Non-Trilinear Chromatographic Time Retention−Fluorescence Emission Data Coupled to Chemometric Algorithms for the Simultaneous Determination of 10 Polycyclic Aromatic Hydrocarbons in the Presence of Interferences

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Multivariate calibration coupled to high-performance liquid chromatography−fast scanning fluorescence spectroscopy (HPLC-FSFS) was employed for the analysis of 10 selected polycyclic aromatic hydrocarbons (PAHs), six of which correspond to heavy PAHs. The goal of the present study was the successful resolution of a system even in the presence of real interferences. Second-order HPLC-FSFS data matrices were obtained in a short time with a chromatographic system operating in isocratic mode. The difficulties in aligning chromatographic bands in complex systems, such as the ones presented here, are discussed. Two second-order calibration algorithms which do not require chromatographic alignment were selected for data processing, namely, multivariate curve resolution−alternating least-squares (MCR-ALS) and parallel factor analysis 2 (PARAFAC2). These algorithms did also achieve the second-order advantage, and therefore they were able to overcome the problem of the presence of unexpected interferences. The study was employed for the discussion of the scopes of the applied second-order chemometric tools, demonstrating the superiority of MCR-ALS to successfully resolve this complex system. The quality of the proposed techniques was assessed on the basis of the analytical recoveries from different types of water and olive oil samples after solid-phase extraction. The studied concentration ranges in water samples were 5.6×10^{-3}−0.20 ng mL^{-1} for heavy PAHs and 0.036−0.80 ng mL^{-1} for light PAHs, while in oil samples the PAHs concentrations were 0.13−9.6 and 2.3−49.5 ng mL^{-1} for heavy and light PAHS, respectively. All real samples were analyzed in the presence of the studied interferences.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous compounds produced by incomplete combustion of carbon-containing materials, such as wood, coal, municipal, and agricultural wastes and the operation of both diesel and gasoline engines.[1] Although PAH exposure in humans is associated with

different diseases, the toxic effect of most concern is cancer. Cancer is especially associated with those PAHs bearing more than four benzene rings in their structures, which are usually called heavy PAHs.[2,3] As a result of the serious consequences of the presence of PAHs in the environment, continuous efforts are devoted to find sensitive and selective methods for PAH residue quantification in natural samples.

High-performance liquid chromatography (HPLC) has been profusely applied to PAH determination.[4,5] Because of the similar chromatographic retention properties of some of these compounds, in certain complex samples it is difficult to achieve their complete separation, even using a solvent gradient mode. In such situations, multivariate data analysis can be used for improving selectivity by mathematical means.

Recently, Ortiz and Sarabia have reviewed quantitative determinations of different analytes of interest using chromatographic analysis and N-way calibration strategies.[6] As described in this latter review, high-order data can be easily obtained coupling the chromatographic systems to either an ultraviolet-diode-array detector (UV-DAD) or a fluorescence detector. The latter is a better choice when high sensitivity is required, as in the case of analyzing PAHs, since their admissible concentration levels in environmental samples are extremely low. Specifically, fast-scanning fluorescence spectrometry (FSFS) has been shown to be a very useful tool for the analysis of complex systems. As regards the analysis of PAHs by HPLC-FSFS multivariate data, a few literature works have been reported. In 1981 Appellof et al.[7] developed a qualitative HPLC analysis of different synthetic mixtures of perylene, fluoranthene, tetracene, and 9,10-dimethyl-anthracene and real mixtures of benzo[a]pyrene, benzo[e]pyrene, and 9-methylanthracene using a videofluorimeter as detector. The research group of Guiteras et al. quantified 14 PAHs in water

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samples coupling HPLC-FSFS to the partial-least-squares (PLS) algorithm.[8] Subsequently, the same group described the application of N-dimensional partial least-squares (N-PLS) and parallel factor analysis (PARAFAC) to the determination of the same compounds from three-dimensional chromatograms obtained by HPLC-FSFS.[9] In these reports, however, the presence of potential interferences was not considered in the analysis. Some second-order methods allow the determination of calibrated analytes in the presence of other uncalibrated components which can be present in real samples. This useful property is named "second-order advantage" and avoids the requirement of either interference removal, as in zeroth-order calibration, or the construction of a large and diverse calibration set as in first-order calibration.[10−13]

The present work describes the simultaneous determination of fluoranthene (FLT), pyrene (PYR), chrysene (CHR), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenz[a,h]anthracene (DBA), indeno[1,2,3-cd]pyrene (IcP), and benzo[g,h,i]perylene (BgP), using HPLC-FSFS under isocratic conditions, which notably reduces the analysis time. With the exception of FLT, the selected PAHs bear four or more aromatic rings in their structures, and most of them are considered of concern by agencies related with the human health like the International Agency for Research on Cancer (IARC)[14] and the United States Environmental Protection Agency (US EPA).[15]

Two fundamental issues distinguish this work from those previously published regarding the determination of PAHs using HPLC-FSFS: (1) the analyses are performed in the presence of two additional heavy PAHs used as interferences [benzo[e]pyrene (BeP) and benzo[j]fluoranthene (BjF)], and (2) the determinations are carried out applying chemometric algorithms which do not require a given component to show the same chromatographic profile in each experimental run. This second point is related to the problems which arise during chromatographic alignment in the presence of interferences and also when both the magnitude and sign of the observed retention time shifts are analyte-specific.

The selected second-order calibration methods which do not require trilinearity and simultaneously achieve the second-order advantage were multivariate curve resolution-alternating least-squares (MCR-ALS)[16] and PARAFAC2.[17] Both procedures were compared in terms of their abilities of rendering reliable results working either with the complete data matrices or evaluating selected chromatographic regions. All models were used for the analysis of PAHs in spiked water and olive oil samples.

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THEORY

Both the MCR-ALS and the PARAFAC2 models have been discussed in detail and thus only a brief description is presented here, suitable when the second-order advantage is to be achieved.

MCR-ALS. In this second-order multivariate method, an augmented data matrix is created from each test data matrix and the calibration data matrices. If all matrices are of size $J \times K$, where J is the number of data points in the dimension of the retention times and K the number of fluorescence wavelengths, the direction of columns is considered the time direction and the direction of rows the spectral direction. Augmentation can be performed in either direction, depending on the type of experiment being analyzed and also on the presence of severe overlapping in a given data mode.[18,19] In the presently studied case, the augmentation was implemented in the time direction, because of the presence of retention time shifts between different chromatographic runs.

In the time augmentation mode, the bilinear decomposition of the augmented matrix is performed according to the expression:

$$\mathbf{D} = \mathbf{GS}^{T} + \mathbf{E} \qquad (1)$$

where the rows of $\mathbf{D}$ contain the spectra measured for different samples at several times, the columns of $\mathbf{G}$ contain the time retention profiles of the intervening species in all experiments which are analyzed together and the columns of $\mathbf{S}$ their related spectra, and $\mathbf{E}$ is a matrix of residuals not fitted by the model. The sizes of these matrices are $\mathbf{D}$, $J(I_{cal} + 1) \times K$; $\mathbf{G}$, $J(I_{cal} + 1) \times N$; $\mathbf{S}$, $K \times N$; $\mathbf{E}$, $J(I_{cal} + 1) \times K$ (N is the number of responsive components). As can be seen, $\mathbf{D}$ contains data for the I_{cal} calibration samples and for a given test sample.

Decomposition of $\mathbf{D}$ is achieved by iterative least-squares minimization of the Frobenius norm of $\mathbf{E}$. The minimization is started by supplying estimated spectra for the various components, which are employed to estimate $\hat{\mathbf{G}}$ (with the "hat" implying an estimated matrix) from eq 1:

$$\hat{\mathbf{G}} = \mathbf{D}(\mathbf{S}^{T})^{+} \qquad (2)$$

where the superscript "+" indicates the generalized inverse. With matrix $\hat{\mathbf{G}}$ from eq 2 and the original data matrix $\mathbf{D}$, the spectral matrix $\mathbf{S}$ is re-estimated by least-squares:

$$\hat{\mathbf{S}} = \mathbf{D}^{T}(\hat{\mathbf{G}}^{+})^{T} \qquad (3)$$

and finally $\mathbf{E}$ is calculated from eq 1 using $\mathbf{D}$ and the estimated $\hat{\mathbf{G}}$ and $\hat{\mathbf{S}}$ matrices. These steps are repeated until convergence, under suitable constraining conditions during the ALS process, in our case, non-negativity in spectral and time profiles and unimodality in the time profiles (except for the background signal). MCR-ALS requires the number of components to be set, while it is recommended and safer to initialize the system with parameters as close as possible to the final results. The number of components can be estimated using principal component analysis on the basis of singular value decomposition of the $\mathbf{D}$

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matrix.[20,21] Finally, the species spectra can be obtained from either pure analyte standards or from the analysis of the so-called "purest" spectra, based on the SIMPLISMA (simple interactive self-modeling mixture analysis) methodology, a multivariate curve resolution algorithm which extracts the purest spectra of the mixture from a series of spectra of mixtures of varying composition.[22] In our case, the best strategy was to supply known pure profiles for the calibrated analytes and positive random numbers for the potential interferences (due to severe spectral overlapping this was preferable to SIMPLISMA profiles).

After MCR-ALS decomposition of $\mathbf{D}$, concentration information contained in $\mathbf{G}$ can be used for quantitative predictions, by first defining the analyte concentration score as the area under the profile for the ith sample:

$$a(i, n) = \sum_{j=1+(i-1)J}^{iJ} G(j, n) \tag{4}$$

where $a(i,n)$ is the score for the component n in the sample i. The scores are employed to build a pseudo-univariate calibration graph against the analyte concentrations, predicting the concentration in the test samples by interpolation of the test sample score.

PARAFAC2. This model is a development of the original PARAFAC1 model, which aims at handling shifted, or more generally, varying profiles in a more efficient manner than PARAFAC1.[23,24] If a three-way data set generated by fluorescence detected chromatographic data has an ideal trilinear structure, which results from a constant retention time for each analyte, the matrix formulation of the corresponding PARAFAC1 model can be expressed as

$$\mathbf{X}_i = \mathbf{B}\mathbf{Y}_i\mathbf{C}^T + \mathbf{E}_i \tag{5}$$

where $\mathbf{X}_i$ is the ith frontal slab of the three-way array and contains the fluorescence values at each retention time (columns) and at each emission wavelength (rows) for the ith sample, $\mathbf{B}$ and $\mathbf{C}$ are the temporal and spectral loading matrices, respectively, for the N components, $\mathbf{Y}_i$ is a diagonal matrix holding the concentrations (scores) of the N analytes in sample i in its diagonal, and $\mathbf{E}_i$ is a residual matrix. Thus, in the PARAFAC1 model, the equation to be fitted is

$$\sigma(\mathbf{B},\mathbf{C},\mathbf{Y}_1, ..., \mathbf{Y}_i) = \sum_{i=1}^{I} \left\| \mathbf{X}_i - \mathbf{B}\mathbf{Y}_i\mathbf{C}^T \right\|^2 \tag{6}$$

where σ represents the fitting error.

However, in real chromatographic systems analyte retention time shifts between different runs, which can be regarded as a violation of the assumption of parallel proportional profiles underlying the PARAFAC1 model. The PARAFAC2 model relaxes the strict trilinearity by allowing profiles to be estimated in one mode for each occasion in the other mode. The matrix formulation of the PARAFAC2 model is

$$\mathbf{X}_i = \mathbf{B}_i\mathbf{Y}_i\mathbf{C}^T + \mathbf{E}_i \tag{7}$$

where $\mathbf{B}_i$ is the matrix holding the elution profiles of the analytes present in sample i and the proposed function to minimize is

$$\sigma(\mathbf{B}_i, \mathbf{C}, \mathbf{Y}_1, ..., \mathbf{Y}_i) = \sum_{i=1}^{I} \left\| \mathbf{X}_i - \mathbf{B}_i\mathbf{Y}_i\mathbf{C}^T \right\|^2 \tag{8}$$

Initialization is usually performed with the best profiles obtained after 10 runs of maximally 80 iterations. It is a rather slow method but is needed to avoid local minima. Regarding algorithmic restrictions, non-negativity was applied in both the concentration and spectral modes of the three-way data array, which allows physically interpretable results to be obtained. However, restrictions cannot be imposed in the retention time direction when modeling varying chromatographic profiles from sample to sample. This is in contrast to PARAFAC1, where all three modes can be restricted to be non-negative but cannot be applied to nontrilinear data sets. It is also in contrast to MCR-ALS, in which both spectral and time retention dimensions can be restricted. As will be shown below, this may be the cause of the better performance of MCR-ALS in the presently studied case.

As with MCR-ALS, analyte quantitation is performed in PARAFAC2 by first building a pseudounivariate calibration line with the analyte scores in the calibration samples and then interpolating the analyte score in the test sample.

Software. The routines employed for MCR-ALS and PARAFAC2 are written in MATLAB and are available on the Internet (refs 25 and 26, respectively).

EXPERIMENTAL SECTION

Reagents and Solutions. Fluoranthene, pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[a,h]anthracene, indeno[1,2,3-cd]pyrene, benzo[g,h,i]perylene, and benzo[e]pyrene were purchased from Aldrich (Milwaukee, WI). Benzo[j]fluoranthene was obtained from Fluka (Buchs, Switzerland). Methanol, acetonitrile, dichloromethane, and hexane were obtained from Merck (Darmstadt, Germany). All reagents were of high-purity grade and used as received.

Stock solutions of all PAHs of about 1000 μg mL^{-1} were prepared in acetonotrile. From these solutions, more diluted acetonitrile solutions (ranging from 1.00×10^{-3} to 10.0 μg mL^{-1}) were obtained. Working solutions were prepared immediately before their use by taking appropriate aliquots of solutions and diluting with acetonitrile and water (85:15 v/v) to the desired concentrations.

The PAHs were handled with extreme caution, using gloves and protective clothing.

Apparatus. HPLC was carried out on a liquid chromatograph equipped with a Waters (Milford, MA) 515 HPLC pump and a

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Varian Cary-Eclipse luminescence spectrometer (Varian, Mulgrave, Australia) as detector. A 200 μL loop was employed to introduce each sample onto a Zorbax SB C18 column (5 μm average particle size, 150 mm × 4.6 mm i.d.). The data matrices were collected with the excitation wavelength fixed at 300 nm, using emission wavelengths from 340 to 580 nm each 2 nm and times from 0 to 7.2 min each 2.7 s. The excitation and emission slit widths were 10 nm and photomultiplier sensitivity was 800 V. The emission-time matrices were the of size 121 × 161 and were saved in ASCII format and transferred to a PC based on AMD Sempron 2800 for subsequent manipulation.

HPLC Procedure. The mobile phase was the same mixture of acetonitrile and water (85:15 v/v) used to prepare the samples. The flow rate was maintained at 1.5 mL min^{-1}. Each chromatographic determination, performed under isocratic conditions, was accomplished in less than 7 min.

Calibration, Validation, and Test Samples. The experimental procedure corresponding to the three-way analysis was developed preparing a calibration set of 18 samples. Sixteen of these samples corresponded to the concentrations provided by a semifactorial design at two levels. The remaining two samples corresponded to a blank solution and to a solution containing all the studied PAHs at an average concentration. The tested concentrations were in the ranges 0.0–500 ng mL^{-1} for FLT and PYR, 0.0–300 ng mL^{-1} for CHR, 0.0–100 ng mL^{-1} for BaA, BbF, and IcP, 0.0–50 ng mL^{-1} for BaP, DBA, and BgP, and 0.0–20.0 ng mL^{-1} for BkF. These ranges were established on the basis of the analysis of the linear fluorescence-concentration range for each analyte. A validation test set was prepared employing concentrations different than those used for calibration and following a random design.

Calibration and validation samples were prepared by measuring appropriate aliquots of standard solutions, placing them in 10.00 mL volumetric flasks to obtain the desired concentrations, and completing to the mark with mobile phase.

As will be demonstrated below, both BeP and BjF have signals significantly overlapped with some of the studied compounds. Therefore, with the purpose of evaluating the proposed strategies in the presence of these two interferences, 30 additional test samples containing random concentrations of the 10 studied PAHs and either one or both interferences were prepared. The concentrations of BeP and BjF in these latter samples were in the ranges 50–1000 ng mL^{-1} and 40–600 ng mL^{-1} for BeP and BjF, respectively.

Water Sample Procedure. Tap, mineral, and underground water samples were prepared by spiking each sample with standard solutions of the studied PAHs, obtaining concentration levels in the range 5.6×10^{-3}–0.20 ng mL^{-1} for heavy PAHs and 0.036–0.80 ng mL^{-1} for light PAHs. These values were selected on the basis of the levels which can be found in different natural waters.[27] In addition, the interferences BeP and BjF were incorporated to these samples at concentrations between 0.15 and 1.0 ng mL^{-1}. These water samples were prepared in duplicate and underwent no previous treatment. Because of the low investigated concentrations of analytes, solid-phase extraction (SPE) had to be applied before the chromatographic determination. The SPE procedure was carried out using SPEC (solid phase extraction concentrator) octadecyl (C18AR) membranes (Ansys Diagnostics, Lake Forest, CA, U.S.A.). Prior to the extraction of 250 mL of the sample, the membrane was conditioned with 2 mL of methanol. The retained PAHs were eluted with hexane, and this solvent was evaporated with a nitrogen stream. Then, the solutions were reconstituted with 0.500 mL of a mixture of acetonitrile and water (85:15 v/v) and subjected to the same chromatographic analysis as the validation samples. In this way, the preconcentration factor was 500.

Oil Sample Procedure. The absence of a legal limit for PAHs in edible oils has led some organizations to set up their own recommendations. Usually, limits of 5 and 25 ng g^{-1} for heavy and total PAHs contents, respectively, are accepted.[28] Taking into account the mean olive oil density value (0.918 g cm^{-3}), the maximum admissible concentration for total PAHs is about of 23 ng mL^{-1}. However, this value can be exceeded in samples coming from contaminated olive fruits. In our experiments, commercial samples of olive oil were spiked with stock solutions of the 10 studied PAHs to obtain concentrations below 50 ng mL^{-1}. The two analyzed interferences, BeP and BjF, were also added at final concentrations between 20 and 40 ng mL^{-1}. Solid-phase extraction was carried out following the method suggested by Moret and Conte.[29] Briefly, 5.00 mL of spiked olive oil were placed in a 10.00 mL volumetric flask and diluted with hexane to the mark. Then, this solution was loaded onto a SPE cartridge packed 5 g of silica phase (Supelco, Bellefonte, U.S.A.) previously washed with 20 mL of dichloromethane, dried by means of vacuum, and conditioned with 20 mL of hexane. PAHs were eluted with a mixture of hexane and dichloromethane 70:30 (v/v). The first 8 mL of eluate were discharged, and the next 8 mL fraction, containing the PAHs, was collected. The solvent of the collected fraction was evaporated with a nitrogen stream, and the residue was dissolved in 0.500 mL of mobile phase and injected into the HPLC apparatus. In this way, the preconcentration factor for the oil samples was 10.

RESULTS AND DISCUSSION

General Considerations. Figure 1A,B shows a three-dimensional and a contour plot, respectively, of the complete landscape of fluorescence intensity as a function of emission wavelength and retention time for a mixture of the 10 studied PAHs. As can be seen, three different regions are distinguished in the time axis: 0–2.5 min, 2.5–4.5 min, and 4.5–6 min. In the first region, the bands of FLT, PYR, CHR, and BaA (group I) are detected. The second one includes BbF, BkF, BaP, and DBA (group II), and the last region involves both the IcP and the BgP (group III) bands. It is clear in this figure that overlapping of different degrees occurs among the bands, and as will be discussed below, the situation becomes more serious if additional PAHs which may overlap with any of the peaks (Figure 1C) are also present. In this latter case, only second-order calibration using suitable algorithms can be applied for the quantitation of the analytes because of the need of achieving the second-order advantage.[10–13]

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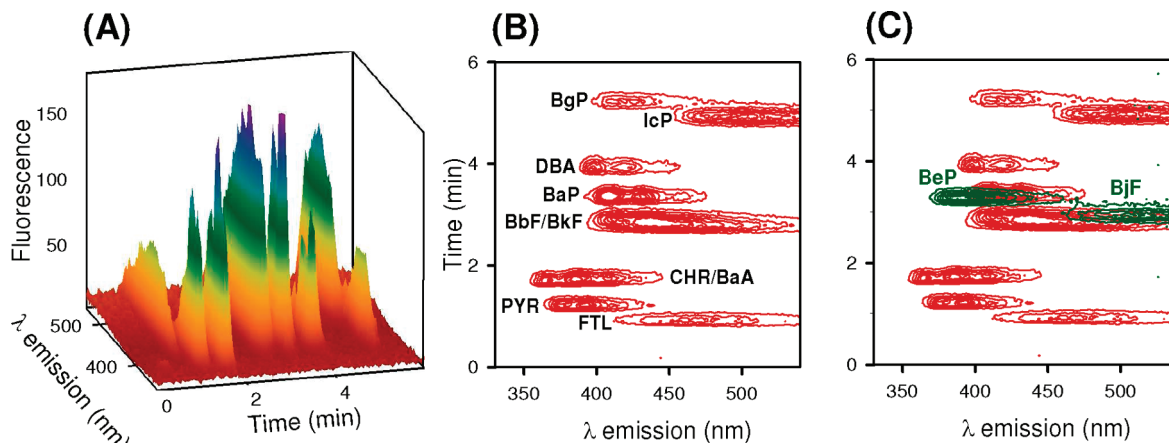(29) Moret, S.; Conte, L. *J. Sep. Sci.* **2002**, *25*, 96–100.

Figure 1. (A) Three-dimensional plot of a typical chromatogram of a sample containing the studied PAHs and (B) the corresponding two-dimensional contour plot. (C) Two-dimensional contour plot of the chromatogram of a sample containing the studied PAHs and BeP and BjF (green lines) as interferences. Concentrations are as follows (all in ng mL^{-1}): FLT, 500; PYR, 500; CHR, 300; BaA, 100; BbF, 100; BkF, 20; BaP, 50; DBA, 50; IcP, 100; BgP, 50; BeP, 300; BjF, 300.
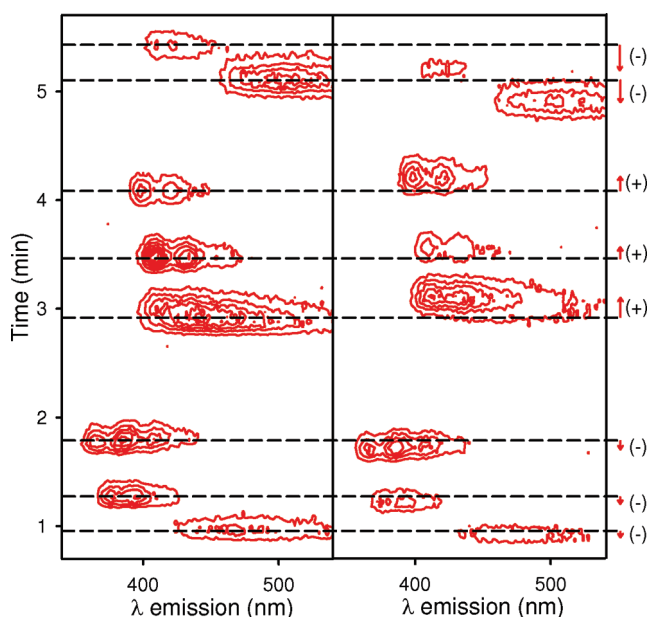

Figure 2. Contour plots in two different chromatographic runs. Dashed lines serve as guide for the eye. Arrows and signs indicate the magnitude and sense of the time-shift suffered by each peak.

Before building the chromatographic time-retention−fluorescence emission matrices to be chemometrically processed, certain particularities of these types of data must be taken into account, which are discussed in the following section.

Time Retention−Fluorescence Emission Data. There are two problems inherent to the obtainment of time retention−fluorescence emission second-order data: (1) the measurement of an emission spectrum of a moving sample, which makes the sample concentration variable during the spectrum acquisition, and (2) the lack of repeatability in the retention times between successive runs.

The first problem can be overcome using a fast-scanning detector, because spectra are obtained in a very short time. In our experimental conditions, each spectrum was recorded in 1.5 s, significantly lower than the base width of each chromatographic peak (about 30 s).

The second point, related with the retention time shift in different runs, is a serious limitation when second-order data are analyzed with algorithms which require that the data show the property of trilinearity. In addition, with increasing shifts the peak widths are also increased. If the shifts are not corrected, the program will consider these changes as modifications in chemical composition and incorrect results will be obtained.

As a result of this fact, several preprocessing methods have been applied to align the chromatographic bands and restore the trilinearity to the system.[30−32] This alignment process is relatively simple when few bands are involved and certain conditions are fulfilled. These conditions are that (1) both the magnitude and the sense (positive or negative) of the shift for all analytes should be the same with respect to a chromatographic run taken as a
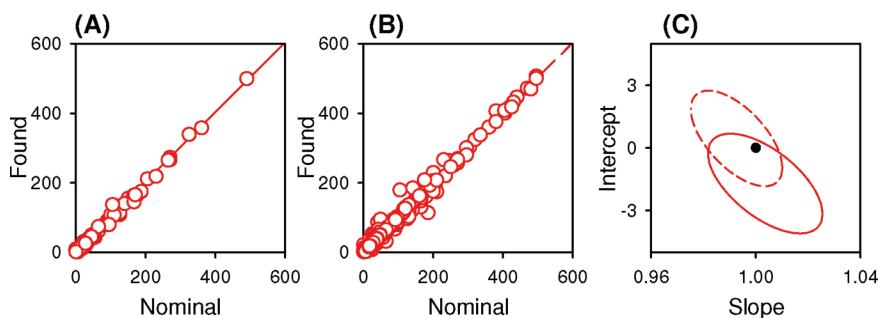
Figure 3. Plots for MCR-ALS predicted concentrations as a function of the nominal values using the entire chromatographic data matrices of the 10 studied PAHs in validation samples (A) and in the presence of BeP and/or BjF (B). (C) Elliptical joint regions (at 95% confidence level) for slope and intercept of the regression for validation (dark red solid line) and for test (dark red dashed line) samples. Black circle marks the theoretical (intercept = 0, slope = 1) point.

Figure 4. Profiles retrieved by MCR-ALS when processing samples without (validation) and with (test) interferences. (A) Spectral profiles. (B) Time profile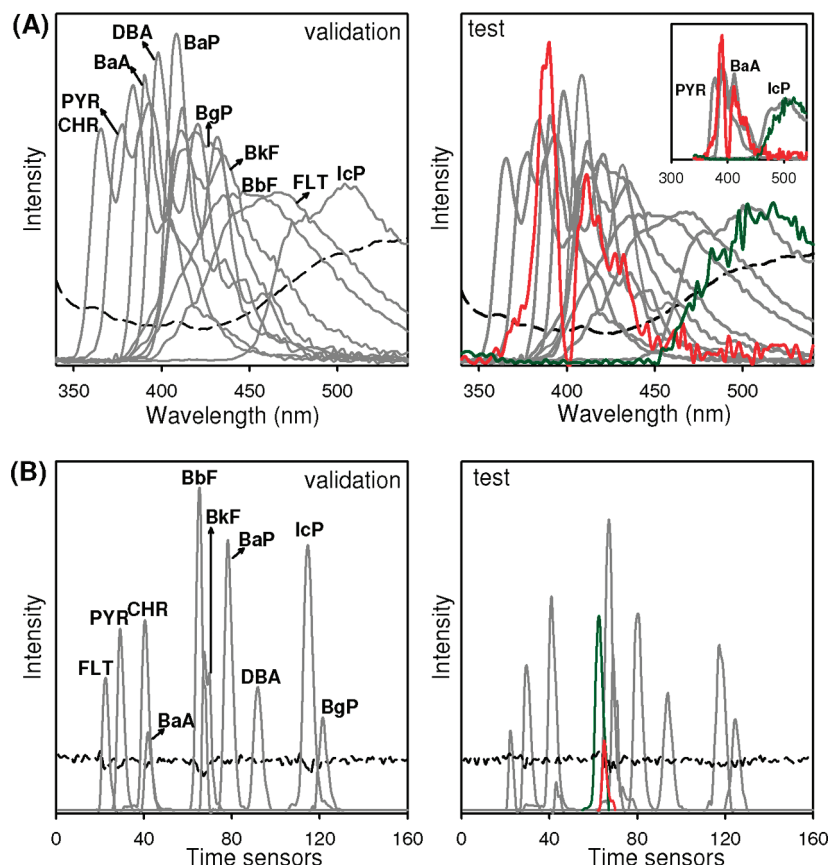s. In both cases, gray dashed, solid red, and solid green lines indicate the signals from background, BeP and BjF, respectively. The inset shows selected spectra implying a significant overlapping.

Table 1. MCR-ALS Statistical Results for the Studied PAHs in Validation Samples and in Samples with BeP and BjF as Interferences Using the Complete Matrix Data

	FLT	PYR	CHR	BaA	BbF	BkF	BaP	DBA	IcP	BgP
				Validation samples[a]						
RMSEP[b]	9	11	5	12	7	1	4	2	5	4
REP[c]	3	4	3	21	13	11	15	9	8	13
LOD[d]	57	42	19	20	10	2	2	2	3	6
				Samples with interferences[e]						
RMSEP[b]	18	34	7	16	8	3	2	2	5	3
REP[c]	6	12	4	28	14	29	7	7	9	11
LOD[d]	64	48	20	25	10	3	2	2	5	6

[a] Number of samples = 10. [b] RMSEP, root-mean-square error of prediction in ng mL^{-1}. [c] REP, relative error of prediction in %. [d] LOD, limit of detection in ng mL^{-1} and calculated according to ref 45. [e] Number of samples = 30.

reference and (2) unexpected compounds with signals strongly overlapped with the calibrated analytes are absent.[33]

Nielsen et al. developed an algorithm based on linear correlation optimized warping (COW), which can successfully resolve the alignment when the first condition is not fulfilled but, unfortunately, it is not able to produce a reliable alignment in the presence of interferences.[34,35] On the other hand, although an algorithm was proposed for the chromatographic alignment in the presence of unexpected compounds,[36,37] this is not able to solve the problem when the magnitude and sense of shift is different for each analyte.

The system here analyzed is complex and presents both of the above-mentioned problems. As an example, Figure 2 shows the chromatographic peaks for the studied PAHs in two runs (corresponding to different samples, hence, having different contours), where the dissimilar shifts can be appreciated. Furthermore, the presence of the interferences included in the present work must be considered. For these reasons, we decided to apply and compare MCR-ALS and PARAFAC2, two different chemometric algorithms which do not require chromatographic alignment.

It is important to note that N-PLS [or N-PLS coupled to residual bilinearization (RBL) when interferences are present], which does not require trilinearity to be strictly fulfilled, did not render good results. A similar result was observed in a previous work devoted to a chromatographic determination of fluoroquinolones,[38] where alignment of chromatographic bands was carried out before applying N-PLS to obtain reliable predictions for the investigated analytes using this latter methodology. This was attributed to the limited number of calibration samples employed for model building. Indeed, it is likely that the present calibration set, which involves 18 different samples, is not representative enough to model the very complex test samples here analyzed to successfully apply N-PLS/RBL.

Entire Chromatographic Data Matrices. Our first attempt was to quantify the 10 PAHs using the complete data matrices, thus involving the full range of retention times and the complete range of emission wavelengths. Preliminary studies carried out with PARAFAC2 showed that it was not possible to obtain reliable
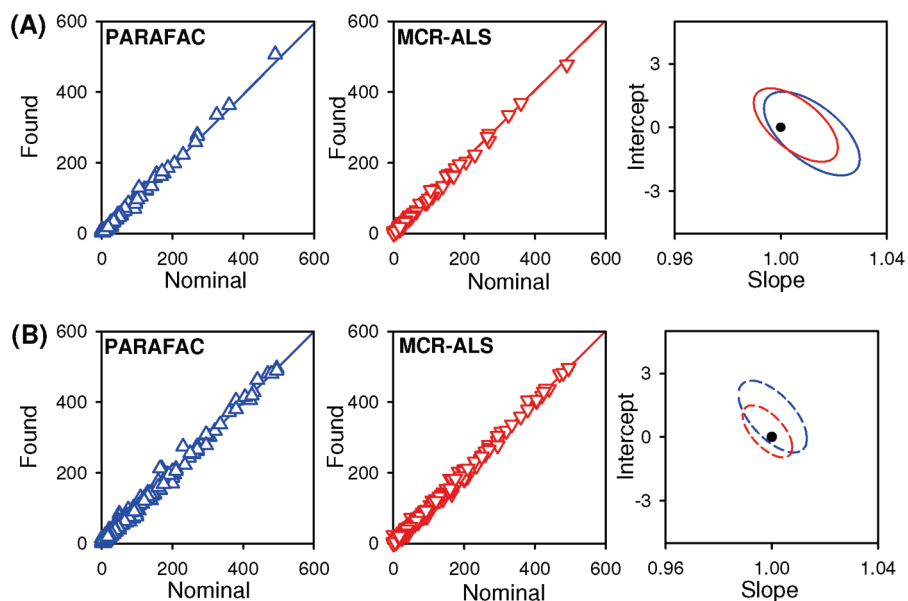
Figure 5. (A) Plots for PARAFAC2 (blue triangle up) and MCR-ALS (dark red triangle down) predicted concentrations as a function of the nominal values of the 10 studied PAHs in validation samples and elliptical joint regions (at 95% confidence level) for slope and intercept of the regression of PARAFAC2 (blue solid line) and MCR-ALS (dark red solid line) results. (B) Plots for the same algorithms applied to samples with BeP and/or BjF and elliptical joint regions (at 95% confidence level) for slope and intercept of the regression of PARAFAC2 (blue dashed line) and MCR-ALS (dark red dashed line) results. Black circles mark the theoretical (intercept = 0, slope = 1) point.
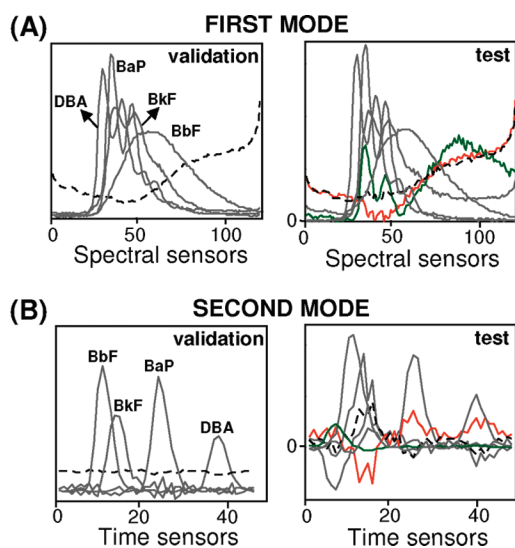

Figure 6. Profiles retrieved by PARAFAC2 for the validation and test sample slabs when processing group II PAHs. (A) Spectral profiles. (B) Time profiles. In both cases, black dashed, solid red, and solid green lines indicate the signals from background, BeP, and BjF, respectively.

results working with the whole chromatogram. This fact represents a limitation of PARAFAC2.

On the other hand, the experiments performed with MCR-ALS, with matrix augmentation in the temporal direction, showed

Table 2. Statistical Results for the Studied PAHs in Validation Samples and in Samples with BeP and BjF as Interferences Using PARAFAC2 and MCR-ALS in Selected Chromatographic Regions[a]

	group I				group II				group III	
	FLT	PYR	CHR	BaA	BbF	BkF	BaP	DBA	IcP	BgP
Validation samples[b]										
PARAFAC2										
RMSEP[c]	7	7	7	9	8	3	4	2	2	3
REP[d]	2	2	4	15	15	26	14	7	4	10
LOD[e]	26	20	14	6	4	2	3	2	5	3
MCR-ALS										
RMSEP[c]	8	7	7	7	3	0.5	4	2	2	2
REP[d]	3	2	4	12	5	5	14	7	4	7
LOD[e]	20	26	10	17	4	1	2	2	3	3
p-value[f]	0.38	0.77	0.85	0.34	0.45	0.02	0.80	0.50	0.97	0.54
Samples with interferences[g]										
PARAFAC2										
RMSEP[c]	17	14	8	6	9	3	4	5	2	2
REP[d]	6	5	5	11	15	25	13	17	3	8
LOD[e]	26	20	15	6	4	3	3	2	5	3
MCR-ALS										
RMSEP[c]	11	12	6	4	5	2	2	1	2	3
REP[d]	4	4	4	7	9	19	7	4	4	11
LOD[e]	20	26	10	17	6	2	2	2	3	3
p-value[f]	0.03	0.77	0.42	0.12	0.07	0.04	0.30	0.003	0.82	0.63

[a] See the text. [b] Number of samples = 10. [c] RMSEP, root-mean-square error of prediction in ng mL^{-1}. [d] REP, relative error of prediction in %. [e] LOD, limit of detection in ng mL^{-1} and calculated according to ref 45. [f] Probability value arising from the randomization test (see text). [g] Number of samples = 30.

encouraging results, and therefore a detailed study applying this algorithm in the complete range of data was carried out. In fact, in previously reported works, MCR-ALS has been shown to be a powerful chemometric tool to cope with strong coelution problems.[39–42] Specifically, complex biocide mixtures in environ-

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Table 3. Probabilities Associated with the Randomization Test for the Comparison of RMSEP Values Obtained by MCR-ALS-Entire Chromatographic Data Matrices and MCR-ALS-Split Chromatographic Data Matrices[a]

	FLT	PYR	CHR	BaA	BbF	BkF	BaP	DBA	IcP	BgP
validation samples	0.64	0.19	0.35	0.07	0.19	0.12	0.30	0.25	0.11	0.15
samples with interferences	0.25	4×10^{-3}	0.68	5×10^{-4}	0.20	0.96	0.24	0.56	2×10^{-3}	0.89

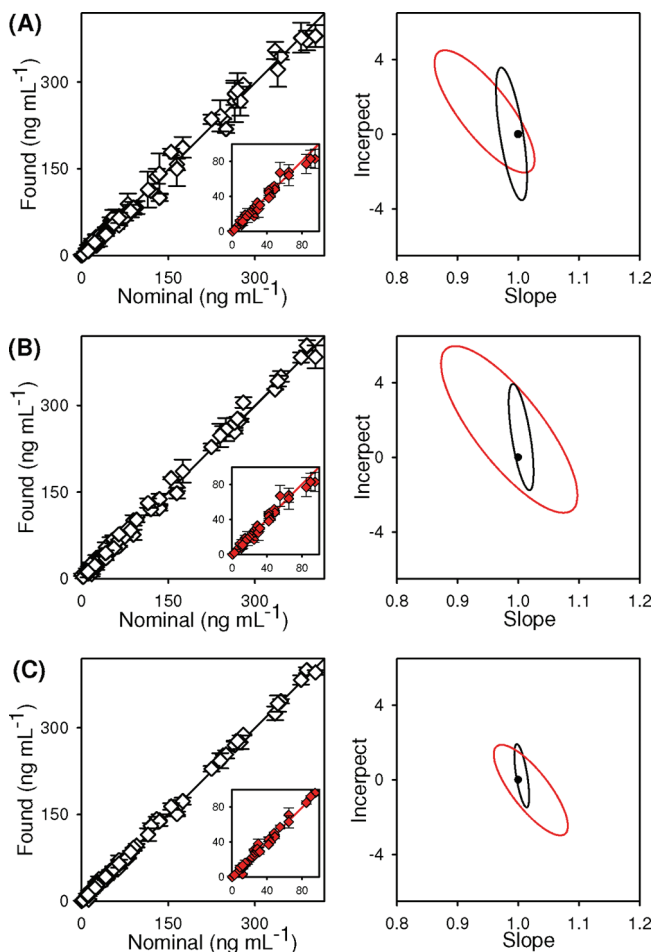[a] Values lower than 0.05 are shown in boldface.

Figure 7. Plots of PAHs predicted concentrations in water samples in the presence of interferences, as a function of the nominal values (the solid lines are the perfect fits) and elliptical joint regions (at 95% confidence level) for the slope and intercept of the regression of the corresponding data. Crosses mark the theoretical (intercept = 0, slope = 1) points. Each inset shows the predictions for PAHs of group II (dark red diamond), and the corresponding ellipse is indicated in a dark red solid line. (A) MCR-ALS using the entire chromatographic data matrices, (B) PARAFAC2 using split chromatographic data matrices, and (C) MCR-ALS using split chromatographic data matrices.

mental samples were resolved coupling MCR-ALS to liquid chromatography with different detection systems such as a diode array (DAD),[39] mass spectrometry (MS),[40] and also using DAD-MS fused data.[41] In addition, different calibration approaches including external calibration, standard addition, and internal standard were proposed to deal with sensitivity changes and

matrix effects encountered in the MCR-ALS analysis of natural environmental samples.[42] As will be demonstrated below, neither of the synthetic or natural samples presently studied showed matrix effects. Therefore, external calibration rendered satisfactory results.

Matrix data for each validation sample were augmented with the calibration data matrices and decomposition according to eqs 1−3 was performed by imposing the restriction of non-negativity in both dimensions and unimodality in the temporal dimension. The number of MCR components was estimated using a principal component analysis.

Figure 3A shows the prediction results corresponding to the application of MCR-ALS to a set of 10 validation samples different from those used for the calibration step. As can be observed, the predictions for the 10 PAHs are in good agreement with the corresponding nominal values. If the elliptical joint confidence region (EJCR)[43] is analyzed for the slope and intercept of the above plot (Figure 3C), we conclude that ellipse includes the theoretically expected values of (1,0), indicating the accuracy of the used methodology.

The power of the proposed method could not be completely appreciated until its ability to overcome the ubiquitous problem of the potential presence of interfering species in the analyzed matrices is demonstrated.[12] Several tests carried out with different heavy PAHs showed that BeP and BjF strongly overlap the spectral signals of PAHs of group II (Figure 1C). The effect of these heavy PAHs, which could be concomitantly present in the samples, was evaluated on the determination of the analytes under study. With this purpose, 30 test samples also containing BeP, BjF, or both were prepared and evaluated with MCR-ALS.

Figure 4 shows the profiles retrieved by MCR-ALS in the spectral (Figure 4A) and temporal (Figure 4B) dimensions for a validaton sample and for a typical sample with interferences. As can be seen, although the system is very complex, the spectra are distinguished, and the chromatographic bands are recognized as belonging to the analytes (present in all samples) or interferences (only present in the test sample).

Figure 3B shows the prediction results corresponding to the application of the assayed algorithm to the different samples containing interferences. Although some of calculated values show a slight dispersion with respect to the perfect fit line, the corresponding ellipse obtained when the EJCR analysis is applied (Figure 3C) implies accurate predictions and the ability of MCR-ALS to resolve highly overlapped analytes.

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Table 4. Recovery Study for the 10 Studied PAHs in Spiked Water Samples in the Presence of BeP and BjF as Interferences Using MCR-ALS and Selected Chromatographic Regions[a]

	group I				group II				group III	
	FLT	PYR	CHR	BaA	BbF	BkF	BaP	DBA	IcP	BgP
water I										
taken	135	335	50	25	25	0	12	15	26	12
found	140(10)	325(12)	46(3)	25(1)	31(1)	0(1)	3(1)	13(1)	30(1)	12(1)
recovery	104	97	92	100	124		25	87	115	100
taken	265	245	165	55	65	12.5	25	26	42	28
found	270(8)	252(3)	162(13)	58(2)	71(8)	10(1)	24(1)	28(1)	40(1)	29(1)
recovery	102	103	98	105	109	80	96	108	95	104
taken	390	165	280	90	95	19.5	48	49	49	49
found	400(5)	151(4)	288(1)	88(1)	96(3)	19(2)	50(1)	46(2)	51(1)	52(2)
recovery	103	92	103	98	101	97	104	94	104	106
water II										
taken	380	345	265	80	90	17.5	42	42	48	47
found	383(8)	346(4)	270(6)	79(3)	92(1)	16(2)	43(2)	43(3)	48(1)	48(2)
recovery	101	100	102	99	102	91	102	102	100	102
taken	250	240	175	60	55	11	31	28	27	27
found	254(7)	243(13)	173(6)	57(1)	57(2)	10(1)	30(4)	30(2)	31(3)	29(2)
recovery	102	101	99	95	104	91	97	107	115	107
taken	130	135	80	25	30	6	11	13	22	13
found	142(4)	138(8)	74(1)	33(1)	38(5)	5(1)	12(1)	14(2)	20(1)	17(1)
recovery	109	102	93	132	127	83	109	108	91	131
water III										
taken	275	270	155	65	65	11.5	29	32	52	32
found	275(12)	277(2)	164(6)	57(1)	63(7)	11(2)	32(2)	29(1)	53(2)	33(1)
recovery	100	103	106	88	97	96	110	91	102	103
taken	340	405	225	75	85	14.5	44	42	56	42
found	342(14)	396(4)	229(5)	71(2)	85(2)	14(1)	40(1)	37(1)	56(1)	42(1)
recovery	101	98	102	95	100	97	91	88	100	100
taken	120	115	65	18	15	2.8	9	12	23	11
found	130(5)	115(11)	65(7)	21(1)	16(3)	2(1)	11(2)	13(1)	22(1)	11(2)
recovery	108	100	100	117	107	71	122	108	96	100

[a] Preconcentration factor = 500 (see text). Water I: tap water from Rosario (Argentina). Water II: mineral water from Mendoza (Argentina). Water III: underground water from Pérez City (Argentina). Concentrations are given in ng mL^{-1}, and recoveries are given in percentage. The found values are means of duplicates. Standard deviation between parentheses. The concentrations of BeP and BjF were in the range 150−1000 ng mL^{-1}.

The statistical results for MCR-ALS applied to the whole chromatogram of samples without and with interferences can be appreciated in Table 1. In general, the highest relative error of prediction (REP) values in validation samples are related to the presence of those analytes which show the largest overlapping in both dimensions: BaA, BbF, BkF, and BaP (see Figure 1). On the other hand, the presence of the interferences BeP and BjF in the samples produces an increase in most of the REP values, which can be ascribed to increasing overlapping in both data dimensions.

The limits of detection do not appear to be significantly affected by the presence of the studied interferences. Although these values are rather large for the studied light PAHs, those corresponding to more concerned heavy PAHs are in the range 2−10 ng mL^{-1}, and therefore the developed method should be adequate for analyzing their presence in environmental samples after a simple preconcentration step.

Split Chromatographic Data Matrices. As indicated above, PARAFAC2 was unable to give reliable results when the full chromatogram was processed. Therefore, we decided to analyze the performance of this algorithm with data matrices processed by parts. Thus, each chromatographic data matrix was divided in three time regions, involving analytes of groups I, II, and III, respectively. The emission range was 340−540 nm for all analytes.

The number of components when PARAFAC2 was applied to the validation samples was selected following two tests: (1) analysis of PARAFAC2 residuals and (2) consideration of both the spectral and the chromatographic profiles produced by the addition of subsequent components. In the latter test, if the addition of a new component generated repeated analyte profiles, suggesting overfitting, the new component was discarded and the previous number of components (i.e., the last one which did not produce overfitting) was selected. The results obtained by both procedures were consistent and established that the estimated number of components was five, five, and three for the groups I, II, and III, respectively, which can be justified taking into account the presence of the number of known analytes in each selected region plus the background signal. Figure 5A shows that calculated concentrations are reasonably close to the nominal values for the same validation set used above, after applying PARAFAC2 to the three different selected subarrays.

The 30 test samples containing the two PAHs (BeP and BjF) used as interferences were also processed with this algorithm. The selection of the number of PARAFAC2 factors was carried out through the same test as in the validation samples. As expected, the number of factors was only different from the validation samples in the region of group II, where either one or two additional components were required.

Figure 6 shows the profiles retrieved by PARAFAC2 in the region involving analytes of group II, in the first (spectral) mode

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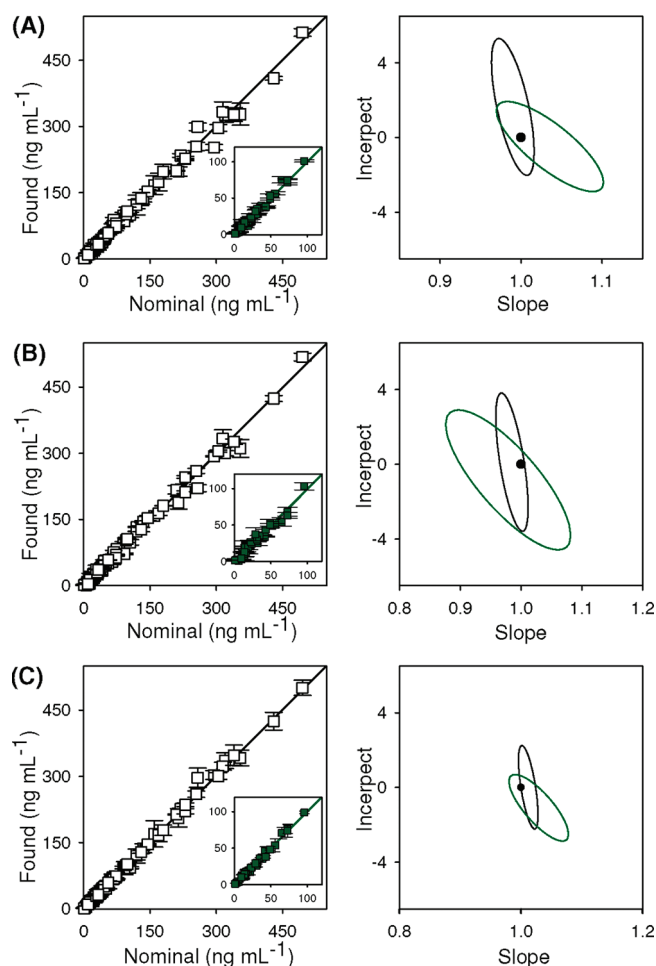
Figure 8. Plots of PAH predicted concentrations in olive oil samples in the presence of interferences, as a function of the nominal values (the solid lines are the perfect fits) and elliptical joint regions (at 95% confidence level) for the slope and intercept of the regression of the corresponding data. Crosses mark the theoretical (intercept = 0, slope = 1) points. Each inset shows the predictions for PAHs of group II (green diamond), and the corresponding ellipse is indicated in a green solid line. (A) MCR-ALS using the entire chromatographic data matrices, (B) PARAFAC2 using split chromatographic data matrices, and (C) MCR-ALS using split chromatographic data matrices.

(Figure 6A) and the second (temporal) dimension (Figure 6B) for a sample without interferences and for a typical test sample with interferences. It should be noticed that in the test sample, while the profiles corresponding to the four analytes of group II are correctly retrieved, those for interferences are recovered as linear combinations of the pure component profiles and the background signal. However, from the strictly analytical point of view, this fact should not influence the successful prediction of the analyte concentrations.

Figure 5B illustrates the prediction results corresponding to the application of PARAFAC2 to the 30 samples containing interferences and the ellipse obtained when the EJCR analysis is applied. The results are good and indicate that PARAFAC2 is also able to resolve the interfering system after splitting the chromatographic data.

To compare analytical results under the same conditions, MCR-ALS was also evaluated working with split chromatograms. Figure 5A,B shows the obtained concentration values when MCR-ALS was applied to both validation and test samples, respectively. An inspection of the sizes of the corresponding ellipses allows one

to asses that a better prediction is obtained for the MCR-ALS calculated values.

Table 2 shows the statistical results when both selected algorithms were applied to samples without and with interferences in the three different chromatographic regions. The relative errors of prediction when PARAFAC2 was applied were higher than those corresponding to MCR-ALS predictions, especially in the cases of the analytes which suffer the largest overlapping. It is interesting to note that the presence of interferences does not significantly modify the REP values of the studied analytes.

The significance of the comparison of RMSEP (root-mean-square error of prediction) values for each PAH using PARAFAC2 and MCR-ALS in both validation and test samples was checked using the randomization approach proposed by van der Voet,[44] and the obtained probability values are shown in Table 2. Most of these values are higher than 0.05, indicating no significant differences between both employed algorithms. However, a better prediction for FLT and DBA in test samples and for BkF in both types of samples is obtained using MCR-ALS (Table 2).

Limits of detection for FLT and PYR are approximately 10 times higher than those obtained for heavier PAHs. This fact can be ascribed to the experimental conditions used (e.g., excitation wavelength) which were selected to optimize the fluorescence of the heavier PAHs investigated.

Entire vs Split Chromatographic Data Matrices. Apparently, processing entire chromatographic data matrices is faster and simpler than working with partial data matrices. However, it is necessary to test if the quality of the prediction of these two different approaches is similar. Since the MCR-ALS algorithm applied to the split chromatograms rendered better results than PARAFAC2, the RMSEP values obtained with the former method in both validation and test samples were compared with the same algorithm applied to the entire chromatograms. With this purpose, the randomization t-test indicated above was employed.[44] As can be concluded from the probability values shown in Table 3, both procedures demonstrate comparable performances regarding the validation samples. On the other hand, the predicted values for PYR, BaA, and IcP in samples with interferences are significantly improved when MCR-ALS is applied in the three selected chromatographic regions. An explanation for this fact is the important overlapping among these analytes and the interferences in the spectral mode when the full data matrices are considered. Indeed, while the fluorescence emission spectrum of BjF is similar to that for IcP, the spectrum of BeP strongly overlaps with the spectra of BaA and PYR (see the inset in Figure 4).

Real Samples. With the purpose of testing the applicability of the investigated methods to real systems, the analysis of different kinds of waters and edible oils was performed.

Water Samples. Because the analyzed water samples did not contain the studied PAHs, they were spiked with all analytes and also with the potential interferences, and a recovery study was carried out. Three different water samples (tap, mineral, and underground) were tested, each containing three different analyte fortification levels, which were in the concentration ranges indicated in the experimental section. The results obtained, in terms of the EJCR accuracy test, are shown in Figure 7, using MCR-ALS (both processing complete and split data matrices) and PARAFAC2 (only with split chromatograms).

Table 5. Recovery Study for the 10 Studied PAHs in Spiked Olive Oil Samples in the Presence of BeP and BjF as Interferences Using MCR-ALS and Selected Chromatogram Regions[a]

	group I				group II				group III	
	FLT	PYR	CHR	BaA	BbF	BkF	BaP	DBA	IcP	BgP
					oil I					
taken	160	170	90	33	32	3.7	14	15	33	17
found	170(30)	167(15)	93(3)	35(3)	28(4)	3(1)	15(1)	16(4)	34(3)	18(1)
recovery	106	98	103	106	88	81	107	107	103	106
taken	320	315	220	66	65	13.7	37	38	38	38
found	336(18)	323(9)	227(1)	67(8)	71(8)	11(4)	37(1)	35(2)	41(4)	36(5)
recovery	105	103	103	102	109	80	100	92	108	95
taken	215	355	105	41	42	5.7	19	22	29	23
found	206(21)	342(18)	93(2)	44(1)	46(6)	4(1)	19(2)	17(4)	33(2)	22(2)
recovery	96	96	89	107	110	70	100	77	114	96
					oil II					
taken	340	210	230	71	73	15	35	43	42	41
found	348(23)	214(1)	221(6)	74(4)	74(6)	15(6)	36(1)	38(2)	43(6)	44(4)
recovery	102	102	96	104	101	100	103	88	102	107
taken	120	135	75	23	19	2.6	11.5	12	27	13.5
found	120(16)	137(10)	74(6)	27(4)	18(5)	2(1)	13(1)	13(4)	32(1)	18(1)
recovery	100	101	99	117	95	77	113	108	119	133
taken	230	255	145	51	56	9.8	24	30	37	26
found	236(4)	260(7)	146(2)	53(7)	54(9)	11(2)	24(1)	26(1)	34(1)	26(7)
recovery	103	102	101	104	96	112	100	87	92	100
					oil III					
taken	430	495	258	93	96	15.8	49	49	51	49
found	425(20)	501(17)	227(22)	92(15)	99(2)	17(1)	48(1)	48(4)	46(3)	53(6)
recovery	99	101	115	99	103	108	98	98	90	108
taken	120	130	68	32	23	1.3	13	14	99	46
found	123(12)	129(3)	68(6)	36(7)	23(1)	1(1)	12(2)	14(6)	101(2)	50(4)
recovery	103	99	100	113	100	77	92	100	102	109
taken	295	305	180	73	73	9.8	31	29	56	33
found	303(10)	301(8)	178(12)	74(5)	74(9)	10(4)	30(2)	29(1)	59(4)	32(2)
recovery	103	99	99	101	101	102	97	100	105	97

[a] Preconcentration factor = 10 (see text). Oils I, II, and III from Córdoba (Argentina), San Juan (Argentina), and Teramo (Italy), respectively. Concentrations are given in ng mL^{-1} and recoveries in percentage. The found values are means of duplicates. Standard deviation between parentheses. The concentrations of BeP and BjF were in the range 200−400 ng mL^{-1}.

To evaluate the influence of the presence of BeP and BjF in the results, the predicted values for those PAHs which suffer their interferences (group II) were also separately considered (see dark red ellipses in Figure 7). Although reliable results were obtained in all cases, the best analytical figures of merit were obtained when MCR-ALS was employed to process the split chromatographic data matrices (see Table 4). The good recoveries are also indicative of the effectiveness of the SPE method and the possibility of quantifying the very low PAHs levels admitted in environmental samples.

Olive Oil Samples. A recovery study by spiking olive oils of different origin with the 10 studied analytes and the two interferences was carried out applying MCR-ALS analysis to the complete data matrices and MCR-ALS and PARAFAC2 to the three selected chromatographic regions. As in the case of water samples, the EJCR were analyzed for slopes and intercepts of the plots of predicted vs nominal concentrations (Figure 8).

On the basis of the obtained results, we can conclude about the accuracy of the employed methodologies. The obtained results suggest that neither of the two investigated foreign PAHs (BeP and BjF) nor other inorganic and organic compounds which may be possibly present in the studied samples produce a significant interference in our analysis. The best analytical figures of merit were again obtained when MCR-ALS was employed to process the split chromatographic data matrices (Table 5).

CONCLUSIONS

Both MCR-ALS and PARAFAC2 combined with high-performance liquid chromatography−fast scanning fluorescence spectroscopy have been demonstrated to be powerful tools to resolve, in a very short time, a complex mixture of analytes of similar structure. The determinations are carried out in the presence of unexpected compounds, without the necessity of a complete chromatographic separation or the requirement of time chromatographic alignment. Two approaches, using either the entire or divided chromatographic data matrices, were compared. It is important to remark that while PARAFAC2 rendered good results only when applying the second approach, MCR-ALS was able to provide successful predictions of the concentration of the 10 studied PAHs using both methods. Therefore, this is a new example of the power and applicability of MCR-ALS. Although the best predictions were obtained when MCR-ALS was applied to split data matrices, the results obtained working with the full chromatographic data matrix were more than satisfactory for most of the studied analytes and highlight the potentiality of this algorithm to solve the complex problem here presented.