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A novel method using high-performance liquid chromatography with fluorescence detection for the determination of betaxanthins

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Abstract

Betaxanthins are natural water-soluble yellow pigments present in plants of the order Caryophyllales. The native fluorescence of these compounds is extensively characterized in this work, with study of the fluorescent properties of 14 different betaxanthins. All the species showed a similar behavior, with excitation maxima between 463 and 475 nm and emission maxima between 506 and 515 nm. Thus, betaxanthins absorb light corresponding to the blue color and emit visible green light. Similarities in excitation and emission spectra point to the responsibility of betalamic acid in fluorescence. The influence of the amine moiety is discussed. For the first time fluorescent properties of betaxanthins are applied to the detection of these pigments after separation by high-performance liquid chromatography. Wavelengths used were 460 nm for excitation and 510 nm for emission, which were suitable for detecting the native fluorescence of all the pigments assayed. Calibration was performed in each case and it exhibited linearity within the range considered, at least 20 μM. The lowest detection limit was 100 nM, corresponding to betaxanthins derived from methionine sulfoxide and leucine. Fluorescence detection was applied to the quantification of betaxanthins present in *Carpobrotus acinaciformis*. The present work opens up new possibilities for the analysis of betaxanthins by improving existing protocols through fluorescence detection.

Keywords: Pigment; Betalain; Betaxanthin; Fluorescence; HPLC

1. Introduction

Betalains are nitrogenous plant pigments and are characteristic of the order Caryophyllales, bestowing yellow and violet coloration [1]. Interest in betalains has grown since their antiradical activity was characterized [2–4] and they are widely used as additives in the food industry because of their natural colorant properties and absence of toxicity [5]. Betalains are divided into two structural groups: betaxanthins (Fig. 1(A)–(D)) where the structural unit betalamic acid is conjugated with an amino acid or amine, and betacyanins (Fig. 1E), in which condensation is made with *cyclo*-DOPA. Betalains are located in different parts of plants [6,7]. Their presence in flowers (*Bougainvillea*, *Celosia*, *Gomphrena*, *Portulaca*, *Mirabilis*,. . .) [8] is especially interesting due to the importance of color, and where a variety of functions has been proposed, including the attraction of animals for pollen transfer.

The growing interest in betalains is demonstrated by the existence of the wide recent literature [1,8,9]. The main characteristic of betalains is color, since they are pigments. The hypsochromic shift that occurs in betacyanins when they are formed through the glycosylation of betanidin or the bathochromic effect when esterification with hydroxycinnamic acids is performed are well known. However, fluorescence in betalains has not been characterized to date. The color in these pigments is attributable to the common resonating double bonds present in their structure due to the betalamic acid moiety [9]. In betacyanins, the electronic resonance is extended to the diphenolic aromatic ring and the absorption maximum is shifted from 480 nm (yellow,

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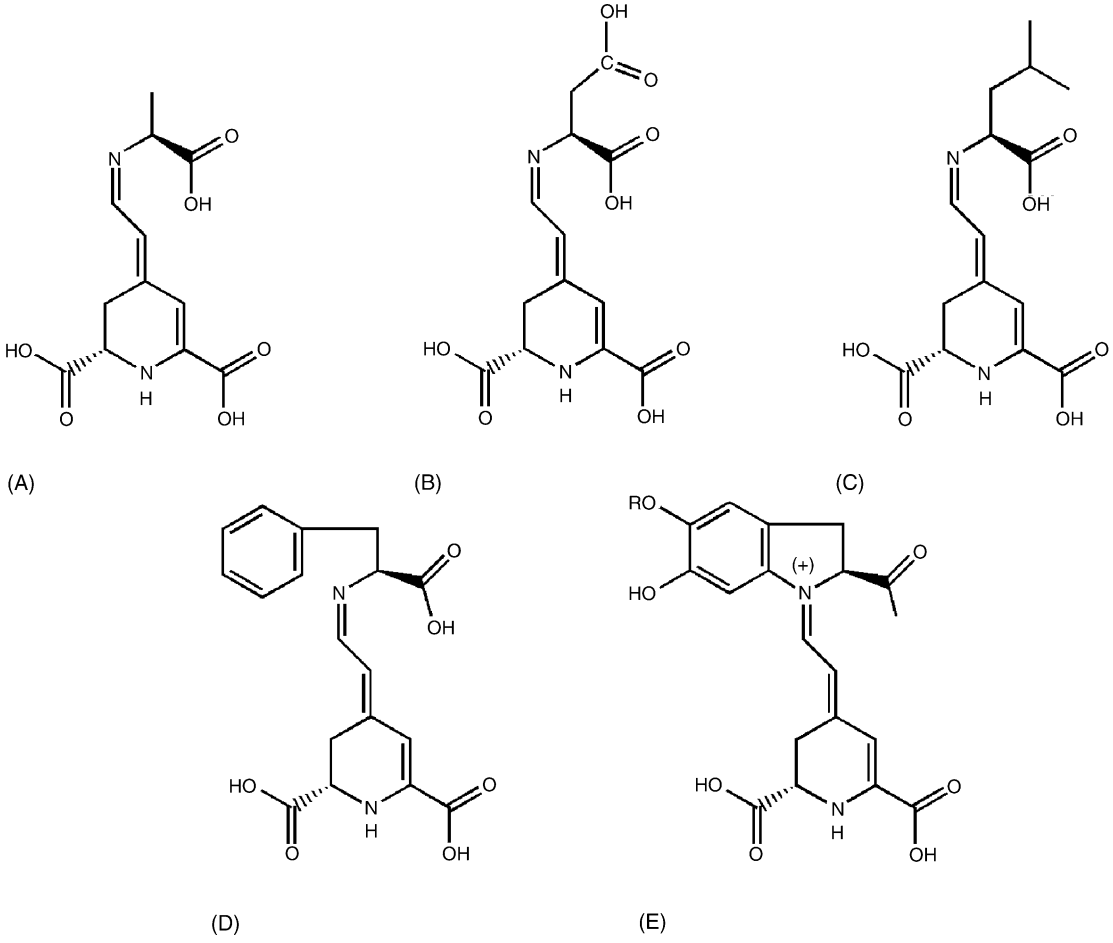

Fig. 1. Structures of betalain pigments. Yellow betaxanthins are exemplified by (A) Ala-betaxanthin, (B) Asp-betaxanthin, (C) Leu-betaxanthin and (D) Phe-betaxanthin. Violet betacyanins structural unit is betanidin (E, R = H). R = glucose, betanin.

betaxanthins) to 540 nm (violet, betacyanins). Betanidin is the basic structural unit of betacyanins, and different structures can be obtained through glycosylation and acylglycosylation of one of the two hydroxyl groups. There are about 15 naturally occurring betaxanthins [8], derived from different amino acids and new structures are continuously being described [10].

Natural pigment identification and quantitative analysis have attracted the attention of researchers in order to analyze the pigment composition of a variety of sources, especially given the use of natural pigments as food colorants [11]. Several HPLC methods have been reported for the analysis of betalains using reversed phase chromatography and different kinds of detection. Absorbance [12,13] and photodiode array detection [7,14–16] are traditionally used for betalain identification, and mass spectrometry [10] and NMR [17] detectors are gaining relevance due to the structural information they are able to provide. To date fluorescence has not been considered for betaxanthin analysis.

Fluorescence detection implies high selectivity of the analysis due to the specific excitation of the compounds and it has been extensively employed for the determination of different kinds of molecules in biosciences. Compounds like vitamins [18,19], amino acids [20,21] and drugs [22,23] have been successfully determined by using fluorescence detection after a chromatographic step. Because few compounds emit a strong fluorescence suitable for direct measurements, a derivatization procedure is normally necessary [20,21,24].

In the present work, fluorescence of betalains is studied in depth, and the fluorescent properties of 14 different betaxanthins are characterized. Application of native fluorescence to the detection of these natural compounds after HPLC separation is described for the first time.

2. Experimental

2.1. Chemicals

Chemicals, solvents and reagents were purchased from Sigma and Fluka (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Labscan Ltd. (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Equipment

The LC system was a Shimadzu LC-10A (Shimadzu Corporation, Kyoto, Japan) unit equipped with an RF-10AXL fluorescence detector (Shimadzu). Alternatively a SPD-M10A photodiode array detector (Shimadzu) was used. Fluorescence spectroscopy was performed in an LS50B apparatus (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). An Uvikon 940 spectrophotometer (Kontron Instruments, Zurich, Switzerland) was used for absorbance spectroscopy. Betanin extraction was performed in a Model 230 Omnimixer (Sorvall Inc., Norwalk, CT, USA). Natural pigments form flowers were extracted by using a Polytron homogenizer (Kinematica AG, Littau, Switzerland). The ultracentrifuge was an Optima LE-80K from Beckman Coulter (Fullerton, CA, USA). An Äkta purifier automated system (Amersham Biosciences, Uppsala, Sweden) was used for betaxanthins purification.

2.3. Sample preparation

2.3.1. Betacyanins

Betanin was obtained from commercial red beet. Roots were peeled and sliced and extraction was performed in 10 mM phosphate buffer, pH 6.0, in a mixer at maximum speed for 10 s. The homogenate was filtered through cheesecloth and centrifuged at 120,000 × *g*. The supernatant was then filtered using a YM-10 membrane (Millipore) to remove proteins. All steps were carried out at 4 °C. The pigment was then purified according to the method described by Escribano et al. [2]. A Sephadex G-25 (Sigma) gel was conditioned and used in a 30 mL column. Elution was performed with a volume of 40 mL of purified water, pH 6.0 and 1 mL fractions were collected. The elution process was followed at 536 and 480 nm. Fractions containing purified betanin were pooled.

Betanidin was obtained enzymatically from purified betanin through β-glucosidase treatment. A betanin solution 4 μM was incubated for 30 min with 14 units/mL of β-glucosidase (Sigma) in 50 mM acetate buffer, pH 5.0 at 25 °C. The enzyme was removed by ultrafiltration through YM-10 membranes. Transformation was complete according to HPLC analysis.

2.3.2. Betaxanthins

Synthetic betaxanthins were obtained as immonium condensation products of betalamic acid with several amino acids (L forms of DOPA, Tyr, Gln, Glu, Asp, Met sulfoxide, Pro, Ala, Phe, Met, His and Leu) and amines (tyramine, dopamine), as previously described by Wyler et al. [25], with some modifications. Briefly, betanin was extracted and purified as described above and used as a source of betalamic acid. Basic hydrolysis (pH 11.4) of 0.15 mM betanin released the betalamic acid [12], which was condensed with different amines or amino acids after reaching pH 5.0. The corresponding betaxanthin was obtained, accompanied by a color change from pale yellow (betalamic acid, λ_m = 424 nm) to deep yellow (betaxanthins, λ_m = 480 nm). Immediately after synthesis, an automated system was used to achieve a purification step based on anionic exchange chromatography to remove the excess of substrates and secondary products. Solvents used were 2,2-bis-(hydroxymethyl)-2,2′,2″-nitrilotriethanol (BisTris) 20 mM, pH 6.0 (solvent A) and BisTris 20 mM, pH 6.0 with 2 M NaCl (solvent B). A 25 mm × 7 mm, 1 mL Q-Sepharose Fast Flow column (cross-linked agarose with quaternary ammonium as exchanger group, 90 μm of particle size) purchased from Amersham Biosciences Inc. was used. The elution process was as follows: 0% B from beginning to 15 mL; then a linear gradient was developed from 0% B to 35% B in 20 mL, collecting 1 mL fractions. Cleaning (7 mL, 50% B) and re-equilibration (7 mL, 100% A) steps were performed between each elution. Injection volume was 1 mL and the flow rate was 0.5 mL/min.

2.3.3. Pigments from flowers

Carpobrotus acinaciformis plants were obtained from a plant nursery and supplier (Eco-Flor, Murcia, Spain), and grown by the authors in Murcia (Spain). Yellow flower samples were collected and petals were carefully removed and washed. Pigments were extracted from 1.0 g petals with 10 mL of phosphate buffer 10 mM, pH 6.0, containing 10 mM ascorbic acid (AA) in a homogenizer (5 s, 2 pulses, at medium speed). The homogenate was filtered through nylon cloth and then centrifuged at 120,000 × *g* for 40 min. The supernatant was filtered through YM-10 membranes (Millipore, Bedford, MA, USA) to remove proteins and the filtrate was used for pigment analysis. The whole process was carried out at 4 °C.

2.4. Fluorescence and absorbance spectroscopy

For fluorescence spectroscopy, samples were diluted in water and excitation and emission spectra were recorded at 25 °C. These conditions were chosen because they are near to the physiological ones [26], and because most of the possible applications of the fluorescence of these pigments will imply an aqueous environment. Quartz cuvettes were used. The final concentration of the pigment was set at 6 μM, except for the MetSO-derived betaxanthin, where the final concentration used was 3 μM. Excitation spectra were recorded in each case by following emission at the maximum wavelength. Emission spectra were obtained by exciting at the corresponding maximum.

Pigment concentration was evaluated through absorbance at 25 °C, by taking a molar extinction coefficient of ε = 48,000 M^{-1} cm^{-1} [14,27] at 480 nm for betaxanthins and ε = 65,000 and 54,000 M^{-1} cm^{-1} at 536 nm for betanin and betanidin, respectively [13].

2.5. HPLC analysis

For reversed phase chromatography, a linear gradient was performed in 25 min from 100% A (water + 0.05% trifluoroacetic acid – TFA) to 65% A and 35% B (acetonitrile +

0.05% TFA). A 5 μm C-18 column (250 mm × 4.6 mm) supplied by Teknokroma (Barcelona, Spain) was used. The flow rate was 1 mL/min, operated at 25 °C. For fluorescence detection, excitation was carried out at 460 nm and emission was followed at 510 nm. The injection volume was 10 μL. All experiments were performed at least in duplicate. Alternatively, photodiode array detection was used under the same conditions. In this case, injection volume was 20 μL.

3. Results and discussion

3.1. Extraction and synthesis of betalains

Betanin (betanidin 5-*O*-β-glucoside) is present in most plants containing the violet betacyanins [28,29]. It was extracted from beet root and purified by G-25 chromatography. HPLC-PDA detection confirmed the purity of the pigment and showed that the diastereoisomeric form isobetanin was present with a betanin:isobetanin ratio 95:5. The presence of diastereoisomers has been extensively reported for betalains [8] and is associated to the chirality of the C-15 carbon present in the betalamic acid moiety.

Betanidin is the structural unit of most betacyanins and a 5-*O*-glucosiltransferase is able to add a glucose residue leading to betanin [30]. The inverse process was followed in this work to obtain betanidin enzymatically, as described in Section 2.

Betaxanthins are imines and can be obtained as condensation products of the building block betalamic acid with different amines or amino acids through a Schiff condensation reaction [25]. The chiral carbon responsible for diasteroisomerism in the synthesis starting material betanin will be maintained. Thus, the compounds obtained will also exist with both isomers, with around 5% in the "*iso*" form (2*S*/*R*). Immediately after synthesis, a purification protocol was carried out. Precursor amines and amino acids as well as *cyclo*-DOPA-glucoside – the other degradation product of betanin – were unable to interact with the column under the working conditions and were totally washed out as an unbound fraction. The pigments were stable for further work and characterization.

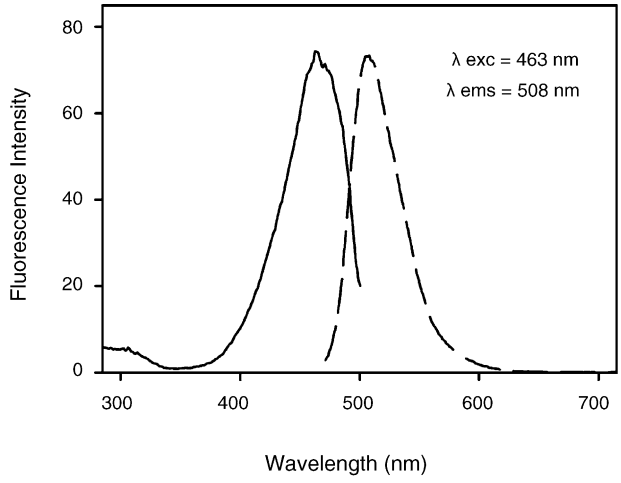

Fig. 2. Fluorescence spectra (—, excitation; - - -, emission) for the betaxanthin derived from Ala. Conditions were as detailed in Section 2.

3.2. Characterization of fluorescence in betaxanthins

The native fluorescence of fourteen different betaxanthins was investigated. Fig. 2 shows the spectra obtained for the simplest betaxanthin studied, derived from the amino acid Ala (Fig. 2), which was recently described as a natural pigment [10]. It exhibits fluorescence with excitation and emission maxima of 463 and 508 nm, respectively. Analogous results were found for other betaxanthins derived from polar, non-polar and aromatic amino acids. All of them exhibited fluorescence and showed similar excitation and emission spectra to those described in Fig. 2. Wavelengths where the spectra are situated correspond to the visible range of the electromagnetic spectrum. Table 1 compiles excitation and

Table 1
Fluorescent properties of betaxanthins

Betaxanthin (trivial name)	Amino acid/amine	λ_m Exc (nm)	λ_m Ems (nm)	Stokes shift (nm)	Relative fluorescence intensity[a] (%)	Exc. width (nm)	Ems. width (nm)
Muscaurin VII	His	465	509	44	128	56.5	47.5
Vulgaxanthin I	Gln	464	509	45	103	60.5	49.5
Miraxanthin I	MetSO	475	509	34	218	56.5	49
Miraxanthin II	Asp	474	507	33	111	58.5	46.5
Vulgaxanthin II	Glu	466	508	42	136	59	46.5
–	Ala	463	508	45	100	61.5	47.5
Indicaxanthin	Pro	463	515	52	88	65	41.5
Dopaxanthin	DOPA	463	510	47	81	63.5	46.5
–	Met	464	509	45	138	59	46.5
Portulacaxanthin II	Tyr	474	509	35	94	58	46.5
Miraxanthin V	Dopamine	465	512	47	54	62.5	67.5
Miraxanthin III	Tyramine	464	506	42	85	61	47
–	Leu	464	509	45	128	61.5	47
–	Phe	464	510	46	133	60.5	45.5

Measurement conditions were as described in Section 2.

[a] Relative fluorescence intensity values are calculated in relation to Ala-derived betaxanthin.

emission maximum wavelengths measured for each of the assayed betaxanthins which present excitation maxima between 463 and 475 nm and emission maxima between 506 and 515 nm. The width for the spectra at half the maximum intensity is also given. Shapes of the spectra obtained in all cases respond to the same pattern. They exhibit a strong fluorescence not characterized by other authors.

Betaxanthins share the betalamic acid moiety, which is known to act as the chromophore due to the presence of the conjugated double bonds [9]. These bonds may also be responsible for the fluorescent properties. The nature of the amine or amino acid moiety seems to have a limited contribution to the final spectral characteristics and there are no significant differences in relation to the chain length or its polarity. However, an intensifying effect when the electron density is withdrawn from the resonating system can be observed. This may explain the high fluorescence exhibited by the pigment derived from Met sulfoxide when compared with that corresponding to Met-betaxanthin (Table 1). The betaxanthins derived from Gln and Glu have the same chain length and the latter present higher fluorescence intensity, presumably due to the withdrawing effect of the carboxyl group. Moreover, in aromatic betaxanthins, those derived from monophenolic structures (Tyr-betaxanthin and tyramine-betaxanthin) present higher intensities than their corresponding diphenolic analogues, which may be due to the drawing effect of the hydroxyl group in the aromatic ring. Furthermore, the presence of the extra carboxylic acid in DOPA and Tyr-betaxanthin strengthens the fluorescence with respect to dopamine- and tyramine-betaxanthin. However, the fluorescence intensity of the pigment derived from Phe is not in line with this argument, indicating that other factors may also be involved in the modulation of fluorescence by the amine moiety. Aromatic amino acids absorb in the UV range of the electromagnetic spectrum and present fluorescence [31]. This phenomenon is not related to the fluorescence of aromatic betaxanthins, which implies visible light for excitation and emission and is due to the electronic resonance in the dihydropiridine moiety.

Betanidin and the derivative pigment betanin were assayed for fluorescence and no positive results were obtained. Both have the same dihydropiridine moiety responsible for fluorescence in betaxanthins, but in these cases *cyclo*-DOPA or its glucoside are attached. The aromatic diphenolic structure resonates with the system responsible for fluorescence and there is a shift in absorbance from 480 to 540 nm and fluorescence disappears for betacyanins.

3.3. Fluorescence detection of betaxanthins after high-performance liquid chromatography

The absence of visible fluorescence in most biomolecules, the existence of two specific wavelengths and the sensitivity of fluorescence detectors may improve the existing methods used for separation, identification and quantification of betaxanthins.

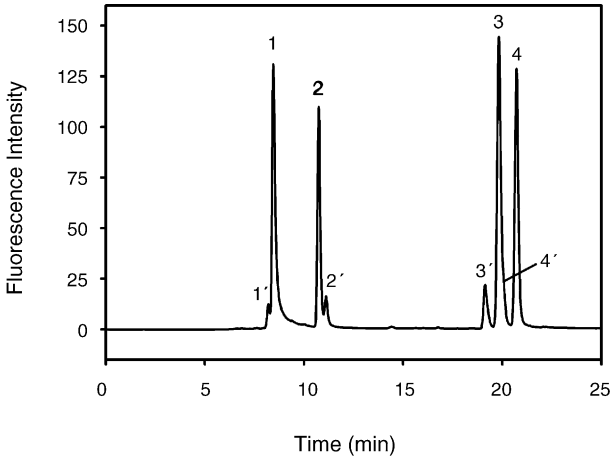

Fig. 3. HPLC profile following fluorescence for the analysis of a betaxanthin mixture 80 μM containing, in the same ratio, the following pigments: Asp-betaxanthin (1), Ala-betaxanthin (2), Leu-betaxanthin (3) and Phe-betaxanthin (4). 2*S*/*S* pigments (main peaks) are accompanied by the 2*S*/*R* diastereoisomeric forms (minor peaks).

HPLC analyses of the synthesized betaxanthins were made with the system described in Section 2. Wavelengths for detecting the fluorescence of betaxanthins after separation were set at 460 nm for excitation and 510 nm for emission. These conditions were appropriate for the detection of all the betaxanthins assayed, independently of the elution time – acetonitrile content. Fig. 3 shows the chromatographic profile obtained for a mixture of selected betaxanthins derived from the amino acids Ala, Asp, Leu and Phe. The wavelengths used for the detector should be considered as standard conditions for future betaxanthin analysis by fluorescence detection.

Due to the successful detection of betaxanthins through native fluorescence, reported here for the first time, calibration graphs were performed for all the betaxanthins assayed. Linear regression analysis was made by plotting concentration (μM) against peak area using the signal obtained from fluorescence. The number of data points used for calibration was five and samples were injected at least in duplicate. The results obtained are summarized in Table 2. The linearity of the detection is maintained in the working range, at least 20 μM and regression factors calculated are comprised between $r = 0.9994$ and 0.9999. Detection limits (LOD) were calculated on the basis of 3σ (σ being the residual SD around the regression line) and the quantitation limits (LOQ) on the basis of 10σ. All values are listed in Table 2. In those pigments where diastereoisomer pairs are separated, data correspond to the calibration of the main isomer (2*S*/*S*). Individual content was determined by HPLC as percentage of peak area.

The calibration parameters and the data estimated for the limits of detection and quantification of betaxanthins through HPLC with fluorescence detection imply a general improvement with respect to the data obtained with the PDA detector (Table 2). Fluorescence allows a reduction in the sample volume and in the minimum concentration suitable for quantification in most of betaxanthins, mainly

Table 2
HPLC calibration parameters for betaxanthins, followed by fluorescence detection

Amino acid/amine[a]	Rt (min)		Calibration			LOD (μM)	LOQ (μM)	LOD[b] (μM)	LOQ[b] (μM)
	2*S*/*S*	2*S*/*R*	Slope (μM^{-1})	Intercept	Corr. coeff.				
His	7.04		162058 ± 3059	33120 ± 20121	0.9995	0.3	1.5	0.8	2.4
Gln	8.02		23285 ± 420	2479 ± 3220	0.9997	0.4	1.6	0.5	1.3
MetSO	8.05		160026 ± 488	−5085 ± 3743	0.9999	0.1	0.3	0.2	0.5
Asp	8.46	8.22	109139 ± 2222	−61983 ± 23077	0.9994	1.4	3.5	1.2	3.7
Glu	9.61		72678 ± 1194	−12392 ± 7085	0.9996	0.6	1.5	0.5	1.8
Ala	10.75	11.12	64195 ± 1296	24945 ± 12269	0.9994	0.4	2.3	1.2	3.7
Pro	12.65	12.20	76269 ± 591	−12831 ± 3872	0.9999	0.3	0.6	0.4	1.0
DOPA	14.46	14.16	37304 ± 99	−4086 ± 3574	0.9999	0.5	1.4	0.5	1.1
Met	16.42	15.73	113127 ± 1081	−5896 ± 2875	0.9999	0.2	0.4	0.3	0.7
Tyr	16.43	16.08	62189 ± 365	−5581 ± 1386	0.9999	0.2	0.4	0.3	0.8
Dopamine	16.62		22474 ± 42	−13769 ± 1973	0.9999	0.8	1.7	0.6	1.8
Tyramine	18.70		35534 ± 127	−9499 ± 2631	0.9999	0.5	1.2	0.6	1.7
Leu	19.90	19.17	107384 ± 762	17957 ± 6551	0.9999	0.1	0.7	0.4	1.2
Phe	20.26	20.01	99641 ± 1382	−5423 ± 8955	0.9997	0.4	1.3	0.6	1.7

Excitation was made at λ = 460 nm and fluorescence was measured for emission at λ = 510 nm.
[a] Betaxanthins are identified by means of the amine moiety.
[b] LOD and LOQ determined for PDA detection are provided for comparative purposes.

in MetSO-betaxanthin, Pro-betaxanthin, Tyr-betaxanthin and Leu-betaxanthin, where the limits are reduced to about 50%. Detection limits are also improved, especially for His-betaxanthin, Ala-betaxanthin and Leu-betaxanthin, whose limits are reduced by 60%.

Pre-existing protocols for betaxanthin analysis can be improved by using fluorescence detection, which may become a powerful tool in a field where new pigment sources are investigated and natural betaxanthins are continually being described [10].

3.4. Application of fluorescence to the determination of betaxanthins present in Carpobrotus acinaciformis

The pigment content of a wide number of species has been investigated in order to evaluate the potential of plants as sources of natural food colors [1,11]. *Carpobrotus* is a genus of plants, native to South Africa, which belongs to the Aizoaceae family. The existence of betalains in the genus *Carpobrotus* has been known since Piattelli and Impellizzeri [32] described the presence of a mixture of betacyanins in the violet flowers of *C. acinaciformis*. However, no report exists on the pigments present in the yellow ones. Extraction of the pigments of yellow flowers of *C. acinaciformis* was performed in phosphate buffer, pH 6.0, as described in Section 2. Fluorescence detection was applied after HPLC separation under the same conditions described for the standards, and the presence of a mixture of betaxanthins was evidenced (Fig. 4). The identity of the pigments corresponding to the main peaks was determined by retention times (Table 2) and confirmed by coelution experiments with synthetic standards. The individual pigment content was determined by means of the calibration curves described above and was as follows: Gln-betaxanthin, 0.028 mg g^{-1} of fresh petals; Glu-betaxanthin, 5.5 μg g^{-1} and Tyr-betaxanthin, 0.011 mg g^{-1}.

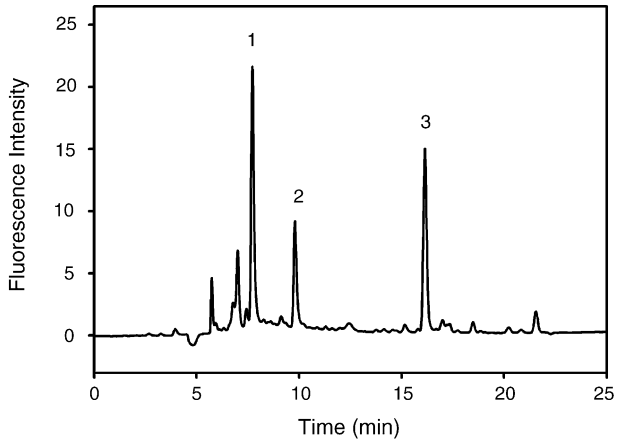

Fig. 4. HPLC profile obtained for yellow *C. acinaciformis* flower extract, measuring fluorescence. Using standards, the main peaks were identified as Gln-betaxanthin (1), Glu-betaxanthin (2) and Tyr-betaxanthin (3).

4. Conclusions

The native fluorescence of 14 different betaxanthins was characterized. All the compounds showed similar excitation and emission spectra. We conclude that the optical properties exhibited are due to the presence of the betalamic acid moiety conjugated with an amine or amino acid, and are general to all betaxanthins. The amine group modulates the fluorescence intensity, with an intensifying effect being observed when withdrawing groups are condensed. The presence of *cyclo*-DOPA in betacyanins extends the resonating system of the dihydropyridine moiety and implies a lack of fluorescence.

A new detection procedure suitable for HPLC analysis has been developed based on the fluorescent properties of betaxanthins. These compounds were detected for the first time through fluorescence, and calibration was performed.

By combining HPLC separation and fluorescence detection, improved selective and sensitive determinations of betaxanthins can be obtained. This can promote research into the field of betalains and may lead to the discovery of new natural betaxanthins. The description of fluorescence in natural and highly hydrophilic pigments may be of interest for food colorant technology as betalains are permitted as a food color additive.

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