	λ_2		λ_3				
				Mean	SD	Mean	SD			
Trollichrome	1	0.178	0.010	419.3	0.6	442.8	0.9	421.5	447.5	[16][g]
Antheraxanthin	2	0.276	0.012	443.2	0.7	470.7	0.4	443	470	[18][h]
cis-Antheraxanthin[f]	3	0.296	0.011	440.7	0.6	467.7	0.9	441.5	471.5	[16]
Neoxanthin	4	0.332	0.013	437.7	0.6	467	0.6	434.5	481.5[e]	[16]
Auroxanthin A[f]	5	0.410	0.009	399.6	0.8	423.9	0.7	400	425	[18]
Auroxanthin B[f]	6	0.437	0.009	399.2	0.6	423.5	0.6	401.5	424.5	[16]
cis-Violaxanthin[f]	7	0.453	0.013	432.7	0.6	462.1	0.7	432	460	[4][i]
Mutatoxanthin A[f]	8	0.536	0.010	425.1	0.7	450.5	0.7	427.5	451.5	[16]
Mutatoxanthin B[f]	9	0.562	0.010	425.3	0.8	450.5	0.4	427.5	451.5	[16]
Lutein	10	0.588	0.009	442.4	0.6	470.4	0.6	445.5	471.5	[16]
Isolutein	11	0.616	0.006	439.9	0.6	466.4	0.6	439.5	467.5	[16]
Zeaxanthin	12	0.634	0.005	449.2	0.3	477	0.6	450	478	[18]
Isolutein+zeaxanthin	12′	0.659	0.012	445.1	1.8	468.1	2.1			
α-Cryptoxanthin	13	0.886	0.005	443.5	0.5	470.9	0.6	445.5	473.5	[16]
β-Cryptoxanthin	14	1.00	0.00	448.9	0.7	475.3	0.7	450.5	477.5	[16]
Phytofluene	15	1.064	0.008	346.4	0.5	364.5	0.7	347	366	[18]
α-Carotene	16	1.213	0.017	443.9	0.9	471.9	0.8	442	472	[18]
ξ-Carotene	17	1.242	0.014	398.8	0.5	423.4	0.6	400	425	[18]
β-Carotene	18	1.286	0.018	449.9	0.8	475.8	0.7	450	476	[18]

[a] Mean of 23 samples.
[b] $\alpha=(t_R-t_{R0})/(t_{R(\beta\text{-cryptoxanthin})}-t_{R0})$.
[c] Eluent, MTBE–methanol–water.
[d] Standard deviation.
[e] In ethanol: 438 nm; 467 nm [18].
[f] Tentatively identified according to [16], unknown.
[g] In MTBE–methanol–water.
[h] In hexane.
[i] In ethanol.

values (% area of total peaks taken in account) and on absolute values (expressed in mass of β-carotene) were observed (2.5 and 3.8%, respectively). The relative standard deviation obtained based on relative values (expressed in % area) is better than that based on absolute values. The limit of quantitation (LOQ) of this method is around 0.01 mg l^{-1} in orange juice.

Figs. 4 and 5 show the carotenoid profiles of two authentic samples of pure valencia orange juice from Belize (Central America) and Spain (Europe), respectively, plotted at 350, 430 and 486 nm. The sample from Spain was differentiated from the Belize sample (shading peaks) by a large amount in phytofluene (**15**) (Figs. 4A and 5A), large amounts of *cis*-violaxanthin (**7**), ξ-carotene (**17**) (Figs. 4B and 5B) and by a large amounts of α- and β-cryptoxanthins (**13**, **14**) (Figs. 4C and 5C). The Belize sample could be differentiated from the Spanish sample by having a large peak of lutein (**10**).

The percentage of carotenoids (**7**, **10**, **13**, **14**–**18**) expressed in mass of β-carotene plus compound **12′** (isolutein+zeaxanthin) are given in Table 3. The Spanish sample is well differentiated from the Belize sample by a different carotenoid profile. Pure valencia orange juice from Spain is characterized by higher contents of **7**, **13**, **14**, **15** and **17** compared with that of Belize origin: compound **7**: 34.6% in

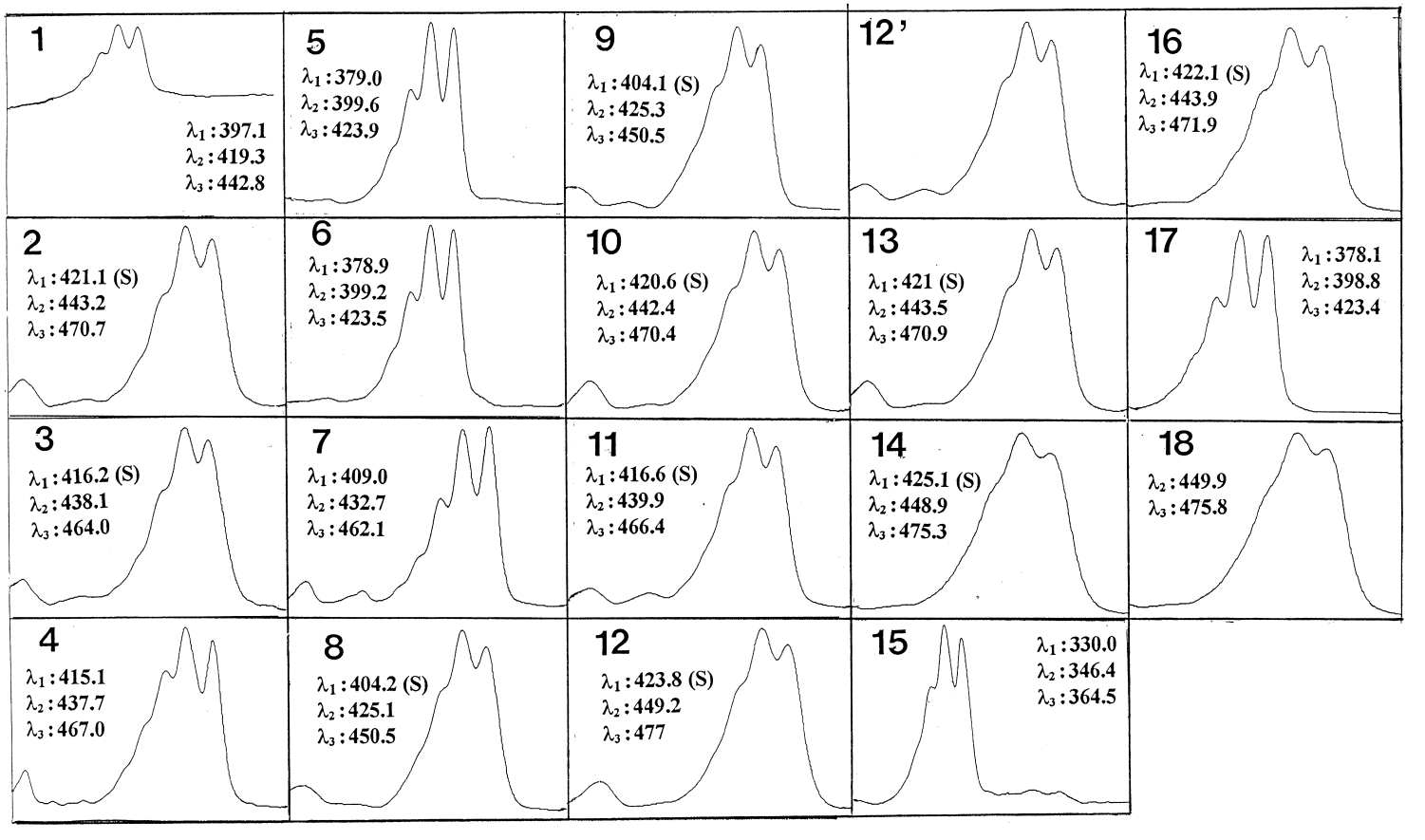

Fig. 3. Spectral characteristics of saponified carotenoids encountered in pure valencia orange juices. For chromatographic conditions and sample preparation, see Experimental and Table 1. For compound identification, see Table 2.

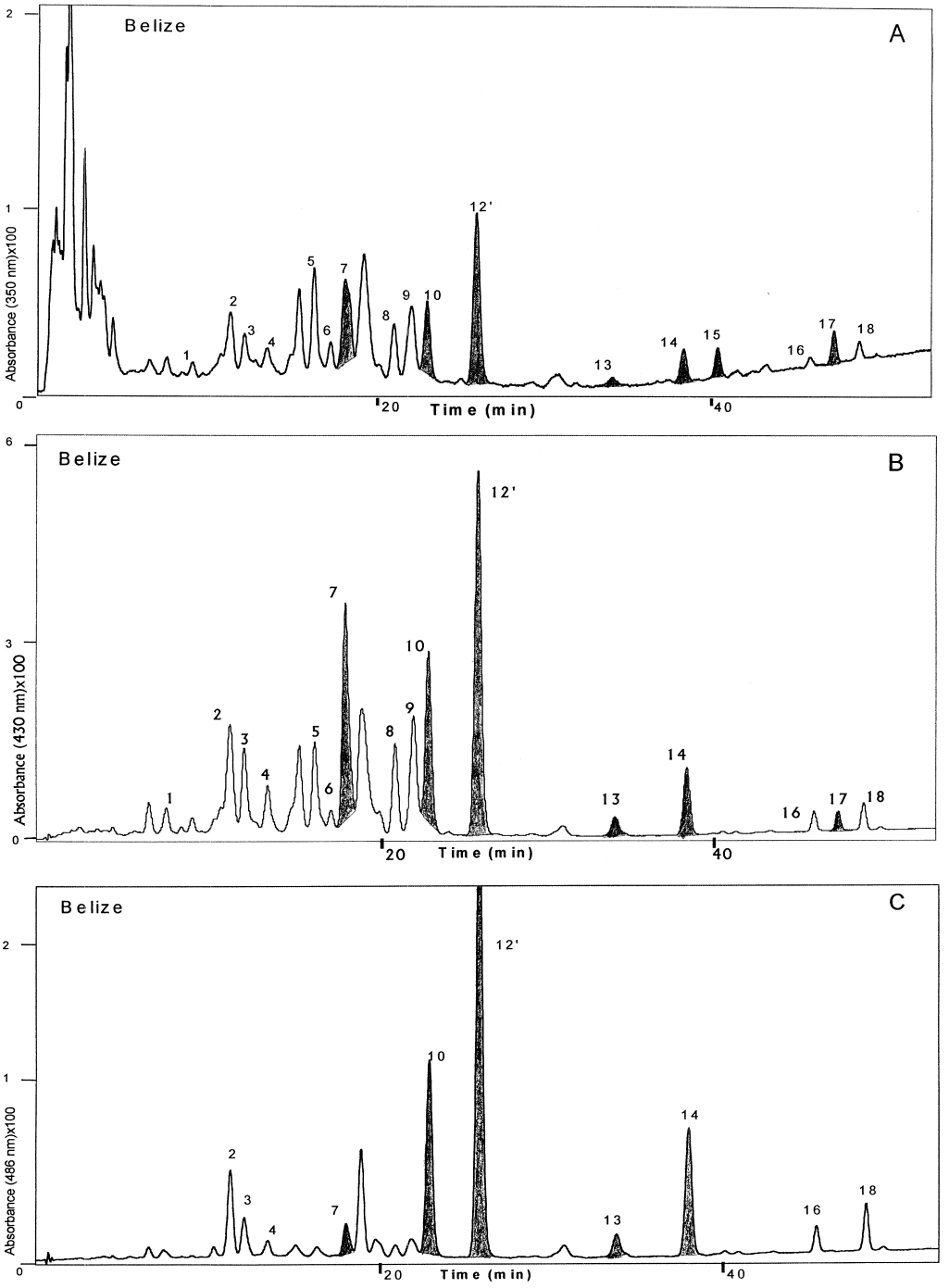

Fig. 4. Carotenoid profile of pure valencia orange juice from Belize, plotted at 350 nm (A), 430 nm (B) and 486 nm (C). For chromatographic conditions and sample preparation, see Experimental and Table 1. For compound identification, see Table 2. Amount injected, 20 μl.

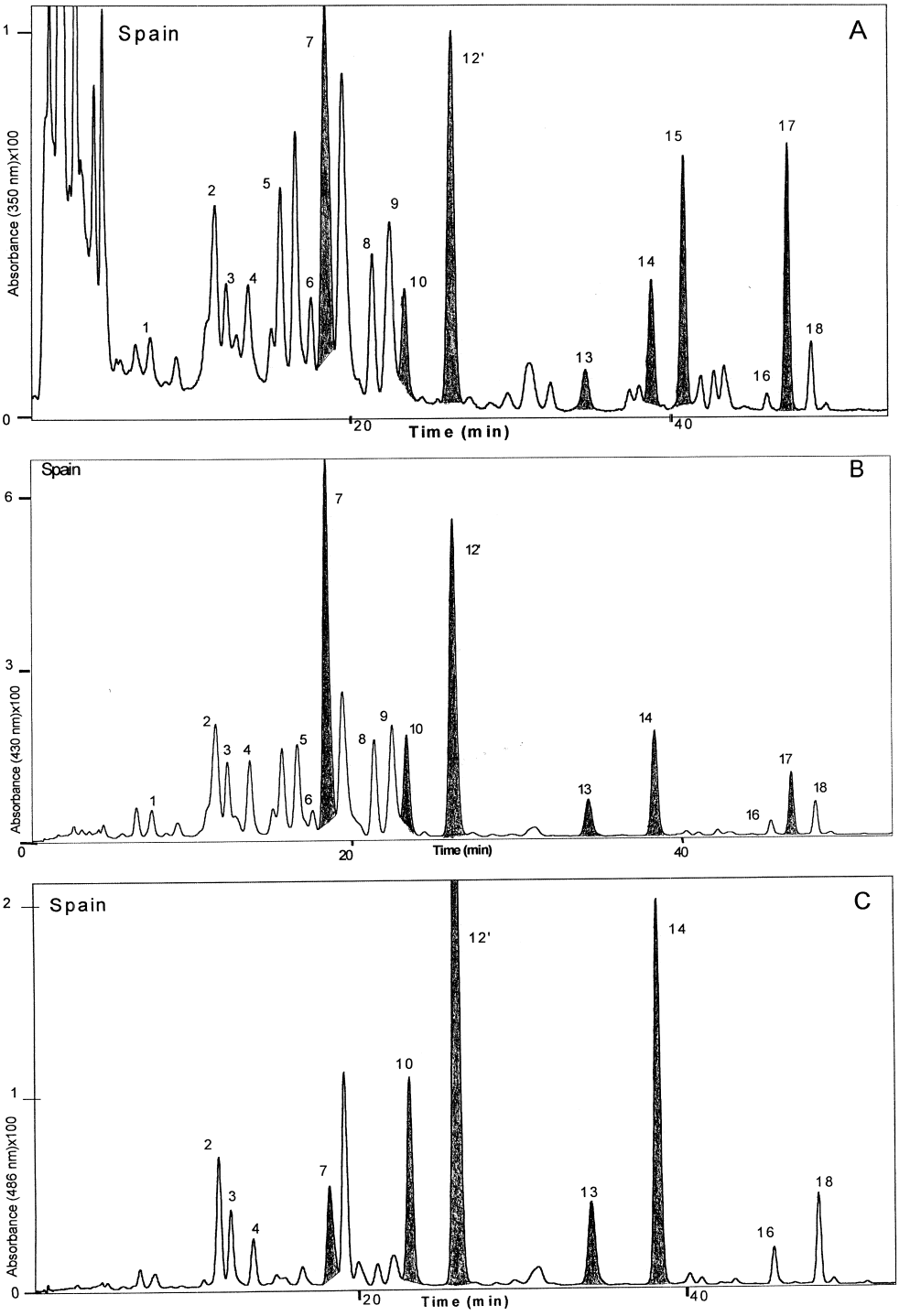

Fig. 5. Carotenoid profile of pure valencia orange juice from Spain, plotted at 350 nm (A), 430 nm (B) and 486 nm (C). For chromatographic conditions and sample preparation, see Experimental and Table 1. For compound identification, see Table 2. Amount injected, 20 μl.

Table 3
Carotenoid contents in various valencia orange juices from two geographical origins.

Compound	Peak No.	Spain				Belize			
		Expressed as a % of the total peak area taken into account		Expressed in mg l^{-1} of β-carotene		Expressed as a % of the total peak area taken into account		Expressed in mg l^{-1} of β-carotene	
		Mean[a]	Min–max	Mean[a]	Min–max	Mean[b]	Min–max	Mean[b]	Min–max
cis-Violaxanthin	7	34.6	21.9–43.6	5.86	3.52–8.20	25.0	22.9–27.2	1.19	0.91–1.47
Lutein	10	6.4	3.8–9.0	1.24	0.73–1.76	10.5	9.2–11.8	0.49	0.35–0.63
Isolutein+zeaxanthin	12′	31.6	28.2–35.0	5.19	3.97–6.41	45.5	44.3–47.0	2.18	1.59–2.77
α-Cryptoxanthin	13	4.9	3.2–6.6	0.93	0.62–1.24	2.4	2.0–2.9	0.12	0.08–0.16
β-Cryptoxanthin	14	12.3	7.6–16.6	1.95	1.18–2.73	8.9	8.0–9.7	0.44	0.32–0.56
Phytofluene	15	1.8	0.9–2.9	0.33	0.15–0.51	0.5	0.4–0.5	0.02	0.02–0.03
α-Carotene	16	1.3	0.9–1.7	0.22	0.12–0.31	2.3	2.0–2.6	0.11	0.08–0.15
ξ-Carotene	17	4.9	3.1–6.7	0.92	0.54–1.30	1.8	1.5–2.0	0.09	0.06–0.11
β-Carotene	18	2.2	1.8–2.7	0.38	0.24–0.52	3.1	2.2–3.9	0.17	0.12–0.21
Total		100		17.0	12.1–22.0	100		4.81	3.75–5.87

[a] Mean of 9 samples of pure valencia orange juices from Spain.
[b] Mean of 9 samples of pure valencia orange juices from Belize.

Spanish sample and 25.0% in Belize sample; compound **13**: 4.9 and 2.4% respectively; compound **14**: 12.3 and 8.9%; compound **15**: 1.8 and 0.5% and compound **17**: 4.9 and 1.8%, respectively. A lower content of compounds **10** and **12′** was also observed in Spanish samples compared to those from Belize: (6.4 and 10.5%m respectively, for compound **10** and 31.6 and 45.5% for **12′**). The pure valencia orange juice from Spain contains a higher total carotenoid content, expressed in β-carotene (12.1–22.0 mg l^{-1}), compared to pure valencia orange juice from Belize (3.8–5.9 mg l^{-1}).

4. Conclusion

Knowledge of the carotenoid compounds in valencia orange juice showed a clear differentiation between those of different geographical origin, i.e., from Spain and Belize. The LC method must be used in conjunction with a photodiode array detection for the judicious identification of the different pigments, by their spectral characteristics, and to obtain various chromatograms at different wavelengths. This method, using a C_{30} column, can be a valuable tool for orange juice quality control and, in addition, for determining polyphenolic profiles for the authentication of citrus juices.

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