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Use of multivariate statistical techniques to optimize the simultaneous separation of 13 phenolic compounds from extra-virgin olive oil by capillary electrophoresis

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ABSTRACT

Characterization of phenolic compounds in olive oil has not been achieved as yet, owing to the complexities of their chemical structures and analytical matrix. The aim of this work is to optimize and validate a method for simultaneous separation and quantification of 13 phenolic compounds from extra-virgin olive oil: tyrosol, hydroxytyrosol, oleuropein glycoside, ferrulic acid, *p*-coumaric acid, cinnamic acid, *p*-hydroxybenzoic acid, gallic acid, caffeic acid, luteolin, apigenin, vanillic acid and 3,4-dihydroxybenzoic acid. A statistical central composite design, response surface analysis and the simultaneous optimization method of Derringer and Suich were used to separate all the peaks. These multivariate procedures were efficient in determining the optimal separation condition, using five peak-pair resolutions and runtime as responses. The optimized method employed a fused-silica capillary of 50 μm i.d. × 60 cm effective length with extended light path, 50 mmol L^{-1} boric acid electrolyte, 10.2 pH, 25 °C, injection of 50 mbar for 25 s with application of reverse voltage (−30 kV for 5 s) before setting the running voltage (+30 kV) with detection at 210 nm and a run time of 12 min. Peak resolutions are found to be very sensitive to pH values outside the 10.15–10.25 range but acceptable electropherograms can be obtained for a wide range of boric acid concentrations within this pH interval.

1. Introduction

The Mediterranean diet includes a high level of olive oil consumption that contains large quantities of phenolic compounds with elevated antioxidant activity, by means of free radical scavenging [1–3]. These phenols also act as anti-inflammatory, antiviral and anticarcenogenic agents [4].

The phenolic compounds that have already been identified and quantified in olive oil belong to the phenylethylalcohols (like hydroxtyrosol and tyrosol), phenolic acids (such as *p*-coumaric and vanillic acids), lignans [(+)-pinoresinol and (+)-1-acetoxypinoresinol], secoiridoids (several aglycone derivatives of oleuropein and ligstroside) and flavonoids (luteolin and apigenin) [5]. It should be noted that the phenolic compounds have not been completely characterized in olive oil owing to the complexity of their chemical natures and the matrix in which they are encountered [6].

Capillary electrophoresis (CE) presents a good compromise between analysis time and satisfactory characterization for the phenolic compounds in olive oil. The speed, resolution and simplicity of CE, combined with its low operational cost and small residue generation, makes this technique an attractive option for the development of analytical methods for food analysis [7,8].

The development of CE methods has recently seen a large number of multivariate statistical design applications [9–12] because several factors, such as running electrolyte concentration, voltage and medium pH affect the separation of analyte signals. Since food samples studied by CE often involve a large number of analytical peaks, many of which must be separated, the optimization process must take into consideration all the critical separations simultaneously. A set of experimental conditions that results in good separations for some peaks may not resolve other peaks that are overlapped. As such multi-criteria methods such as the one proposed by Derringer and Suich [13] are very convenient to use if accurate response surfaces have been determined from experimental results of a statistical design. This experimental strategy has been recently applied to the optimization of analytical systems in high performance liquid chromatography [14,15] and to simultaneously improve peak resolutions and minimize analysis times in CE for the determination of resveratrol in a nutraceutical [16], for the chiral separation of peptides [17], for a glucagon competitive immunoassay [18], for determination of epinastine hydrochloride in human serum [19], and for antibiotic residue quantitations in milk [20].

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In the work reported here a central composite design, response surface analysis and the Derringer–Suich desirability function were applied to separate 13 phenolic compounds in extra-virgin olive oil using capillary electrophoresis with a diode array detector (CE-DAD). For food matrices the analysis challenge is intimidating owing to the complexity of sample matrix as well as the low concentrations of the analytes of interest. The 13 phenolic compounds chosen to make up the mixture are representative of a variety of compounds encountered in olive oils of different producing countries so the optimized conditions would have wide applicability. The experimental factors varied were the concentration of boric acid, the running electrolyte, pH and voltage. The pH plays an extremely relevant role in electrophoretic separation since small pH variations can improve resolution, or even invert the order of peak elution. Furthermore the pH can interact with the factors being optimized making it especially suitable for multivariate optimization. The difficulty of optimizing pH has been pointed out by Orlandini et al. [16] in a multi-criteria response surface study for resveratrol determination. The pH factor was studied with univariate experiments and not included in their multivariate design. Besides including this factor in the central composite design six different responses were simultaneously optimized in our investigation compared with four responses for the resveratrol study [16].

2. Experimental

2.1. Reagents

Hexane p.a. (Synth, Brazil), methanol p.a. (Synth, Brazil) and HPLC grade methanol (J.T. Baker, USA) were used, as well as boric acid (Ecibra, Brazil) and sodium hydroxide p.a. (Nuclear, Brazil). Water was purified in a Milli-Q system (Millipore, USA). Standards of tyrosol, gallic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, caffeic acid, 3,4-dihydroxybenzoic acid, cinnamic acid, vanillic acid, ferrulic acid, luteolin and apigenin were acquired from Sigma–Aldrich (EUA). The hydroxytyrosol standard was obtained from Cayman Chemical (USA) and the oleuropein glycoside standard was acquired from Extrasynthese (France). The solutions were filtered through 0.45 μm Millipore filter and placed under ultrasound during 5 min before injection.

The standard stock solutions were prepared in HPLC grade methanol, filtered through 0.45 μm membranes and stored at −18 °C and protected from light.

In order to execute the optimization experiments a working methanol:water solution (30:70), containing 6.7 mg L^{-1} of each one of the analyte compounds was prepared, except for caffeic, gallic and 3,4-dihydroxybenzoic acids, whose concentrations were 10.4 mg L^{-1}; luteolin, whose concentration was 13.3 mg L^{-1}; and tyrosol which had a 28.3 mg L^{-1} concentration.

2.2. Equipment

An Agilent G1600AX (Agilent Technologies, Germany) capillary electrophoresis system equipped with a diode array detector (DAD), automatic injector and temperature control system adjusted to 25 °C was used in this study. A fused-silica capillary of 50 μm diameter and 60 cm of effective length with extended light path (Agilent Technologies, Germany) was also used. The detection was made at 210 nm and data treatment was performed with HP ChemStation software.

New capillaries were activated and conditioned by washing under 1 bar pressure using 1 mol L^{-1} NaOH for 30 min, followed by 10 min of water. At the beginning of each workday, the capillary was conditioned for 5 min with 1 mol L^{-1} NaOH, followed by 5 min with water and 10 min with electrolyte. At the end of the day the capillary was washed for 5 min with 1 mol L^{-1} NaOH and 5 min with water. The capillary was stored in water during the night.

2.3. Experimental design and data treatment

Most methods developed for the analysis of phenolic compounds by CE employ capillary zone electrophoresis with basic solutions controlled by a borate buffer. The borate buffer also can complex with the hydroxyl groups of phenolic compounds, which promotes peak separation [21–24].

For this reason boric acid was chosen as a running electrolyte. Before multivariate optimization, a preliminary study was carried out varying the boric acid concentration between 50 and 250 mmol L^{-1} in order to determine an adequate concentration range for further investigation. Smaller concentrations were then chosen for further studies since they shortened the experimental run time, improved peak symmetry and diminished noise. In all these tests injections were carried out at 5 mbar for 5 s with a temperature of 25 °C and a voltage of 30 kV.

The central composite design investigated changes in the boric acid concentration (BOR), pH and voltage. Levels varied from 33.2 to 66.8 mmol L^{-1} for (BOR), from 9.86 to 10.54 for pH and between 24 and 30 kV for applied voltage. All the central composite design experiments were made with injections at 50 mbar for 5 s, 25 °C and detection at 210 nm. The design center point was executed in triplicate resulting in a total of 17 experiments that were executed in random order. Before each experimental run the capillary was conditioned for 5 min with 1 mol L^{-1} NaOH, 5 min with water and 10 min with the appropriate running electrolyte. Each design experiment was injected twice with capillary conditioning for 2 min with the running electrolyte in between runs.

Elementary resolution, R_S, was chosen as the response variable. Since it resulted in acceptable statistical models permitting an adequate assessment of the quality of peak separation for all the design experiments, alternative response variables were not investigated. Resolutions were calculated between pairs of peaks that co-eluted in at least one of the design conditions resulting in five responses to be optimized. Run time was added as a sixth response of interest.

Response values were calculated using:

$$R_S = \frac{2(t_2 - t_1)}{w_2 + w_1}$$

for which t_1 and t_2 are migration times and w_1 and w_2 are the corresponding widths of the bases of the pair of adjacent peaks.

The models were validated by mean of the analysis of variance (ANOVA) at the 95% confidence level. Then the optimum conditions to separate all the 13 peaks were determined examining response contour graphs and using the multi-criteria response technique of Derringer and Suich. Desirability values were established for each individual response and they were combined into their recommended global desirability function [14].

The individual desirabilities were defined to maximize the resolutions and to minimize the runtime. For maximization of the resolutions:

$$d_i = 0 \quad \text{if} \quad R_{Si} < R_{Si}^{\min} \tag{1}$$

$$d_i = \frac{R_{Si} - R_{Si}^{\min}}{R_{S\,\text{targ}} - R_{Si}^{\min}} \quad \text{if} \quad R_{Si}^{\min} \le R_{Si} \le R_{S\,\text{targ}} \tag{2}$$

$$d_i = 1 \quad \text{if} \quad R_{Si} > R_{S\,\text{targ}} \tag{3}$$

To minimize the runtime:

$$d_i = 1 \quad \text{if} \quad t_i < t_{\text{targ}} \tag{4}$$

$$d_i = \frac{t_i - t_i^{\max}}{t_{\text{targ}} - t_i^{\max}} \quad \text{if} \quad t_i^{\max} \le t_i \le t_{\text{targ}} \tag{5}$$

$$d_i = 0 \quad \text{if} \quad t_i > t_i^{\max} \tag{6}$$

where d_i corresponds to the i^{th} desirability (between 0 and 1), R_{Si} and t_i are values predicted from the statistical models for the i^{th} response, $R_{S\,targ}$ and t_{targ} are target values for the responses, R_{Si}^{min} is the minimum resolution value and t_i^{max} the acceptable maximum runtime.

For resolution maximization $R_{Si}^{min} = 3$ was chosen for all the pairs of peaks since this value would provide adequate peak separation for all the peak pairs. The value of $R_{S\,targ}$ was considered individually for each peak pair as being the highest resolution observed experimentally for that peak pair in the statistical design experiments. In this way the algorithm uses the statistical models to maximize the resolutions of the five peak pairs while not accepting any theoretical resolution value below three and above the maximum value experimentally obtained, since in this case it would probably be out of the experimental region for the investigated factors. For runtime values of t_{targ} = 12 min and $t_i^{max} = 15$ min were used, because they cover the smallest runtimes observed in the central composite design. The algorithm will then search for maximum resolutions and minimum runtime. Data treatment was carried out using the Design Expert 6.0.10 (Minneapolis, EUA) software.

2.4. On-line pre-concentration (stacking)

In order to increase detectability as well as reduce solvent consumption in the liquid–liquid extraction procedure used to extract phenolic compounds from olive oil, an on-line pre-concentration method called stacking was evaluated [25,26].

Knowing the optimum separation conditions, ionic strength-mediated stacking, large-volume sample stacking (LVSS) and reverse electrode polarity stacking modes (REPSM) were studied, by means of dissolving the standards in methanol:water (low-conductivity sample), enlarging the sample injection volume and applying a reverse voltage. To increase the injection volume the same 50 mbar pressure was maintained with variation of injection times (10, 15 and 25 s) considering that the original injection lasted just 5 s. The application of a −30 kV voltage, evaluated between 3 and 6 s of application, was necessary for the 15 and 25 s applications. The tests were performed with mixtures of the 13 phenolic standards. The electrolyte was changed after three runs.

To determine the effect of pre-concentration on the samples, methanolic extracts of extra-virgin olive oil were prepared by liquid–liquid extraction [27,28]. Extractions using 2.0 g and 15.0 g samples were made to determine if the increase in the sample quantity was significant.

2.5. Capillary electrophoresis method validation

The method was validated as prescribed by the United States Pharmacopeia [29] and requirements of Brazilian legislation [30,31]. Method repeatability was determined injecting a solution containing the 13 phenolic compounds. This procedure was carried out consecutively 10 times in 1 day. Intermediate precision was determined repeating this procedure on 3 consecutive days. System linearity was verified individually for each compound with calibration curves made up of seven points. A lack of fit test for each calibration curve was performed as recommended by Danzer and Currie [32]. The limits of detection (LOD) and quantification (LOQ) were estimated as being 3 and 10 times the signal to noise ratio.

3. Results and discussion

3.1. Variation of boric acid concentration

An increase in boric acid concentration resulted in an increase in run time, peak broadening and a loss of peak symmetry. Peak broadening and asymmetry do not favor peak separation and increase runtime. For this reason the region of lower boric acid concentrations was studied since the use of 50 mmol L^{-1} BOR resulted in a runtime of only 9 min and very symmetric peaks.

3.2. Model validation for resolution (R_S)

Resolutions were calculated for all the pairs of peaks that co-eluted under any of the experimental conditions of the central composite design. In all, five resolutions were calculated for the pairs: oleuropein glycoside/tyrosol (OLE/TYR), vanillic acid/3,4-dihydroxybenzoic acid (VAN/3,4-D), gallic acid/*p*-hydroxybenzoic acid (GAL/p-HYD), caffeic acid/*p*-coumaric acid (CAF/p-CUM) and 3,4-dihydroxybenzoic acid/gallic acid (3,4-D/GAL). Runtime (RTIM) was also included as a response since it should be minimized. Table 1 summarizes the results for the six response values for

Table 1
Experiments of the central composite design and respective responses.

Experiment	Variables[a]			Resolution (R_S)[b]					RTIM (min)
	BOR (mmol L^{-1}) (x_1)	pH (x_2)	Voltage (kV) (x_3)	OLE/TYR	VAN/3,4-D	GAL/p-HYD	CAF/p-CUM	3,4-D/GAL	
1	−1	−1	−1	0.00	6.61	6.70	4.40	0.00	13.73
2	1	−1	−1	1.58	6.60	10.83	7.09	0.00	18.13
3	−1	1	−1	11.19	1.29	1.17	6.80	10.25	15.70
4	1	1	−1	7.95	0.80	6.96	11.34	10.74	21.68
5	−1	−1	1	2.13	6.77	6.72	4.25	0.00	11.72
6	1	−1	1	1.60	6.74	10.90	7.18	0.00	15.30
7	−1	1	1	8.00	1.59	2.04	6.97	9.35	12.85
8	1	1	1	11.55	1.11	4.27	9.37	12.11	18.47
9	−1.68	0	0	2.22	4.18	5.87	6.33	3.49	12.02
10	1.68	0	0	3.98	2.92	12.68	12.21	5.40	20.50
11	0	−1.68	0	6.95	9.76	7.29	2.03	2.02	13.53
12	0	1.68	0	13.30	1.00	0.83	7.62	16.32	17.37
13	0	0	−1.68	2.09	3.77	9.16	9.09	3.97	17.73
14	0	0	1.68	2.65	3.61	9.16	9.14	4.62	13.79
15	0	0	0	2.06	4.06	9.23	8.93	3.73	15.41
16	0	0	0	2.99	3.07	9.02	9.67	5.21	15.88
17	0	0	0	1.84	3.78	9.29	9.62	4.41	15.95

[a] Codified values of experimental factors: $x_1 = ([BOR] - 50)/10$; $x_2 = (pH - 10.2)/0.2$; $x_3 = (V - 27)/2$.
[b] Responses: OLE/TYR, oleuropein glycoside/tyrosol; VAN/3,4-D, vanillic acid/3,4-dihydroxybenzoic acid; GAL/p-HYD, gallic acid/*p*-hydroxybenzoic acid; CAF/p-CUM, caffeic acid/*p*-coumaric acid; 3,4-D/GAL, 3,4-dihydroxybenzoic acid/gallic acid; RTIM, runtime.

Table 2
Summary of the ANOVA considering the statistical significance of the regression and the lack of fit of linear and quadratic models, employing resolution and runtime as responses.

Responses[a]	Regression		Lack of fit	
	MS_R/MS_r	$F_{9,7,95\%}$	MS_{lof}/MS_{pe}	$F_{5,2,95\%}$
OLE/TYR	32.31	3.68	5.63	19.30
VAN/3,4-D	69.29		0.49	
GAL/p-HYD	18.03		80.02	
CAF/p-CUM	15.22		9.43	
3,4-D/GAL	163.35		0.32	
RTIM	245.02		0.53	

MS_R, mean square of regression; MS_r, mean square of residual; MS_{lof}, mean square lack of fit; MS_{pe}, mean square pure error.

[a] OLE/TYR, oleuropein glycoside/tyrosol; VAN/3,4-D, vanillic acid/3,4-dihydroxybenzoic acid; GAL/p-HYD, gallic acid/*p*-hydroxybenzoic acid; CAF/p-CUM, caffeic acid/*p*-coumaric acid; 3,4-D/GAL, 3,4-dihydroxybenzoic acid/gallic acid; RTIM, runtime.

each of the central composite design experiments. As such, this CE optimization involved more responses than those previously treated by response surface-desirability function analysis [16–20]. For example, in the work reported by Orlandini et al. [16], three peak pairs presented separation problems, while in the work from Vera-Candioti et al. [20] only 5 compounds were separated.

Linear and quadratic models were determined for each of the six responses. Model preferences and validation were accomplished by analysis of variance (ANOVA) at the 95% confidence level. Table 2 shows the ANOVA results for the models for each response and Table 3 shows the corresponding model coefficients.

Table 2 shows that all the peak pair models except the one for the gallic acid/*p*-hydroxybenzoic acid (GAL/p-HYD) peak pair did not show evidence of lack of fit since their mean square lack of fit/mean square pure error ratios are smaller than the 95% critical value for five lack of fit and two pure error degrees of freedom. The Derringer and Suich desirability function may not work properly if the models inputted into the optimization algorithm are not accurate and suffer from lack of fit. However the diagnostic residuals vs. predicted value graph for the GAL/p-HYD model results did not present evidence that the residual behavior was not normal or suffered from heteroscedasticity. Moreover, the MS_{pe} value for this pair resulted in a much lower value (0.020) than the MS_{pe} values for the other pairs (OLE/TYR = 0.37; VAN/3,4-D = 0.26; CAF/p-CUM = 0.17; 3,4-D/GAL = 0.54). So, the MS_{lof}/MS_{pe} quotient for the GAL/p-HYD pair could be inflated as a result of underestimation of its MS_{pe} value. As will be seen shortly this did not adversely affect the search for the optimum conditions. Furthermore all the models have mean square of regression/residual mean square ratios that are comfortably larger than the $F_{9,7}$ 95% confidence value. In fact the calculated ratios are 4–60 times larger than the critical value. This is a good indication of the accuracies of the validated models given in Table 3.

Table 3 contains the model coefficients that are significant at or above the 95% confidence level. As can be seen the linear coefficients for pH are significant for all the resolutions. Curvature effects are also significant at this level for all the models except for the OLE/TYR one. Interestingly no binary interaction effect involving pH is significant. Boric acid concentration has significant linear effects on the GAL/p-HYD, CAF/p-CUM and 3,4-D/GAL resolutions, no significant curvature effects and participates with applied voltage in one significant interaction for the OLE/TYR resolution. As such applied voltage and boric acid concentration should not be optimized using conventional univariate approaches because the optimized value of either one of these factors depends on the level employed for the other. Applied voltage has only this significant interaction effect. All its linear, quadratic and other interaction effects are not significant.

Interpretation of the model coefficients in Table 3 can help the researcher understand how peak elution is affected by changing the experimental design factor levels. Increasing [BOR] in the range investigated here increases the resolution of (GAL/p-HYD) and (CAF/p-CUM) pairs that have positive model gradients of 2.03 and 1.64. The increase in the 3,4-D/GAL resolution is much smaller with a linear coefficient of only +0.47.

The effect of changing pH is much more complicated as was anticipated in the beginning of this investigation. The linear coefficients for all the resolution responses have different algebraic signs. These linear terms contribute to increases in resolution of the (OLE/TYR), (CAF/p-CUM) and (3,4-D/GAL) pairs while provoking decreases in the resolutions of the (VAN/3,4-D) and (GAL/p-HYD) pairs. However the quadratic coefficients for pH are also important, especially for the (GAL/p-HYD) and (CAF/p-CUM) peak pairs. Both of them are negative indicating the existence of rising ridges with local maxima for their response surfaces. As such the resolutions of these two pairs are very sensitive to small changes in pH. On the other hand the quadratic coefficients for the VAN/3,4-D and 3,4-D/GAL resolution models are positive but not as large in absolute value as those for the (GAL/p-HYD) and (CAF/p-CUM) peak pairs. In any case the complexity of the behavior of these resolutions on changes in pH certainly indicate that the researcher must be careful to have a precise control over pH in order to find optimized resolutions for these peak pairs.

Finally runtime seems to follow a linear model. Increasing both [BOR] and pH increases runtime whereas increasing the applied voltage reduces it, as expected.

Inspection of the electropherograms of the central composite design experiments revealed that experiment 14 in Table 1 resulted in the desired resolutions and runtime. The experiment was performed with a 50 mmol L^{-1} boric acid concentration, a 10.2 pH value and with 30 kV of applied voltage. These experimental conditions result in the following experimental and theoretical resolutions for the various peak pairs (theoretical values from models in Table 3 are given in parenthesis): OLE/TYR, 2.65 (2.98), VAN/3,4-D, 3.61 (3.60), GAL/p-HYD, 9.16, (8.27), CAF/p-CUM, 9.14 (9.04),

Table 3
Significant model coefficients and their standard errors for peak-pair resolutions and runtime.

Pair of compounds	Significant coefficients ± standard error									
	Intercept	*A* (BOR)	*B* (pH)	*C* (V)	A^2	B^2	C^2	*AB*	*AC*	*BC*
OLE/TYR	2.22 ± 0.73	–	5.72 ± 0.34	–	–	–	–	–	1.11 ± 0.45	–
VAN/3,4-D	3.66 ± 0.24	–	−2.68 ± 0.11	–	–	0.55 ± 0.12	–	–	–	–
GAL/p-HYD	9.26 ± 0.61	2.03 ± 0.29	−2.31 ± 0.29	–	–	−2.08 ± 0.32	–	–	–	–
CAF/p-CUM	9.39 ± 0.63	1.64 ± 0.29	2.04 ± 0.29	–	–	−2.28 ± 0.32	–	–	–	–
3,4-D/GAL	4.45 ± 0.30	0.47 ± 0.14	5.37 ± 0.14	–	–	0.95 ± 0.16	–	–	–	–
RTIM	15.74 ± 0.14	2.48 ± 0.07	1.19 ± 0.07	−1.28 ± 0.07	0.21 ± 0.07	–	–	0.45 ± 0.09	–	–

Pair of compounds: OLE/TYR, oleuropein glycoside/tyrosol; VAN/3,4-D, vanillic acid/3,4-dihydroxybenzoic acid; GAL/p-HYD, gallic acid/*p*-hydroxybenzoic acid; CAF/p-CUM, caffeic acid/*p*-coumaric acid; 3,4-D/GAL, 3,4-dihydroxybenzoic acid/gallic acid; RTIM, runtime.

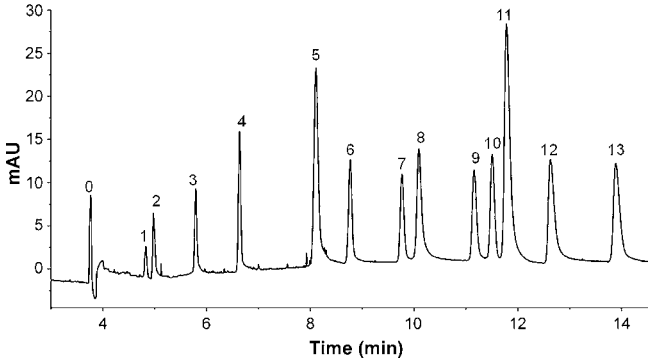

Fig. 1. Electropherogram for the optimal condition of separation of the 13 phenolic compounds from extra-virgin olive oil by capillary zone electrophoresis. Fused-silica capillary of 50 μm i.d. × 60 cm effective length with extended light path, 50 mmol L^{-1} of boric acid electrolyte, 10.2 pH, 30 kV, 25 °C, injection of 50 mbar for 5 s and detection at 210 nm. Peak identification: 0, solvent; 1, oleuropein glycoside; 2, tyrosol; 3, hydroxytyrosol; 4, cinnamic acid; 5, luteolin; 6, apigenin; 7, ferulic acid; 8, caffeic acid; 9, *p*-coumaric acid; 10, vanillic acid; 11, 3,4-dihydroxybenzoic acid; 12, gallic acid; 13, *p*-hydroxybenzoic acid.

3,4-D/GAL, 4.62 (4.46). The poorest agreement occurs for the resolution of the GAL/p-HYD peak pair, but it must be remembered that the model for this peak pair suffered from lack of fit. The runtime for this experiment was 13.79 min, whereas a runtime of 13.83 min is predicted by the model in Table 3. Fig. 1 contains the corresponding electropherogram with all the peaks neatly separated.

The voltage and boric acid concentration factors are involved in an interaction effect. For this reason the response contour graph, shown in Fig. 2, was made for the experimental design domain of the boric acid concentration and *V* while holding the pH constant at its optimum value, 10.2. The interaction is characterized by the inclined contour curves in the figure. The shaded area corresponds to all the boric acid concentrations and *V* values having acceptable (greater than three) peak resolutions and analysis times that are less than 14 min.

Although the contour lines are much different for the boric acid concentration vs. pH graph the shape of the overlay region of acceptable values for all the responses is very similar to the one shown in the figure. This region falls between pH values of 10.15 and 10.25 at lower boric acid concentrations expanding a bit to a pH of about 10 in the region of the center point. The acceptable response region occurs for a long but narrow region on the overlaid response surfaces being extremely sensitive to pH values but not to boric acid concentrations. Small pH increases or decreases

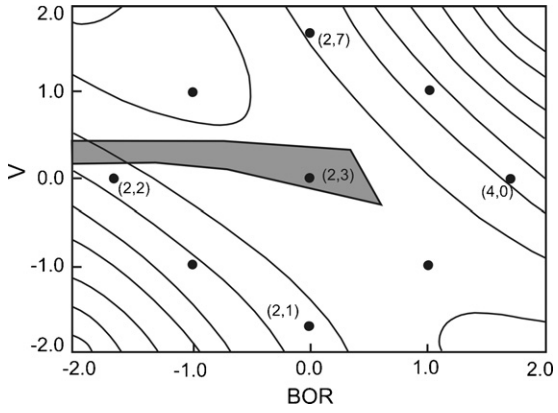

Fig. 2. Contour line graph for the boric acid concentration and V showing the experimental region that results in acceptable electropherograms. The pH was held constant at 10.2. The central point shows the optimum conditions. Experimental values for some design points are in parenthesis.

outside the 10.15–10.25 range result in at least one resolution with an unacceptable value below three. Increasing boric acid concentrations inside this pH range, however, increases runtime but does reduce the peak resolutions.

The Derringer–Suich algorithm was then applied to see if another set of experimental conditions might give an electropherogram of even better quality. The algorithm encountered six sets of experimental conditions that satisfied the established criteria but all were slight variations from the conditions employed in run 14. These small variations can be attributed to errors propagated into the model coefficients in Table 3 owing to the experimental errors in the response values in Table 1. The global desirability function value was around 0.2 for all these possible experimental conditions, which is probably due to the very restrictive criteria employed in this application.

3.3. On-line pre-concentration (stacking)

The most promising conditions for stacking standards were a 10 s injection without applying reverse voltage and a 25 s injection with a 5 s application of −30 kV reverse voltage. The latter resulted in a 6-fold increase in peak areas (compared to the same concentrations injected during 5 s) and a 4-fold increase in peak heights, considerably improving detectability. These conditions were tested on the olive oil extracts obtained from 2.0 and 15.0 g of extra-virgin olive oil together with injection using the original method with a 5 s injection time. When the extract obtained from 2.0 g was injected with reverse voltage it was possible to obtain a higher detectability than when injecting the extract obtained from 15 g during 5 s. Thus it was possible to use 7.5 times smaller quantities of sample, methanol and hexane by simply increasing the injection time and applying reverse voltage for a short time. Injection time and reverse voltage are easily controlled by the system software. Under these conditions runtime was also reduced to 12 min.

3.4. Method validation

All the tests made using only standards were conditioned for 2 min with electrolyte between runs. However when tests were carried out on stacking by increasing injection volume this conditioning was not sufficient to stabilize the runs. So several types of conditioning were tested using 1 mol L^{-1} NaOH, water and electrolyte. The runs stabilized with a conditioning of 1 min of 1 mol L^{-1} NaOH + a 1 min wait + 1 min of water + 1 min of electrolyte + a 1 min wait + 1 min of electrolyte, totaling 6 min of conditioning time between runs. With this procedure conditioning was not necessary at the start of the workday.

Validation, after the conditioning and stacking investigations, resulted in the data shown in Table 4. The values for repeatability and intermediate precision given in the table are for area measurements. The relative standard deviation (RSD) values for the migration times of all the compounds stayed below 1.19% for repeatability (in 1 day) and below 1.43% for intermediate precision (between days). The method satisfies all the necessary validation requirements including the lack of fit criterion for linearity [29–32]. Fig. 3 shows an electropherogram of the 13 standards under the final method conditions after pre-concentration and validation.

There are several papers in the literature reporting the separation of phenolic compounds in extra-virgin olive oil by capillary electrophoresis. Considering those where CE-DAD was employed [28,33–36], the set of phenolic compounds assayed in this study were not separated. Moreover, all the optimizations were done by means of univariate experiments. Some authors have separated a larger number of peaks [28,33], however, with lower resolution between several peak pairs. The methods proposed in these other works did not use stacking as a tool for improving detectability and

Table 4
Figures of merit for the validation of the method for separation of 13 phenolic compounds from extra-virgin olive oil.

Compounds	Linearity ($mg\,L^{-1}$)	R^2	p-value (lack of fit test)[a]	Repeatability ($n=10$)[b]	Intermediate precision ($n=3$)[b]	LOD ($mg\,L^{-1}$)	LOQ ($mg\,L^{-1}$)
Oleuropein glycoside	1.0–13.0	0.9975	0.313	4.51	5.65	0.15	0.49
Tyrosol	1.0–13.0	0.9929	0.217	4.86	2.20	0.22	0.74
Hydroxytyrosol	1.0–15.0	0.9851	0.052	5.41	6.96	0.09	0.29
Cinnamic acid	1.0–13.0	0.9981	0.290	2.22	3.05	0.07	0.24
Luteolin	3.0–13.0	0.9951	0.052	2.58	3.77	0.85	2.84
Apigenin	2.0–15.0	0.9996	0.134	2.80	3.81	0.59	1.98
Ferulic acid	1.0–13.0	0.9981	0.156	2.77	3.03	0.10	0.33
Caffeic acid	1.0–15.0	0.9977	0.059	2.95	3.74	0.08	0.25
p-Coumaric acid	1.0–13.0	0.9976	0.325	2.66	2.98	0.05	0.18
Vanillic acid	1.0–13.0	0.9978	0.274	2.78	3.34	0.08	0.25
3,4-Dihydroxybenzoic acid	1.0–13.0	0.9966	0.102	2.98	3.45	0.04	0.13
Gallic acid	1.0–13.0	0.9912	0.482	6.96	3.62	0.13	0.43
p-Hydroxybenzoic acid	1.0–13.0	0.9979	0.299	2.88	3.12	0.08	0.25

[a] The probability value of the lack of fit test should be greater than 0.05.
[b] The repeatability and intermediate precision parameters were evaluated by calculating the relative standard deviation, RSD (%). LOD, limit of detection; LOQ, limit of quantification.

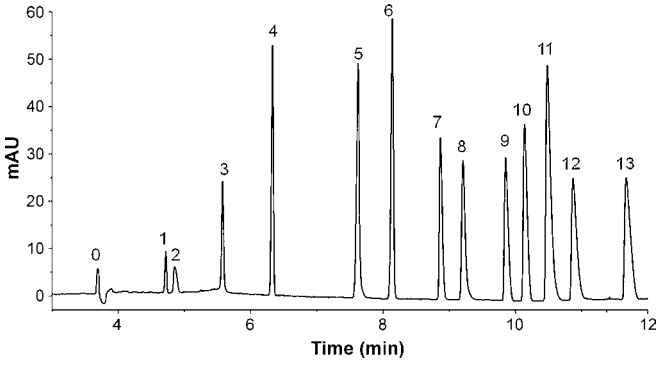

Fig. 3. Electropherogram for the 13 phenolic compounds after overall optimization. Standards mixture containing 12.9 $mg\,L^{-1}$ of each compound. Fused-silica capillary of 50 μm i.d. × 60 cm effective length with extended light path, 50 $mmol\,L^{-1}$ of boric acid electrolyte, 10.2 pH, 30 kV, 25 °C, injection of 50 mbar for 25 s with application of reversed voltage (−30 kV for 5 s) and detection at 210 nm. Peak identification can be seen in Fig. 1.

lowering toxic organic solvent consumption and waste generation. As such, the method developed in this study improved resolution between some critical peak pairs, in a shorter runtime, as well as presented lower reagent costs and residue generation.

4. Conclusion

Central composite design, response surface analysis and the Derringer–Suich multi-criteria method were used to optimize the electrophoretic separation of 13 phenolic compounds from extra-virgin olive oil providing maximum resolution between peaks and a shorter runtime. Response surface models provided useful information on the nature of peak migrations caused by changes in electrolyte concentration, pH values and applied voltages. The use of on-line pre-concentration (stacking) resulted in a 6-fold area increase and 4-fold increase in peak height. Moreover it reduced consumption of sample and organic solvents, methanol and hexane, diminished residue generation and environmental impact without affecting analytical quality.

Final conditions consist of using a 50 μm i.d. × 60 cm effective length capillary with extended light path, 50 $mmol\,L^{-1}$ boric acid electrolyte, 10.2 pH, 25 °C, 50 mbar injection for 25 s with application of a reverse voltage (−30 kV for 5 s) before applying the run voltage (+30 kV) and detection at 210 nm. The separation of 13 peaks occurred in 12 min. Although the peak resolutions are very sensitive to pH fluctuations outside the 10.15–10.25 range, boric acid concentration changes do not reduce them within this pH range. The validation study showed that the method satisfies official requirements for the quantification of phenolic compounds in samples of extra-virgin olive oil.

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References

[1] J. Perona, R. Cabello-Moruno, V. Ruiz-Gutierrez, J. Nutr. Biochem. 17 (2006) 429.
[2] C. Sánchez, A. González, M. García-Parrilla, J. Granados, H. García De La Serrana, M. Martínez, Anal. Chim. Acta 593 (2007) 103.
[3] O. Lee, B. Lee, Y. Kim, K. Shetty, Y. Kim, J. Food Sci. 73 (2008) C519.
[4] M. Savarese, E. De Marco, R. Sacchi, Food Chem. 105 (2007) 761.
[5] A. Carrasco-Pancorbo, A. Gómez-Caravaca, L. Cerretani, A. Bendini, A. Segura-Carretero, A. Fernández-Gutiérrez, J. Sep. Sci. 29 (2006) 2221.
[6] A. Bendini, L. Cerretani, A. Carrasco-Pancorbo, A. Gómez-Caravaca, A. Segura-Carretero, A. Fernández-Gutiérrez, G. Lercker, Molecules 12 (2007) 1679.
[7] A. Carrasco-Pancorbo, L. Cerretani, A. Bendini, A. Segura-Carretero, T. Gallina-Toschi, A. Fernández-Gutiérrez, J. Sep. Sci. 28 (2005) 837.
[8] D. Lima, A. Duarte, V. Esteves, Talanta 72 (2007) 1404.
[9] G. Box, J. Hunter, W. Hunter, Statistics for Experimenters, Wiley, New York, 2005.
[10] R.E. Bruns, I.S. Scarminio, B. Barros-Neto, Statistical design – Chemometrics, Elsevier, Amsterdam, 2006.
[11] R.G. Peres, G.A. Micke, M.F.M. Tavares, D.B. Rodriguez-Amaya, J. Sep. Sci. 32 (2009) 3822.
[12] A. Meinhart, C. Bizzotto, C. Ballus, M. Prado, R. Bruns, J. Teixeira Filho, H. Godoy, Food Chem. 120 (2010) 1155.
[13] G. Derringer, R. Suich, J. Qual. Technol. 12 (1980) 214.
[14] M. Breitkreitz, I. Jardim, R. Bruns, J. Chromatogr. A 1216 (2009) 439.
[15] M. Zecevic, B. Jocic, L. Zivanovic, A. Protic, Chromatographia 68 (2008) 911.
[16] S. Orlandini, I. Giannini, S. Pinzauti, S. Furlanetto, Talanta 74 (2008) 570.
[17] Y. Loukas, S. Sabbah, G. Scriba, J. Chromatogr. A 931 (2001) 141.
[18] A. Lomasney, C. Guillo, A. Sidebottom, M. Roper, Anal. Bioanal. Chem. 394 (2009) 313.
[19] L. Vera-Candioti, A. Olivieri, H. Goicoechea, Anal. Chim. Acta 595 (2007) 310.
[20] L. Vera-Candioti, A. Olivieri, H. Goicoechea, Talanta 82 (2010) 213.
[21] P. Morin, F. Villard, M. Dreux, J. Chromatogr. 628 (1993) 153.
[22] P. Morin, F. Villard, M. Dreux, J. Chromatogr. 628 (1993) 161.
[23] O. Maman, F. Marseille, B. Guillet, J. Disnar, P. Morin, J. Chromatogr. A 755 (1996) 89.
[24] P. Jác, M. Polásek, M. Pospísilová, J. Pharm. Biomed. Anal. 40 (2006) 805.
[25] Z. Malá, A. Slampová, P. Gebauer, P. Bocek, Electrophoresis 30 (2009) 215.
[26] M. Moraes, S. Moraes, E. Pereira, M. Tavares, Quim. Nova 32 (2009) 1041.
[27] F. Pirisi, P. Cabras, C. Cao, M. Migliorini, M. Muggelli, J. Agric. Food Chem. 48 (2000) 1191.
[28] M. Bonoli, M. Montanucci, T. Gallina-Toschi, G. Lercker, J. Chromatogr. A 1011 (2003) 163.
[29] The United States Pharmacopeia, 31st Revision, The United States Pharmacopeial Convention, Rockville, MD, 2008.

[30] Inmetro (Instituto Nacional de Metrologia, Normalização e Qualidade Industrial), Orientações sobre Validação de Métodos de Ensaios Químicos, DOQ-CGCRE-008, Rio de Janeiro, 2003.
[31] M. Ribani, C. Bottoli, C. Collins, I. Jardim, Quim. Nova 27 (2004) 771.
[32] K. Danzer, L. Currie, Pure Appl. Chem. 70 (1998) 993.
[33] A. Bendini, M. Bonoli, L. Cerretani, B. Biguzzi, G. Lercker, T. Gallina-Toschi, J. Chromatogr. A 985 (2003) 425.
[34] A. Carrasco-Pancorbo, C. Cruces-Blanco, A. Segura-Carretero, A. Fernández-Gutiérrez, J. Agric. Food Chem. 52 (2004) 6687.
[35] A. Carrasco-Pancorbo, A. Gómez-Caravaca, L. Cerretani, A. Bendini, A. Segura-Carretero, A. Fernández-Gutiérrez, J. Agric. Food Chem. 54 (2006) 7984.
[36] A. Carrasco-Pancorbo, A. Gómez-Caravaca, A. Segura-Carretero, L. Cerretani, A. Bendini, A. Fernández-Gutiérrez, J. Sci. Food Agric. 89 (2009) 2144.