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Novel combination of non-aqueous capillary electrophoresis and multivariate curve resolution-alternating least squares to determine phenolic acids in virgin olive oil☆

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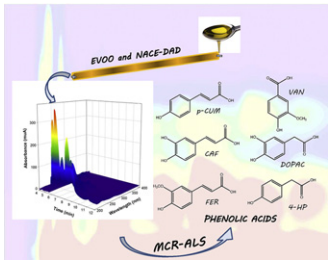
HIGHLIGHTS

- Novel combination of NACE and MCR-ALS for determination of phenolic acids in EVOO.
- Good results are achieved in less time than other CE method for these compounds.
- Resolution and quantitation without to be necessary a complex experimental work.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper presents the development of a non-aqueous capillary electrophoresis method coupled to UV detection combined with multivariate curve resolution-alternating least-squares (MCR-ALS) to carry out the resolution and quantitation of a mixture of six phenolic acids in virgin olive oil samples. *p*-Coumaric, caffeic, ferulic, 3,4-dihydroxyphenylacetic, vanillic and 4-hydroxyphenilacetic acids have been the analytes under study. All of them present different absorption spectra and overlapped time profiles with the olive oil matrix interferences and between them. The modeling strategy involves the building of a single MCR-ALS model composed of matrices augmented in the temporal mode, namely spectra remain invariant while time profiles may change from sample to sample. So MCR-ALS was used to cope with the coeluting interferences, on accounting the second order advantage inherent to this algorithm which, in addition, is able to handle data sets deviating from trilinearity, like the data herein analyzed. The method was firstly applied to resolve standard mixtures of the analytes randomly prepared in 1-propanol and, secondly, in real virgin olive oil samples, getting recovery values near to 100% in all cases. The importance and novelty of this methodology relies on the combination of non-aqueous capillary electrophoresis second-order data and MCR-ALS algorithm which allows performing the resolution of these compounds simplifying the previous sample pretreatment stages.

Abbreviations: VOO, virgin olive oil; NACE, non-aqueous capillary electrophoresis; CZE, capillary zone electrophoresis; BGE, background electrolyte; p-CUM, *p*-coumaric acid; CAF, caffeic acid; FER, ferulic acid; DOPAC, 3,4-dihydroxyphenylcacetic acid; VAN, vanillic acid; 4HP4, -hydroxyphenylaceitc acid; LOD, limit of detection; LOQ, limit of quantitation.

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1. Introduction

Under the denomination "phenolic compounds" there are more than 4000 compounds divided in 12 subclasses [1]. Currently, these compounds are receiving considerable attention, fundamentally due to its antioxidant activity, strongly related to the prevention of cancer, inflammatory disorders and cardiovascular diseases [2,3]. They are part of the minor components of virgin olive oil (VOO), one of the most important foods in the Mediterranean diet which has associated many benefits for the human health, essentially due to its content in these compounds [4]. In addition, phenolic compounds and their strong natural antioxidant activity contribute to the stability of VOO against oxidation and influence in its organoleptic characteristics and nutritional qualities [5]. The composition of phenolic compounds in VOO is related to agronomic and technological aspects [6].

For the quantitation of phenolic compounds in VOO it is important to carry out a complete extraction of this fraction from the oil. Table 1 shows the most used procedures (both traditionally and nowadays) and a comparison between them. Both the liquid–liquid extraction (LLE) and the solid phase extraction (SPE) procedures are complex, tedious and time consuming. In addition, it is habitual to have a great consumption of toxic solvents, like hexane [1]. Nowadays, the traditional methods for detection and quantitation of phenolic compounds have been replaced by separation techniques, such as gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) coupled to different detectors [12–15,20,21]. CE is getting importance and popularity for the analysis of food components, mainly due to the combination of short analysis time and high separation efficiency [22]. In addition, and especially in the case of non-aqueous matrices as those of olive oil samples, the pretreatment of the sample can be greatly simplified using the non-aqueous capillary electrophoresis (NACE) mode. Compared with aqueous capillary zone electrophoresis (CZE), NACE has the advantages of wide bore capillary as a consequence of a minor Joule effect, fast analysis since it is possible to use a higher separation voltage, low adsorption on the capillary wall, and high separation selectivity by selecting the adequate non aqueous background electrolyte (BGE) [23].

Ideally, in optimized conditions, electrophoretic experiments lead to total analytes separation, i.e. each peak belongs to a single compound. It is interesting to note that, although a complete separation of the peaks could not be performed, second order data coupled to chemometrics can be used to achieve selectivity by mathematical means, allowing for resolution and quantitation of overlapped analytes [24,25]. The information provided by the second-order signals, adequately decomposed by suitable second-order algorithms, can be uniquely ascribed to the analyte of interest, even in the presence of unexpected components not considered in the calibration stage. This property is called the second-order advantage and avoids the requirement of physically removing interferences [26,27]. Among the available second order algorithms, MCR-ALS and PARAFAC2, a variant of PARAFAC (parallel factor analysis) [28], are those able to handle second order data deviating from trilinearity, i.e. when changes in shape and/or position of component profiles from sample to sample occur, which is commonly found in capillary electrophoresis data [29–32]. To overcome this challenge, MCR-ALS was performed in the so-called extended mode [33], which involves building an augmented data matrix by appending calibration and test data matrices in the time direction, i.e. the rows represent spectra and the columns time profiles, because this alleviates the problems associated with sample-to-sample differences in this dimension.

Regarding the published works in this context, Sentellas and Saurina reviewed in 2003 the application of chemometrics in CE, in which the methods for data analysis [34] and optimization [35] were introduced. In later years, both first- and second-order methods have been also used for quantification in CE, including principal component regression (PCR), partial least squares regression (PLS), multiple linear regression (MLR), artificial neural networks (ANN) [36–38] and MCR-ALS [24,32,39]. CE coupled with chemometric methods enhances its ability of separation and analysis tremendously. Regarding the combination of chemometric and CE for food analysis, many papers have been published in the authentication and characterization field [36,37]. However, to the best of our knowledge, no paper has been published regarding the use of second order data and CE in the food analysis field for resolution and quantitation. In this context, we pretend innovatively to develop a non-aqueous capillary electrophoresis method coupled to UV detection (NACE-DAD) and combine it with the MCR-ALS algorithm to carry out the resolution and quantitation of a complex mixture of six phenolic acids in VOO samples, in a short period of time and without being necessary a complex experimental work.

2. Theory

2.1. Baseline correction adapted to second order data

Generally, the elimination of baseline is crucial for reducing the number and complexity of the unexpected components. In this work, baseline correction was carried out according to the asymmetric least-squares methodology proposed by Eilers [40] and adapted to second-order data [41], which consists in the minimization of the cost function:

$$Q = \sum_i v_i (y_i - f_i)^2 + \lambda \sum_i (\Delta^2 f_i)^2 \quad (1)$$

in which y is the experimental signal, f is a smooth trend (the baseline approximation), and v is a prior weight. The elements of v are 1 in all places where y is observed or allowed to influence f, while, in all other places, these elements are 0. The positive parameter λ sets the second term weight. It acts as a roughness penalty: the larger λ, the smoother f will be. Δ denotes the derivative of f.

Taking into account the following choice of asymmetric weights: $v_{JK} = p$ if $y_{JK} > f_{JK}$ and $v_{JK} = 1 - p$ if $y_{JK} \leq f_{JK}$ with $0 < p < 1$, positive deviation from the trend will get weights different from negative residuals. Experience demonstrates that a quick and reliable solution could be achieved in about 10 iterations, starting from $v \cong 1$ and iterating between the two computations.

2.2. MCR-ALS

MCR-ALS is an algorithm capable of handling data sets deviating from trilinearity, i.e. data in which migration time shifts or peak shape changes occur for analytes from sample to sample. This can be done due to the strategy of augmenting matrices along the mode which is suspected of breaking the trilinear structure, i.e. if matrix-to-matrix variation of profiles occurs along the column direction, a column-wise augmented matrix is created. The bilinear decomposition of the augmented matrix **D** is performed according to the expression:

$$\mathbf{D} = \mathbf{C} \times \mathbf{S}^{\mathbf{T}} + \mathbf{E} \quad (2)$$

in which the rows of **D** contain the UV–Vis spectra (K wavelengths), as a function of time (J times), the columns of **C** contain the time profiles of the N compounds involved in the process, the columns of **S** their related spectra, and **E** is a matrix of residuals not fitted by the model. Decomposition of **D** is achieved by iterative least-squares minimization of $||\mathbf{E}||$, under suitable constraining conditions, i.e. non-negativity in the spectral profiles, unimodality and non-negativity in the time profiles, correspondence among

Table 1
Commonly utilized procedures to carry out the extraction of phenolic compounds from VOO samples.

Extraction procedure	Characteristics	References
Liquid–liquid extraction (LLE)	Oil previously dissolved in hexane Several portions of methanol/water as extracting solvent	[7,8]
Solid phase extraction (SPE)	C_{18} cartridges Methanol as elution solvent Phenols are separated from VOO for the first time	[9]
LLE vs. SPE	LLE according to Montedoro et al. [7] SPE, Alltech C_{18} Extract – Clean High Capacity cartridges, methanol as elution solvent SPE more efficient to separate simple phenols The recovery of the secoiridoid derivatives using LLE is higher	[10]
	LLE using methanol/water 60:40 (v/v) and VOO dissolved in hexane SPE using C_8 cartridges and acetonitrile as elution solvent Significant differences in the phenols recovery were not found	[11]
	SPE using C_8, modified C_8, C_{18} or Diol cartridges and LLE were examined Diol-SPE and LLE showed higher recoveries of total phenols than other extraction procedures	[12]
	LLE with SPE using C_{18}, Diol or Sax cartridges were compared Diol-SPE and LLE methods were found more effective for the extraction of tyrosol, hydroxytyrosol, secoiridoids and lignans	[13]
LLE and Diol-SPE	The most utilized procedure to carry out the extraction of these kinds of compounds from olive oil samples.	[5,14–19]

species and samples in the case of samples containing uncalibrated interferents.

Typically **D** is built by placing one on top of another the calibration submatrices and each of the test data submatrices. While the pure spectrum of each compound should be the same in all experiments and the spectral mode must be selective, the temporal profiles in the different **C** submatrices need not share a common shape. This is the reason why electropherographic runs can be analyzed together even in the presence of migration time shifts from sample to sample, i.e. non-trilinear data.

It is necessary to point out that MCR-ALS requires initialization with system parameters as close as possible to the final results. In our case, the analyte and interference spectra are required, because the resolution is based on the selectivity in the spectral mode. In this work, the latter were obtained by a combination of the selection of the purest spectra for the interferents based on SIMPLISMA (simple interactive self-modeling mixture analysis) [42] and the introduction of the real spectra for the analytes.

3. Materials and methods

3.1. Chemicals and reagents

For all experiments, analytical reagent grade chemicals and solvents were used. Ultrapure water was obtained from a Millipore Milli-QA10 System (Waters, Germany). *p*-Coumaric acid (*p*-CUM), caffeic acid (CAF), ferulic acid (FER), 3,4-dihydroxybenzoic acid (DOPAC), vanillic acid (VAN) and 4-hydroxyphenylacetic acid (4HP) were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Boric acid (ACS quality) was provided by Carlo Erba (Italy) and potassium hydroxide (PA quality) by Merck (Germany). All employed solvents were HPLC grade, ethanol was provided by Panreac (Spain), 1-propanol by Sigma–Aldrich (USA) and methanol by Scharlau (Spain).

3.2. Standards and samples

1.00 mg mL^{-1} stock solutions of each compound were prepared in volumetric flasks by dissolving the suitable amount of the commercial products and diluting to the mark with 1-propanol. These solutions were stored at 4 °C, avoiding exposure to direct light. Fresh solutions of lower concentrations were prepared by appropriate dilution of the stock solution with the selected solvent. Olive oil samples were acquired from the market and were kept at 4 °C avoiding exposure to direct light. It is important to clarify that these samples are characterized as a particular group of VOO, called extra virgin olive oil (EVOO), since they own an acidity $\leq$2.0%.

3.3. CE method

CE was performed using a capillary electrophoresis system ^{3D}CE (Agilent Technologies, Waldbronn, Germany) equipped with temperature control devices in the sample tray (by a thermostatic bath) and in the capillary (by forced air) and a DAD (Agilent Technologies, Germany). Fused-silica capillaries of 49 cm in length and 75 μm inner diameter (375 μm outer diameter) were used (Agilent Technologies, Germany). The software package ChemStation was used to control the instrument, and for acquisition of signals. The instrumental and chemical separation conditions [43] were the following: separation voltage, +20 kV; hydrodynamic injection, −30 mbar for 6 s in the cathode; separation temperature, 35 °C; temperature of the sample tray, 20 °C; BGE, 18 mM KOH and 25 mM boric acid in a 74:26 v:v 1-propanol:methanol medium (pH* = 11.2). In these conditions all analytes are negatively charged and migrate to the anode against of EOF spending a maximum time of 10–12 min.

At the beginning of the day, the capillary was rinsed with water for 3 min (2 bar), flushed with aqueous NaOH 0.1 M for 1 min and rinsed with water and 1-propanol, successively, for 3 min each (2 bar). For the separation, the capillary was previously flushed with BGE for 3 min (900 mbar) and after the separation, in the post conditioning, it was rinsed with 1-propanol (3 min, 2 bar), water (5 min, 2 bar) and 1-propanol again (2 min, 2 bar). Every three injections the post conditioning was changed as follows: rinse with 1-propanol for 3 min and water for 2 min (2 bar), flush with aqueous NaOH 0.1 M (900 mbar, 0.2 min) and rinse with water and 1-propanol, successively, for 2 min each (2 bar).

For each electropherogram, spectra were registered in the range 200–400 nm each 2 nm, at regular steps 0.8 s for a total time of 12 min. The matrices were built by placing the wavelengths in columns and the times in rows (therefore with dimensions of 901 × 102, although selected regions were subsequently employed for multivariate calibration).

3.4. Software

Data were saved in ASCII format, and transferred to a PC for subsequent manipulation by chemometric programs. All

Table 2
Prediction results of the validation samples using MCR-ALS.

Sample	*p*-CUM		CAF		FER		DOPAC		VAN		4HP	
	Actual	Predicted[a]	Actual	Predicted[a]	Actual	Predicted[a]	Actual	Predicted[a]	Actual	Predicted[a]	Actual	Predicted[a]
1	9.90	9.33 (94.3)	9.54	7.72 (80.9)	9.09	7.56 (83.2)	10.4	8.75 (83.8)	9.90	9.30 (93.9)	3.09	3.41 (110)
2	3.30	3.45 (104)	9.54	8.33 (87.3)	9.09	7.09 (78.0)	10.4	9.64 (92.4)	9.90	9.63 (97.3)	9.27	7.71 (83.3)
3	3.30	3.16 (95.6)	3.18	2.70 (84.8)	9.09	7.74 (85.2)	3.48	4.07 (117)	9.90	10.1 (102)	9.27	8.30 (89.5)
4	3.30	3.11 (94.2)	3.18	2.67 (84.1)	3.03	2.35 (77.4)	10.4	9.12 (87.4)	3.30	3.04 (92.2)	9.27	7.65 (82.5)
5	9.90	9.37 (94.6)	3.18	2.66 (83.8)	3.03	2.26 (74.4)	3.48	3.71 (107)	9.90	9.91 (100)	9.27	7.55 (81.5)
6	9.90	9.81 (99.0)	9.54	8.36 (87.6)	9.09	7.62 (83.9)	3.48	3.76 (108)	9.90	9.99 (101)	3.09	3.39 (110)
7	3.30	3.21 (97.3)	9.54	8.87 (93.0)	9.09	7.77 (85.4)	10.4	9.68 (92.8)	3.30	3.63 (110)	9.27	7.50 (80.9)
8	9.90	9.43 (95.3)	3.18	2.61 (82.0)	9.09	7.98 (87.8)	10.4	9.44 (90.4)	9.90	9.46 (95.5)	3.09	3.14 (102)
9	3.30	3.18 (96.4)	3.18	2.94 (92.6)	3.03	2.40 (79.1)	3.48	3.54 (102)	3.30	3.49 (106)	3.09	3.35 (108)
10	6.60	6.41 (97.2)	6.36	5.24 (82.4)	6.06	4.98 (82.2)	6.96	5.86 (84.2)	6.60	5.93 (89.8)	6.18	5.04 (81.6)
RMSE[b]		0.31		0.95		1.24		0.90		0.36		1.17
REP[c]		5.2		15.8		20.7		15.0		6.08		19.4

[a] Concentrations are given in $\mu g\,mL^{-1}$ and recoveries (between parentheses) are given in percentage.

[b] RMSE (root mean square error) $= \left[\sum_1^I (c_{act} - c_{pred})^2/1\right]^{1/2}$, where $I = 10$.

[c] REP (relative error of prediction) $= 100 \times RMSE/\bar{c}$, where $\bar{c}$ is the mean calibration concentration.

employed algorithms were implemented in MATLAB 7.6 [44]. Those for applying MCR-ALS are available in the Internet at http://www.mcrals.info/. Homemade routines based on the Eilers algorithm were applied to perform the second-order data baseline correction [40].

3.5. Calibration and model validation procedure

For calibration, a set of standard samples of each analyte was prepared by triplicate in a concentration range from 2 to 10 $\mu g\,mL^{-1}$ by dilution of known amounts of the phenolic acids stock solutions in 1-propanol. For computing figures of merit, the peak areas of the phenolic compounds predicted by MCR-ALS were plotted against the nominal concentrations and the lines were fitted by a least-squares method. Then, the figures of merit were calculated according to Saurina et al. [45].

For model validation, 10 standard mixtures were randomly prepared in concentrations between 3 and 9 $\mu g\,mL^{-1}$ (Table 2) in 1-propanol, taking into account the concentration levels of these compounds in olive oil [46]. To perform the MCR resolution, a **D** augmented matrix was built by appending the second order data gathered for these 10 mixtures together with those for the calibration samples (see Section 4.3 for more details). Then, the nominal concentrations of each analyte were compared with those founded taking into account the areas retrieved by MCR-ALS and the pseudounivariate external standard calibration plots.

3.6. Virgin olive oil analysis

VOO samples (5 g) were accurately weighed in a centrifuge tube and extracted with 1.00 mL of ethanol stirring for 2 min in an ultrasonic bath [47]. Later, the samples were centrifuged and, after the separation of the phases, the ethanolic phase was directly injected in the capillary. The electrophoretic analysis was carried out using the conditions described in Section 3.3.

To perform the determination of the phenolic compounds by the standard addition calibration method coupled to MCR-ALS, second order data for VOO samples (5 g) spiked with variable and growing concentrations of phenolic acids by triplicate were registered. In this case, the **D** augmented matrix was composed of the VOO sample and its additions appended with the calibration data (see Section 4.4 for more details). The standard addition calibration plots were built by representing the peak areas retrieved by MCR-ALS versus the added concentration of each compound, in $\mu g\,g^{-1}$ olive oil. The recovery tests were carried out using the same data than in

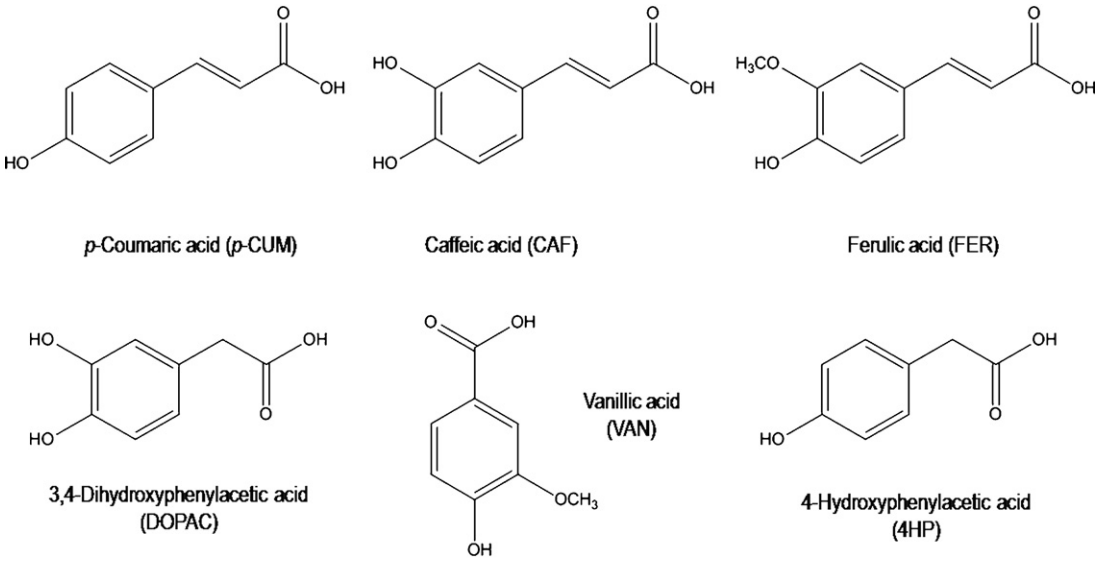

Fig. 1. Chemical structures of the studied phenolic acids.

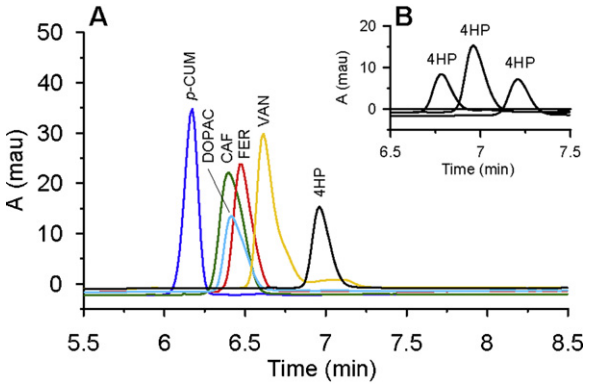

Fig. 2. Electropherograms for 20 μg mL^{-1} solutions of the phenolic acids in 1-propanol (A) and different 4HP standards of 10 and 20 μg mL^{-1} (B). BGE: 18 mM KOH, 25 mM boric acid in a 1-propanol:methanol 74:26 v:v medium.

the standard addition method, but subtracting the signal of found phenolic acids in the olive oil to the rest of signals.

4. Results and discussion

4.1. General considerations

As mentioned in Section 1, the main objective of the present work is to develop a chemometric method in combination with the NACE technique for the resolution and determination of several phenolic acids from a VOO sample in a short period of time and without being necessary a complex experimental work. In this sense, our research was performed in two steps. Firstly, the prediction ability of the selected algorithm according to the analytical problem under consideration was studied by applying MCR-ALS to resolve and quantitate ten standard mixtures of the six studied phenolic acids (Fig. 1) randomly prepared at different concentrations. Then, the application of the method for the resolution and determination of these compounds in a real VOO sample was carried out. The electrophoretic separation and detection were performed using the conditions described in Section 3.3.

4.2. Electropherographic analysis

In Fig. 2 the electropherograms corresponding to different standards of each analyte are shown. It can be seen an important overlap between the different peaks and, in addition, peak shifts occur between different runs. Some of them, as in the case of 4HP, suffer a marked shift (Fig. 2B).

The high complexity of the analytical problem under study can also be seen in Fig. 3, which shows two contour plots of the complete landscape of absorbance as a function of wavelength and migration time for a calibration sample composed of 20 μg mL^{-1} of the six phenolic acids (Fig. 3A), and for an ethanolic extract of a VOO sample spiked with them at the same concentrations (Fig. 3B).

In the present work, MCR-ALS was chosen for data processing because this algorithm achieves the second-order advantage without requiring that the electropherograms remain invariant between different runs for each analyte.

4.3. Implementation of NACE–MCR-ALS to resolve standard mixtures of phenolic acids

MCR-ALS is able to handle data sets deviating from trilinearity, like the NACE data herein analyzed. Therefore and to exploit this advantage, the electropherographic data was augmented in the temporal mode, namely spectra remain invariant while time

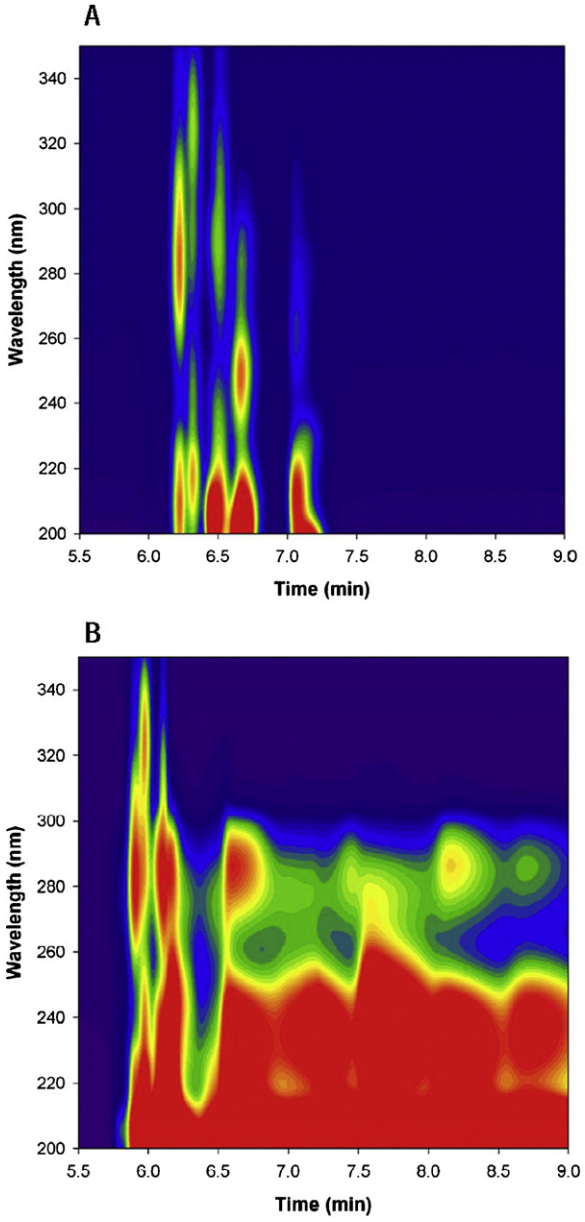

Fig. 3. Two dimensional contour plots for a 20 μg mL^{-1} standard of the six phenolic acids (A) and an ethanolic extract of a VOO sample spiked with the six analytes at the same concentrations (B).

profiles may change from sample to sample. In this way, a matrix-to-matrix variation of profiles occurs along the column direction and thus, a column-wise augmented matrix was created (see Section 2.2).

It is important to point out that the performance of the resolution strongly depends on the knowledge of the global and local properties of the data set, particularly on those related to the mathematical and chemical rank [48]. When applying SVD to determine the correct number of compounds in the model validation matrices, it was not capable of detecting the right number of contributing components which explain the variance of the system. This fact can be ascribed to some similarities in the species spectra but especially because of some identical migration times, i.e. such as in the case of DOPAC and FER (see Fig. 2), leading to rank-deficient matrices. This problem was overcome by resorting to the strategy of matrix augmentation [34,49], i.e. to append matrices of pure analyte standards, in this case the calibration data, to the rank deficient matrices

Table 3
Analytical figures of merit [45].

Analyte	Lineal range ($\mu g\,mL^{-1}$)	Intercept ± SD[a]	Slope ± SD[a] ($mL\,\mu g^{-1}$)	Determination coefficient (r^2)	% linearity	(Analytical sensitivity, γ)$^{-1}$ ($\mu g\,mL^{-1}$)	LOD[b] ($\mu g\,mL^{-1}$)	LOQ[c] ($\mu g\,mL^{-1}$)
p-CUM	2.20–11.0	−30 ± 15	105 ± 2	0.998	98	0.2	0.4	1
CAF	2.12–10.6	−34 ± 25	89 ± 3	0.990	96	0.4	0.8	2
FER	2.02–10.1	178 ± 12	53 ± 2	0.995	96	0.3	0.8	2
DOPAC	2.32–11.6	−43 ± 11	33 ± 1	0.989	96	0.4	0.9	3
VAN	2.20–11.0	−40 ± 11	68 ± 2	0.997	97	0.2	0.4	1
4HP	2.06–10.3	−40 ± 16	52 ± 3	0.986	95	0.4	0.9	3

[a] SD, standard deviation.
[b] LOD, limit of detection.
[c] LOQ, limit of quantitation, calculated as (10/3)LOD.

under study. This strategy, combined with the inclusion of information about the correspondence among species in each submatrix, leads to the successful MCR resolution.

The external standard calibration plots were built between 2 and 10 $\mu g\,mL^{-1}$ of each phenolic acid in 1-propanol. In addition, for model validation, a set of 10 samples was randomly prepared (Table 2). All these solutions were injected in the capillary and, the obtained data were then modeled by MCR-ALS. For this, a column-wise augmented **D** data matrix was built by placing on top of each other all the calibration and validation data matrices, without performing a region selection. As they were available, the real spectra of the phenolic acids were provided to be used as initial estimations.

Decomposition was performed by imposing the restrictions of non-negativity in spectral profiles and unimodality and non-negativity in concentration profiles. Besides, successful MCR-ALS was also aided by the inclusion of information about the correspondence among species in each matrix (i.e. information as to whether a given component exists or not in a given sample) [48].

The analytical figures of merit, calculated making used of the pseudounivariate calibration curves established with the relative areas extracted for the calibration samples as described in Section 3.5 are shown in Table 3. These pseudounivariate calibration curves allowed us to predict the concentrations of the analytes in the model validation samples (Table 2). The recovery values as well as the relative errors of prediction are satisfactory taking into account the complexity of the data, i.e. very overlapped peaks with marked time shift between runs (Fig. 2).

4.4. Application of NACE–MCR-ALS to the analysis of virgin olive oil

The proposed method was then applied to the resolution and quantitation of the six phenolic acids in a VOO sample. The determination of these compounds was experimentally carried out by using a simple LLE with ethanol (Section 3.6). The ethanolic extract was directly injected in the capillary and the separation was performed in the optimized electrophoretic conditions (Section 3.3). Despite the simplicity of the experimental work, the standard addition calibration method was necessary, since it is not possible to accurately know the final volume of the extract, and subsequently the amount of the analytes in it. Thus, for the experimental work, aliquots of 5 g of a VOO were spiked by triplicate with increasing concentrations of the phenolic acids, extracted with ethanol and directly injected in the capillary. The detection was performed with the DAD by recording the spectral data between 200 and 400 nm in a time not higher than 12 min.

The importance of the matrix influence is reflected in Fig. 3B, where several unknown substances appear in the olive oil matrix, making necessary to model the data with a second order algorithm capable of exploiting the second order advantage, like MCR-ALS. In addition, a considerable baseline drift is present in these samples (Fig. 4) and also in the standards (data not shown). Therefore a

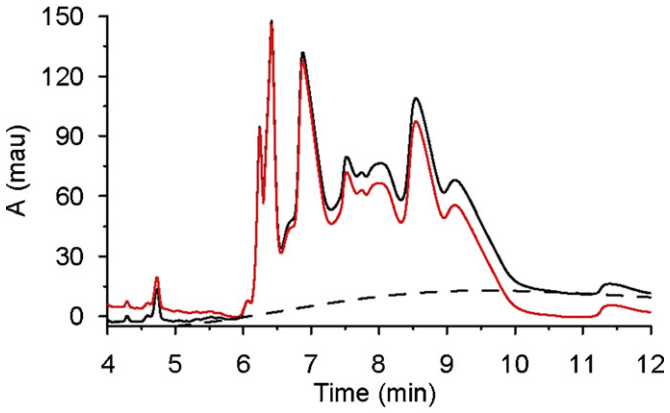

Fig. 4. Electropherogram (at 220 nm) of an ethanolic extract of a VOO sample spiked with 25 $\mu g\,mL^{-1}$ of the six analytes (black solid line), the baseline calculated at the same wavelength (black dashed line), and the corrected electropherogram, by subtraction of the baseline to the original electropherogram (red solid line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

baseline correction both for standards and VOO samples was performed (Section 2.1). The original electropherogram, the computed baseline, and the corrected electropherogram for an ethanolic extract of a VOO sample spiked with the phenolic acids (25 $\mu g\,mL^{-1}$ of all of them) are shown in Fig. 4.

In the resolution of the phenolic acids from VOO samples not only the great influence of the olive matrix, but also the peaks shift, which also appears in the samples, difficult the analytical problem. It can be appreciated in Fig. 5 the electropherograms for the VOO sample spiked with the phenolic acids at increasing concentration levels. In Fig. 5A, where the original electropherograms are shown, the peak shifts can be observed and in Fig. 5B, the region in which the analytes migrate has been expanded to show a better sight of the problem under consideration. In order to simplify the resolution of the sample, due to the great interference of the olive oil matrix, the total electropherographic data was divided in different regions: region 1 to resolve *p*-CUM and CAF, region 2 for FER, region 3 for DOPAC, region 4 for VAN and, finally region 5 to resolve 4HP. The regions of temporal sensors in which the total electropherographic data was split in order to build individual MCR-ALS models can be seen in Table 4. However, previously to the regions establishment, a manual alignment of the samples was applied in order to simplify the MCR-ALS resolution. The final alignment of the sample electropherograms can be seen in Fig. 5B, as well as the selected regions.

Once the data was obtained and treated as it has been mentioned, column-wise augmented **D** data matrices were built to resolve the analytes by MCR-ALS. Taking into consideration the rank deficiency of the real VOO sample data, the resolution was also conducted appending the calibration data to the real sample data. To build the initial estimations, the spectra of the interferences

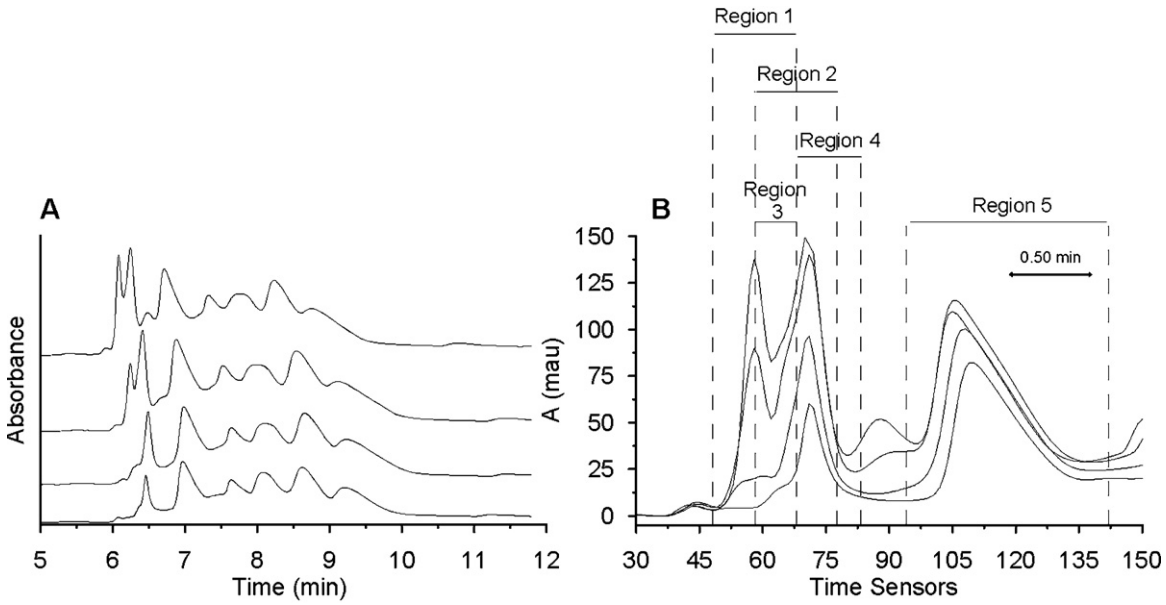

Fig. 5. (A) Original electropherograms of a VOO sample and it spiked with increasing concentration levels of the phenolic acids (220 nm). (B) Expanded and aligned electropherograms of the different additions to the VOO sample. Regions in which the total electropherographic data were divided in order to simplify the analysis: region 1 (*p*-CUM and CAF), region 2 (FER), region 3 (DOPAC), region 4 (VAN) and region 5 (4HP).

Table 4
Regions in which the total electropherographic VOO sample data was split in order to build individual MCR-ALS models after the manual alignment and MCR-ALS necessary factors.

Region	Analytes	Time sensors	Spectral region (nm)	MCR-ALS factors
1	*p*-CUM/CAF	48–68	220–400	8
2	FER	58–78	220–400	10
3	DOPAC	58–68	220–400	9
4	VAN	68–83	220–400	12
5	4HB	94–142	220–400	15

were obtained from the analysis of the purest spectra based on the SIMPLISMA methodology [42] applied to the VOO matrix without phenolic acids additions, and combined with the known spectra of the pure analytes. Then, each one of the established regions (Fig. 5B) was successively resolved for the VOO sample and its corresponding phenolic acids additions by imposing the restrictions of non-negativity in spectral profiles, unimodality and no-negativity in concentration profiles and correspondence among species in each matrix. The latter restriction, which denotes the presence or absence of species in each appended submatrix, was implemented taking into account the unexpected components present in each of the 5 regions. Table 4 includes the number of factors needed to perform the resolution of each analyte in its region, which evidences the great complexity of the sample matrix, considering that it was perform in the presence of 6–14 non calibrated interferences. As an example of how each sample was analyzed, the extracted time profiles of region 4 are shown in Fig. 6. In this region, which corresponds to the analyte VAN, twelve components were necessary to model the system. As can be seen in the four sub-figures, several profiles were extracted in the VOO sample, showing a severe overlapping between them. The decomposition of the data into the relevant contributions, by using the MCR-ALS algorithm, allows using the areas under the extracted temporal profiles for quantitative purposes. Thus, the isolation of the areas corresponding to VAN for this region (black solid line), in each studied sample, can be used for accurate analyte quantitation. In Fig. 7 the normalized real spectrum of VAN and those extracted by MCR-ALS for VAN and all the components of region 4 are shown. As can be seen, there is a reasonable agreement between the real VAN spectrum and the profile extracted by MCR (correlation coefficient equal to 0.999). It is relevant to mention that a good concordance is obtained for all the studied analytes, i.e. correlation coefficients equal to 0.999,

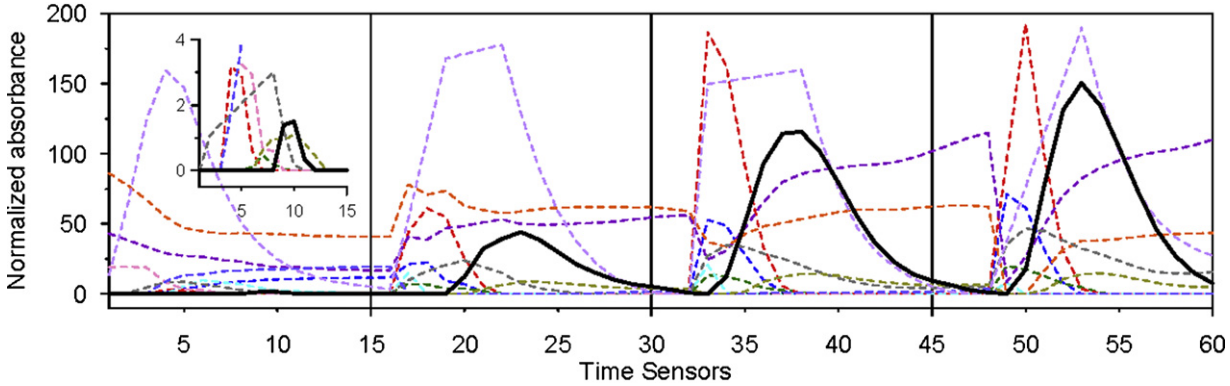

Fig. 6. Electropherograms retrieved by MCR-ALS processing of a VOO sample and successive additions of the phenolic acids. The profiles correspond to VAN (black solid line) and different components (dashed lines).

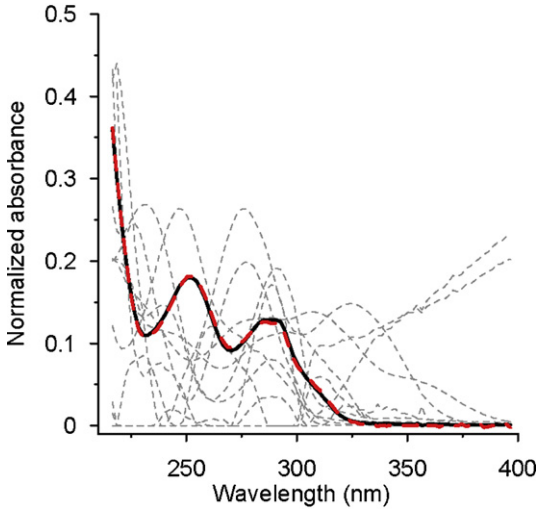

Fig. 7. MCR-ALS spectral profiles for region 4: predicted VAN spectrum (black solid line), real VAN spectrum (red dashed line) and different components spectra (gray dashed lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 5
MCR-ALS predictions of VOO spiked with the analytes.

Analyte	Nominal concentration[a]	Found[a]	Recovery[a]	CV[a]
p-CUM	1.09	1.14	104.6	7.0
CAF	1.04	1.01	97.1	11.6
FER	1.04	0.92	88.5	5.3
DOPAC	1.08	1.15	106.5	14.7
VAN	1.17	1.22	104.3	14.7
4HP	1.10	1.11	100.9	8.2

[a] Concentrations are given in $\mu g\,g^{-1}$ and recoveries and coefficients of variations (CV) are given in percentage.

0.994, 0.995, 0.996 and 0.998 for *p*-CUM, CAF, FER, DOPAC and 4HP, respectively.

Pseudounivariate standard addition calibration curves, by triplicate, allowed us to predict the concentrations of the analytes in the VOO sample. Recovery assays were also carried out by comparing the predicted concentration with the nominal concentration of the first spiked VOO sample, by subtracting the signal of the found phenolic acids in the olive oil to the rest of signals. *p*-CUM and CAF were found to be no detectable, FER and VAN no quantifiable and 0.262 (±0.080) and 1.13 (±0.22) $\mu g\,g^{-1}$ olive oil were the concentrations (±standard deviations in parentheses) found for DOPAC and 4HP, respectively. Predictions for the six analytes, in the first spiked VOO sample are displayed in Table 5, together with recoveries which were computed taking into account the nominal concentrations spiked in the VOO sample, and coefficients of variations for the triplicates. It is important to note that predictions, in most of the samples, may be considered satisfactory, taking into consideration the complexity of the analytical problem. Regarding the found concentration of the phenolic acids, they are in the order expected for the VOO samples, according to previous reports by other authors [46], and taking into account that the phenolic content in olive oil is influenced by different factors, such as the olive variety, location, environmental conditions or degree of ripeness, as well as agronomic and technological aspects of production [6].

Finally, it is important to highlight the advantages of the present method, not only in the good obtained results, but also in the time reduction achieved. In first place, the combination of the NACE technique with MCR-ALS algorithm allows performing the resolution of these compounds by simplifying the previous sample pretreatment stages. On the other hand, the total electrophoretic run time is also reduced. In fact, it has been previously carried out the determination of phenolic compounds by NACE without chemometrics [47], and the total necessary time for a run was about 20–24 min, while if chemometrics is used a time not higher than 10–12 min is spent. In addition, the use of chemometrics has allowed to carry out the quantitation of the phenolic acids herein studied, which could not be determined by NACE without chemometrics, due to problems in the peaks resolution.

5. Conclusions

A non-aqueous capillary electrophoresis method, based on modeling diode array detection second-order data with the multivariate curve resolution alternating least squares algorithm, was presented for the simultaneous determination of six phenolic acids in virgin olive oil samples. It was shown that MCR-ALS is one of the most versatile algorithms available for the management of complex data without trilinearity. In addition, the non aqueous capillary electrophoresis technique allows the analysis of complex samples without being necessary an exhaustive pretreatment and, on the other hand, in comparison with the electrophoresis methods which do not use chemometrics, the NACE/MCR-ALS method herein proposed supposes less total time than the required to optimize the complete electrophoretic resolution of similar systems.

This is the first time that non-aqueous capillary electrophoresis data is combined with a second order algorithm in the food analysis field, in general, and for the resolution and quantitation of phenolic compounds in virgin olive oil, in particular. Most of the previous literature reports based in capillary electrophoresis for the analysis of this kind of compounds in olive oil require long analysis times, while in the present report good results can be achieved in less time due to the combination of the non aqueous capillary electrophoresis and the MCR-ALS algorithm.

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