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Solving liquid chromatography mass spectrometry coelution problems in the analysis of environmental samples by multivariate curve resolution

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Abstract

Multivariate curve resolution-alternating least squares (MCR-ALS) is shown to be a powerful tool to resolve coelution problems in liquid chromatograpy–mass spectrometry (LC–MS) in scan mode. This investigation was performed using two types of LC columns, one traditional LC column of 25 cm length with a slow gradient and a shorter LC column of 7.5 cm with a rapid gradient which allowed much faster analysis and save of reagents and solvents. Mixtures of multiple biocide compounds were simultaneously analyzed in standard mixtures and in environmental samples (sediment and wastewater samples) with little sample pretreatment. Using the more traditional LC 25 cm column, all biocide compounds were properly resolved by MCR-ALS and quantitatively analyzed with estimated errors always below 20%. When fast chromatography (LC column of 7.5 cm) was used, MCR-ALS resolution of the more strongly coeluted compounds was also achieved but limitations were found in their simultaneous quantitative determination, specially for environmental samples.

Keywords: Multivariate curve resolution; Alternating least squares; MCR-ALS; LC–MS; Coelution; Short column; Fast chromatography; Biocides; Environmental samples

1. Introduction

An extensive development of multivariate data analysis techniques and chemometrics in different branches of analytical chemistry and in particular in chromatography has been performed in recent years [1]. Many of these studies have been applied to liquid chromatography with diode array detection (LC-DAD) [2–9], to gas chromatography with mass spectrometry detection (GC–MS) [10–13], and less frequently to liquid chromatography with mass spectrometry detection (LC–MS). In particular, in recent years different research groups have been developing and applying chemometrics methods to LC–MS at all levels [14–17]. Windig et al. [14] used CODA and COMPARELCMS methods for a fast interpretation of complex LC–MS data and to increase the productivity in the instrument. Seto et al. [15] have optimized, with a minimal amount of experimentation, LC–MS conditions by means of a chemometric experimental design approach. Fleming et al. [16] have improved signal to noise ratios in both chromatography and mass spectrometry data acquired in peptide mapping of recombinant DNA derived proteins. Koh et al. [17] have explained how chemometrics and other analytical advances are being employed in all stages of drug discovery for the prevention and treatment of human diseases. Idborg et al. [18] have used PCA, PARAFAC and N-PLS for metabolite pattern recognition.

In this work, the chemometrics method known as multivariate curve resolution-alternating least squares (MCR-ALS) [6–7] is applied to solve LC–MS (in scan mode) coelution problems. The application of an alternating regression (AR) strategy to solve coelution problems was also proposed by Karjalainen [19] some years ago. However AR did only consider non-negativity constraints, and it did not consider the simultaneous analysis of multiple chromatographic runs

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using a matrix augmentation data arrangement like the here proposed, nor it did consider in detail rank deficiency problems nor local rank conditions for resolution. MCR-ALS is a much more powerful and simple to use strategy both for resolution and for quantitative purposes. MCR-ALS is applied first to resolve coelutions in the analysis of standard mixture samples where matrix interferences are minimal. And secondly, MCR-ALS is applied to the analysis of strong coelutions that appear in the analysis of environmental samples where the presence of impurities and matrix interferences is very significant. In a previous work [20] the same type of problem was investigated by means of LC-DAD with 25 and 7.5 cm LC columns. Successful resolution and quantitation of standard mixture samples was achieved using both LC columns. Limitations of MCR-ALS resolution were found when complex environmental samples were analyzed with the short 7.5 cm LC column due to the lack of selectivity of the DAD detector. In the present work these difficulties are investigated using LC–MS in scan detection mode instead of using UV diode array spectrometric detection (LC-DAD). Different types of sample matrices are also investigated: pure standard solutions, standard mixtures and environmental samples (sediments and waters). And, they are also analyzed using LC columns of two different lengths, 25 and 7.5 cm. Separation of 10–15 compounds is achieved in 50 min in the 25 cm LC column with a slow gradient, and in less than 7 min in the case of use a 7.5 cm LC column with a rapid gradient. However, using the short LC column, coelutions increase significantly and full resolution and quantitative determinations are more difficult to achieve. Implicit with this research, there is also the investigation about some current problems in LC–MS analytical determinations, like ion identification and quantification problems in MS, the presence of coeluting ions both from other compounds and from the sample matrix, the lack of correspondence between ion abundances in environmental and standard samples, and also the possible presence of ion suppression effects.

2. Experimental work

2.1. Chemicals and reagents

Biocides methomyl, deethylsimazine, deethylatrazine, carbendazim, carbofuran, simazine, atrazine, alachlor, chlorpyrifos-oxon, terbutryn, chlorfenvinphos, pirimiphos-methyl and chlorpyrifos were obtained from Sigma-Aldrich (St. Louis, USA). Stock individual standard solutions (500 μg/mL for carbendazim, 2000 μg/mL for the others) were prepared dissolving accurate amounts of pure standards in methanol. Standard mixture samples of all of them were prepared at different concentration levels: 20, 10, 5, 1, 0.5 and 0.1 μg/mL, in methanol. Methanol and HPLC grade water were obtained from Merck (Darmstadt, Germany). Extraction cartridges (OASIS, 60 mg) were from Waters (Milford, MA, USA).

2.2. Samples analysed

Three types of samples were investigated (i) pure standards; (ii) mixture standards and (iii) spiked environmental wastewater (WWTP of La Llagosta, North-East Spain) and sediment (Aznalcóllar mine, South Spain) sample. Both extracts were spiked at 10 μg/mL with target biocides. In the case of the wastewater sample, only a fast solid phase extraction without further clean-up was performed [21]. For the sediment extract, a more exhaustive sample pretreatment consisting of Soxhlet extraction followed by alumina clean up was performed, according to experimental conditions described in detail in a previous work [22].

2.3. Chromatographic analysis

HPLC instrumentation system consisted of an HP1100 autosampler with a 100 μL loop and an HP 1100 LC double binary pump, both from Hewlett-Packard (Palo Alto, CA, USA). HPLC separation was achieved in two different columns: (a) on a 5 μm, 250 mm × 4 mm i.d. (LiChroCART) C_{18} reversed phase column (Purospher STAR RP-18 endcapped) with a guard column 5 μm, 4 mm × 4 mm i.d. (LiChrospher 100 RP-18); and (b) on a 5 μm, 75 mm × 4 mm i.d. C_{18} reversed phase column (LiChrospher 100 RP-18) with the same guard column as previously.

Detection was carried out using an MS HP1100 detector from Hewlett-Packard (Palo Alto, CA, USA), with electrospray interface and the following conditions: nebulizer pressure, 60 psig; fragmentation, 90 V; capillary voltatge, 5500 V, in positive mode; drying gas flow, 13 L/min; and drying gas temperature, 280 °C. The MS detector was operated in Scan mode (70–400 amu) with a 1.93 s/cycle. Data acquisition program was LC/MSD CHEMSTATION ver. A.08.03 (847).

All chromatographic separations were performed under gradient elution conditions using methanol and water. For the 25 cm column, biocides were separated using the following solvent gradient programming: from methanol–water (20:80) to (95:5) in 45 min at a flow-rate of 1 mL/min and then returning to the initial conditions in 5 min. For the 7.5 cm column, biocides were separated using methanol–water (50:50) to 100% of methanol in 2.5 min at a flow-rate of 1 mL/min, then this condition was kept isocratic for 1 min, and finally returning to initial conditions in 1 min.

Figs. 1 and 2 give respectively the reconstructed total ion current (TIC) chromatograms obtained with the 25 and 7.5 cm LC columns of (A) a standard mixture sample, (B) a sediment sample and (C) a water sample. Noticeable differences among these chromatograms were obtained for the 25 cm and the 7.5 cm LC columns. TIC chromatograms of the In-WWTP sample (Figs. 1C or 2C, for 25 and 7.5 cm LC columns, respectively) were the most different because of the extreme complexity of the matrix of this sample.

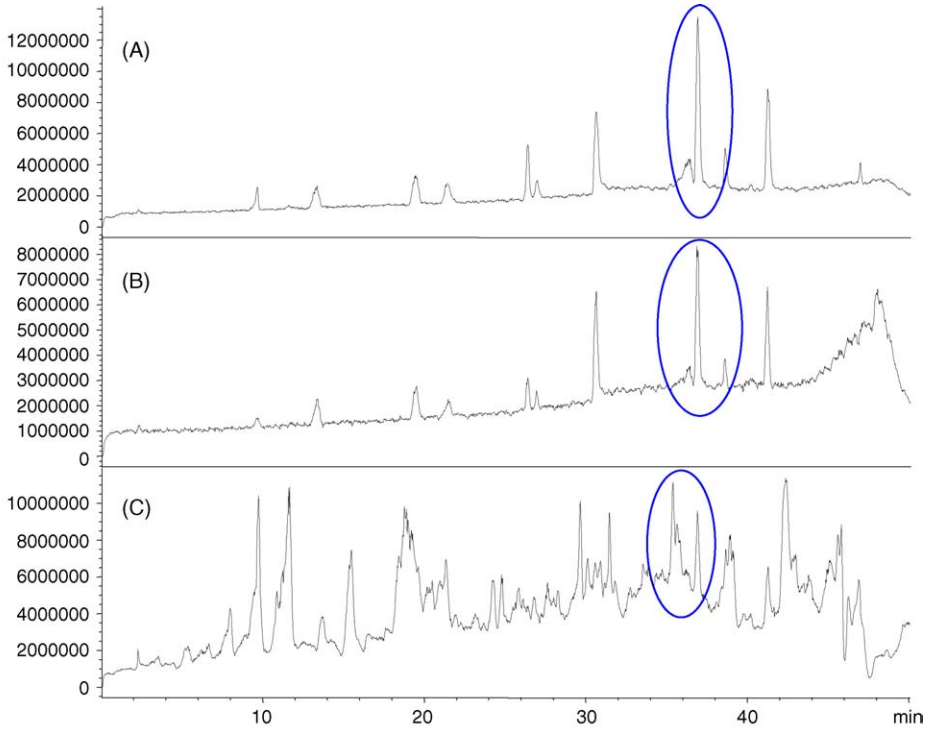

Fig. 1. Reconstructed total ion current (TIC) chromatograms obtained with the 25 cm LC column. Encircled the coelution region of the studied compounds is showed: alachlor, chlorpyrifos-oxon and terbutryn (in elution time order). (A) Chromatogram of a standard mixture sample of the 13 biocides under study at concentrations of 20 μg/mL. (B) Chromatogram of the Aznalcóllar sediment sample spiked with the same 13 biocides at 10 μg/mL. (C) Chromatogram of the In-WWTP water sample from La Llagosta spiked with the 13 biocides at of 10 μg/mL.

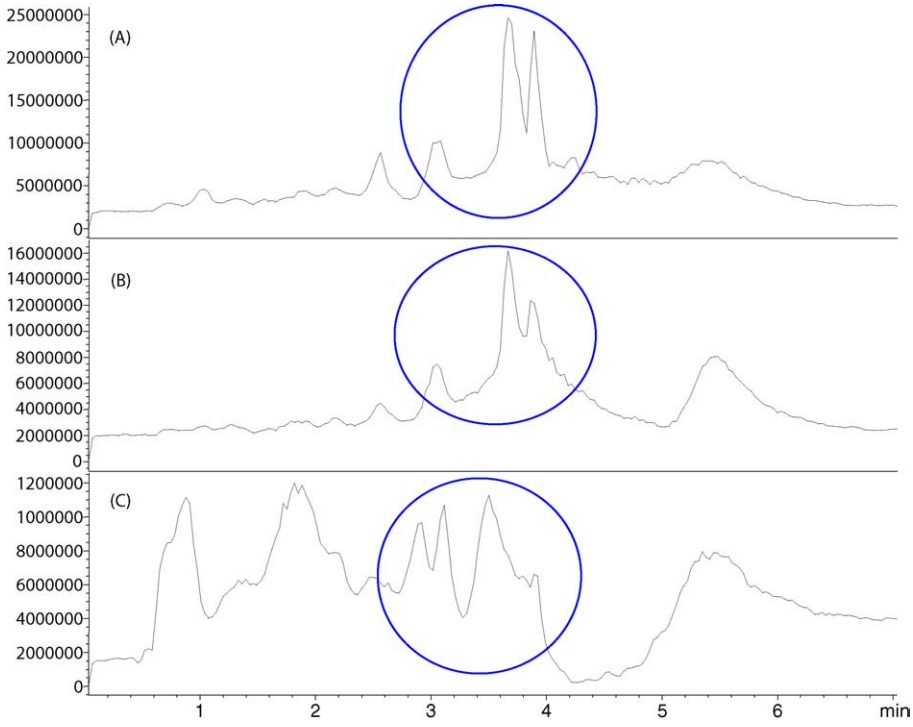

Fig. 2. Reconstructed total ion current (TIC) chromatograms obtained with the 7.5 cm LC column. Encircled the coelution region of the studied compounds is showed: alachlor, chlorpyrifos-oxon, terbutryn, chlorfenvinphos and pirimiphos-methyl (in elution time order). (A) Chromatogram of standard mixture sample of the 13 biocides under study at concentrations of 20 μg/mL. (B) Chromatogram of the Aznalcóllar sediment sample spiked with the 13 biocides at concentrations of 10 μg/mL. (C) Chromatogram of the In-WWTP water sample from La Llagosta spiked with the 13 biocides at 10 μg/mL.

3. Chemometrics

3.1. Bilinear data model

Data obtained in the LC–MS chromatographic analysis of one sample in scan mode provides an array of numbers which are ordered in a data table or data matrix with a number of rows equal to the number of elution times and with a number of columns equal to the number of mass (m/z). Whereas for all the chromatographic runs, the same m/z range was selected (70–400 amu with 3301 m/z points, with a m/z resolution of 0.1 U), a different number of elution times were selected for each data set under study depending on the elution time of the compounds of interest (analytes). Thus, every data matrix had the same number of columns but they differed in the number of rows. Every chromatographic run recorded at different concentration of analytes gave a new data matrix, $\mathbf{D}_k$ ($k = 1, 2, \ldots, N$), which can be described by a bilinear model.

$$\mathbf{D}_k = \mathbf{C}_k\mathbf{S}^{\mathrm{T}} + \mathbf{E}_k \quad k = 1,2,\ldots,N \tag{1}$$

Rows of matrix $\mathbf{D}_k$ are the mass spectra recorded at different elution times and columns of matrix $\mathbf{D}_k$ are the chromatographic elution profiles recorded at different m/z. $\mathbf{C}_k$ is the matrix of the elution profiles of the compounds resolved during a particular chromatographic run in the analysis of sample k and $\mathbf{S}^{\mathrm{T}}$ is the matrix of their corresponding pure mass spectra. Resolved mass spectra allow the identification of the coeluted compounds. Finally, $\mathbf{E}_k$ is the background and noise contribution not modeled by the resolved compounds in $\mathbf{C}_k$ and $\mathbf{S}^{\mathrm{T}}$ (see Fig. 3A).

The same type of data analysis based on a bilinear model can also be carried out simultaneously over several chromatographic runs (several samples analyzed), setting the corresponding data matrices $\mathbf{D}_k$ one on top of each other (column data matrix augmentation) and keeping their columns (m/z) the same for all of them. The new column-wise augmented data matrix $\mathbf{D}_{\mathrm{aug}}$ can be decomposed similarly using the bilinear model equation:

$$\mathbf{D}_{\mathrm{aug}} = \begin{bmatrix}\mathbf{D}_1\\ \mathbf{D}_2\\ \vdots\\ \mathbf{D}_N\end{bmatrix} = \begin{bmatrix}\mathbf{C}_1\\ \mathbf{C}_2\\ \vdots\\ \mathbf{C}_N\end{bmatrix}\mathbf{S}^{\mathrm{T}} + \begin{bmatrix}\mathbf{E}_1\\ \mathbf{E}_2\\ \vdots\\ \mathbf{E}_N\end{bmatrix}$$

$$= \mathbf{C}_{\mathrm{aug}}\mathbf{S}^{\mathrm{T}} + \mathbf{E}_{\mathrm{aug}} \tag{2}$$

This new augmented data matrix $\mathbf{D}_{\mathrm{aug}}$ has a number of rows equal to the total number of recorded elution times considered for the analysis in the different chromatographic runs ($k = 1, 2, \ldots, N$), and it has a number of columns equal to the number of considered m/z. As previously stated, whereas the number of m/z is equal for all considered chromatographic

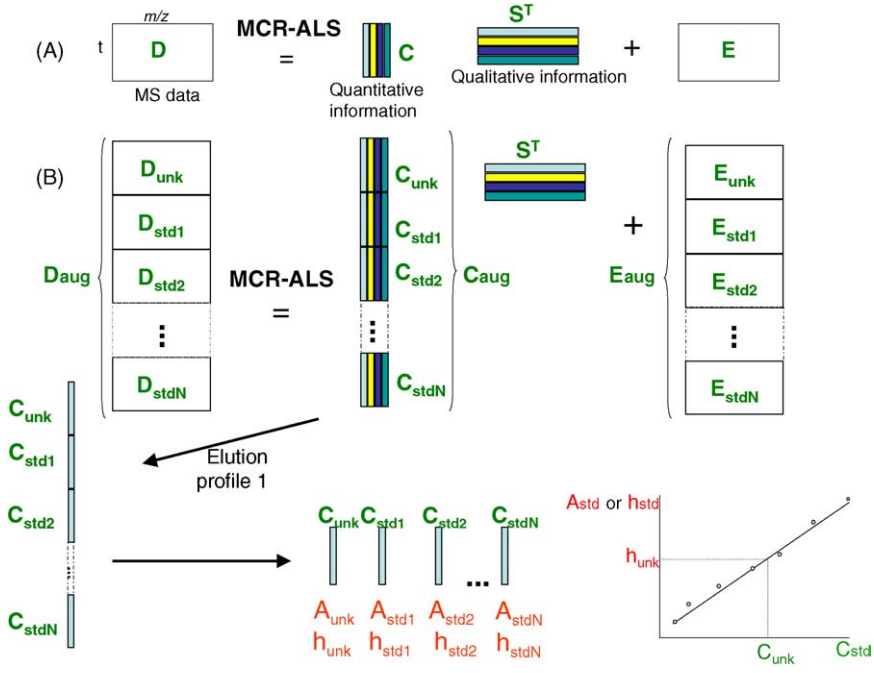

Fig. 3. (A) MCR-ALS resolution (Multivariate Curve Resolution- Alternating Least Squares) applied to the single data matrix **D**. Elution profiles, **C**, gives quantitative information. Spectra matrix, $\mathbf{S}^{\mathrm{T}}$, gives qualitative information. (B) Multivariate curve resolution-alternating least squares resolution (MCR-ALS) applied to the column-wise augmented data matrix $\mathbf{D}_{\mathrm{aug}}$. $\mathbf{D}_{\mathrm{unk}}$ is the unknown sample data matrix. $\mathbf{D}_{\mathrm{std}}$ are the standard mixture sample matrices. Caug is the column-wise augmented elution profiles matrix. $\mathbf{C}_{\mathrm{unk}}$ and $\mathbf{C}_{\mathrm{std}}$ are the MCR-ALS resolved elution profiles matrices from the unknown and standard mixture samples, respectively. From the comparison of their heights or areas of the resolved elution profiles in the different standard mixtures samples, quantitative information from calibration curves can be obtained (section 2.6). $\mathbf{S}^{\mathrm{T}}$ matrix is the pure spectra of MCR-ALS resolved compounds, which allows their identification. **E** and $\mathbf{E}_{\mathrm{aug}}$ allow fit and diagnostics evaluation.

runs, the number of elution profiles considered in each chromatographic run is different, since different chromatographic ranges could be analyzed in each case. This is the reason why column-wise augmentation was selected in this case, i.e. to keep the mass spectral common vector space (pure component spectra) the same among different chromatographic runs, but allowing each chromatographic run to be described (both in elution shape and in retention time) by a different set of elution profiles, even if they belong to the same compound in different chromatographic runs. Therefore, $\mathbf{C}_{aug}$ is the augmented matrix of the resolved elution profiles in the different chromatographic runs, formed by multiple $\mathbf{C}_k$ submatrices ($k = 1, 2, \ldots, N$, see Eq. (2)), each one of them with the elution profiles of the coeluted compound in a particular chromatographic run, $\mathbf{D}_k$. $\mathbf{S}^T$ is (like in Eq. (1)), the matrix of pure mass spectra of the resolved coeluted compounds, and **E** matrix is the noise and background signal absorption not explained by the model described by $\mathbf{C}_{aug}$ and $\mathbf{S}^T$ (see Fig. 3B).

3.2. Alternating least squares

To solve the bilinear models expressed by Eqs. (1) and (2), an alternating least squares approach is used [6–9,23]. A particular elution time range is selected covering the chromatographic region of interest containing the cluster of coeluted peaks. Before starting the alternating least squares iterative process, the number of coeluted compounds in a particular peak cluster is estimated. This is usually accomplished by principal component analysis (PCA) [24] or by singular value decomposition (SVD) [25]. It is assumed that variance explained by eluted chemical components is larger than noise variance, and therefore that only larger components are needed to explain sufficiently well the experimental data. Moreover, only a first preliminary estimation is required, since the correct number of components finally used in the resolution process will be corroborated afterwards by the ALS optimization. Also to start this optimization, initial estimates of these components either in **C** or in $\mathbf{S}^T$ matrices are required. This can be accomplished in many different ways. It is sensible to start with the best possible estimates available, i.e. with the mass spectra of components if they are known. At the beginning of the study of a new unknown sample, when nothing is known about the nature of the coeluted compounds, evolving factor analysis (EFA) [26] or SIMPLISMA [27] may provide good initial estimates. EFA is especially good for sequentially evolving systems, as it is often the case of well behaved chromatographic systems. However, in the analysis of environmental samples with very complex sample matrices, the use of pure variables detection methods, like those provided by the SIMPLISMA method is usually better.

Once the number of coeluted components and an initial estimation of their elution profiles or mass spectra are available, the alternating least squares constrained optimization starts. Different constraints have been already described in the literature [6–9,23]. These constraints are applied to avoid the presence of rotational and intensity ambiguities, i.e. to avoid obtaining solutions fitting equally well Eqs. (1) and (2) but without physical meaning. Conditions to obtain unique solutions in curve resolution have been investigated in previous works [6,23,28,29]. In this particular study, the applied constraints have been non-negativity, unimodality, selectivity and normalization.

Non-negativity constraint is applied both to concentration and mass spectra profiles, because chemical concentrations and mass spectra are only defined to be positive or zero. Unimodality is another constraint frequently applied to chromatographic elution profiles in **C** matrix to force them to have a single peak shape. The application of this constraint avoids the appearance of elution profiles with double peaks when components with similar mass spectra coelute. In some cases however, its application is not reasonable. like for resolution of solvent gradient contributions or for resolution of isomer species having similar MS spectra. Selectivity constraint could be applied either to concentration or mass spectra profiles. In this case, it has been applied only to concentration profiles to determine the absence of species in a certain elution range. Finally, a normalization constraint has been applied to mass spectra profiles to fix scale indeterminacy during the ALS resolution. In this way, all the components in a particular chromatographic run are assumed to have the same relative signal contribution in the spectral domain. All the differences in concentration of the components in the mixtures will be expressed in the intensity/area of elution profiles.

Outcomes from ALS optimization (Fig. 3B) are estimations of **C**, $\mathbf{S}^T$ and **E** matrices. **C** matrix gives MCR-ALS resolved elution profiles, from which quantitative information may be obtained. $\mathbf{S}^T$ matrix gives MCR-ALS resolved pure species mass spectra, from which identification and qualitative information is obtained. Finally, **E** matrix gives MCR-ALS residuals, i.e. variance not explained by the bilinear model and/or not fitted by the constrained ALS optimization procedure. It is useful for model fit and diagnostics evaluation.

Resolution of natural environmental samples is greatly improved when they are simultaneously analyzed together with individual and mixture standard samples. Strongly overlapped elution profiles in the analysis of environmental and of standard mixture samples could not be resolved without ambiguities if these samples were analyzed separately run by run (i.e. using Eq. (1). Resolution conditions for the analytes [5,23,28,29] are greatly improved if individual standard samples are also included in the simultaneous analysis of them (i.e. using Eq. (2). Since background solvent gradient (see Section 2) has an important contribution which is difficult to subtract (specially if coelutions are strong), they should be considered in the ALS resolution of the **C** concentration profiles matrix. In the $\mathbf{S}^T$ matrix, mass spectra of coeluted biocide compounds under analysis plus the solvent and other possible interferences were obtained.

3.3. Quantitative estimations

Like in traditional chromatographic analysis, when a single chromatographic run is analyzed, estimation of relative concentrations of the different eluted compounds within the same chromatographic run is only possible if some assumption about their spectra/signal contribution is used and that this assumption is true. Otherwise, such estimations are scale ambiguous and no quantititative information can be derived from the analysis of a single chromatographic run (intensity/scale ambiguity [6]). On the other hand, and also as in currently used external calibration procedures with standards, relative quantitative information of one particular compound is easily derived from comparison of the resolved elution profiles of this component in the different chromatographic runs simultaneously analyzed. Under the assumption of a linear relationship between relative peak areas/heights of the ALS resolved profiles for a considered component and their relative concentrations in the different analyzed samples (runs), calibration curves and relative quantitative estimations are obtained. From these calibration curves, figures of merit may be also calculated and concentration of unknown samples obtained by inverse regression (Fig. 3B).

3.4. Software

Data treatments, algorithms, computer programs, graphics and software were run under the MATLAB computer and visualization environment (Release 13, The Mathworks, Natick MA, USA). More details about homemade MCR-ALS programs are given in [6,23] and software implementation and download in [30].

4. Results and discussion

Biocide compounds under study were first analyzed individually by LC–MS in scan mode. The goal was to know without ambiguities their chromatographic behavior and their characteristic mass spectra, for an afterwards easier identification of them in more complex mixtures at the same experimental chromatographic conditions. Resolution of pure standards samples also provided the characteristic chromatographic profiles of the analyzed biocides. In the resolution of these individual standard samples, the contribution of the solvent was always simultaneously resolved.

4.1. Resolution and quantitation of coeluted biocides by LC–MS (scan mode) and MCR-ALS for 25 cm LC column

Standard mixture samples of the thirteen different biocides at the different concentration levels of 20, 10, 5 and 1 μg/mL, were analyzed by LC–MS (scan mode) using the 25 cm LC columns with a slow gradient. With this 25 cm LC column, elution of all the compounds lasted approximately 50 min (Fig. 1). MCR-ALS was applied to column-wise augmented data matrices (Eq. (2)) formed the different standard mixture samples at the four concentration levels (20, 10, 5 and 1 μg/mL), together with the different individual standard samples, previously analyzed (see Fig. 3).

Using the 25 cm LC column at the optimal experimental conditions of analysis (Fig. 1A) some coelutions appeared. Three compounds were coeluted in a small cluster of two peaks in all the chromatographic runs of the standard mixture samples (encircled region in Fig. 1A for chromatogram of the standard mixture at 20 μg/mL). Although these three compounds could probably be properly resolved by mass spectrometry using selected ion monitoring (SIM) mode approach, the present study wants to show that LC–MS in scan mode plus MCR-ALS provides a fast and accurate simultaneous determination of multiple LC coeluted compounds. Four different independent contributions were resolved by MCR-ALS in this peak cluster region. In Fig. 4, MCR-ALS resolved elution profiles of these species are given for this 25 cm LC column. To achieve this resolution, non-negativity, unimodality and normalization constraints were applied during the ALS resolution. Three of these contributions were identified (see spectra) as alachlor, chlorpyrifos-oxon and terbutryn compounds. Between chlorpyrifos-oxon and terbutryn, the coelution was stronger and their chromatographic resolution was very low, approximately around 0.1 (see Table 1). The same elution profiles at different concentration levels, with the same species and with the same time elution order were obtained for all standard mixture samples. The fourth resolved contribution was the solvent gradient profile, always overlapping with the other compounds. The shape of the solvent gradient profile showing a continuous linear increase during elution is in agreement with solvent composition changes during the chromatographic run (see Fig. 4, and experimental conditions for gradient elution in Section 2.3). Normalized pure mass spectra and characteristic mass peaks of each coeluted compound were confirmed from previous LC–MS analysis of pure standard samples using conventional methods (alachlor m/z: 292; clorpyrifos-oxon m/z: 356, 358; terbutryn m/z: 242, 186). In Fig. 4, MCR-ALS spectra of these components are also given.

MCR-ALS simultaneous analysis of data matrices of the four standard mixture samples ((Eq. (2)) at different concentrations (20, 10, 5 and 1 μg/mL), allowed the quantitative determination of coeluted biocides using the procedure described in the method section and in Fig. 3. Linear relationships between peak heights or areas (y-axis) and concentrations (x-axis) were investigated to build calibration curves. Better linear relationships between peak heights and concentrations than between peak areas and concentrations were found in all the cases, with good correlation coefficients (see Table 1). This result confirms, that, on one side, MCR-ALS resolution of LC–MS analysis (scan mode) of standard mixture samples was successful and, that, on the other side, quantitation results were as good as those usually obtained by means of full separation chromatographic methods when no coelution problems exist. It might be argued that sim-

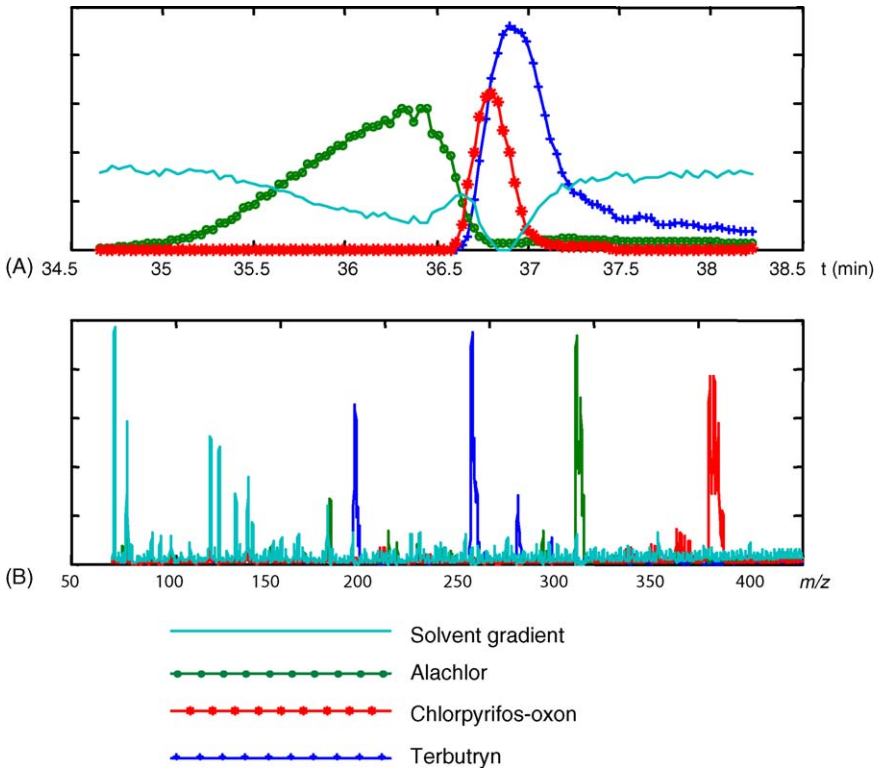

Fig. 4. MCR-ALS resolution of the standard mixture of biocides at concentration of 20 μg/mL, in the 25 cm LC column (Fig. 1A). (A) MCR-ALS resolved elution profiles. Resolved compounds in elution order: alachlor, chlorpyrifos-oxon and terbutryn. (B) MCR-ALS resolved MS spectra. Compound identification: alachlor *m/z:* 292*;* chlorpyrifos-oxon *m/z:* 356, 358; terbutryn *m/z*: 242, 186.

ilar or even better results could have been achieved when LC–MS is performed using SIM mode, however the advantage here is that a single analysis is performed simultaneously for the multiple coeluted compounds. Estimation errors for concentration of the three analytes in standard mixture samples were always well below 20%. This is only an estimation of high boundary quantitation errors, which could be better estimated using an improved design of the reference standard samples including independent changes of analyte concentrations. The analysis of complex environmental samples is more problematic since apart from previously described biocide and solvent coelutions, now, a much larger contribution of the matrix of the sample with multiple unknown interferences was also present making peak identification, resolution and quantitation of analytes much more difficult. Two different environmental samples were analyzed: one sediment sample and one wastewater sample (see Section 2), both spiked with biocides at concentrations of 10 μg/mL. In Fig. 1B and C, the reconstructed total ion current chromatograms obtained using the 25 cm LC column are shown respectively for the sediment sample and for the wastewater sample. In Fig. 5A, MCR-ALS resolution of the sediment sample in the chromatographic region previously explored in the analysis of standard mixture samples (Fig. 4) is given. Elution profiles are similar to those previously found in the analysis of standard mixture samples. Clorpyrifos-oxon and terbutryn were practically totally embedded one inside the other with a resolution lower than 0.1 (Table 1). Alachlor was well resolved and less coeluted than it was expected for a complex sediment sample matrix like the one analyzed here. MCR-ALS resolution of the wastewater sample is shown in Fig. 5B. In this case, a very broad unknown matrix interference profile was resolved. Clorpyrifos-oxon was totally embedded inside terbutryn ($R_s < 0.1$). Alachlor had also a weak coelution with some other compounds, like in the analysis of the standard mixture and sediment samples. Terbutryn was the easiest compound to resolve because its peak emerged outside the matrix interference profile; differently to alachlor which was totally inside the matrix interference.

Quantitation of coeluted compounds, alachlor, chlorpyrifos-oxon and terbutryn, in sediment and wastewater samples was carried out using calibration curves obtained from standard mixture samples (see above). The MCR-ALS strategy of simultaneous resolution of multiple data matrices was used to analyse environmental samples, standard mixture samples and pure standard samples together. Once the whole system was resolved, correlation between resolved peak heights and known concentrations of standards was used to build a calibration curve, from which the concentration of analytes in environmental samples were estimated. In Table 1, a summary of quantitation errors of the studied coeluted compounds for sediment and wastewater samples using the LC 25 cm column are given. In all the cases quantitation errors were below 20%, which were considered rather good taking into account the

Table 1
Figures of merit obtained in the MCR-ALS resolution and quantitation of coeluted compounds in 25 cm and 7.5 cm LC columns

Compounds	25 cm LC column[a]					7.5 cm LC column[b]				
	r^2[c]	Resolution[d]	% average relative error[e]	MCR-ALS concentration[f] (% relative error[e])		r^2[c]	Resolution[d]	% average relative error[e]	MCR-ALS concentration[f] (% relative error[e])	
			Standards	Sediment	Water			Standards	Sediment	Water
Atrazine[a]	–	–	–	–	–	0.9468	0.45[g]	26	0.20 (58)	Not found
Alachlor	0.9709	0.17[h]	15	11.28 (12)	10.87 (8)	0.9238	<0.1[h]	23	0.17 (66)	0.13 (74)
Chlorpyrifos-oxon	0.9515	0.10[i]	19	11.63 (16)	10.51 (5)	0.9982	<0.1[i]	3	0.06 (88)	0.62 (26)
Terbutryn	0.9282	0.19[j]	19	11.72 (16)	11.54 (14)	0.9564	<0.1[j]	17	0.52 (3)	Not found
Chlorfenvinphos[a]	–	–	–	–	–	0.9586	<0.1[k]	18	0.19 (60)	0.19 (60)
Pirimiphos-methyl[a]	–	–	–	–	–	0.9688	0.17[l]	14	0.07 (87)	Not found

[a] In 25 cm LC column, atrazine, chlorfenvinphos and pirimiphos-methyl were not coeluted, so they were not considered. Calibration curves have been built with standard mixture samples at concentration levels of 20, 10, 5 and 1 μg/L.

[b] In 7.5 cm LC column, calibration curves have been built with standard mixture samples at concentration levels of 1, 0.5, 0.1 μg/L.

[c] r^2 correlation coefficient obtained between peak heights of MCR-ALS resolved coeluted peaks and concentrations for the considered compounds in the analysis of their standard mixture samples.

[d] Resolution evaluation: $R_s = 2\Delta t_{AB}/(w_A + w_B)$. Where Δt_{AB} is the time difference between the two maxima of the resolved peaks and w_A and w_B are the respective resolved peak widths at 5% of peak height.

[e] $\%\,\text{Relative error} = \left(\sqrt{\sum_i (c_i - \hat{c}_i)^2}/\sqrt{\sum_i c_i^2}\right) 100$ where c_i is the known reference concentration in the standard i and $\hat{c}_i$ is its calculated value using the calibration equation with the ALS resolved concentration profiles.

[f] In 25 cm LC column, sediment and water samples were spiked at 10 μg/mL (alachlor: 10.06 μg/mL, chlorpyrifos-oxon: 9.99 μg/mL and terbutryn: 10.12 μg/mL). In 7.5 cm LC column, sediment and water samples were spiked at 0.5 μg/mL (atrazine: 0.48 μg/mL, alachlor: 0.50 μg/mL, chlorpyrifos-oxon: 0.49 μg/mL, terbutryn: 0.50 μg/mL, chlorfenvinphos: 0.47 μg/mL and pirimiphos-methyl: 0.50 μg/mL).

[g] Chromatographic resolution between atrazine and alachlor.

[h] Chromatographic resolution between alachlor and chlorpyrifos-oxon.

[i] Chromatographic resolution between chlorpyrifos-oxon and terbutryn.

[j] Chromatographic resolution between terbutryn and alachlor.

[k] Chromatographic resolution between chlorfenvinphos and terbutryn.

[l] Chromatographic resolution between pirimiphos-methyl and chlorfenvinphos.

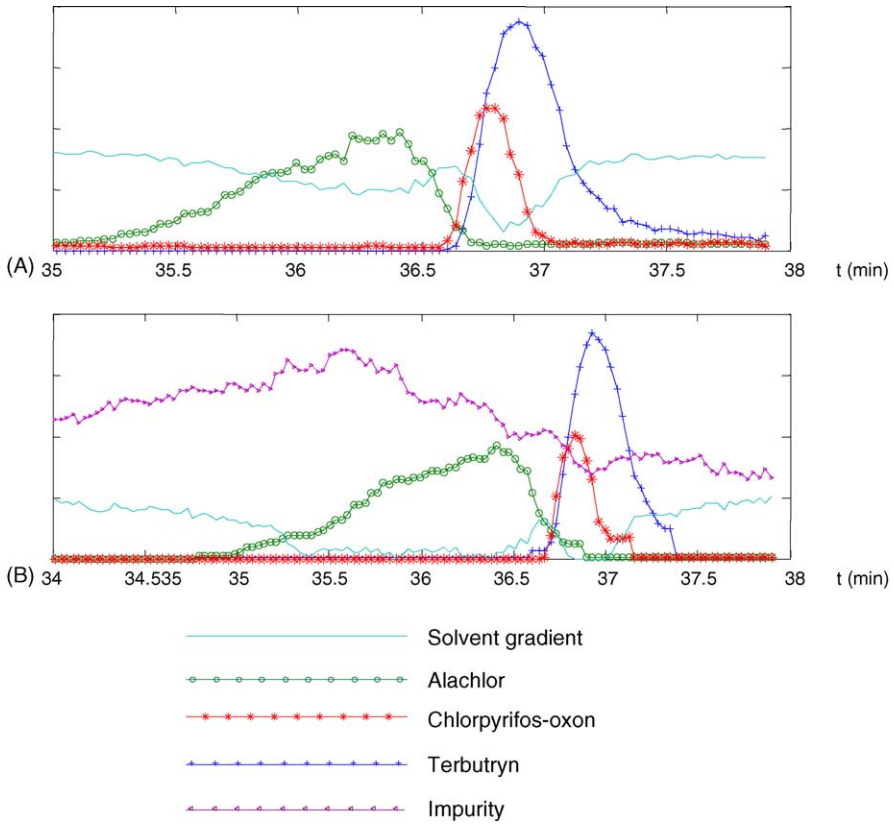

Fig. 5. MCR-ALS resolution of environmental samples spiked with biocides at 10 μg/mL, in the 25 cm LC column. (A) MCR-ALS resolved elution profiles of Aznalcóllar sediment sample (Fig. 1B). Resolved compounds in elution order: alachlor, chlorpyrifos-oxon and terbutryn. Solvent gradient is included. (B) MCR-ALS resolved elution profiles of In-WWTP water sample from La Llagosta (Fig. 1C). Resolved compounds in elution order: alachlor, chlorpyrifos-oxon and terbutryn. Solvent gradient and a very large sample matrix interference are included.

complexity of the analyzed environmental samples with little sample pretreatment. Specifically, alachlor, chlorpyrifos-oxon and terbutryn gave 12.1%, 16.5% and 15.7% relative errors, respectively in the sediment sample; and 7.9%, 5.3% and 13.9% relative errors in the wastewater sample.

4.2. Resolution and quantitation of coeluted biocides by LC–MS and MCR-ALS for 7.5 cm LC column

Standard mixture samples of the thirteen different biocides at different concentration levels of 20, 10, 5, 1, 0.5 and 0.1 μg/mL, were also analyzed using the 7.5 cm LC column with a rapid gradient. Whereas in the 25 cm LC column, elution of all the compounds lasted approximately 50 min (Fig. 1), in the 7.5 cm LC column, the same compounds in the same mixture samples were eluted in less than 5 min (Fig. 2). However, and as it is clearly apparent from comparison of Figs. 1 and 2, chromatographic resolution was worse when short columns were used. As before, for the 25 cm LC column, MCR-ALS was applied to column-wise augmented data matrices formed by standard mixture samples and individual standard samples (Eq. (2)).

In the analysis of standard mixture samples with the 7.5 cm LC column, coelution problems became significantly stronger and were more difficult to resolve than using the 25 m LC column. Only results obtained in the investigation of the coelution area, encircled in black in Fig. 2A, are given in detail for brevity and to compare with previous results obtained using the 25 cm LC column. In Fig. 6, results of MCR-ALS analysis of Fig. 2 data are shown. For all standard mixture samples, six coeluted compounds were identified and resolved. Alachlor, chlorpyrifos-oxon, terbutryn and chlorfenvinphos were coeluting approximately at the same elution time (Fig. 6). The first eluted compound was atrazine, and the sixth and last one was pirimiphos-methyl. Atrazine tail was coeluted with alachlor ($R_s = 0.45$), which was partially embedded inside clorpyrifos-oxon and terbutryn elution profiles. Chlorpyrifos-oxon and terbutryn were coeluted practically at the same time ($R_s < 0.1$), and partially coeluted at high elution times with chlorfenvinphos. Chlorfenvinphos was present as its two isomers (E, Z) with identical mass spectrum but giving two peaks of different height, the shorter and first one around 3.5 min and the higher and second one around 3.8 min. The resolution of these two peaks of cholrfenvinphos was possible because unimodality constraint was not applied in this case The first peak of chlorfenvinphos was totally inside alachlor. Since there was a complete overlap between these two compounds using the short 7.5 cm LC column, the same resolution as with MCR-ALS of scan mode data could only have been possible using MS SIM mode. Therefore, the use of the pro-

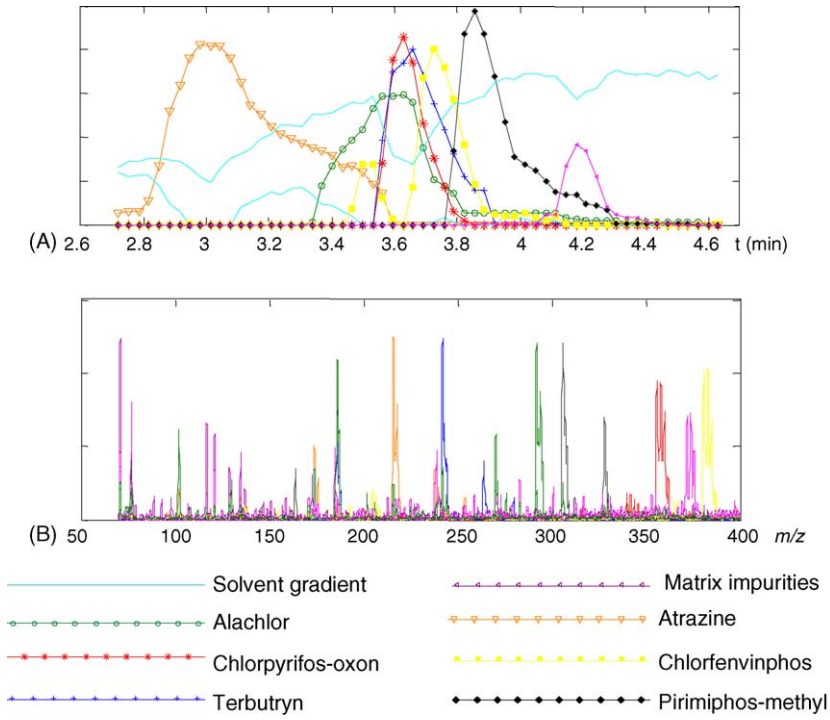

Fig. 6. MCR-ALS resolution of the standard mixture of biocides at concentration of 20 μg/mL in the 7.5 cm LC column (Fig. 2A). (A) MCR-ALS resolved elution profiles. Resolved compounds in elution order: atrazine, alachlor, chlorpyrifos-oxon, terbutryn, chlorfenvinphos and pirimifos-methyl. (B) MCR-ALS resolved MS spectra. Compound identification: atrazine *m/z*: 216, 238, 174; alachlor *m/z:* 292*;* chlorpyrifos-oxon *m/z:* 356, 358; terbutryn *m/z*: 242, 186*;* chlorfenvinphos *m/z:* 381, 383*;* pirimiphos-methyl *m/z:* 306, 164, 108.

posed MCR-ALS resolution method gives an alternative possibility to solve this type of extreme coelutions. The second chlorfenvinphos peak was coeluted with pirimiphos-methyl profile ($R_s = 0.16$). Finally all of them had also the solvent gradient interference and a big unknown matrix interference or impurity which is coeluted with all the compounds and with the solvent. The solvent gradient profile in this case was not resolved as well as for the 25 cm LC column, probably due to the presence of other unknown interferences not explicitly taken into account in the model and to its different experimental implementation (see Section 2.3). In this case solvent gradient needed more than one contribution (dot lines in Fig. 6) to be modelled. In Fig. 6, MCR-ALS resolved MS spectra are also shown. Although most of coelutions present in the samples were untreatable by MS scan mode chromatography, all peaks could be identified and quantified by MCR-ALS (atrazine *m/z*: 216, 238, 174; alachlor *m/z*: 292; chlorpyrifos-oxon *m/z*: 356, 358; terbutryn *m/z*: 242, 186; chlorfenvinphos *m/z*: 381, 383; pirimiphos-methyl *m/z*: 306, 164, 108; solvent ions were not seen in the range under study (70–400 amu); there were plenty of not identified matrix and interference ions from 70 to 400 amu.

Pure and mixture standard samples at different concentrations levels were analyzed together (Eq. (2)). MS signals of the standards at the higher concentrations (20, 10 and 5 μg/mL) did not follow a linear increase with concentration since they nearly did not change of height when their concentration in the mixtures increased. It was not possible to build any calibration curve with them Standard mixture samples at lower concentrations (1, 0.5 and 0.1 μg/mL) had a better signal to concentration relation, which allowed building calibration curves for quantitative estimations at these low concentrations using the 7.5 cm LC column. Correlation coefficients were always higher than 0.9 units (see Table 1). Estimated relative quantitative errors were always below 30%.

The two different environmental samples (sediment and waste water samples) spiked with the biocides at 10 μg/mL, were also analyzed using the 7.5 cm LC column as for the 25 cm LC column. In Figs. 2B and C, the corresponding chromatograms obtained using the 7.5 cm LC column are shown. Wastewater sample was more difficult to resolve than the sediment sample because of the larger amount of interferences present in this sample which was submitted to very little sample pretreatment (see Section 2.2). However, even in this complex case MCR-ALS resolution was also possible.

In both cases, all compounds were totally coeluted with matrix interferences. As it is possible to see in Fig. 7A, the sediment sample had all compounds coeluted in a cluster but atrazine. Atrazine was only coeluted, in its tail, with alachlor. Chlorpyrifos-oxon and terbutryn were strongly coeluted with a $R_s < 0.1$, one embedded inside the other. Alachlor was also coeluted with both of them as well as pirimiphos-methyl, which was the last and less coeluted compound of the cluster. Chlorfenvinphos was eluted in the middle of the cluster, and coeluted with all other four compounds. Chlorfenvinphos isomers, E and Z, could be resolved. Obviously unimodality

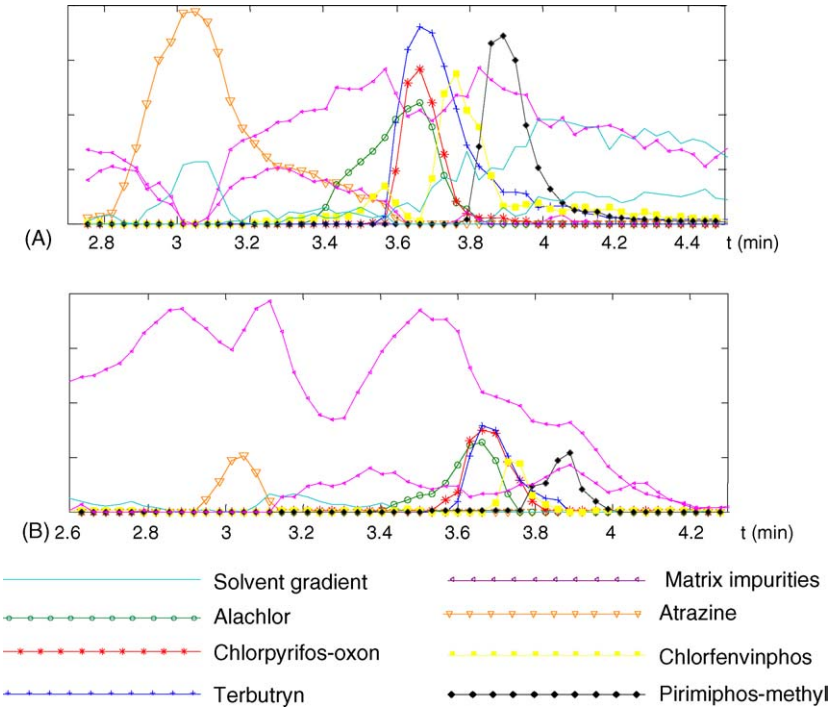

Fig. 7. MCR-ALS resolution of the environmental sample spiked with biocides at 10 μg/mL, analyzed using the 7.5 cm LC column. (A) Aznalcóllar sediment sample (Fig. 2B). Resolved compounds in elution order: atrazine, alachlor, chlorpyrifos-oxon, terbutryn, chlorfenvinphos and pirimifos-methyl. Solvent gradient and several sample matrix interferences are included. (B) In-WWTP water sample from La Llagosta (Fig. 2C). Resolved compounds in elution order: atrazine, alachlor, chlorpyrifos-oxon, terbutryn, chlorfenvinphos and pirimifos-methyl; solvent gradient and several matrix interferences with a large one embedding all the other compounds, are included.

constraint was not applied for this compound, in this case to allow the resolution of its two peaks with practically the same MS spectrum. The first peak of chlorfenvinphos was coeluted half inside alachlor ($R_S = 0.3$), and half inside chlorpyrifos-oxon and terbutryn ($R_S = 0.4$ and 0.5, respectively). The second peak of chlorfenvinphos was coeluted with the peaks of four other compounds; these from alachlor, chlorpyrifos-oxon, terbutryn and pirimiphos-methyl, respectively. Elution profiles of the wastewater sample are also shown in Fig. 7B. Elution order is the same as for the standard mixture and sediment samples, but in this case atrazine was not coeluted with any other of the studied compounds. Alachlor was coeluted with chlorpyrifos-oxon ($R_S < 0.1$) and with terbutryn ($R_S < 0.1$), which were also practically totally coeluted, one inside the other ($R_S < 0.1$). These three compounds were also coeluted with chlorfenvinphos. Chlorpyrifos-oxon and terbutryn were also coeluted with pirimiphos-methyl ($R_S = 0.35$). Finally, all these coeluted compounds were embedded inside a broad and large matrix impurity, responsible of the main trends of the shape of the reconstructed total ion current chromatogram of Fig. 2C.

Attempts to perform the quantitation of environmental samples spiked at 10 μg/mL were not possible because of signal saturation of the MS detector and no calibration with standard samples between 5 and 20 μg/mL was possible as mentioned above. Sediment and wastewater samples spiked at lower concentrations, under 0.5 μg/mL were investigated. In this case also (like for environmental samples spiked at 10 μg/mL), good resolution of studied compounds was achieved. Semi quantitative analysis was then attempted using calibration curves obtained from standard mixture samples at similar concentration levels. Quantitation errors of studied compounds are given for the sediment and wastewater samples in Table 1. In all cases, errors obtained for the 7.5 cm LC column were higher than errors obtained for the same samples in the 25 cm LC column.

In future works, alternative methods of calibration will be explored and adapted to the MCR-ALS strategy, including those based in the use of standard additions and of internal standards. Special attention will be paid to the development of these strategies to simultaneous quantitative determinations of multiple coeluted compounds in short column (fast) chromatography.

5. Conclusions

Complex environmental samples (sediment and wastewater samples) were analyzed by LC–MS in scan mode. Strong coelutions and matrix interferences found in the analysis of these samples have been solved by means of the multivariate curve resolution-alternating least squares chemometrics method. Once simpler standard mixture samples at different concentration levels were properly resolved, environmental

samples were also resolved and quantified. Quantitation errors in environmental samples were always below 20% for a traditional 25 cm LC column. When a 7.5 cm LC column (fast chromatography) was used instead of a 25 cm LC column, coelution and matrix effect problems increased significantly making their resolution and quantitation by MCR-ALS more challenging. In spite of these difficulties, the analyzed biocides were also properly resolved by MCR-ALS in both standard mixture samples and in sediment and wastewater samples although their concentrations could only be estimated at low concentrations due to the lack of linearity of the detector response at concentrations higher than 1 μg/mL. Combination of complex environmental matrices, embedded peaks, very strong coelutions and ion suppression effects were the main reasons why better quantitative estimations of coeluted compounds could not be possible for the 7.5 cm LC column.

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