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Simultaneous extraction and analysis by high performance liquid chromatography coupled to diode array and mass spectrometric detectors of bixin and phenolic compounds from annatto seeds

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ABSTRACT

This study was designed to identify and quantify the carotenoids and phenolic compounds from annatto seeds using high performance liquid chromatography coupled to diode array and mass spectrometer detectors (HPLC–DAD-MS/MS). Furthermore, using response surface methodology, an optimized procedure for simultaneous extraction of these compounds was established. In addition to bixin, known to be the main carotenoid in annatto seeds, hypolaetin and a caffeoyl acid derivative were identified as the main phenolic compounds. The optimized procedure involved 15 extractions using acetone:methanol:water (50:40:10, v/v/v) as solvent, a solid–liquid ratio of 1:9 (m/v) and an extraction time of 5 min. Validation data indicated that the HPLC method proposed provided good linearity, sensitivity, procedure accuracy, system precision and suggested its suitability for the simultaneous analysis of phenolic compounds and carotenoids in annatto seeds.

1. Introduction

Brazil is one of the major producers and exporters of the annatto seeds and extracts. These are used as coloring agents in the food, pharmaceutical and cosmetic industries [1] and are known for their antioxidant capacity, which is due to carotenoids and phenolic compounds.

Bixin (methyl (9-*cis*)-hydrogen-6,6′-diapo-Ψ,Ψ-carotenedioate) (Fig. 1), accounting some 80% of the carotenoids present [2], is responsible for the reddish-orange color of the annatto seeds and their extracts. Its antioxidant capacity results from its ability to quench singlet oxygen, deactivate the excited triplet state of sensitizers and scavenge free radicals [3–5].

Although the seeds are reported to contain phenolic compounds [6], no information about the composition of phenolic compounds present in the seeds and extracts were found. According to Harbone [7], the leaves were found to contain the flavones apigenin 7-glucoside, apigenin 7-bisulphate, luteolin 7-glucoside, luteolin 7-bisulphate as the major compounds, and the flavonoid hypolaetin 8-bisulphate as the minor one (Fig. 1).

Both carotenoids and phenolic compounds are considered to promote human health, since they are responsible for critical biological functions [8,9]. These compounds are associated with a decrease in the risk of development of inflammations and cataracts, as well as various chronic degenerative diseases, such as cancer, cardiovascular diseases and macular degeneration [10–12]. In general, the reduction in the risk of these diseases involves the inhibition of oxidative reactions via the quenching of singlet oxygen and scavenging of free radicals.

Response surface methodology (RSM), introduced by Box and Wilson [13], is useful for the evaluation of the effects of multiple factors and their interactions and can be effectively used to find the combinations of these factors, which will produce an optimal response. One of the main advantages of this methodology is that it generally requires fewer experiments than would be necessary for a traditional full factorial design, yet provides statistically valid results. The central composite rotational design (CCRD) is the most popular form of RSM and it has been utilized to optimize the extraction process of secondary metabolites [14–16].

The objective of this study was the development and validation of a simple and reliable method for simultaneous separation, identification and quantification of the main carotenoids and phenolic compounds in annatto seeds and extracts using high performance liquid chromatography coupled to diode array and mass spectrometer detectors (HPLC–DAD-MS/MS). Moreover, the conditions for

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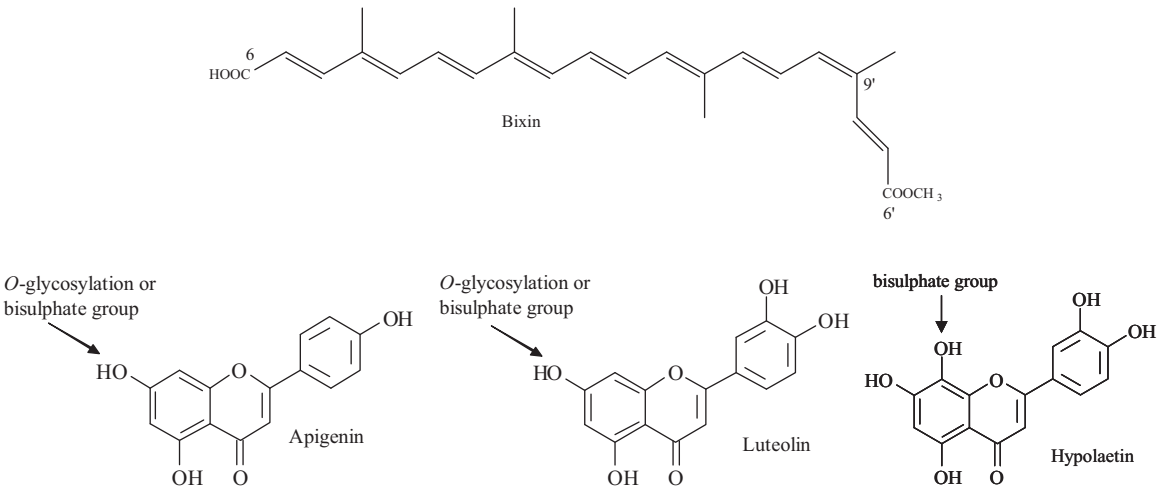

Fig. 1. Structures of bixin ($C_{25}H_{30}O_4$), apigenin, luteolin and hypolaetin with common *O*-glycosylation and bisulphate positions indicated with an arrow.

simultaneous extraction of compounds from both carotenoids and phenolic classes were optimized by response surface methodology.

2. Materials and methods

2.1. Materials

In August 2008, annatto seeds were obtained from the local market in Campinas, São Paulo, Brazil. The seeds were portioned (500 g), vacuum packed and stored under light-free conditions at room temperature until analysis. The acetone and methanol (P.A.) used on the extraction procedure were obtained from Synth (São Paulo, Brazil). Methanol and acetonitrile of chromatographic grade were obtained from J.T. Baker (Phillipsburg, USA) and ultrapure water was obtained from the Millipore system (Billerica, USA). Formic acid was purchased from Merck (Darmstadt, Germany). The standards quercetin and rutin were purchased from Sigma–Aldrich Co. (St. Louis, USA), and gallic acid from Extrasynthèse (Lyon Nord, France). The bixin standard was isolated in our laboratory [17] and re-crystallized to achieve 98% purity, as determined by HPLC–DAD.

2.2. Extraction procedure

The phenolic compounds and carotenoids were extracted from about 1 g of annatto seeds using different ratio values of methanol:water (8:2, v/v) in acetone solution at 25 ± 1 °C using ultrasound equipment (Unique, São Paulo, Brazil). The proportion of methanol:water (8:2) in acetone varied from 10 to 90%, and the solid (g)–liquid (mL) ratio from 1:1 to 1:9 (m/v); extraction time and number of extractions were also investigated (from 5 to 25 min and from 5 to 25 extractions, respectively). After extraction, the extracts were stored at −36 °C until analysis.

2.2.1. Experimental design

The optimal conditions for the extraction of bixin and phenolic compounds from annatto seeds were determined by response surface methodology (RSM) and analyzed using Statistica® 6.0 software [18]. A central composite rotational design (CCRD) with two levels and four factors (solvent composition, number of extractions, solid–liquid ratio and extraction time) was used (Table 1). The acetone is the standard solvent used for quantitative determination of bixin content in annatto seeds [19] and methanol:water solution (8:2, v/v) is the standard solvent for the quantitative extraction of phenolic compounds [20]. However, in this study they were used combined in order to verify the improvement of the simultaneous extraction of bixin and phenolic compounds. The responses of the experimental design were carotenoid content (quantified as bixin) and phenolic compounds content. Regression coefficients were determined for the experimental data by fitting to a quadratic model (Eq. (1)):

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} b_{ij} X_i X_j \quad (i = 1-3, j = 1-3) \tag{1}$$

where Y the predicted response; b_0 a constant; b_i the linear coefficient; b_{ii} the quadratic coefficient; and b_{ij} the interaction coefficient of variables; i and j, and X_i and X_j are independent variables; and k the number of tested variables. The adequacy of the model was determined by evaluating lack of fit, coefficient of determination (R^2) and Fisher test value (F-value) obtained from the analysis of variance (ANOVA) generated by the software. Statistical significance of the results of the model and variables was determined at 5% and 10% ($\alpha = 0.05$ and $\alpha = 0.10$). The quadratic model equation shown above is used to build the response surfaces, with three-dimensional response surface plots and contour plots generated by maintaining one of the response variables at its optimal level and plotting it against two independent variables. The levels of the independent variables used in the analysis are given in Table 2. The CCRD consisted of a 2^4 factorial design plus 8 axial points and 4 repetitions at the central point (Table 2) totalizing 28 extractions.

2.2.2. Verification of the model obtained by RSM

The optimal conditions for the simultaneous extraction of bixin and total phenolic compounds were determined by the response surface models. Experiments were conducted in triplicate under

Table 1
Central composite rotational design: independent variable levels (original and coded).

Independent variable		Level				
		−2	−1	0	+1	+2
Acetone in methanol/water 8:2 (Acetone:X)	X_1	10%	30%	50%	70%	90%
Number of extractions	X_2	5	10	15	20	25
Solid–liquid ratio (1:X)	X_3	1	3	5	7	9
Time (min)	X_4	5	10	15	20	25

Table 2
Central composite rotational design and experimental responses of bixin and total phenolic contents.

Experiment	Independent variable levels				Response	
	X_1	X_2	X_3	X_4	Bixin (mg/g)	TPC (mg/g)
1	−1	−1	−1	−1	9.7	1.7
2	1	−1	−1	−1	7.6	2.4
3	−1	1	−1	−1	4.0	2.4
4	1	1	−1	−1	3.5	2.1
5	−1	−1	1	−1	12.0	2.7
6	1	−1	1	−1	9.4	2.4
7	−1	1	1	−1	13.6	2.4
8	1	1	1	−1	11.0	2.5
9	−1	−1	−1	1	12.3	2.1
10	1	−1	−1	1	9.4	1.7
11	−1	1	−1	1	12.7	2.1
12	1	1	−1	1	12.0	2.3
13	−1	−1	1	1	9.6	2.2
14	1	−1	1	1	10.6	3.1
15	−1	1	1	1	10.2	2.7
16	1	1	1	1	11.5	2.6
17	−2	0	0	0	10.9	2.1
18	2	0	0	0	9.2	2.3
19	0	−2	0	0	10.3	1.5
20	0	2	0	0	9.8	3.0
21	0	0	−2	0	11.4	1.3
22	0	0	2	0	13.4	2.7
23	0	0	0	−2	10.7	2.4
24	0	0	0	2	11.2	2.0
25 (CP)	0	0	0	0	11.0	2.4
26 (CP)	0	0	0	0	11.0	2.1
27 (CP)	0	0	0	0	11.7	2.7
28 (CP)	0	0	0	0	12.8	2.6

X_1 = acetone in methanol/water (%), X_2 = number of extractions, X_3 = solid–liquid ratio (m/v), X_4 = time (min). CP: central point; TPC: total phenolic compounds.

the optimal conditions and the results were compared with the predicted values to check the reliability of the predictive extraction model.

2.2.3. Bixin quantification

The bixin concentration of the seeds and of the 28 extracts was determined using an adaptation of the methodology described by FAO/WHO [19]. For the seeds, 1 g was weighed and the pigment thoroughly extracted with acetone until the seeds were colorless. Aliquots (0.1 mL) of the 28 extracts were evaporated under N_2 flow and re-suspended to 10 mL with acetone. Absorbance was measured with a UV-visible spectrophotometer (Agilent, Santa Clara, USA) at 487 nm and the bixin concentration was calculated according to the Lambert–Beer law, using $E^{1\%}_{1\,cm} = 3090$ [19].

2.2.4. Total phenolic compounds quantification

For the annatto seeds, 5 g were weighed and extracted five times with methanol:water (8:2, v/v) in ultrasound equipment (Unique model, São Paulo, Brazil) for 10 min at 25 °C. After extraction, the extracts were evaporated under vacuum ($T < 40$ °C), transferred with 5 mL of methanol to 25 mL volumetric flask and filled with distilled water. For the 28 extracts obtained by RSM, aliquots (2.5 mL) were evaporated under N_2 flow, re-suspended in 2.5 mL of methanol, transferred to 10 mL volumetric flask and filled with distilled water. These extracts were then put in the freezer for 20 min before centrifugation at $290 \times g$ for 20 min. The total phenolic content of the seeds and extracts was determined using the Folin-Ciocalteu colorimetric method [20], and was expressed as milligrams of gallic acid equivalent (GAE) per mL of extract. All measurements were performed in triplicate.

2.3. HPLC–DAD-MS/MS analysis

Simultaneous extraction of bixin and phenolic compounds under the optimal conditions, established by RSM, produced an extract which was subjected to solvent evaporating under N_2 flow, dissolved in methanol and injected into a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps (LC-20AD), a degasser unit (DGU-20A5), a Rheodyne injection valve with a 20 μL loop, and diode array detector (DAD) (SPD-M20A) connected in series to a mass spectrometer (MS/MS) from Bruker Daltonics (Esquire 4000 model, Bremen, Germany), with an electrospray ionization source (ESI) and an ion-trap analyzer. The compounds were separated on a C_{18} Luna column (5 μm, 250 mm × 4.6 mm, Phenomenex) at 0.9 mL/min of flow, column temperature at 29 °C, with a mobile phase consisting of water:formic acid (98:2, v/v) (solvent A) and methanol:formic acid (98:2, v/v) (solvent B) in gradient from A:B 70:30 to 40:60 in 15 min; then from 40:60 to 20:80 in 10 min; and finally from 20:80 to 5:95 in 10 min. This latter ratio (5:95) was maintained for an additional 10 min. The column eluate was split to allow only 0.15 mL/min to enter the ESI interface. The spectra were obtained between 200 and 600 nm with the chromatograms processed at 320 nm (phenolic compounds) and 459 nm (bixin). The mass spectra were acquired with a scan range from 100 to 800 m/z; the MS parameters were set as follows: ESI source in positive ion mode; capillary voltage: 1500 V, end plate offset: −500 V, capillary exit: 120 V, skimmer 1: −10 V, skimmer 2: −5 V, dry gas (N_2) temperature: 325 °C; flow rate: 8 L/min; nebulizer: 30 psi; MS/MS fragmentation energy: 1.4 V. The bixin and phenolic compounds were identified by comparison of elution order in the reverse phase column and retention time of the peaks in relation to standards, and UV-visible and mass spectra features. Bixin and phenolic compounds were quantified by comparison to external standards using seven-point calibration curves based on standard solutions (measurements in duplicate), with concentrations varying from 0.5 to 12.0 μg/mL for bixin, 0.6 to 12.5 μg/mL for rutin, and 0.4 to 10 μg/mL for quercetin.

2.4. Validation of the HPLC–DAD method

The results were validated to show compliance with international requirements for analytical methods for the quality control of pharmaceuticals [21] using measures of linearity, limits of detection (LOD) and quantification (LOQ), recovery and repeatability. Linearity was revealed by the coefficient of determination (R^2) of the seven-point calibration curves of the standard solutions. The recovery analysis for bixin and phenolic compounds was conducted simultaneously using two levels of addition (5.7 and 10 μg/mL) for bixin, rutin and quercetin standards, with 6 replicates for each level. Repeatability was evaluated based on the values of relative standard deviation (RSD %) in relation to values of the standards injected (bixin, rutin and quercetin).

3. Results and discussion

3.1. Validation of HPLC–DAD method

This is the first study to fully validate a HPLC method to be applied for the simultaneous analysis of secondary metabolites from *Bixa orellana* (annatto) seeds. These validation parameters included linearity, selectivity, procedure accuracy, system precision, recovery and stability. For the target compounds, linear regression analyses were performed by using external calibration curves. The parameters for the calibration curves (slope, intercept, relative standard deviation of slope, relative standard deviation of intercept and correlation coefficient) are shown in Table 3.

Table 3
Statistical analysis for external calibration curves of rutin, quercetin and bixin.

Compound	Linearity range (μg/mL)	Slope (a)	Intercept (b)	R^2	LOD (μg/mL)	LOQ (μg/mL)
Rutin	0.6–12.5	41,655 (1.6%)	2586 (24.0%)	0.9996	0.0049	0.0151
Quercetin	0.4–10.0	98,726 (1.1%)	19,681 (24.4%)	0.9994	0.0019	0.0058
Bixin	0.5–12.0	195,141 (5.0%)	45,697 (43.8%)	0.9958	0.1850	0.5630

Experimental conditions described in Section 2. LOD: limit of detection; LOQ: limit of quantification. For each curve, the equation is $y = ax + b$, where y is the peak area, x the concentration of the analyte (μg/mL), a the slope, b the intercept and R^2 the coefficient of correlation. Relative standard deviation (RSD) values are given in parentheses.

Table 4
Recovery values for rutin, quercetin and bixin added to extracts of annatto seeds.

Compound	Level 1				Level 2			
	Original (μg/mL)	Spiked (μg/mL)	Found (μg/mL)	Recovery (%)	Original (μg/mL)	Spiked (μg/mL)	Found (μg/mL)	Recovery (%)
Rutin	nd	5.7	5.5 ± 0.21	96.3 (3.7)	nd	10	9.7 ± 0.14	94.3 (2.9)
Quercetin	nd	5.7	5.9 ± 0.16	103.2 (2.8)	nd	10	10.1 ± 0.22	102.4 (4.3)
Bixin	188.3 ± 0.06	5.7	193.9 ± 0.54	95.2 (2.0)	215.9 ± 0.2	10	226.7 ± 0.65	105.2 (0.7)

nd: not detected. Mean ± standard deviation for the amount found, $n = 6$. Mean (relative standard deviation) for recovery, $n = 6$.

Excellent linearity was found for all of the analytes in the peak areas for the concentrations tested. The LOD and LOQ values were experimentally verified by the LOD and LOQ concentrations of the standard solutions of rutin, quercetin and bixin injected. These results indicate that the proposed HPLC method is sufficiently selective to quantify bixin and phenolic compounds in extracts of annatto seeds.

The accuracy of the analytical procedure was evaluated using the recovery test. This involved the addition of known quantities of standard reference compounds to the sample weighed of annatto seeds and analyzed using the optimal conditions. The percentage of recovery obtained by comparing the results from the original samples and the fortified samples are reported in Table 4. Since the recovery rates obtained were close to 100% in almost all cases, this method can be considered accurate.

The precision of the chromatographic system was tested by performing intra and inter-day multiple injections of a solution containing the standards of rutin, quercetin and bixin, and then checking the RSD of retention times and peak areas. Six injections were performed each day in two different days. The intra and inter-day RSD values for both retention times and peak areas (Table 5) indicate the high precision of the chromatographic system.

Stability was tested with the standard solutions. These were stored for two months in amber glass flasks under air at −36 °C prior to analysis. The analytes in solution did not show any appreciable change in chromatographic profile over the two-month period, showing a standard deviation of the concentration of 0.3 for rutin, 0.3 for quercetin and 1.4 for bixin. No degradation products were detected by HPLC–DAD.

The validation data indicated that the proposed HPLC method provides good linearity, sensitivity, procedure accuracy, system precision, as well as highlighting its suitability for the simultaneous analysis of phenolic compounds and bixin in annatto seeds.

3.2. Characteristics of annatto seeds

The commercial seeds of annatto used in this study presented 14.1 ± 1.9 mg bixin/g and 1.7 ± 0.05 mg GAE/g of phenolic compounds, both on wet basis, measured spectrophotometrically. Although the total phenolic compounds levels for annatto seeds were not found in the literature, the average concentration of bixin is reported to vary from 12 to 23 mg/g, depending on environmental factors such as temperature, illumination, rainfall, soil, and cultivar [22,23].

3.3. Optimization of simultaneous extraction of bixin and phenolic compounds using RSM

The concentration obtained at the RSM central point for bixin and total phenolic compounds revealed little variation (RSD = 7.2% and 11.2%, respectively) indicating a good repeatability of the extraction procedure. Table 2 shows the variation in bixin content (from 3.5 to 13.6 mg/g of seeds) and total phenolic content (from 1.3 to 3.1 mg GAE/g of seeds). The bixin determined, at the central point conditions, accounted for 77–90% of the bixin in the raw seeds. On the other hand, the total phenolic content of the extracts obtained by RSM showed that the extraction seems to be more efficient than that from the seeds (only methanol:water (8:2, v/v) without acetone), since their values exceeded the original value in more than 90% of the experiments. This efficiency can be attributed to the use of the acetone combined with methanol:water solution, as previously observed [24].

The efficiency of solvents in the extraction of bixin and total phenolic compounds was studied by Cardarelli et al. [6], who found that the polarity of the solvents is crucial and that bixin has greater affinity for solvents with medium polarity. In relation to the total phenolic compounds, minimum values were obtained with hexane (0.30 mg GAE/g) and maximum with

Table 5
Intra and inter-day precision values for retention times (t_R) and peak area obtained with extracts of annatto seeds.

Compound	Intra-day precision (n = 6, mean)								Inter-day precision (n = 12, mean)			
	Day 1				Day 2				t_R (min)	RSD (%)	Area (mAU)	RSD (%)
	t_R (min)	RSD (%)	Area (mAU)	RSD (%)	t_R (min)	RSD (%)	Area (mAU)	RSD (%)				
Rutin	12.4 ± 0.1	0.4	12,387 ± 210	1.7	12.4 ± 0.3	2.4	12,487 ± 299	2.4	12.4 ± 0.1	0.4	12,437 ± 870	7.0
Quercetin	18.1 ± 0.0	<0.1	39,105 ± 860	2.2	18.1 ± 0.1	0.5	38,095 ± 647	1.7	18.1 ± 0.1	0.3	38,600 ± 1582	4.1
Bixin	38.6 ± 0.1	0.1	3,737,612 ± 85,965	2.3	38.6 ± 0.1	0.2	3,827,653 ± 72,725	1.9	38.6 ± 0.1	0.1	3,782,633 ± 139,957	3.7

Experimental conditions described in Section 2. Mean ± standard deviation; RSD: relative standard deviation.

Table 6
Chromatographic and spectroscopic characteristics of compounds isolated from extracts of annatto seeds.

Peaks[a]	Compound	Concentration (μg/g)[b]	t_R range (min)	λ_{max} (nm)[c]	$[M+H]^+$ (*m/z*)	MS^2 (+) (*m/z*)[d]	$[M-H]^-$ (*m/z*)	MS^2 (−) (*m/z*)[d]
1	Hypolaetin	316.8 ± 5.6	13.5–13.8	321	303	**285 [M+H-18]$^+$**, 275 [M+H-28]$^+$, 257 [M+H-18-28]$^+$, 231 [M+H-72]$^+$	301	283 [M−H-18]$^-$, **257 [M−H-18-26]$^-$**, 229, 185
2	Caffeoyl acid derivative	228.6 ± 5.8	15.1–15.6	316	nd	365 [M+H-18]$^+$[e], 347 [M+H-18-18]$^+$[f], 305[f] [M+H-18-18-42]$^+$[f], **203 [M+H-18-162]$^+$**[f], 185 [M+H-18-180]$^+$[f]	381	337 [M−H-44]$^-$, 313$^-$, 247, **201 [M−H-180]$^-$**, 179, 135
3	Bixin	21.353.5 ± 325.7	38.5–38.8	430, 459, 487	395	377 [M+H-18]$^+$, 363 [M+H-32]$^+$, **335 [M+H-32-28]$^+$**, 317 [M+H-32-28-18]$^+$, 282, 260, 209, 157	393	361 [M−H-32]$^-$, **349 [M−H-44]$^-$**, 317 [M−H-32-44]$^-$

[a] Numbered according to the chromatogram shown in Fig. 2.
[b] Peaks 1 and 2 were quantified as equivalent to quercetin and peak 3 was quantified as bixin (five replicates for all compounds).
[c] Solvent: gradient of 2% formic acid in water and methanol with 2% formic acid.
[d] In the MS^2, the most abundant ion is shown in boldface.
[e] In-source detected fragment. nd: not detected.
[f] MS/MS from the fragment with 365u.

methanol:water (1.84 mg GAE/g), while intermediate levels of total phenolic compounds were obtained using ethanol and ethyl acetate [6].

The model proposed for bixin extraction (Eq. (2)) fits the experimental data, with $R^2 = 0.80$ (data available in Tables 1 and 2 of the Supplementary data). This indicates that the model can be used to predict responses, and represents adequately the effect of the independent variables:

$$\text{Bixin (mg/g)} = 11.62 - 0.53X_1^2 - 0.53X_2^2 + 0.93X_3 + 0.68X_4 + 0.71X_2X_3 + 1.06X_2X_4 - 1.73X_3X_4 \quad (2)$$

The model showed that for bixin extraction, the solid–liquid ratio and extraction time effects were significant ($p < 0.05$) and positive, i.e., there was an increase in the bixin content extracted as the solid–liquid ratio and the extraction time increased. On the other hand, an increase in the percentage of methanol:water in the acetone led to a decrease in bixin content extracted. This result was expected, since the official method published by the FAO/WHO [19] recommends acetone alone as the solvent for the extraction of bixin.

According to the response surface model (shown in Fig. 1 of Supplementary data) the best condition for bixin extraction was 15 extractions with 50% methanol:water (8:2, v/v) in acetone, solid–liquid ratio of 1:9 and extraction time of 5 min.

For the total phenolic compounds extraction, the effect of the number of extractions and the solid–liquid ratio were found to be significant ($p < 0.10$) and positive, i.e., the extraction efficiency improving at higher solid–liquid ratios and with the numbers of extraction. The results of the ANOVA (data available in Tables 1 and 2 of Supplementary data) reveal a relatively low coefficient of determination ($R^2 = 0.51$), so the model proposed (total phenolic (mg GAE/g) = $2.45 + 0.14X_2 + 0.28X_3$) is unable to explain adequately the behavior of the experimental data. However, the optimal conditions for the bixin extraction also gave a high yield of phenolic compounds. The response surface were plotted using the coefficients of the two significant linear variables to illustrate the tendency of the process; the optimum point for the phenolic compound extraction suggest a solid–liquid ratio close to 1:9 (available in Fig. 2 of Supplementary data).

3.4. Validation of RSM model for simultaneous extraction of bixin and phenolic compounds

The experimental validation of the models proposed for the simultaneous extraction of bixin and the phenolic compounds were performed, in triplicate, at the optimal conditions (50% of methanol:water (8:2, v/v) in acetone, 15 extractions, a solid–liquid ratio of 1:9 (m/v) and 5 min extraction). The bixin content was found to be 11.1 ± 0.3 mg/g (RSD = 3.2%) and the phenolic compounds was 1.9 ± 0.08 mg/g (RSD = 4.3%). There were no significant differences ($p < 0.05$) between the results of the optimization experiments and those predicted by the models, confirming the good prediction capacity of the models for the extraction of bixin and phenolic compounds from annatto seeds.

3.5. Application of the validated HPLC–DAD-MS/MS to annatto extracts

Fig. 2 presents the chromatogram of the extract obtained at the optimal conditions established by RSM. Two different compositions of the mobile phase were tested, one consisting of acetonitrile and

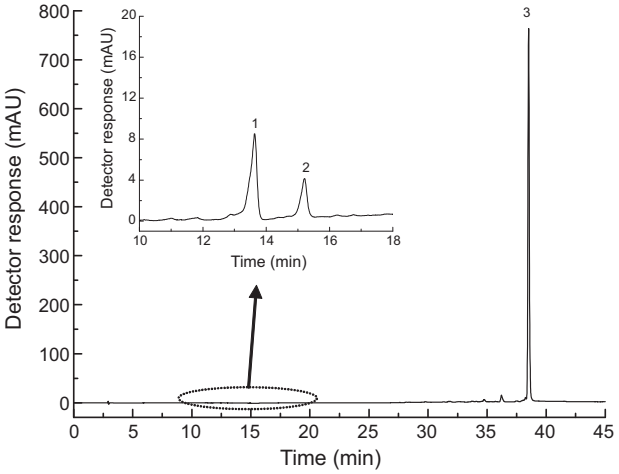

Fig. 2. Chromatogram obtained by HPLC–DAD of phenolic compounds and bixin from annatto seed extracts. Chromatographic conditions: see text. Processed at 459 nm; figure inset processed at 320 nm (peak characterization is given in Table 6).

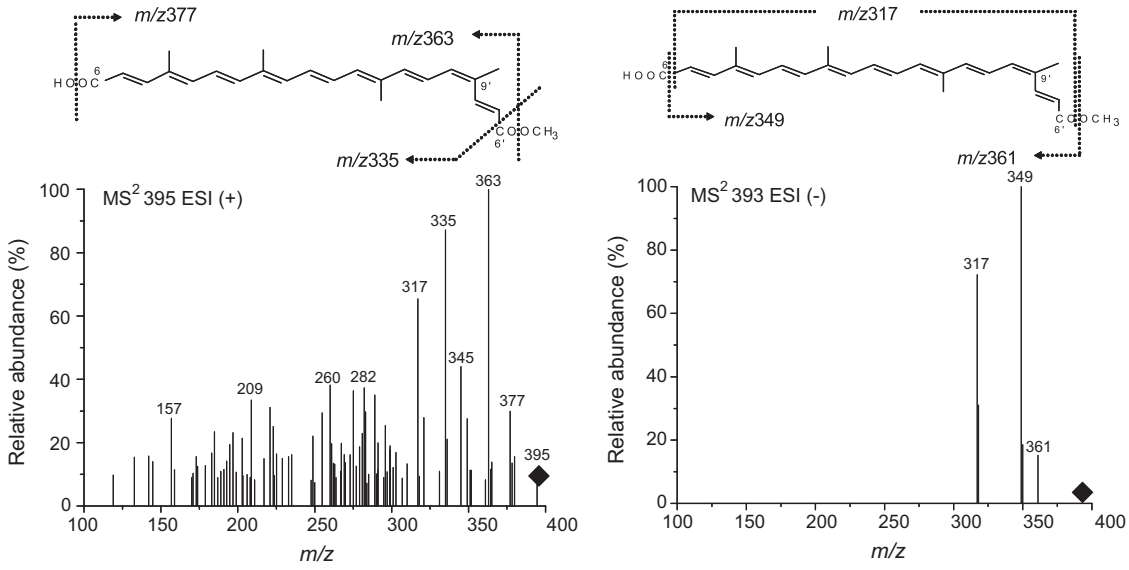

Fig. 3. MS^2 spectra of bixin obtained on ion trap instrument equipped with ESI source (positive and negative ion mode) showing main fragmentation pattern.

water both with 2% formic acid (data not shown) and the other of methanol and water both with 2% formic acid. The applied chromatographic elution method (see Section 2) with the two organic solvents led to the separation of 2 phenolic compounds, as well as carotenoids, especially bixin. The discussion focuses on the identification of the two phenolic compounds and the carotenoid bixin.

Peak 1 was tentatively identified as a pentahydroxyflavone, MW 302, based on UV-visible and MS spectra features (Table 6). In the positive ion mode, peak 1 revealed the presence of the protonated molecule $[M+H]^+$ at *m/z* 303, and the MS/MS showed consecutive losses of H_2O (*m/z* 285), CO (*m/z* 275), CO_2 (*m/z* 209), H_2O+CO (*m/z* 257), and $CO+CO_2$ (*m/z* 231) from the protonated molecule. The molecular weight was confirmed by the negative ion mode spectrum, with the deprotonated molecule ($[M-H]^-$) at *m/z* 301 and the MS/MS showing neutral losses of H_2O at *m/z* 283, and of $H_2O+C_2H_2$ at *m/z* 257. Such fragmentations are typically observed for flavones and hydroxyflavones [25–27]. Since Harbone [7] reported the presence of hypolaetin derivatives in annatto leaves, peak 1 was identified as hypolaetin (Fig. 1).

Peak 2 was tentatively identified as a caffeoyl acid derivative, MW 382, with a deprotonated molecule $[M-H]^-$ at *m/z* 381 in the negative ion mode, whereas in the positive ion mode the protonated molecular ion was not observed, but a strong in-source fragmentation was observed at *m/z* 365 $[M+H-18]^+$. According to Table 6, the identification was confirmed by the fragments obtained from the ion at *m/z* 365, such as neutral losses of H_2O (*m/z* 347), of $H_2O+C_2H_2O$ (*m/z* 305), of caffeoyl moiety (*m/z* 203), and of caffeic acid (*m/z* 185), all in the positive ion mode. Moreover, in the negative ion mode, the MS/MS showed neutral losses of CO_2 (*m/z* 337) and caffeic acid (*m/z* 201) from the deprotonated molecule (*m/z* 381). The specific structure of the compound eluted as peak 2 could not be determined. However, these fragmentations are typically observed for caffeoyl acid derivatives [28–30].

Peak 3 was identified as bixin (MW 394), the major carotenoid present in annatto seeds. In the positive ion mode (Table 6), peak 3 showed the protonated molecule at *m/z* 395 and MS^2 showing losses of H_2O (*m/z* 377), CH_3OH (*m/z* 363), CH_3OH+CO (*m/z* 335) and $CH_3OH+CO+H_2O$ (*m/z* 317) (Fig. 3). The negative ion mode spectrum confirmed the assignment of the molecular weight, with the deprotonated molecule ($[M-H]^-$) at *m/z* 393 and MS/MS showing losses of CH_3OH (*m/z* 361), $COO^\bullet$ (*m/z* 349) and $CH_3OH+COO^\bullet$ (*m/z* 317) (Fig. 3). This fragmentation pattern for bixin has already been reported in the literature [31–33]. The identification of peak 3 as bixin was also confirmed by co-elution with bixin standard (98% purity), and by the UV-visible features (%III/II = 33 and $\%AB/A_{II} = 10\%$), in accordance with many studies [5,17,34].

4. Conclusions

In summary, the proposed method for the simultaneous extraction, separation, identification and quantification of phenolic compounds and bixin were successfully validated. The optimized procedure for simultaneous extraction of these compounds by response surface methodology provided the best conditions to extract phenolic compounds and bixin from annatto seeds. Additionally, hypolaetin and caffeoyl acid derivative were identified for the first time in annatto seeds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.094.

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