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Quantification from highly drifted and overlapped chromatographic peaks using second-order calibration methods

Enric Comas, R. Ana Gimeno, Joan Ferré*, Rosa M. Marcé, Francesc Borrull, F. Xavier Rius

Department of Analytical Chemistry and Organic Chemistry, Rovira i Virgili University, Pl. Imperial Tarraco, 1, 43005- Tarragona, Spain

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Abstract

For determining low levels of pesticides and phenolic compounds in river and wastewater samples by high performance liquid chromatography (HPLC), solid phase extraction (SPE) is commonly used before the chromatographic separation. This preconcentration step is not necessarily selective for the analytes of interest and it may retain other compounds of similar characteristics as well. In this case, we present, humic and fulvic acids caused a large baseline drift and overlapped the analytes to be quantified. The inaccurate determinations of the area of the peaks of these analytes made it difficult to quantify them with univariate calibration. Here we compare three second-order calibration algorithms (generalized rank annihilation method (GRAM), parallel factor analysis (PARAFAC) and multivariate curve resolution–alternating least squares (MCR–ALS)) which efficiently solve this problem. These methods use second-order data, i.e., a matrix of responses for each peak, which is easily obtained with a high performance liquid chromatography–diode array detector (HPLC–DAD). With these methods, the area does not need to be directly measured and predictions are more accurate. They also save time and resources because they can quantify analytes even if the peaks are not resolved. GRAM and PARAFAC require trilinear data. Biased and imprecise concentrations (relative standard deviation, %R.S.D. = 34) were obtained without correcting the time-shift. Hence, a time-shift correction algorithm to align the peaks was needed to obtain accurate predictions. MCR–ALS was the most robust to the time-shift. All three algorithms provided similar mean predictions, which were comparable to those obtained when sulfite was added to the samples. However, the predictions for the different replicates were more similar for the second-order algorithms (%R.S.D. = 3) than the ones obtained by univariate calibration after the sulfite addition (%R.S.D. = 13).

Keywords: Peak overlap; Second-order calibration; GRAM; PARAFAC; MCR–ALS; Water analysis; Uncertainty reduction; Pesticides; Phenolic compounds

1. Introduction

High performance liquid chromatography with diode array detection (HPLC–DAD) is routinely used for the qualitative and quantitative analysis of natural samples. In optimized separation conditions, each chromatographic peak ideally corresponds to a single compound. Actually, peaks may overlap, particularly when the samples are environmental and biological and have a complex matrix. In this case, quantification with univariate calibration requires special attention in order to neither incorporate bias nor reduce precision.

One such case is shown in Fig. 1. The chromatogram is of a water sample from a sewage treatment plant, which is studied in this paper. The analytes of interest are two phenolic compounds (resorcinol and phenol) and two pesticides (oxamyl and methomyl). These compounds are potentially hazardous for the environment and human health, so they are regulated by the European Union (EU) to ensure good quality bathing [1] and drinking water [2]. Because of their low concentrations, a preconcentration step by solid phase extraction (SPE) is carried out before the chromatographic separation [3,4]. The SPE process also retained humic and fulvic acids because their polarity was similar to that of the analytes of interest. This caused a large peak at the beginning of the chromatogram (around 3–4 min) and baseline drift. This baseline drift considerably increases the uncertainty of the predicted concentration of resorcinol if univariate calibration is used, since it is not possible to know where the peak starts and finishes. Since the baseline cannot be defined precisely, both the area and the height of the peak will be uncertain. Moreover, univariate calibration requires

* Corresponding author. Tel.: +34-977-559564; fax: +34-977-559563.
E-mail address: ferre@quimica.urv.es (J. Ferré).

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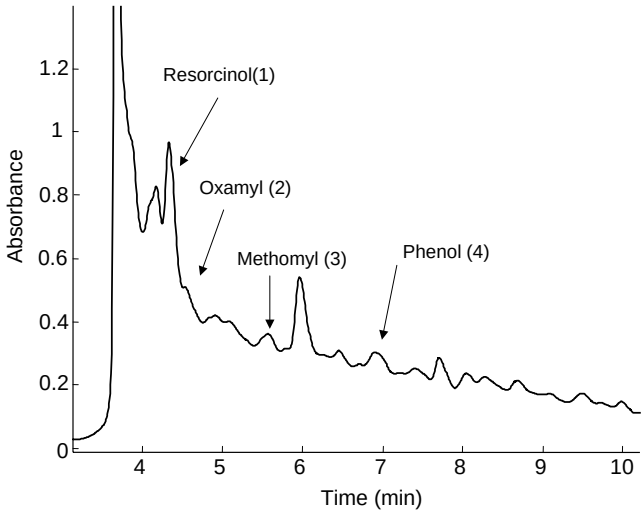

Fig. 1. Chromatographic profile of the sewage treatment plant sample measured at 240 nm. The analytes of interest are indicated.

selective measurements, i.e., the area or height of the peak must be due only to the analyte of interest. Here, it is difficult to check whether other compounds of similar polarity coeluted with the analyte of interest, since the spectrum at each retention time also contains the contribution of the humic and fulvic acids. Hence, the peak purity parameter that is commonly found in the software of the HPLC instrument will fail.

The analytes of interest can be determined more precisely by changing the experimental conditions to achieve full resolution. This involves spending time and resources and there is no guarantee that the separation will be complete. In particular, resorcinol is difficult to isolate from humic and fluvic acids because their chemical properties are similar.

A second option is to add sodium sulfite (Na_2SO_3) to the sample before it is preconcentrated [5]. This compound reacts with the humic and fulvic acids and makes them elute separately from the analytes of interest. However, the effect of sodium sulfite depends on the sample matrix and in some cases, such as the analyses of water from a sewage treatment plant (see below), it is not useful.

In this paper, we study and apply a third solution: the chemometric processing of the peak, in order to obtain the net contribution of the analyte of interest. This can be done with a variety of mathematical approaches. Basically, when the detection is based on absorbance responses in the UV-Vis region, they can be classified into two groups: those based on mono-channel detection, i.e, one absorbance value measured at each retention time; and those based on multi-channel detection, i.e., a UV-Vis spectrum measured at each retention time.

The approaches that use mono-channel detection include neural networks [6], genetic algorithms [7], differential signal detection [8] and the development of a set of equations that model the chromatographic peak [9,10]. One of the drawbacks of these methods is that they must assume that the chromatographic profile has a particular shape and that each peak has a number of analytes. Meyer [11,12] fully discussed how the area of the peak should be measured for different experimental situations. However, these conditions were limited to overlapping peaks containing only the analyte of interest and a single interference.

Here, we show that multi-channel detection with HPLC–DAD instruments can be used to treat this problem in a more

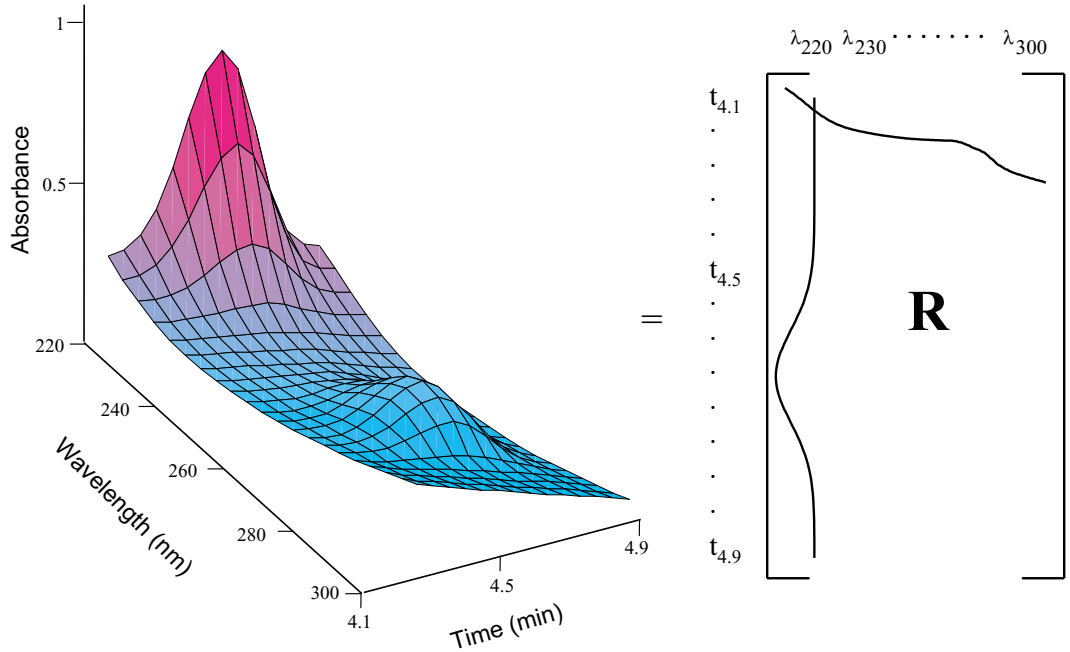

Fig. 2. Second-order data and its equivalence in matrix notation for the resorcinol peak.

efficient way. Since we can measure the spectrum at each retention time, a matrix of absorbances can be obtained for each peak analyzed: a second-order data matrix. Each row of the matrix is a spectrum measured at each retention time. Each column is a chromatographic profile at one wavelength. Fig. 2 shows the second-order data matrix of the resorcinol peak.

Several algorithms can be used to predict the analyte concentration in a non-resolved peak using second-order data [13–17]. Here, we compare the performance of the three that are most commonly used: generalized rank annihilation method (GRAM) [13], parallel factor analysis (PARAFAC) [14] and multivariate curve resolution–alternating least squares (MCR–ALS) [15]. They make quantification possible even if the test sample contains interferences that are not considered in the calibration samples. This is known as "the second-order advantage" [18]. This advantage is particularly looked for in our case where the interferences in the sample are the humic and fulvic acids.

Mitchell and Burdick [19] argued that PARAFAC and MCR–ALS have better properties and that their results are more reliable than those of GRAM. Recently, Faber [20] compared them in a simulation study and concluded that GRAM can also be a useful option in many cases. Here, we extend Faber's study to a real case: the analysis of water samples from the Ebre river (Spain), and from a sewage treatment plant in Tarragona (Spain). Hence, the objective of this paper is two-fold: (a) to demonstrate that GRAM, PARAFAC and MCR–ALS can be used to quantify from highly drifted and overlapping peaks and (b) to point out in which situations one method is better than the others. Their results were externally validated by a reference methodology based on chromatographic optimization and univariate calibration.

2. Experimental section

2.1. Reagents and standards

The compounds studied were: (1) resorcinol (Sigma, Madrid, Spain), (2) oxamyl (Riedel-de-Haën, Seelze, Germany), (3) methomyl (Riedel-de-Haën), (4) phenol (Aldrich Chemie, Beere, Belgium), (5) 4-nitrophenol (Aldrich Chemie), (6) 2,4-dinitrophenol (Aldrich Chemie). They are all more than 97% pure. Standard solutions at a concentration of 2000 mg l^{-1} were prepared in acetonitrile (SDS, Peypen, France) for compound 1 and methanol (SDS) for the other compounds. These solutions were stored at 4 °C. All the working solutions were prepared by diluting these standard solutions. Analytes 1 to 4 were to be determined. Analytes 5 and 6 were included to test the reproducibility of the system.

HPLC gradient grade acetonitrile (SDS) was used for the mobile phase in the chromatographic separation and the extraction process. Ultra pure water was prepared by ultra filtration with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Hydrochloric acid (Probus, Barcelona, Spain) was used to adjust the pH of the mobile phase and the samples. In the validation of the results obtained by the second-order algorithms, sodium sulfite (Probus) was added to reduce the peak at the beginning of the chromatogram caused by humic and fulvic acids in the water samples.

2.2. Samples

Samples were collected from the Ebre River (Spain) and from the output of the sewage treatment plant in Tarragona (Spain) in precleaned amber glass bottles. The pH of these samples was adjusted to 2.5 with hydrochloric acid in order to prevent the compounds of interest from being in ionic form. They were filtered through a 0.45 μm membrane filter and kept at 4 °C until analysis.

The analytes of interest have only occasionally been found in this kind of samples [4]. To ensure that they were actually present, the samples were spiked at different levels of concentrations. One aliquot of the river-water sample was spiked at 5 μg l^{-1} for resorcinol and at 1 μg l^{-1} for the other analytes. This sample was taken as the test sample. In the same way, one aliquot was spiked at 20 μg l^{-1} for resorcinol and at 5 μg l^{-1} for the other analytes. This sample was taken as the calibration sample.

The sample from the sewage treatment plant was treated in the same way. Here the levels were 20 μg l^{-1} for resorcinol and 5 μg l^{-1} for the other analytes in the test sample, and 80 μg l^{-1} for resorcinol and 20 μg l^{-1} for the other analytes in the calibration sample.

2.3. Instrumental

The chromatographic separation was carried out using an HP1100 system (Agilent Technologies, Waldbronn, Germany). This system consisted of a degasser, two isocratic pumps, a manual injector provided with a 20 μl loop, a column oven and a DAD. Each pump was used to deliver one fraction of the mobile phase. Separation was carried out using a 25 cm × 0.46 cm Kromasil 100 C_{18} chromatographic column with a 5 μm particle size (Teknokroma, Barcelona, Spain).

For on-line SPE, an Applied Biosystems pump (Ramsey, USA) was used to preconcentrate samples through a stainless steel precolumn (10 mm × 3 mm, i.d.) (Free University, Amsterdam, The Netherlands), which was laboratory-packed with isolute ENV + sorbent (International Sorbent Technology, Mid. Glamorgan, UK).

Chromatographic and extraction systems were on-line coupled by means of a Rheodyne 7010 valve. The set-up of the system allowed the compounds retained in the extraction cartridge to be eluted with only the organic part of the mobile phase [21]. This set-up was used to prevent the peaks from broadening out because of the low elutropic force of the mobile phase.

2.4. Experimental conditions

2.4.1. Separation

Chromatographic separation was performed under gradient conditions. The mobile phase consisted of acetonitrile and Milli-Q water (pH 3 adjusted with hydrochloric acid to prevent the column degradation). The gradient started with 20% of acetonitrile and it was linearly increased to 55% in 20 min and then to 100% in 5 min. This percentage was maintained for 10 min to return to the initial conditions in 5 min. The column was equilibrated for 5 min. The temperature of the column was 65 °C and the mobile phase flow rate was 1 ml min^{-1}. The spectrum of the effluent was recorded between 220 and 300 nm every 0.4 nm. For univariate calibration, absorbance at 240 nm was used.

2.4.2. Solid phase extraction

The on-line solid phase extraction was as follows: the precolumn was first washed with 10 ml of acetonitrile and then with 10 ml of Milli-Q water (pH 2.5 adjusted with hydrochloric acid) at 4 ml min^{-1}; the position of the valve was changed and the tubes were then purged with the sample; finally, the appropriate volume of sample was preconcentrated at 4 ml min^{-1}. The retained analytes were eluted in back-flush mode by means of the acetonitrile of the mobile phase when the valve position was changed again. The sample volume preconcentrated was 100 ml for the river-water and 25 ml for the sewage treatment plant water.

For univariate calibration, 1 ml of sodium sulfite 10% (w/v) solution was added to the sample before it was preconcentrated in order to decrease the high peak that appears at the beginning of the chromatogram when the river-water was preconcentrated.

2.5. Algorithms

Three second-order calibration methods were considered: generalized rank annihilation method, parallel factor analysis and multivariate curve resolution–alternating least squares. The three methods decompose the chromatographic peak into pure chromatographic profiles and their corresponding spectra. By including samples with known concentration, they can be used as calibration methods. So, we tested how well they predicted the concentration of the analytes of interest when the baseline drift was large, this drift being caused by the presence of the humic and fulvic acids.

The equations can be found elsewhere [13–15]. Briefly, GRAM only needs the peak of the analyte from a calibration sample (which can be either a pure standard [22] or a spiked sample [23]), and the peak of interest in the test sample. This is very attractive for the routine use of chromatography, since there is no need to measure additional samples, which is an important saving of time and resources. The algorithm is non-iterative and based on the resolution of an eigenvalue problem. It is very fast (less than 1 s on a Pentium IV 1.4 GHz) and figures of merit can be calculated easily [24,25].

PARAFAC and MCR–ALS are iterative methods and can work with more than two samples. They need initial estimations of the chromatographic profiles or the spectra to start the iterative process [26]. Here we used, as initial chromatographic profiles, the solutions of the evolving factor analysis [27] applied to the test sample. An attractive property of PARAFAC is that the decomposition of the peak is unique, with no rotational ambiguities. To improve the solutions from PARAFAC and MCR–ALS, constraints in the iterative process are imposed, based on the chemical knowledge of the system. For chromatographic peaks, we imposed that the chromatographic profiles and the spectra had to be non-negative and that the chromatographic profile of each analyte had to be unimodal (one maximum only).

GRAM and PARAFAC require perfect trilinear data whereas MCR–ALS does not. Trilinearity can be viewed as an extension of Beer's law to second-order data. This amounts to assuming that the measured peak is the sum of the individual peaks of each analyte and that the profile and the spectrum of one analyte are proportional in all the samples. However, trilinearity is not always accomplished in chromatography. For it to be so, the profile of the analyte of interest must elute at exactly the same retention time in all the samples. In practice, time-shift is usual in this kind of analysis [22] because of imprecision in the injection or fluctuations of pressure and temperature in the on-line system. Moreover, as the chromatographic separation is done in gradient mode, time-shift is even more significant than when isocratic conditions are used. Several methods have been proposed for correcting the time-shift [28,29]. Prazen et al. [28] plotted the eigenvalues of the augmented matrix containing the calibration sample peak and the test sample peak, for different time windows of the test sample. A minimum in the plot indicated the optimal window. Comas et al. [29] selected the time window of the test sample after the deconvolution of the calibration and the test samples independently, using a curve resolution method, the iterative target transformation factor analysis (ITTFA). Both methods were tested in a preliminary step and provided the same results. The one described by Comas et al. [29] was used.

2.6. Validation of the results from second-order algorithms

Validation of the predictions from second-order calibration algorithms is currently an active area of research [22,30]. The philosophy underlying these algorithms is different than for multivariate calibration methods such as partial least squares (PLS) or principal components regression (PCR). In multivariate calibration, calibration and prediction are independent steps. Hence, we can check the performance of the model before it is used for prediction. In second-order calibration, both calibration and prediction are performed in one step, and both calibration and prediction samples are used at the same time. That is to say, a

new model is calculated for each sample analyzed. Hence, methods are needed to check that the model is calculated correctly and to guarantee as far as possible the accuracy of the predicted concentration in the test sample. This process, which is called internal validation, is possible thanks to the fact that the three methods studied provide the pure spectrum and the chromatographic profile of each analyte. If the calculated spectra are comparable with the true ones (known from standards), and the estimated chromatographic profiles are non-negative and unimodal, the confidence that the predictions are correct is greater.

2.7. Software

The PARAFAC routine belongs to the N-way toolbox of R. Bro and C. Andersson and was downloaded from their website [31]. The MCR–ALS routine belongs to the MCR toolbox of R. Tauler and A. de Juan and was downloaded from their website [32]. We made the GRAM and ITTFA algorithms subroutines in house for MATLAB version 6 [33].

2.8. Data acquisition and data processing

The following procedure was used:

(1) The reproducibility of the on-line preconcentration and separation system was estimated before the second-order calibration methods were applied and validated. Poor reproducibility would make the study meaningless.
(2) Each sample was analyzed by the on-line SPE–HPLC–DAD method, and the second-order chromatogram was recorded.
(3) For both the calibration (with known concentration of the analytes of interest) and test samples, we manually selected the time window in which each analyte of interest eluted. When the start and the end of the peak was uncertain (e.g., resorcinol in Fig. 1), we considered a wider range. The start and end of the peaks need not be precisely estimated for second-order calibration algorithms. These algorithms also make quantification possible with only a fraction of the peak.
(4) The chromatographic profiles were aligned with a time-shift correction algorithm [29]. This was necessary for GRAM and PARAFAC.
(5) GRAM, PARAFAC and MCR–ALS were applied to the corrected peaks.
(6) The predictions were internally validated by checking that the predicted spectra were similar to the spectra of the pure analytes, and that the chromatographic profiles were non-negative and unimodal. This gave confidence in the predictions.
(7) The predictions were externally validated. They were compared to the predictions obtained by adding sodium sulfite to the sample and using univariate calibration. External validation was only possible for the river-water sample. Sodium sulfite had no effect on the water from the sewage treatment plant.

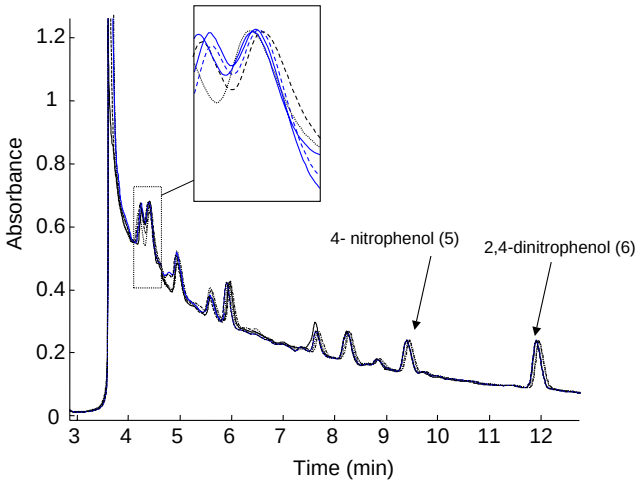

Fig. 3. Five replicates of the river-water sample with absorbance measured at 240 nm and used to check the reproducibility of the analytical system.

3. Results and discussion

3.1. River-water sample

In order to check the reproducibility of the analytical procedure, we analyzed five replicates of the calibration sample (i.e., five aliquots of the same spiked sample). The reproducibility was checked both graphically and by measuring the area of the peaks. Measuring the area in highly drifted peaks was not easy so we added analytes 5 (4-nitrophenol) and 6 (2,4-dinitrophenol) to the sample. These compounds are less polar so they eluted at 9.5 and 12 min, respectively, far from the peak of the humic and fulvic acids. Fig. 3 shows the chromatographic profile of the five replicates measured at 240 nm.

Table 1 shows the mean value of the area calculated by the integration algorithm of the HPLC instrument and its relative standard deviation (R.S.D.) expressed as a percentage. Taking into account the low concentration levels determined, the reproducibility of the on-line system is acceptable for this kind of analysis, and it is similar to what has already been reported [4].

A closer look at the peak of resorcinol in Fig. 3 shows that the maximum of the peak in the different replicates was not at the same retention time, but that the maximum absorbance was the same. This time-shift is usual in this kind of analysis

Table 1
Area of the peaks in the different replicates of river-water

Analyte	Mean value	R.S.D. (%)
Resorcinol	523.2	10.0
Oxamyl	741.2	2.5
Methomyl	663.5	3.4
Phenol	409.2	3.1
4-Nitrophenol	665.5	2.1
2,4-Dinitrophenol	1777	1.1

Table 2
Time range selected for each analyte

Analyte	Initial time (min)	Final time (min)
Resorcinol	4.16	4.83
Oxamyl	4.78	5.30
Methomyl	5.44	5.85
Phenol	8.05	8.66
4-Nitrophenol	9.11	9.75
2,4-Dinitrophenol	11.84	12.30

and had to be corrected before GRAM and PARAFAC were applied.

Once the reproducibility had been assessed, the test samples were analyzed under the same conditions. The selected time ranges where each analyte eluted are shown in Table 2.

GRAM, PARAFAC and MCR–ALS were run with only two matrices, i.e., one calibration and one test sample. Since we used two replicates for the calibration sample and two for the test samples, we built four models for each algorithm and analyte. In MCR–ALS, the matrices were considered column-wise, i.e, the spectra were considered to be common in both matrices. In all cases the number of factors needed to run these algorithms corresponded to the sum of the number of analytes in both matrices. Several methods have been developed to determine the number of factors [30,34–36]. The one used here was the *F*-test [36]. In all cases the number of factors was either 2 or 3, but never 1, which is what is required for univaritate calibration. Table 3 shows the mean predicted concentration (from the four models) and its relative standard deviation (%) when the same time window was considered for the calibration and test samples (before SC in Table 3) and after the time-shift had been corrected (after SC). When the time-shift was not corrected, the three methods gave substantially different predictions, especially for resorcinol and oxamyl. Also, the predicted concentrations are very dissimilar among replicates, resulting in an increase in the R.S.D. value. The reason for this is that GRAM and PARAFAC require trilinear data, whereas ALS does not. When the time-shift was corrected, the predictions of the three methods were similar and the R.S.D. for each analyte was considerably reduced. GRAM and PARAFAC predicted very similar concentrations, and the four models provided close predictions. On the other hand, the predictions made by MCR–ALS with and without correction of the time-shift are very similar. This was to be expected since MCR–ALS does not require the data to be trilinear in the time mode.

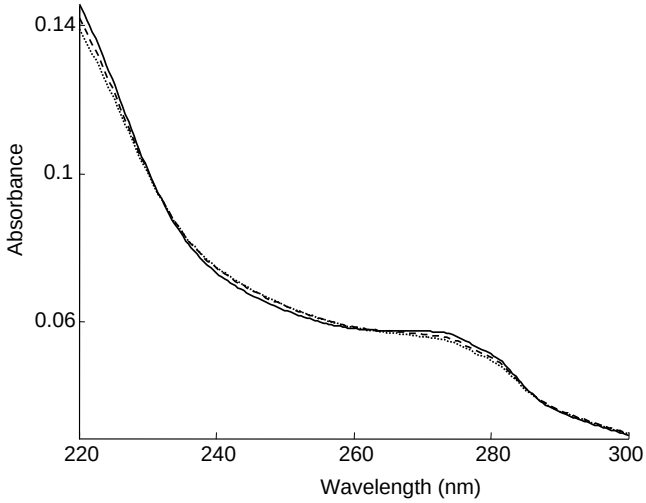

Fig. 4. Spectra provided by GRAM (–), PARAFAC (· · ·) and MCR–ALS (- -).

The reliability of the results was first checked by internal validation. Fig. 4 compares the spectra of resorcinol obtained with GRAM, PARAFAC and MCR–ALS. All three spectra are very similar, with correlation coefficients higher than 0.999 which shows that the results of the three methods are similar. An extensive study is being carried out in our laboratory to test which is the threshold value in the correlation coefficient to be confident of the predictions.

Finally, the predictions were externally validated with univariate calibration. Sodium sulfite was added to the sample to decrease the large band corresponding to the humic and fulvic acids. Fig. 5 shows the chromatogram at 240 nm of the same sample before and after sodium sulfite had been added. The sulfite was successful at removing the peak of fulvic and humic acids and univariate calibration could be used since the area of each peak was determined more accurately.

The river-water sample was spiked at different concentration levels. Those samples were analyzed in the same conditions as the previous samples and the univariate models were constructed. For each spiked aliquot, three replicates

Table 3
Mean value ($\mu g\,l^{-1}$) and its R.S.D. (%) of the predicted concentration with second-order calibration methods, considering the same time range in the calibration and test sample (before SC) and after the time-shift had been corrected (after SC)

Analyte	GRAM				MCR–ALS				PARAFAC			
	Before SC		After SC		Before SC		After SC		Before SC		After SC	
	Mean	R.S.D.	Mean	R.S.D.	Mean	R.S.D.	Mean	R.S.D.	Mean	R.S.D.	Mean	R.S.D.
Resorcinol	10.33	21.3	4.39	8.2	5.41	5.8	5.11	6.1	4.18	34.1	4.20	12.6
Oxamyl	0.98	3.6	0.95	3.4	1.18	13.0	1.12	1.8	0.89	21.6	0.96	3.8
Methomyl	0.98	5.1	1.00	3.1	1.04	1.1	1.04	1.0	0.95	5.7	1.00	3.9
Phenol	1.28	6.4	1.31	3.0	1.21	1.5	1.20	1.2	1.26	8.4	1.30	2.9

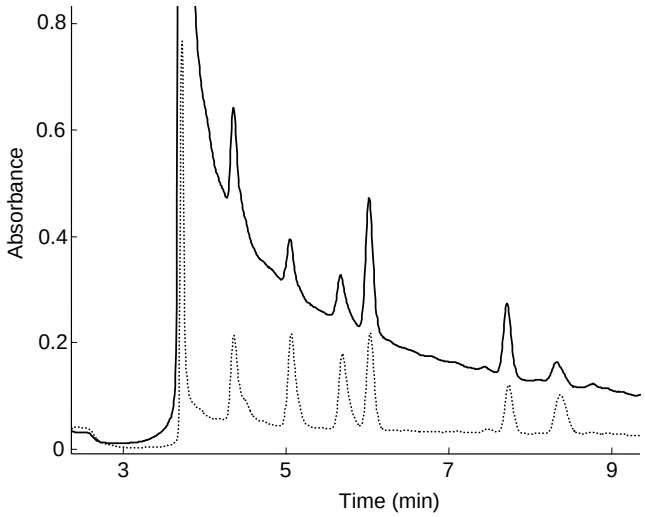

Fig. 5. Chromatographic profiles of the river-water measured at 240 nm before (–) and after (···) sodium sulfite was added.

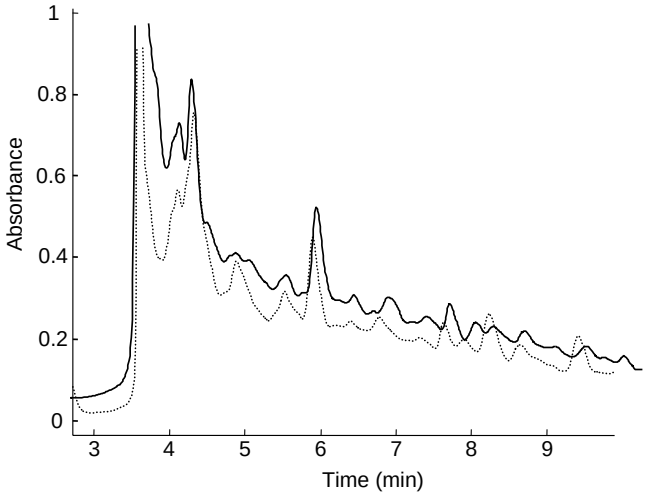

Fig. 6. Chromatographic profiles of the sewage treatment plant water measured at 240 nm before (–) and after (···) sodium sulfite was added.

were analyzed and the compounds studied were quantified. Table 4 shows the results.

As we can see, the results are similar to those in Table 3. A two-sided *t*-test was used to compare the results obtained by the different methods with those obtained with univariate calibration. In all cases, the results were similar for a confidence interval of 95%. This validates the results obtained from the second-order calibration methods.

Hence, any of the three methods can be used, but the MCR–ALS has the advantage that the shift is not a problem as it is in GRAM and PARAFAC. As far as practical aspects of the algorithms are concerned, GRAM is faster and no initial estimations are needed, while MCR–ALS and PARAFAC are iterative and the time needed for completion depends on how similar the initial estimation and the final solution are.

3.2. Sewage plant water

Three calibration samples and three test samples were analyzed in accordance with the conditions in the Experimental section. Unlike the river-water samples, the addition of sodium sulfite had hardly any effect on the organic matter that produced the high band at the beginning of the chromatogram (Fig. 6). Hence, the peaks of the analytes could not be resolved chemically and the external validation could not be done as it was for the river-water sample.

Table 4
Mean predicted concentration ($\mu g\,l^{-1}$) and its R.S.D. found by univariate calibration in the water sample with added sodium sulfite

Analyte	Univariate calibration	
	Mean value	R.S.D. (%)
Resorcinol	5.60	25.1
Oxamyl	1.09	13.2
Methomyl	1.10	8.1
Phenol	1.26	8.6

Table 5
Mean predicted concentration in sewage water ($\mu g\,l^{-1}$) and its R.S.D.

Analyte	GRAM		MCR–ALS		PARAFAC	
	Mean value	R.S.D. (%)	Mean value	R.S.D. (%)	Mean value	R.S.D. (%)
Resorcinol	68.65	7.4	66.86	3.9	62.58	14.1
Oxamyl	5.21	1.7	5.28	7.8	4.96	2.0
Methomyl	6.87	49.2	6.61	35.1	6.39	13.7
Phenol	7.74	0.6	8.08	4.6	7.71	1.0

The concentration of the analytes of interest could only be determined using second-order calibration. Table 5 shows the predictions in $\mu g\,l^{-1}$. For methomil, there was a large difference because when it eluted it was largely overlapped with other interferences. For the three methods, the predicted spectra were similar to the ones measured with standards, with correlation coefficients higher than 0.999. Unlike the river-water, where we recovered approximately the spiked amount, the predicted concentration for resorcinol was significantly larger than what we spiked. To check whether the analyte was present in that sample, we applied GRAM, PARAFAC and MCR–ALS using the non-spiked sample as a test sample. The predicted concentrations were 48.6, 47.3 and 44.4 $\mu g\,l^{-1}$, respectively. This agrees with the obtained value presented in Table 5, which corresponds to the concentration found in the non-spiked sample, plus the amount spiked (20 $\mu g\,l^{-1}$).

4. Conclusions

GRAM, PARAFAC and MCR–ALS were able to quantify overlapped and highly drifted chromatographic profiles. Such profiles can be found in the determination of

compounds at very low concentrations ($\mu g\,l^{-1}$) in natural samples. With these methods it is not critical to assess where the peak starts or finishes. Of the three second-order calibration methods, GRAM is fast, and requires only two matrices and no initial estimations of the chromatographic profiles and the spectra of the analytes. Also the figures of merit can be easily calculated. On the other hand, PARAFAC and MCR–ALS are iterative. GRAM and PARAFAC require trilinear data, which is difficult to achieve in this kind of data because of the time-shift in the chromatographic profiles.

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