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Optimization of a method for determination of phenolic acids in exotic fruits by capillary electrophoresis

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ABSTRACT

In this work, the separation of nine phenolic acids (benzoic, caffeic, chlorogenic, *p*-coumaric, ferulic, gallic, protocatechuic, syringic, and vanillic acid) was approached by a 3^2 factorial design in electrolytes consisting of sodium tetraborate buffer (STB) in the concentration range of 10–50 mmol L^{-1} and methanol in the volume percentage of 5–20%. Derringer's desirability functions combined globally were tested as response functions. An optimal electrolyte composed by 50 mmol L^{-1} tetraborate buffer at pH 9.2, and 7.5% (v/v) methanol allowed baseline resolution of all phenolic acids under investigation in less than 15 min. In order to promote sample clean up, to preconcentrate the phenolic fraction and to release esterified phenolic acids from the fruit matrix, elaborate liquid–liquid extraction procedures followed by alkaline hydrolysis were performed. The proposed methodology was fully validated (linearity from 10.0 to 100 μg mL^{-1}, $R^2 > 0.999$; LOD and LOQ from 1.32 to 3.80 μg mL^{-1} and from 4.01 to 11.5 μg mL^{-1}, respectively; intra-day precision better than 2.8% CV for migration time and 5.4% CV for peak area; inter-day precision better than 4.8% CV for migration time and 4.8–11% CV for peak area; recoveries from 81% to 115%) and applied successfully to the evaluation of phenolic contents of abiu-roxo (*Chrysophyllum caimito*), wild mulberry growing in Brazil (*Morus nigra* L.) and tree tomato (*Cyphomandra betacea*). Values in the range of 1.50–47.3 μg g^{-1} were found, with smaller amounts occurring as free phenolic acids.

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1. Introduction

Phenolic acids are naturally occurring plant secondary metabolites belonging to a broader class of phenolic compounds, widely spread into a variety of taxonomic groups. Structurally, phenolic acids derive from either the hydroxycinnamic or the hydroxybenzoic acid skeletons. The most abundant hydroxybenzoic derivatives in plants are caffeic, *p*-coumaric, vanillic, ferulic and protocatechuic acids [1]. A few plants present moderate amounts of gentisic and syringic acids as well. Among the hydroxycinnamic acid derivatives, chlorogenic acid, an ester formed by caffeic acid and the sugar moiety of quinic acid, is the most ubiquitous [2]. Within the plant, phenolic acids are physically distributed among its several compartments: fruits, leaves, bark, seeds, and roots. In general a smaller fraction of phenolic acids remains unassociated, free form, whereas a larger amount is bound to cellulose, lignins, proteins, or conjugated with sugars, flavonoids and terpenes, among other substances [2]. Fig. 1 depicts the structures of the nine phenolic acids under consideration in this work.

In recent years, great attention has been paid to natural substances with antioxidant activity (flavonoids, carotenes, vitamins C and E, phytate and phytoestrogens, among others), to help overcoming the increasing incidence of serious pathologies such as cancer, cardiovascular diseases, circulatory problems and inflammation, in part attributed to the harmful effects of free radicals (oxidative damage) [3–5]. Although phenolic acids represent one third of the total phenolic compounds, class of recognized and well documented antioxidant activity, the literature reveals a great interest in establishing the antioxidant properties of phenolic acids and their beneficial effects on health [6–8]. As a result, the consumption of fruits, the major source of phenolic acids in the diet, in addition to vegetables and grains, has been encouraged. Interestingly, fruit extracts richer in phenolic acids usually present a larger antioxidant activity than the corresponding pure compounds or even vitamins, an evident synergistic effect [9].

The determination of phenolic acids in fruits and plant extracts has been predominantly conducted by high-performance liquid chromatography (HPLC) in reversed phase under UV detection [10–13]. The coupling of liquid chromatography with mass spectrometry (LC-MS) *via* electrospray ionization (ESI) has been an important tool in the characterization of phenolic acids in fruits

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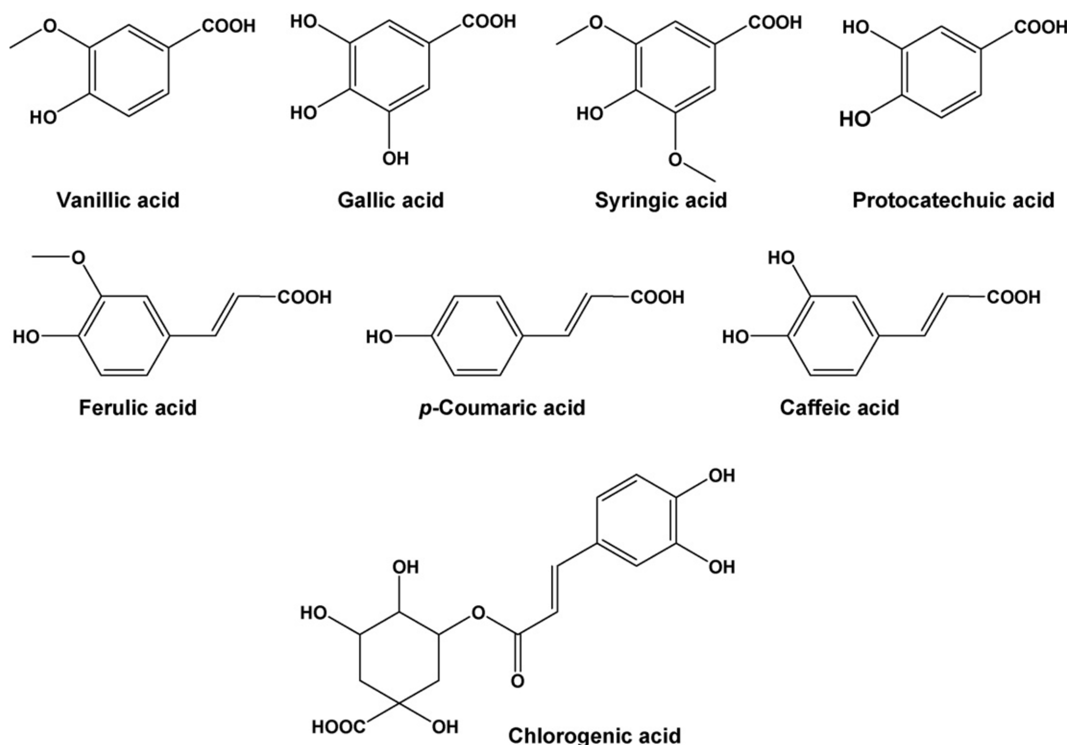

Fig. 1. Structural representation of the phenolic acids under consideration.

and natural products [14–16]. Gas chromatography (GC) with flame ionization (FID) and MS detection has also been considered in the analysis of fruits [17–20]. Although GC related methods present high sensitivity and selectivity, due to low volatility of phenolic acids, derivatization steps must be incorporated in the sample preparation procedure.

In more recent years, capillary electrophoresis (CE) has gained a prominent position in the analysis of natural products, especially those with ionic or highly polar constituents, not as easily handled by other complementary separation techniques [21,22]. Particularly for the determination of phenolic compounds in complex matrices such as fruits, plants and foods, the versatility and advantageous characteristics of CE and its diverse separation modes have been extensively documented. Capillary zone electrophoresis (CZE) in high pH buffers, modified by organic solvents or other additives, under UV direct detection has been the technique of choice for determination of phenolic compounds in fruits [23,24], vegetables [25,26], olive oil [27,28] and herbal extracts [29,30]. Other studies involving CE determination of phenolic acids include the use of electroosmotic flow inverters [31–33] and on-line preconcentration strategies [34].

When a large number of compounds must be assessed simultaneously, the use of micellar electrokinetic chromatography (MEKC) is usually devised as a means of increasing selectivity. MEKC has been applied to the determination of phenolic compounds in herbal extracts [35] and beverages (wine and coffee) [36,37].

Capillary electrophoresis coupled to mass spectrometry (CE–MS) has also been considered for food characterization due to the intrinsic selectivity and structural information the hyphenated technique provide. CE–ESI–MS methods have been developed and applied to the characterization of the phenolic fraction of olive oil [38] and walnuts [39].

Considering the complexity of fruit extracts and the diversity by which phenolic compounds might be present in the fruit matrix, dependable methods for screening and quantitative determination of constituents are in great demand. In this work, a method for the determination of free and bound phenolic acids in exotic fruits has been proposed. The selected fruits, abiu-roxo (*Chrysophyllum caimito*), wild mulberry growing in Brazil (*Morus nigra* L.) and tree tomato (*Cyphomandra betacea*) are part of the Brazilian rich biodiversity and have not been fully characterized in terms of phenolic acid contents yet.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade, solvents were of chromatographic purity and water was purified by deionization (Milli-Q system, Millipore, Bedford, MA, USA).

Stock solutions of the standards Chlorogenic and Benzoic acids, obtained from Sigma Aldrich (St Louis, MO, USA), Vanillic, Ferulic, Protocatechuic, and *p*-coumaric acids, obtained from Fluka (Neu-Ulm, Germany), Syringic acid, obtained from Avocado Research Chemical Ltda (Heisham, Lancs, UK), and Gallic and Caffeic acids, obtained from Spectrum (Gardena, CA, USA), were prepared in ethanol at 1000 $\mu g\,mL^{-1}$ concentration and stored at 4 °C until use. Working solutions containing each acid at 30.0 $\mu g\,mL^{-1}$ concentration were prepared by dilution of the stock solutions during optimization procedures. Likewise, for the analytical curves, standard solutions from 10.0 to 100 $\mu g\,mL^{-1}$ of each acid (except chlorogenic acid, 10.0–75.0 $\mu g\,mL^{-1}$) were prepared by appropriate dilution of the stocks.

The electrolyte solutions were prepared fresh daily from sodium tetraborate (Merck, Darmstadt, Germany) stock solutions at 100 $mmol\,L^{-1}$ concentration. An optimal electrolyte composed of 50 $mmol\,L^{-1}$ sodium tetraborate (STB) at pH 9.2, and 7.5% (v/v) methanol (MeOH) was used for sample analysis.

2.2. Sample preparation

Samples of Abiu-roxo (*C. caimito*), wild mulberry growing in Brazil (*M. nigra* L.) and tree tomato (*C. betacea*) were acquired from

local markets. The extraction of phenolic acids from the whole fruit adopted in this work followed, with minor modifications, the procedure proposed by Krygier et al. for isolation of free, esterified and insoluble-bound phenolic acids in oilseeds [40]. Pieces of *in natura* fruits were weighed and grinded in an ultra-turrax® homogenizer (Ika, Germany) containing c.a. 10 mL methanol. The methanolic extract was then submitted to sonication for 5 min and centrifuged at 10,000 rpm for 10 min. The uppernatant was collected and evaporated. The resulting aqueous suspension was acidified to pH 2 with concentrated HCl to protonate the free phenolates. The acidic suspension was saturated with NaCl and extracted with diethyl ether (1:1 proportion); this solvent extraction procedure was repeated in triplicate. All three organic layer fractions were then combined and evaporated under nitrogen flow. The dry residue was suspended in 1 mL of 1:1 ethanol:deionized water and filtered in a 0.45 μm membrane to give the free phenolic acid fraction.

The remaining aqueous suspension with saturated NaCl was hydrolyzed for 4 h in a 4 mol L^{-1} NaOH solution containing 10 mmol L^{-1} EDTA and 1% ascorbic acid at room temperature. The alkaline hydrolysis condition followed Nardini et al. who introduced EDTA and ascorbic acid in the medium to prevent phenolic acid decomposition [41]. After hydrolysis, the suspension was acidified at pH 2 with HCl, extracted with diethyl ether in triplicate, followed by evaporation and resuspension as described above, to give the hydrolysate fraction.

2.3. Instrumentation

All experiments were conducted in a capillary electrophoresis system (model P/ACE 5510, Beckman Instruments, Fullerton, CA, USA) equipped with a diode array detector set at 200 nm, a temperature control device maintained at 25 °C and an acquisition and data treatment software supplied by the manufacturer (Beckman P/ACE System Gold® Software). Samples were injected hydrodynamically (at 0.5 psi for 5 s; 1 psi = 6.8927 kPa) and the system was operated under normal polarity of +30 kV.

Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with dimensions 40.2 cm total length, 30.0 cm effective length and 50 μm i.d. and 375 μm o.d. were used. New capillaries were preconditioned by 20 psi flushes with 1 mol L^{-1} NaOH (30 min) followed by deionized water (30 min). At the beginning of each day, the capillary was conditioned by 20 psi flushes with 1 mol L^{-1} NaOH (10 min), deionized water (10 min) followed by electrolyte solution (5 min). In between runs, the capillary was reconditioned with the electrolyte solution (3 min flush at 20 psi). At the end of the day, the capillary was rinsed with 1 mol L^{-1} NaOH solution and deionized water, 5 min each at 20 psi.

3. Results and discussion

3.1. Optimization of the separation

In order to evaluate the separation quality of the nine phenolic acids under consideration in terms of resolution, peak shape, and overall migration time, a few preliminary experiments were run testing different electrolyte systems (phosphate and tetraborate) and solvents (methanol and acetonitrile). The combination of sodium tetraborate (STB) at pH 9.2 and methanol (MeOH) performed better and these components were selected for further optimization of electrolyte composition. In high pH tetraborate buffers, all phenolic acids are fully dissociated (pK_a 4.1–4.5) and separation can be modulated by complexation with tetraborate.

Table 1
3^2 Factorial design levels and results.

[STB] (mmol L^{-1})	%v,v MeOH	$Rs_{1,2}$	$Rs_{2,3}$	$Rs_{3,4}$	$Rs_{4,5}$	$Rs_{5,6}$
−1	−1	4.1	1.4	3.2	3.1	1.7
0	−1	4.9	1.6	2.8	4.7	2.3
+1	−1	8.1	2.0	3.0	7.9	2.7
−1	0	2.9	1.8	5.2	2.4	1.7
0	0	4.7	1.9	5.1	3.5	2.2
+1	0	7.8	2.7	6.5	5.3	2.3
−1	+1	3.4	2.2	7.3	1.4	1.7
0	+1	4.5	2.6	8.2	2.0	1.9
+1	+1	6.4	3.1	8.0	2.5	2.0
0	0	4.4	2.0	4.8	3.8	2.0
0	0	4.8	2.0	5.0	3.6	2.3

STB, sodium tetraborate; MeOH, methanol; Rs, resolution between adjacent solute pairs numbered as in Fig. 2; results correspond to factorial design B. Factorial design A: STB at pH 9.2 at 10 mmol L^{-1} (−1), 20 mmol L^{-1} (0) and 30 mmol L^{-1} (+1) and % v,v MeOH at 10% (−1), 15% (0) and 20% (+1). Factorial design B: STB at pH 9.2 at 30 mmol L^{-1} (−1), 40 mmol L^{-1} (0) and 50 mmol L^{-1} (+1) and % v,v MeOH at 5.0% (−1), 7.5% (0) and 10% (+1).

Two 3^2 factorial designs, A and B, were implemented with STB and %MeOH as independent variables at 3 variation levels (+, 0 and −), according to Table 1. Nine experiments were performed with triplicate of the central point (0) for each design. Resolutions of the six first eluting solutes and their adjacent peaks were used preliminarily as response function (organized in Table 1 for the factorial design B). Fig. 2 depicts the corresponding factorial design electropherograms.

In the factorial design A (Fig. 2A), STB varied from 10 mmol L^{-1} (−) to 30 mmol L^{-1} (+) and %MeOH, from 10% v,v (−) to 20% v,v (+), in addition to the central point. In all conditions of Fig. 2A it is clearly noticed that solutes 1–6 bunch together in two closely spaced groups and their separation is particularly difficult whereas solutes 7–9 are well resolved all the time. Moreover, the increase of %MeOH caused significant changes in selectivity including migration order inversion for some of the six first eluting peaks. A borderline condition is achieved at high STB and low %MeOH, (+), (−); however, even at that condition, resolution at the 1–6 peak bunches barely reached baseline, which it is not adequate for method validation (Rs = 2.0 is usually preferred), especially when complex matrix samples such as fruit extracts must be assessed.

The factorial design A data treatment (not shown) indicated that an increase in STB concentration and decrease in %MeOH would favor separation quality. Therefore, the factorial design B was implemented (Fig. 2B), in which STB varied from 30 mmol L^{-1} (−) to 50 mmol L^{-1} (+) and %MeOH, from 5% v,v (−) to 10% v,v (+), in addition to the central point. As it can be observed in Fig. 2B, baseline resolution of all solute peaks was achieved at all conditions. As expected, at low STB concentration, separations are faster (smaller compression of the double layer at the capillary surface, increasing electroosmotic flow). At low to intermediary %MeOH, all peaks are more equally spaced; again, increasing %MeOH causes change in selectivity of peaks 1–6, although in much less extent than it occurred in the factorial design A.

The results of factorial design B were fitted by regression into a quadratic model to generate response surfaces, according to the equation [1]:

$$\hat{y} = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 \quad (1)$$

where $\hat{y}$ are the resolutions of Table 1 (peaks 1–6), x_1 is the STB concentration and x_2 is the %MeOH; the coefficients b_i define the surface curvature and are listed in Table 2 with respective standard errors. Partial *F* and *p*-values for the coefficients were also included in Table 2. All models were validated by ANOVA and lack of fitting was ruled out. For each pair, the best model was selected with basis on the smallest *p*-value. For the pairs 1,2 and 3,4, a linear model was

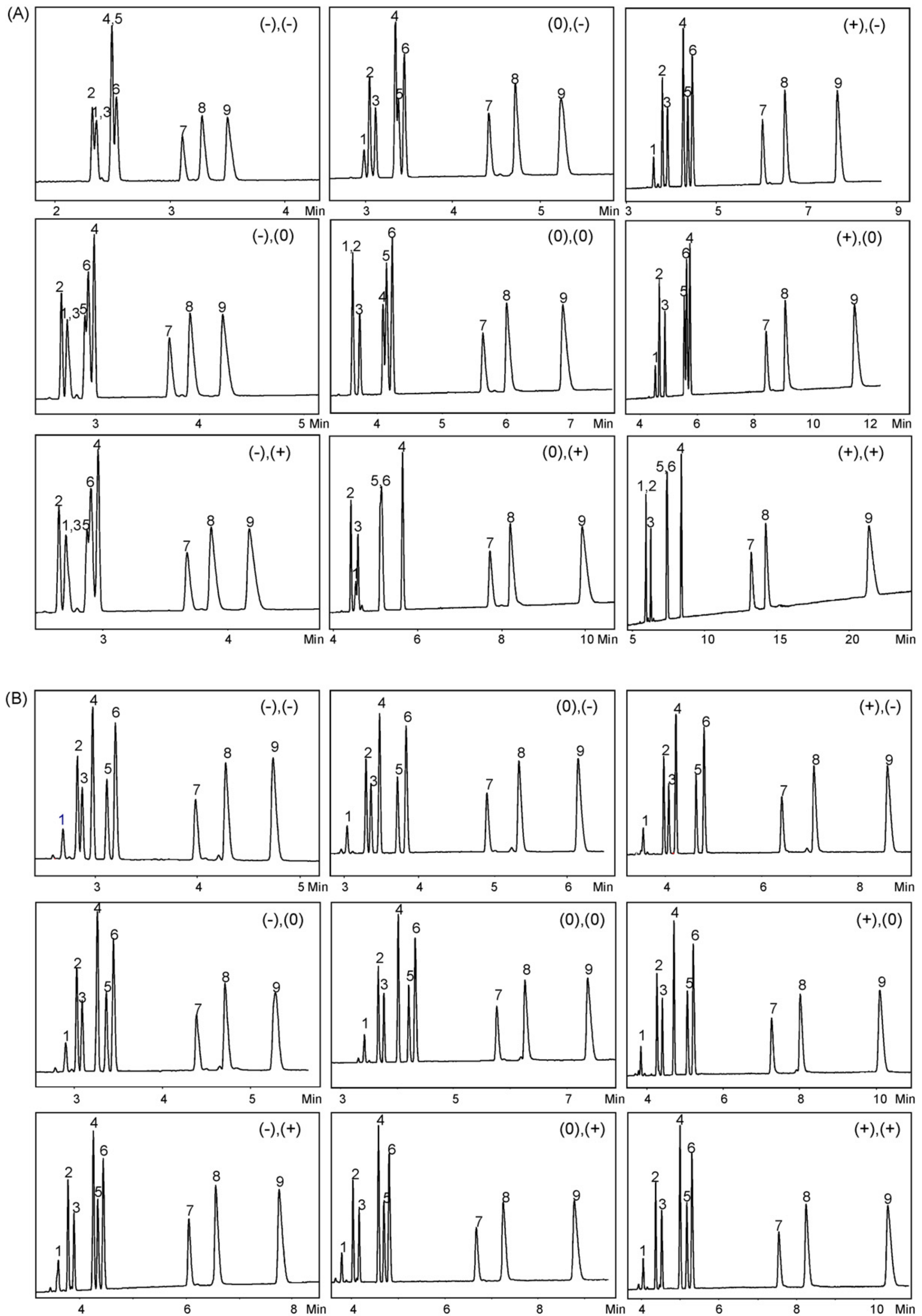

Fig. 2. Factorial designed electropherograms. Electrolyte composition: sodium tetraborate at pH 9.2 at 10 mmol L^{-1} (−), 20 mmol L^{-1} (0) and 30 mmol L^{-1} (+) and % v,v methanol at 10% (−), 15% (0) and 20% (+) in (A) and sodium tetraborate at pH 9.2 at 30 mmol L^{-1} (−), 40 mmol L^{-1} (0) and 50 mmol L^{-1} (+) and % v,v methanol at 5.0% (−), 7.5% (0) and 10% (+) in (B). Analytical conditions: 0.5 psi, 5 s hydrodynamic injection; 25 °C; +30 kV applied voltage; direct detection at 200 nm. Peak legends: chlorogenic acid (1), syringic acid (2), ferulic acid (3), benzoic acid (4), *p*-coumaric (5), vanillic acid (6), caffeic acid (7), gallic acid (8) and protocatechuic acid (9).

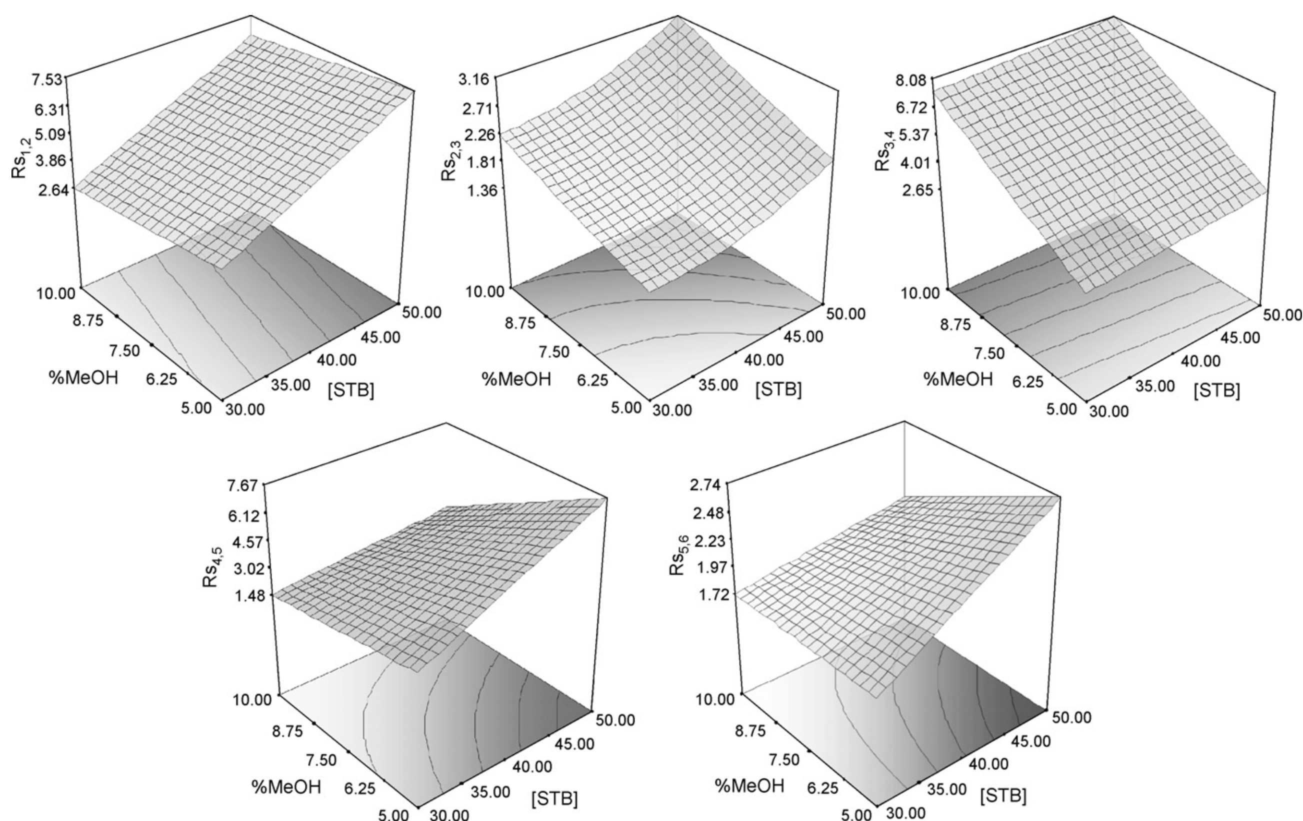

Fig. 3. Resolution of adjacent solute pairs as a function of electrolyte composition (factorial design B of Fig. 2). Peak numbers as in Fig. 2.

better fitted. Although a full quadratic model might be selected, not all coefficients are statistically significant. Coefficients with $p < 0.05$ were disregarded.

The response surfaces are depicted in Fig. 3. As it can be seen in Fig. 3, when different solute pairs are considered, distinct behaviors take place, making it difficult to select a single optimal condition for all solute pairs. Therefore, to select an overall optimal separation condition, a multicriteria decision making approach must be applied. Derringer's desirability functions for each response (Rs, peaks 1–6) and their geometric average, termed the global desirability, were then calculated as a function of STB and %MeOH conditions [42,43]. Derringer's desirability functions are dimensionless scales ranging from zero, a completely undesirable response, to one, a fully desirable response, above which further improvements would have no relevance. A plot of the global desirability as a function of conditions is depicted in Fig. 4.

The inspection of Fig. 4 indicates that an improvement on the separation quality would result from an increase in STB concentration, inclusive surpassing the studied interval, and at intermediary values of %MeOH. The maximum global desirability value (0.656) within the experiment boundaries occurred at 50.0 mmol L^{-1} and 7.46 %MeOH. An increase of STB concentration passed 50 mmol L^{-1} would promote an unnecessary increase in current counts (over 100 mA), compromising the system stability and method endurance. A typical electropherogram for the separation of the nine phenolic acids at the optimal conditions is depicted in Fig. 5. This condition is virtually the same of Fig. 2B (+),(0) electropherogram. However, the electropherogram of Fig. 5 was obtained with a newly cut capillary, and migration times, especially for the last eluting peaks, varied to some extent. It was observed that with capillary use, a faster electroosmotic flow develops, which can be attributed to structural changes at the inner surface. Nevertheless, the condition of Fig. 5 was used in the inspection of phenolic acids in real fruit extracts.

3.2. Method validation

A few figures of merit of the proposed method have been determined according to the AOAC (Association of Analytical Communities) guidelines [44]. The method selectivity was established by the baseline separation of the phenolic acids under investigation (electropherogram of Fig. 5).

Precision has been characterized by triplicate injection of standard solutions of all phenolic acids at three concentration levels

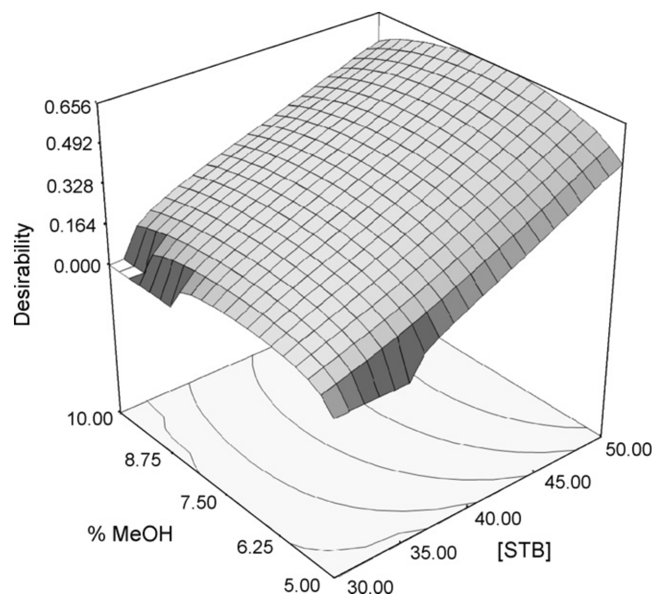

Fig. 4. Response surface based on the desirability function (factorial design B of Fig. 2).

Table 2
Resolution functions (Eq. (1)) and corresponding statistics.

$\hat{y}$	b_0	b_1 ($F_{partial}$, p)	b_2 ($F_{partial}$, p)	b_{11} ($F_{partial}$, p)	b_{22} ($F_{partial}$, p)	b_{12} ($F_{partial}$, p)	Adj. R^2	F	p
$Rs_{1,2}$	−1.42 ± 0.18	0.198 ± 0.24 (68, 0.0001)	−0.187 ± 0.24 (3.9, 0.086)	–	–	–	0.88	36	0.0001
$Rs_{2,3}$	2.84 ± 0.045	−0.129 ± 0.036 (125, 0.0001)	0.0459 ± 0.036 (185, 0.0001)	0.00193 ± 0.056 (12, 0.018)	–	–	0.97	65	0.0001
$Rs_{3,4}$	−3.09 ± 0.14	0.0313 ± 0.19 (2.8, 0.1355)	0.961 ± 0.19 (163, 0.0001)	–	–	–	0.94	83	0.0001
$Rs_{4,5}$	−8.34 ± 0.089	0.422 ± 0.12 (148, 0.0001)	0.817 ± 0.12 (182, 0.0001)	–	–	−0.0367 ± 0.15 (39, 0.0004)	0.97	123	0.0001
$Rs_{5,6}$	−0.890 ± 0.041	0.0873 ± 0.055 (34, 0.0006)	0.219 ± 0.055 (11, 0.012)	–	–	−0.00733 ± 0.067 (7.4, 0.030)	0.83	18	0.0012

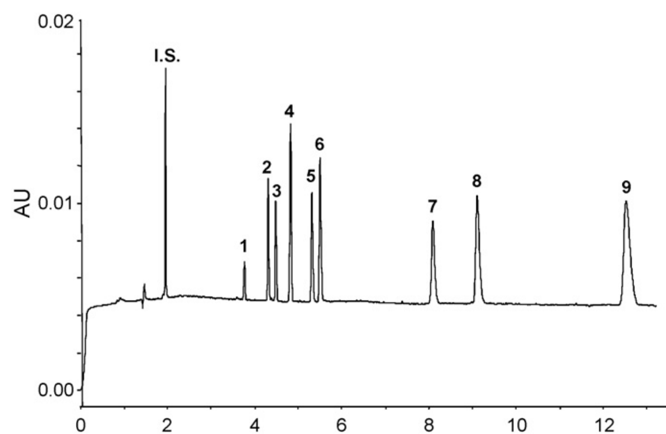

Fig. 5. Optimal condition electropherogram for the separation of phenolic acids. Electrolyte: sodium tetraborate at pH 9.2 at 50 mmol L^{-1} and 7.5 % v,v methanol. Analytical conditions: 0.5 psi, 5 s hydrodynamic injection; 25 °C; +30 kV applied voltage; direct detection at 200 nm. Peak legend as in Fig. 2.

within a single day (repeatability or intra-day precision) and at three different days (intermediary or inter-day precision). The results are displayed in Table 3. Repeatability of migration times and peak area ratios were better than 2.8% and 5.4% CV, respectively. For intermediary precision, the results for migration time were better than 4.8% CV. For peak area ratio at the three concentration levels, precision got worse with CV ranging from 4.8% to 11%; these last results indicate that the capillary surface has been altered with time and usage implying that for quantitative purposes daily calibrations must be performed.

An evaluation of accuracy (recovery tests) was conducted by spiking both free phenolic acid and hydrolysate extracts of the three fruits under investigation with a mixture of phenolic acids standards at 30.0 μg mL^{-1} concentration (Table 4). Values between 81% and 115 % were obtained, which are considered acceptable recoveries for food matrices. Although these values give us an estimate of matrix effects on the method recovery they do not serve to evaluate the extraction performance. A more thorough investigation of the extraction procedure by spiking *in natura* fruits and the resulting fractions at each extraction step is currently being undertaken in our group.

The method linearity was determined by the quality of the statistical data compiled in Table 5. Analytical curves in the concentration range from 10.0 μg mL^{-1} (the approximate limit of quantification) to 100 μg mL^{-1} (except chlorogenic acid, upper limit of 75.0 μg mL^{-1}), with triplicate injection at each of the five calibration levels, were built by plotting peak area ratio (tryptophan was used as internal standard at 40.0 μg mL^{-1}) *versus* concentration. Large F values, small regression errors (SE) and coefficients of determination (R^2) better than 0.999 were obtained (Table 5) indicating good linearity. Limits of detection (LOD) and quantification (LOQ) were calculated from the regression equation statistics as the ratio of the regression error (SE) over the slope, times either 3.3 (LOD) or 10 (LOQ). LOD in the range of 1.32–3.80 μg mL^{-1} were obtained whereas LOQ varied from 4.01 to 11.5 μg mL^{-1} (Table 5).

3.3. Sample analysis

The proposed method has been applied to the determination of phenolic acids in free phenolic acid extracts and hydrolysates of abiu-roxo, wild mulberry and tree tomato. Typical electropherograms are depicted in Fig. 6. The screening of food composition is usually challenged by the complexity of sample matrices. However, as observed in the electropherograms of Fig. 6, the extraction procedure adopted in this work provided good separation capability

Table 3
Method validation regarding precision.

Phenolic acid	Intra-day precision % CV ($n=3$)				Inter-day precision % CV ($n=9$)			
	Migration time	Peak area ratio			Migration time	Peak area ratio		
		10.0 μg mL^{-1}	50.0 μg mL^{-1}	100 μg mL^{-1}		10.0 μg mL^{-1}	50.0 μg mL^{-1}	100 μg mL^{-1}
Chlorogenic acid	0.44	0.93	4.0	3.3	1.6	4.8	6.3	4.9
Syringic acid	0.39	2.3	2.3	2.3	2.0	5.9	8.2	5.8
Ferulic acid	0.39	2.2	0.60	3.3	3.5	5.7	7.4	6.0
Benzoic acid	0.91	2.4	2.5	2.6	3.6	11	7.5	8.5
p-Coumaric acid	0.46	4.4	5.4	2.4	2.7	8.3	10	7.5
Vanillic acid	0.46	3.6	4.5	1.1	4.4	7.5	9.2	5.9
Caffeic acid	1.7	2.2	0.43	1.3	4.5	7.3	7.6	6.2
Gallic acid	2.0	1.3	0.81	1.3	4.2	5.3	4.3	5.2
Protocatechuic acid	2.8	0.47	1.7	1.9	4.8	6.6	11	11

Table 4
Method validation regarding accuracy (recovery tests).

Phenolic acid	Abiu-roxo		Wild mulberry		Tree tomato	
	Free phenolic acid fraction	Hydrolysate fraction	Free phenolic acid fraction	Hydrolysate fraction	Free phenolic acid fraction	Hydrolysate fraction
Chlorogenic acid	97.5 ± 1.0	97.2 ± 2.5	85.3 ± 6.1	84.6 ± 0.88	88.3 ± 3.5	90.2 ± 1.4
Syringic acid	100.2 ± 0.94	98.2 ± 1.2	99.5 ± 1.3	98.4 ± 1.2	95.8 ± 0.67	85.8 ± 3.5
Ferulic acid	98.9 ± 1.2	97.5 ± 2.2	98.5 ± 0.93	98.3 ± 1.5	99.7 ± 0.92	98.4 ± 2.4
p-Coumaric acid	99.5 ± 0.44	98.3 ± 1.7	99.2 ± 1.9	87.2 ± 1.4	98.3 ± 1.1	84.7 ± 2.4
Vanillic acid	99.8 ± 1.1	93.7 ± 2.2	98.7 ± 2.2	95.3 ± 2.2	97.1 ± 0.85	84.1 ± 2.5
Caffeic acid	100.5 ± 0.88	99.2 ± 0.56	97.5 ± 2.2	87.2 ± 3.5	95.4 ± 1.2	81.3 ± 2.4
Gallic acid	98.9 ± 1.5	89.2 ± 5.7	92.6 ± 1.1	95.7 ± 2.0	94.5 ± 1.7	115.0 ± 3.2
Protocatechuic acid	102.0 ± 2.6	110.3 ± 6.4	105.3 ± 5.7	96.5 ± 2.3	94.8 ± 5.9	95.0 ± 1.3

Table 5
Method validation regarding linearity, limits of detection (LOD) and quantification (LOQ).

Phenolic acid	Intercept	Slope	R^2	F	SE	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Chlorogenic acid	−0.0083 ± 0.0055	0.0110 ± 0.0001	0.9996	7,036	0.008	2.30	6.98
Syringic acid	−0.068 ± 0.027	0.0353 ± 0.0005	0.9993	5,528	0.040	3.76	11.4
Ferulic acid	0.027 ± 0.013	0.0296 ± 0.0002	0.9998	16,600	0.019	2.17	6.57
Benzoic acid	0.004 ± 0.018	0.0541 ± 0.0003	0.99986	28,984	0.026	1.64	4.97
p-Coumaric acid	−0.029 ± 0.023	0.0397 ± 0.0004	0.9996	9,717	0.034	2.83	8.59
Vanillic acid	−0.072 ± 0.030	0.0606 ± 0.0005	0.9997	12,711	0.045	2.48	7.51
Caffeic acid	0.025 ± 0.018	0.0551 ± 0.0003	0.99986	29,253	0.027	1.63	4.95
Gallic acid	0.045 ± 0.022	0.0815 ± 0.0004	0.99991	44,461	0.033	1.32	4.01
Protocatechuic acid	0.086 ± 0.10	0.136 ± 0.002	0.9993	5,402	0.16	3.80	11.5

SE, regression standard error.
Analytical curves: peak area ratio versus concentration; tryptophan was used as internal standard at 40.0 μg mL^{-1}.
Concentration interval: 10.0–100 μg mL^{-1} (except chlorogenic acid, 10.0–75.0 μg mL^{-1}).

between the phenolic acids under investigation and the matrix constituents. Migration time of phenolic acids in the extracts slightly differ from what it was obtained with standard solutions possibly due to matrix effects. Therefore, peak identification was accomplished by spiking the extracts with standard solution aliquots and by comparison of on-line UV spectra of each peak in the sample electropherogram and database built with standard solutions.

The corresponding quantitative results of phenolic acids in the fruits under investigation are displayed in Table 6. Abiu-roxo, also known as star apple or caimito is a tropical fruit found in South America. According to Table 6 results, abiu-roxo contains ferulic,

Table 6
Phenolic acid contents of *in natura* fruits (μg g^{-1}).

Phenolic acid	Abiu-roxo		Wild mulberry		Tree tomato	
	Free phenolic acid fraction	Hydrolysate fraction	Free phenolic acid fraction	Hydrolysate fraction	Free phenolic acid fraction	Hydrolysate fraction
Chlorogenic acid	n.d.	n.d.	5.21 ± 0.50	8.03 ± 0.88	5.28 ± 0.83	n.d.
Syringic acid	n.d.	n.d.	n.d.	n.d.	n.d.	5.2 ± 1.0
Ferulic acid	n.d.	6.3 ± 1.3	n.d.	n.d.	n.d.	1.51 ± 0.36
Benzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
p-Coumaric acid	n.d.	2.03 ± 0.43	n.d.	7.19 ± 1.2	n.d.	0.76 ± 0.45
Vanillic acid	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
Caffeic acid	n.d.	n.d.	n.d.	47.3 ± 9.8	n.d.	44.5 ± 9.3
Gallic acid	8.38 ± 0.25	11.4 ± 1.8	n.d.	n.d.	n.d.	n.d.
Protocatechuic acid	n.d.	n.d.	n.d.	6.60 ± 0.98	n.d.	n.d.

n.d. non detectable, <LOD.

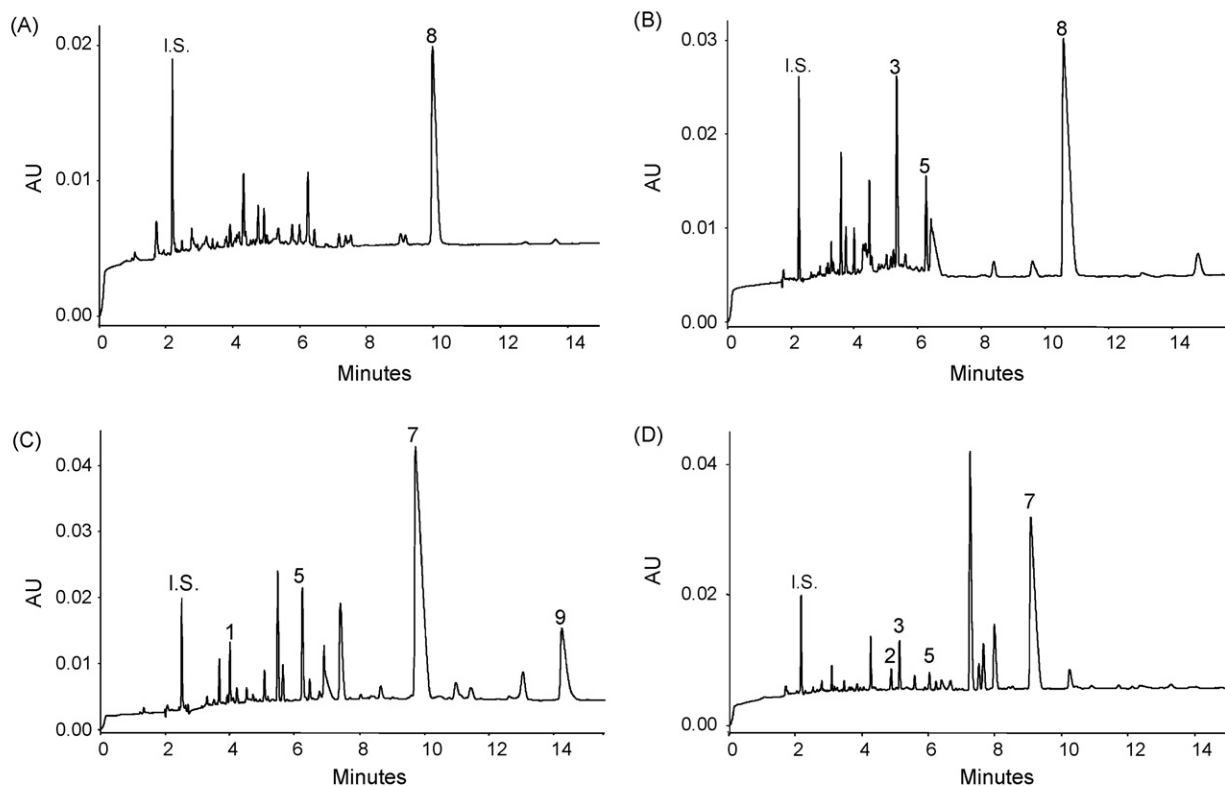

Fig. 6. Electropherograms of the exotic fruit extracts: abiu-roxo (A, free phenolic acids fraction), abiu-roxo (B, hydrolysate fraction), wild mulberry (C, hydrolysate fraction) and tomato tree (D, hydrolysate fraction). Extraction conditions in experimental part. Electrolyte and analytical conditions as in Fig. 5. Peak legend as in Fig. 2.

p-coumaric and relatively large amounts of gallic acids; wild mulberry growing in Brazil is rich in caffeic acid and contains equivalent amounts of chlorogenic, *p*-coumaric, and protocatechuic acids; likewise, tree tomato, a subtropical fruit, is also rich in caffeic acid and contains varied amounts of chlorogenic, syringic, ferulic, and *p*-coumaric acids.

In general, the hydrolysate fractions were richer in phenolic acids than the free phenolic acid fractions, indicating that in the fruit, phenolic acids are bound in larger extent. Another striking features of Table 6 include: chlorogenic acid occurs as free phenolic acid in both wild mulberry and tree tomato whereas gallic acid occurs only in abiu-roxo, as free and bound acid. None of the other studied phenolic acids were detected in the free phenolic acid extracts. *p*-Coumaric acid occurred in the hydrolysate fractions of all studied fruits. Wild mulberry is the only source of bound protocatechuic acid. Wild mulberry and tree tomato are good sources of bound caffeic acid.

There are not much data in the literature regarding the composition and properties of the fruits under investigation. However, a few interesting reports were found. Luo et al. [45] evaluated the antioxidant activity of abiu-roxo using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, demonstrating that an ethyl acetate extract of the fruit exhibited enhanced activity. Furthermore, the polyphenol compounds were fractioned by thin-liquid chromatography in a silica gel column and the spots identified by NMR and ESI-MS. Gallic acid and other flavonoids were the predominant phenolic compounds. No information on bound phenolic acids is available for abiu-roxo in the consulted literature.

Studies on the phenolic composition of different species of mulberry indicate high contents of anthocyanins, the flavonoid subgroup that accounts largely for the fruit antioxidant property [46,47]. However, other nonanthocyanin phenolic compounds also presented accentuated antioxidant activity using free radical scavenge tests [48]. In this latter work, protocatechuic acid, chlorogenic acid, 4-caffeoylquinic acid and 3,5-dicaffeoylquinic acid among other flavonoids were readily identified in mulberry (*Morus alba* L.) by LC–MS.

Information on the antioxidant activity of tree tomato is available [49,50]. An alcoholic extract of *in natura C. betacea* exhibited high activity in the capture of DPPH free radicals. Also, this same fraction presented potential inhibition effect on the oxidation of LDL *in vitro* and stress-induced cytotoxicity on neural PC12 cells [50]. No data on the phenolic composition of tree tomato was found.

In order to help establishing the nutraceutical efficacy of the fruits examined in this work it would be useful to compare their overall phenolic acids contents with other fruits of recognized health promoting characteristics, such as the berry fruits [51]. Ayaz et al. reported a total phenolic acid content of 11.3 μg/g of fresh weight in a little known blueberry (*Vaccinium arctostaphylos* L.) [52] whereas Ehala et al. indicated values comprising cinnamic, ferulic, *p*-coumaric, caffeic and chlorogenic acids ranging from 19 to 58 μg/g of fresh weight for a variety of berries [24]. The total phenolic acid contents of abiu-roxo (28.1 μg/g), wild mulberry (74.3 μg/g) and tree tomato (57.3 μg/g) investigated in this work (Table 6) reveal that the studied fruits are excellent sources of phenolic acids and therefore candidate as nutraceuticals.

4. Conclusions

In this work a simple, reliable and relatively fast CE method for the determination of phenolic acids in exotic fruits has been developed and validated. The electrolyte optimization was successfully assisted by a 3^2 factorial design where a multicriteria decision making approach (based on Derringer's desirability functions) was used as response function. Although the extraction procedure is somehow time consuming, it allowed to discriminate the free and bound phenolic acids in the examined fruits. The method overall analyti-

cal performance proved it suitable for inspecting the phenolic acid composition of fruit matrices and it is probably a good starting point method for implementation in the quality control of fruit derived products.

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