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Development and validation of a method for the determination of regulated fragrance allergens by high-performance liquid chromatography and Parallel Factor Analysis 2



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ABSTRACT

This work presents the development and validation of a multivariate method for quantitation of 6 potentially allergenic substances (PAS) related to fragrances by ultrasound-assisted emulsification microextraction coupled with HPLC-DAD and PARAFAC2 in the presence of other 18 PAS. The objective is the extension of a previously proposed univariate method to be able to determine the 24 PAS currently considered as allergens. The suitability of the multivariate approach for the qualitative and quantitative analysis of the analytes is discussed through datasets of increasing complexity, comprising the assessment and validation of the method performance. PARAFAC2 showed to adequately model the data facing up different instrumental and chemical issues, such as co-elution profiles, overlapping spectra, unknown interfering compounds, retention time shifts and baseline drifts. Satisfactory quality parameters of the model performance were obtained ($R^2 \geq 0.94$), as well as meaningful chromatographic and spectral profiles ($r \geq 0.97$). Moreover, low errors of prediction in external validation standards (below 15% in most cases) as well as acceptable quantification errors in real spiked samples (recoveries from 82 to 119%) confirmed the suitability of PARAFAC2 for resolution and quantification of the PAS. The combination of the previously proposed univariate approach, for the well-resolved peaks, with the developed multivariate method allows the determination of the 24 regulated PAS.

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1. Introduction

Fragrances are important components of many products present in our daily life such as perfumes, cosmetics, personal care items, household and laundry products. Nowadays, there are more than 2500 fragrance ingredients, and although they are generally innocuous, they can sometimes cause skin irritation or allergic reactions. Indeed, currently, there is a list of 24 chemical compounds and two natural moss extracts related to fragrances with a well-known potential to cause allergic reactions [1,2]. These are known in the literature as potentially allergenic substances (PAS), and their usage in cosmetics must be declared on the label if their concentration is higher than 0.001% in leave-on products, and 0.01% in rinse-off products [3]. In addition, as PAS are present in all kind of cosmetics, they can be easily transferred to water in contact

with human body, where their widespread occurrence is notorious [4–6]. Therefore, there is a great interest in the development of sensitive analytical methods for PAS determination in cosmetic and water samples in order to check compliance with the current cosmetic legislation and to assure the quality of the water.

The usual analytical methods for the determination of PAS are mainly based on Gas Chromatography-Mass Spectrometry (GC-MS) systems [7–10]. To enhance the separation capability of this technique in complex samples, different methods based on multidimensional chromatography have been proposed [11–13], or more complex detectors have been used [14,15]. Furthermore, GC-MS systems have also been widely coupled to diverse sample pre-treatment techniques in order to preconcentrate and/or separate the analytes prior to determination. Used sample preparation techniques include liquid extraction [16,17], matrix solid-phase dispersion (MSPD) [18], micro-MSPD [15], solid-phase microextraction (SPME) [4], headspace (HS) SPME [6,19,20], pressurized liquid extraction [21,22], dispersive liquid-liquid microextraction [23] ultrasound-assisted emulsification microextraction [5]

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and dispersive solid phase extraction [24]. Far less often, GC-olfactometry has been used as supplement to GC-MS analysis [25] and the use of GC with flame ionization detector has been proposed coupled to SPME as well [26,27].

Apart from GC, other techniques such as liquid chromatography-MS [28], microemulsion electrokinetic chromatography-diode array detection (MEEKC-DAD) [29], micellar electrokinetic chromatography-DAD (MEKC-DAD) [30], attenuated total reflectance infrared spectroscopy (ATR-IR) [31] and high performance liquid chromatography-DAD (HPLC-DAD) [32] have also been used for determination of PAS. However, these alternative techniques were less sensitive than GC-MS systems. Besides, up to our knowledge, only HPLC-DAD has been successfully applied with a pre-treatment procedure in order to improve the sensitivity of the method [33]. In this previous work, we applied ultrasound-assisted emulsification microextraction followed by the solidification of the floating organic drop (USAEME-SFOD) as a sample pre-treatment technique to determine 18 PAS by posterior analysis with HPLC coupled with diode array detection (DAD). This simple pre-treatment allowed decreasing the limits of detection (LODs) in the PAS determination by HPLC-DAD, showing all the advantages of USAEME and SFOD techniques: analyte extraction was fast and efficient due to the high superficial area between the extraction solvent and the sample, and the technique was non-expensive and environmentally friendly due to the use of low volumes of practically non-toxic solvents [34,35]. Nevertheless, the main drawback of this method was its limited capability to univariately quantify only 18 out of the 24 PAS corresponding to well-defined chemical substances giving well-defined chromatographic peaks.

Ideally, well-resolved and not-overlapped peaks are desired for all the analytes present in a sample measured by chromatography, so that the quantification can be relatively easily achieved by univariate regression. However, this situation is not always possible and it is frequent to have partially (or even totally) overlapped peaks due to the complexity of the sample matrices, technical limitations, or simply due to the fact that faster analysis times are preferred. These instrumental and chemical issues hamper the performance of univariate methods for chromatographic data analysis, as occurs when the method developed to determine 18 PAS [33] was tested to quantify all the 24 PAS simultaneously. Fortunately, multi-way data analysis methods can overcome these issues providing adequate quantitative and qualitative information if proper data are available [36].

Hyphenated chromatographic systems, like HPLC-DAD, combine online separation techniques and spectroscopic detection methods to exploit the advantages of both approaches. These systems generate two-dimensional data (matrix) per sample by recording the signal intensity as a function of the spectral and elution dimensions. In this way, several samples (Fig. 1a) can be arranged together into three-way array structures (data cube), where the recorded signal (absorbance) is a function of the sample (K), the retention time (I) and the wavelength (J). This type of data is known as “second-order” data and it may be treated with multivariate methods that hold the so-called “second-order advantage”, which is the property of quantifying analytes even if there are unknown interfering compounds not previously modelled [37–40].

These three-way data arrays can be studied using a family of resolution models called three-way methods [36,38]. Amongst all the possible techniques, PARAllel FACTor Analysis (PARAFAC) has been frequently used to model multi-way chromatographic data [36,41,42]. PARAFAC is a decomposition method with which data arrays are decomposed as a sum of triple products of vectors [43]. These vectors are called loadings and each triplet of loading vectors is called “component”. Each component represents a source of data variation (it can be a compound, baseline...), and when chromato-

graphic data is decomposed, loadings of a component represent its chromatographic profile, its spectral profile and its relative weight in the different samples. The different loading vectors for all components can be arranged into three matrices (A–C), each one defining one mode (chromatographic, spectral and sample modes). Fig. 1b. shows a typical graphical representation of PARAFAC decomposition of data into a model with R components. As it can be seen, each component is defined by a unique chromatographic (retention time) loading in all samples (as example, the chromatographic loading of the first component is depicted by a blue curve and the one of the R component by a green curve). However, retention time shifts from sample to sample are frequent in chromatography as well as peak-shape changes and baseline drifts [44] and, therefore, all samples cannot always be explained by the same chromatographic loadings. In these cases, in order to make the data analyzable by PARAFAC, proper data pre-processing must be done prior to analysis. For example, correlation optimized warping (COW) can be used for peak alignment [45,46]. Nevertheless, data pre-processing can be complex and time consuming. An alternative to face this problem is to use PARAFAC2, which obtains different (but related) elution profiles for each of the analyzed samples [47–49]. Fig. 1c. shows a graphical representation of PARAFAC2 decomposition. In this case, for figure clarity, only the curves of the different chromatographic loading of two components have been depicted.

In this work, the applicability of PARAFAC2 for the extension of the USAEME-SFOD method coupled to HPLC-DAD [33] to determine hydroxycitronellal, coumarin, lylal, eugenol, citronellol and farnesol when the other 18 regulated PAS are present has been studied. These 6 compounds showed overlapped peaks when the 24 PAS were analysed simultaneously and quantification could not be done univariately even though different chromatographic conditions were probed. The application of a pre-concentration technique together with chemometrics to the analysis by HPLC-DAD is expected to improve both sensitivity and selectivity, spreading out the applications of the method.

2. Materials and methods

2.1. Reagents and standards

Hydroxycitronellal (95%), coumarin (99%), eugenol (99%), citronellol (95%), farnesol (98%), anisyl alcohol (98%), benzyl alcohol (99%), cinnamyl alcohol (98%), cinnamal (95%), isoeugenol (98%), geraniol (98%), linalool (97%), citral (96%), methyl-2-octynoate (99%), benzyl benzoate (99%) benzyl salicylate (99%), benzyl cinnamate (99%), lilyal (98%), amyl cinnamal (97%), α -isomethyl ionone (85%) hexyl cinnamal (95%) and limonene (96%) were all purchased from Sigma-Aldrich (Barcelona, Spain). Exceptions were lylal (97%) and amylcinnamyl alcohol (45.5%), which were supplied by Dr. Ehrenstorfer (Augsburg, Germany).

Acetonitrile (HPLC grade) was obtained from Romil (Cambridge, UK), 2-dodecanol (99%) was acquired from Sigma-Aldrich (Madrid, Spain), acetonitrile was obtained from Panreac (Barcelona, Spain) and doubly distilled water was used throughout the whole work.

2.2. Instrumentation, experimental procedure, chromatographic conditions and intervals selection

Experimental procedure, instrumentation and chromatographic conditions were reproduced as in [33]. In brief, the experimental procedure consisted on an ultrasound-assisted emulsification microextraction technique followed by the solidification of the floating organic drop and the coupling to HPLC-DAD.

The USAEME-SFOD procedure started with the addition of 50 μ L of 2-dodecanol (extraction solvent) to a previously prepared appro-

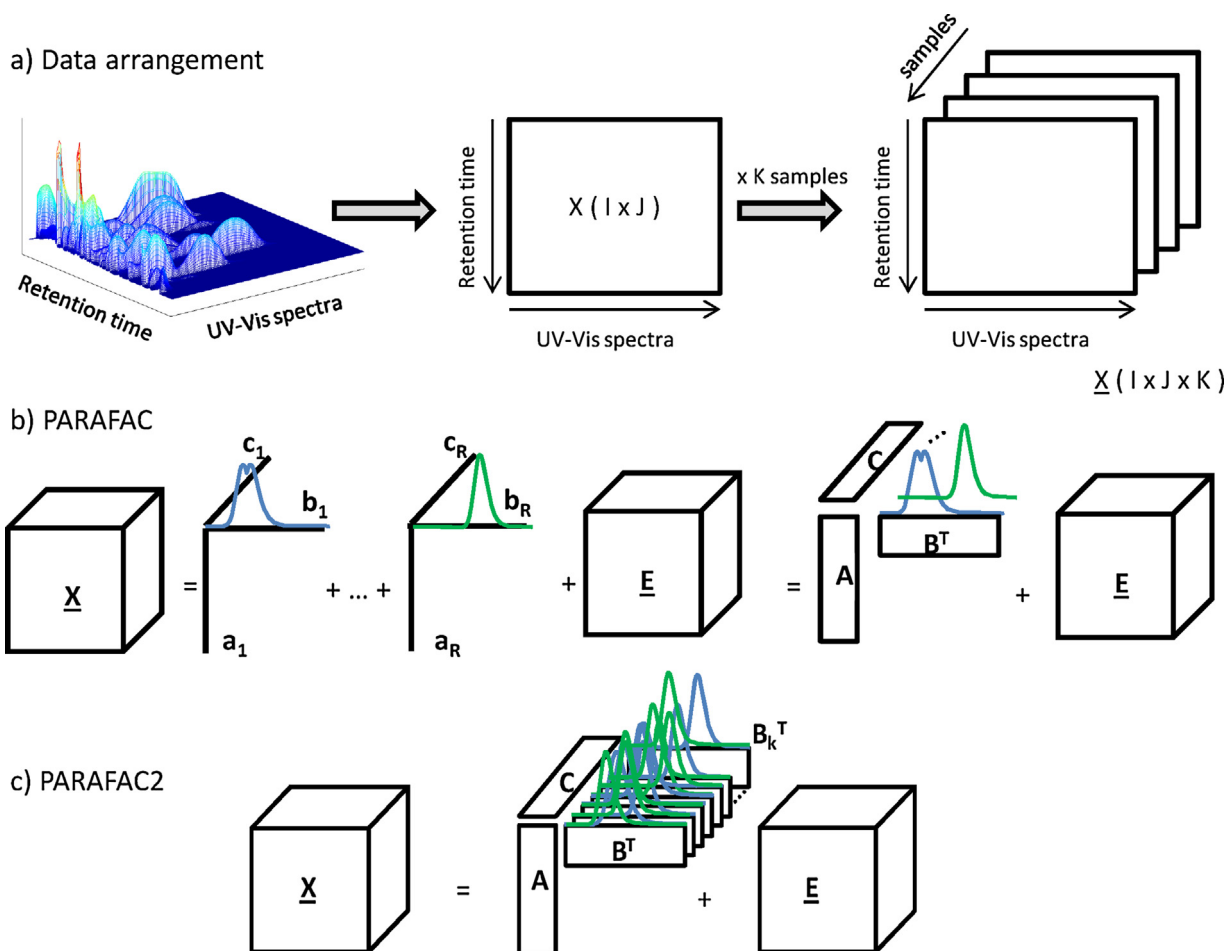


Fig. 1. Representation of: a) three-way data array obtained combining several samples from hyphenated chromatographic systems like HPLC-DAD, b) PARAFAC decomposition of the three-way data array into a R factor model and c) PARAFAC2 decomposition of the three-way data array into a two factors model.

appropriate 10 mL solution containing the analytes and 150 g/L salt. After preparation, the solution was gently shaken by hand and sonicated at 35 kHz and $35 \pm 1^\circ\text{C}$ during 5 min, to form an emulsion of extraction solvent in water. This emulsion was later disrupted by centrifugation at 4500 rpm for 15 min, and the vial was then placed into a thermostatic bath at 3°C for 15 min, for the solidification of the resulting organic drop. Once solidified, the drop was collected with a spatula, transferred to an Eppendorf vial and let melt at room temperature. Then, 25 μL of that drop was collected and mixed with 20 μL of methanol to make it compatible with the HPLC mobile phase. Finally, the mixture was injected in the HPLC and subjected to analysis.

In the chromatographer, gradient elution with water and acetonitrile and changes in flow were used for separation along the XDB-C18 column. Specifically, the initial mobile phase (50% acetonitrile) was held for 6.5 min, and then acetonitrile content was increased to reach 60% in min 15, 80% in min 32 and 90% in min 32.5, where it was maintained for a min. Then, acetonitrile composition was decreased in 0.5 min to reach the starting conditions again, which was maintained from min 34–41 to obtain the last peaks and homogenize the system. Regarding the flow, it was maintained at 0.5 mL/min for 15 min, it was raised to reach 1 mL/min in min 16, and then it was kept until the end.

Fig. 2a shows in black a typical HPLC-DAD chromatogram (at 200 nm) of a mixed standard sample containing the 24 regulated PAS. In this figure, analytes represented by numbers are the ones determined univariately in [33] and analytes represented by letters are the ones to be multivariately determined herein. The identifica-

tion of the peaks was done by comparison of the retention time and the UV-vis spectrum of each peak in the chromatogram with those of the single reference standards. In the same figure, in grey, a blank chromatogram obtained after applying the entire procedure to a sample without analytes has also been represented. In the blank some peaks probably due to the extractant can be observed, but they do not overlap with analytes marked with letters (to be multivariately determined). In addition, although some of them overlap with the analytes marked with numbers (univariately quantified), they do not difficult their determination, as peaks are reproducible from sample to sample.

As it can be seen in Fig. 2a, there are four regions with problems of severely overlapped peaks (intervals 1–4). In Fig. 2b a zoom-in of each of those intervals is presented together with the UV-vis spectrum of each present compound.

2.3. Datasets

Chromatographic runs of 40 min were performed, recording a complete spectrum between 190 and 800 nm every 1.2 nm and every 0.01 min. From them, only the time intervals with overlapping peaks (intervals 1–4) were considered, as the efficiency of PARAFAC2 in terms of both, interpretability and computation time, is increased when only local intervals are taken [50]. Selected intervals were 8.2–9.6 min (interval 1), 13.5–15.2 min (interval 2), 19.8–21.3 min (interval 3) and 30.5–32.5 min (interval 4) min respectively. A different spectral region was considered for each interval: From 197 to 355 nm (interval 1), from 197 to 336 nm

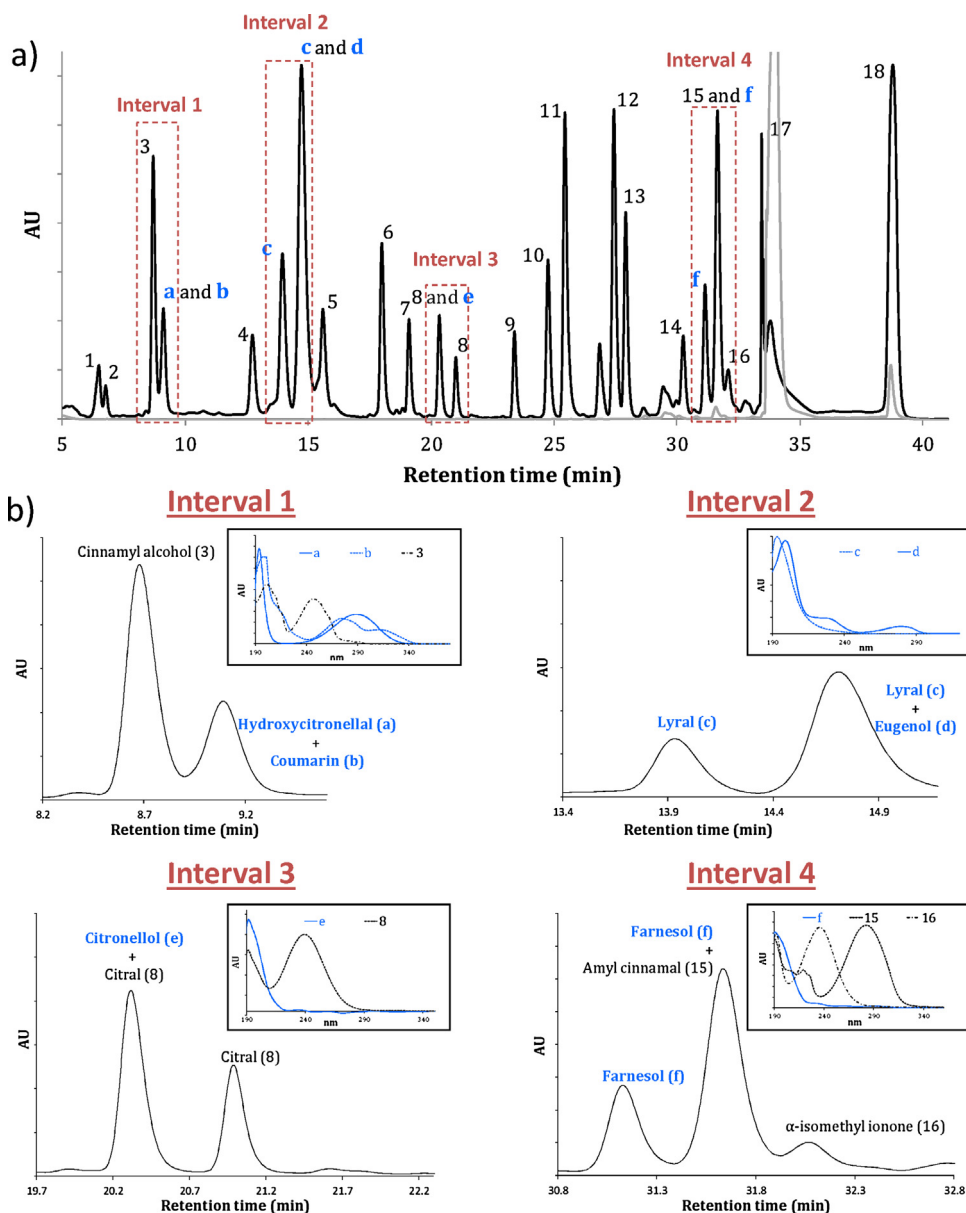


Fig. 2. a) In black: HPLC–DAD chromatogram at 200 nm of a standard mixture of the 24 regulated PAS. Concentration level was 0.8 $\mu\text{g/mL}$ for all the analytes except for hydroxycitronellal (136 $\mu\text{g/mL}$), lyrall (8.37 $\mu\text{g/mL}$) and farnesol (1.98 $\mu\text{g/mL}$). Assignment of peaks: 1: anise alcohol; 2: benzyl alcohol; 3: cinnamyl alcohol; 4: cinnamal; 5: isoeugenol; 6: geraniol; 7: linalool; 8: citral; 9: methyl-2-octynoate; 10: amylcinnamyl alcohol; 11: benzyl benzoate; 12: benzyl salicylate; 13: benzyl cinnamate; 14: linal; 15: amyl cinnamal; 16: alpha-isomethyl ionone; 17: hexyl cinnamal; 18: limonene; a: hydroxycitronellal; b: coumarin; c: lyrall; d: eugenol; e: citronellol; f: farnesol. In grey: blank chromatogram obtained after applying all the procedure to a sample without analytes. b) Zoom-in of the intervals with problems of overlapped peaks and UV–vis spectra of the analytes present in them.

(interval 2), from 200 to 405 nm (interval 3), and from 197 to 335 nm (interval 4). The cut-off point of the mobile phase (short wavelengths) [51] and the zone of the spectra with no absorbance (long wavelengths) were then avoided. Formed sample matrices (I $\times$ J) were of size: 133 $\times$ 128 (interval 1), 158 $\times$ 113 (interval 2), 140 $\times$ 165 (interval 3) and 188 $\times$ 113 (interval 4), where I symbolizes the number of elution times considered and J the number of wavelengths. Under these defined conditions, the following different datasets with diverse conditions of sample complexity were measured, where only the concentration values corresponding to the 10 analytes in the selected intervals are specified:

2.3.1. Calibration set of standards

A set of 10 calibration standards was measured in triplicate. Concentrations of the analytes were c.a. 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.6,

0.8 and 1 $\mu\text{g/mL}$. Exceptions were hydroxycitronellal (15.9, 19.7, 25.1, 47.8, 60.3, 77.1, 97.7, 117, 137 and 153 $\mu\text{g/mL}$) lyrall (10.5, 8.37, 6.29, 4.19, 2.10, 1.05, 0.524, 0.293, 0.0880 and 0.0880 $\mu\text{g/mL}$) and farnesol (3.96, 1.98, 0.991, 0.793, 0.595, 0.396, 0.198, 0.158, 0.0793 and 0.0369 $\mu\text{g/mL}$).

2.3.2. Validation set of standards

For validation at intervals 1, 2 and 4, a set of 36 standards was prepared and measured at two different concentration levels. Levels were 0.08 $\mu\text{g/mL}$ and 0.8 $\mu\text{g/mL}$ for all the analytes, with the exception of hydroxycitronellal studied at 59.9 and 136 $\mu\text{g/mL}$, lyrall at 1.05 and 8.37 $\mu\text{g/mL}$, and farnesol at 0.396 and 1.98 $\mu\text{g/mL}$, respectively.

For interval 3, a set of 54 standards was prepared at three concentration levels. Low and high levels were around 0.08 and

0.8 µg/mL respectively for both analytes and medium level was 0.457 µg/mL for citronellol and 0.261 µg/mL for citral.

2.3.3. Real set samples

Real samples comprised four *eau de toilettes* (ET1, ET2, ET3 and ET4), one *eau de cologne* (EC), one *eau de parfum* (EP), one body milk (BM), one swimming pool water (SPW) and two different baby bath waters (BBW1 and BBW2). Baby bath waters are waters taken from bathtubs after babies had been bathed. Non-aqueous samples were diluted before injection in the HPLC for making the samples compatible with the instrument (as direct injection of that kind of samples is not recommendable). Given the different concentrations of fragrance allergens even inside the same sample, dilutions were carried out in order to be able to determine all the present analytes in all the samples. Dilutions ranged from 0.0029 g to 1.0077 g in 50 mL. No dilution was applied for SPW, BBW1 and BBW2 samples.

2.3.4. Recovery set samples

This dataset consisted of six real samples (triplicates of BBW1 and EC) spiked with all the analytes. In general, EC samples were prepared by dissolving 1 g of EC in 50 mL with the appropriate salt [33]. However, EC contains quantifiable amount of citral, and thus, for quantification in interval 3, only 0.01 g of EC were used. Salt addition but no dilution was necessary for BBW1 samples in all the intervals. All samples were spiked to have final concentrations around 0.5 µg/mL for all the analytes, except for hydroxycitronellal, lylal and farnesol, which had concentrations of 110, 5.83 and 0.888 µg/mL, respectively.

2.4. Software

All PARAFAC2 analyses were performed using the PLS-Toolbox (Version 8.1) (Eigenvector Research, Wenatchee, USA) under MATLAB environment (version R2013b, Mathworks, Natick, USA).

3. Methodology

For the different intervals, previous defined datasets were used in order to evaluate and validate the performance of the proposed method as follows:

Step 1: The calibration dataset was firstly studied in order to assess the feasibility of PARAFAC2 to correctly resolve the chromatographic and spectral profiles of the studied PAS and quantify them. The performance of the calibration models for the different PAS is evaluated through statistical quality parameters.

Step 2: The evaluation of the predictive ability of PARAFAC2 was carried out using the validation dataset as an external dataset of similar nature to the one used in calibration.

Step 3: The set of real samples was used to quantitatively analyze the selected PAS in real samples and test the performance of the models in the presence of various not previously modelled interfering compounds.

Step 4: Finally, the validation of the performance of the method for PAS quantification was accomplished using real spiked samples (recovery dataset).

In steps 2–4 the different datasets are modelled together with the calibration dataset in order to perform the quantitative analysis of the selected PAS using the calculated calibration curve. Then, for each interval and step, a three-way data array $\mathbf{X}$ ($I \times J \times K$) was constructed and analyzed by PARAFAC2, where K is the number of samples (I and J have been previously defined). The main problem when different datasets were analyzed together (steps 2–4) was the retention time-shifts observed between chromatograms, which can be attributable either to loss of column resolution between early data (calibration and validation) and late data (recovery), to time passage (6 months) or to room temperature variations.

The application of PARAFAC2 to the available datasets in different steps was selected because the models obtained showed a better performance than the ones obtained when all the samples were simultaneously used. This is probably due to the different characteristics of each type of samples, each showing a different problem. This procedure has been previously applied with satisfactory results [52,53].

3.1. PARAFAC2

PARAFAC2 [47,48] is a powerful tool for the resolution of chromatographic data with varying retention time profiles [54] based on PARAFAC. It decomposes the three-way data into different matrices; two of those matrices express information in two of the modes (one matrix per mode, in chromatography spectral and sample mode), and for the other mode the algorithm is able to extract a different matrix for each sample, which allows describing different retention time profiles for each of the analyzed chromatograms. PARAFAC2 is graphically represented in Fig. 1c, where each sample is modelled as in Eq. (2).

$$\mathbf{X}_K = \mathbf{A} \mathbf{D}_K \mathbf{B}_K^T + \mathbf{E}_K \quad (2)$$

Where $\mathbf{X}_K$ ($I \times J$) represents the chromatographic data related to the k^{th} sample, $\mathbf{A}$ ($J \times R$) is the loading matrix of one mode (holding in this case the resolved UV spectra for the R components), $\mathbf{D}_K$ ($K \times R$) is the diagonal matrix with the k^{th} row of the sample mode (C) on its diagonal, $\mathbf{B}$ ($I \times R$) holds a loading matrix for each sample of this mode (in this case, an estimate of the individual elution profiles for each of the R factors), and $\mathbf{E}_K$ is the matrix of residuals. In this way, a different chromatographic profile can be used for modelling each of the samples, allowing dealing with different common chromatographic artifacts [44]. It has to be remarked that although in PARAFAC2 we can obtain a different B profile for each sample, the possible B s are restricted, as the cross-product of $\mathbf{B}_k$ has to be constant over all the samples. This implies that the elution profiles of the different samples may differ but should be somehow related [47,54]. Along this work, chromatographic loadings will be referred as first mode (since it is the one which can handle variations), spectral loadings as second mode and sample mode as third mode.

Moreover, the application of different constraints to PARAFAC2 models can help to find stable solutions with real chemical meaning [55,56]. In this case, non-negativity was applied in the second (UV spectra) and third mode (samples), since no constraints in the first mode (elution profiles) can be imposed. Convergence criteria were set as a relative change in fit of 10^{-9} or an absolute change in fit of 10^{-12} . For initialization, the best fitting model of 20 small runs was used.

The correct determination of the number of components is a critical aspect in any curve resolution methodology. In this work, a combination of different criteria was used: core consistency test (CC, %), percentage of variance explained by the model (EV, %), lack of fit (LOF, %), visual analysis of the residuals, chemical meaning of the loadings and correlation between the loadings and the real measured signals [52,54,57]. Specifically, visual evaluation was used to assess if loadings in the first mode looked similar to the measured chromatograms. Besides, correspondence of the spectral loadings with the measured spectra was assessed by calculating the correlation coefficient (r). EV and LOF were defined as in Eqs. (3) and (4).

$$EV (\%) = 100 \times (1 - SSE/SSX) \quad (3)$$

$$LOF (\%) = 100 \times \sqrt{SSE/SSX} \quad (4)$$

Where SSE is the sum of the squares of the residuals and SSX is the sum of the squares of the elements in the original array.

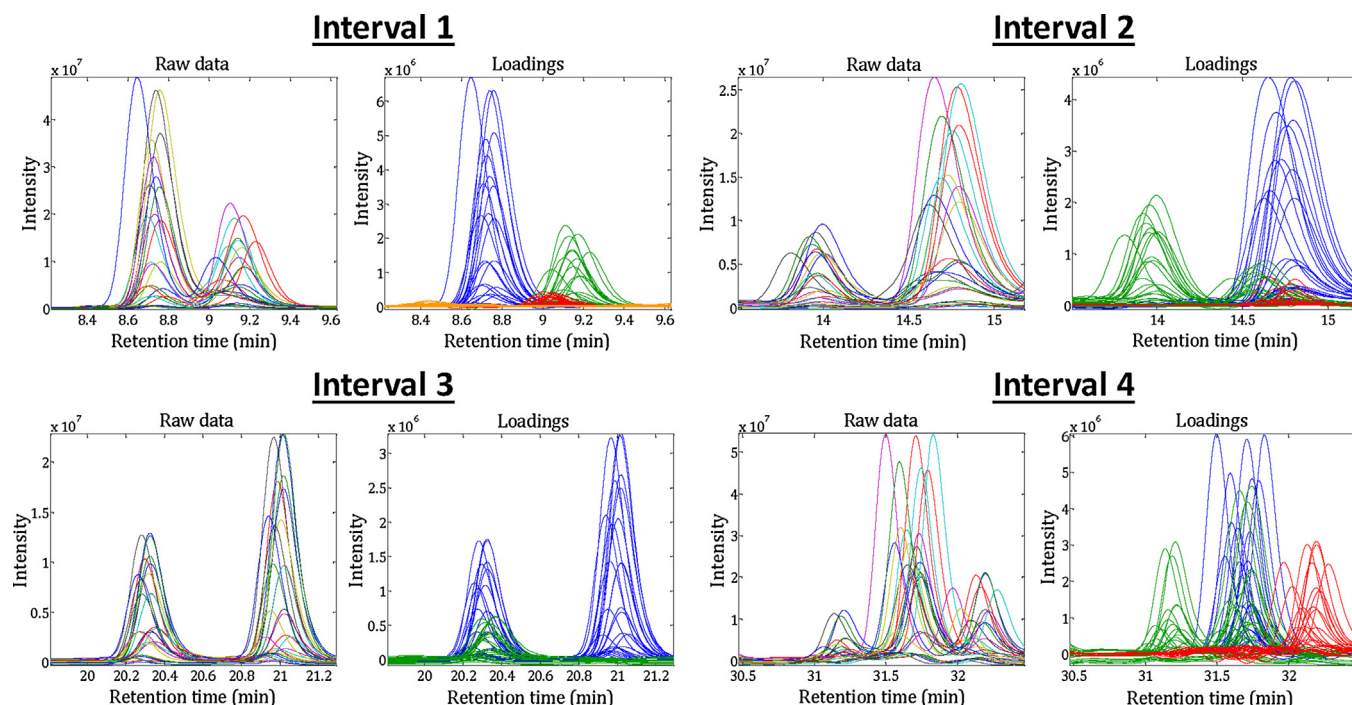


Fig. 3. Raw elution profile data and obtained PARAFAC2 chromatographic loadings for each interval with the calibration samples. In all cases, the first PARAFAC2 component is presented in blue, the second in green, the third in red and the fourth in orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Once a suitable PARAFAC2 model has been built, the score matrix contains the relative concentration of each component in each sample, allowing the use of PARAFAC2 decomposition for quantification purposes. In this work, different statistical quality parameters were calculated for each step described in the methodology section, including limits of detection (LODs) and limits of quantification (LOQs), which were calculated based on the residual standard deviation of the pseudo-univariate linear regression curves (S_{xy}). Specifically, LOD was 3.3 times S_{xy} divided by the value of the slope, and LOQ 10 times S_{xy} divided by the value of the slope [58,59].

4. Results and discussion

In this work, the determination of 10 analytes (PAS) in the regions of the chromatogram with problems of overlapped peaks (see Fig. 2) was accomplished. Among them, 6 PAS (hydroxycitronellal, coumarin, lylal, eugenol, citronellol and farnesol) cannot be determined in a univariate manner due to high spectral and chromatographic overlap, so multi-way data analysis was carried out in order to adequately quantify them. The remaining 4 compounds (cinnamyl alcohol, citral, amyl cinnamal and alpha-isomethylionone) were properly determined by univariate calibration in [33] because either their chromatographic peaks are only partially overlapped (cinnamyl alcohol), or their spectra have a maximum absorption where interfering analytes practically do not absorb (citral and amyl cinnamal), or both (alpha-isomethyl ionone). Nevertheless, the quantification of these 4 analytes by multivariate methods will also be discussed as it will allow comparing the performance of both, multivariate and univariate approaches, giving a better understanding of the quality of the models.

As it can be observed (Fig. 2), some of the analytes (citral, lylal and farnesol) in the selected intervals show double chromatographic peaks. This is probably due to the presence of different isomers of these compounds in the samples. In those cases, the

signal of both peaks together was taken into account for their quantification and therefore, the obtained concentration for each compound is the sum of the concentration of its isomers.

The way to proceed along the work was to construct different PARAFAC2 models with increasing number of components for each interval (see Section 2.2) and step (see Section 3). The optimum number of factors for each model was selected comparing several quality parameters of the model performance as it is indicated in Section 3.1. Specifically, the optimum number of components in each case was initially assessed attending mainly to the chemical meaning of the obtained loadings in spectral and elution time profiles (the model with major chromatographic and spectral similarity with the raw data was selected). After, residuals were checked searching for systematic behaviours (that could suggest that an additional component is necessary). Then, the CC, the EV and the LOF of the models were also evaluated in order to verify that good models were selected. EV normally increases with the number of components but low LOF can be an indicative of the overfitting of the model. Therefore, selected models should have high explained variance with a high value of core-consistency (and with low LOF).

Table 1 shows the statistical parameters calculated for the optimum model in each case. As it can be observed, the obtained models were robust enough, explaining more than the 98% of the variance and with a core consistency over 80% in all cases.

For quantitative purposes, the related scores of the PAS corresponding to the calibration standards in each optimum model were used for building the corresponding calibration curves and for prediction purposes in the rest of samples and standards. In the following lines the assessment results of the method development and validation are discussed.

4.1. Calibration

Calibration models were constructed for the defined intervals using the calibration set of standards in order to assess the qualitative and quantitative performance of PARAFAC2 handling the

Table 1
Quality parameters of the PARAFAC2 models obtained in each interval and each step.

		Components	LOF (%)	EV (%)	CC (%)
Step 1 Calibration	Interval 1	4	1	99.98	98
	Interval 2	3	2	99.97	93
	Interval 3	2	4	99.8	100
	Interval 4	3	12	98	100
Step 2 Validation of PARAFAC2 predictive ability	Interval 1	4	1	99.98	91
	Interval 2	3	1	99.98	96
	Interval 3	2	3	99.90	100
	Interval 4	5	1	99.98	99
Step 3 Quantitation of PAS in real samples	Interval 1	5	2	99.96	98
	Interval 3	4	3	99.89	98
Step 4 Validation of the method performance	Interval 1	4	2	99.95	84
	Interval 2	4	2	99.95	97
	Interval 3	2	4	99.8	100
	Interval 4	5	4	99.8	97

LOF: Lack of fit; EV: Explained variance; CC: core-consistency.

Table 2
Quality parameters of the calibration models constructed for each analyte and interval (step 1).

	R ²	r	LOD (μg/mL)	LOQ (μg/mL)	EF
Interval 1					
Hydroxycitronellal	0.990	0.996	6	19	16
Coumarin	0.990	0.97	0.03	0.10	16
Cinnamyl alcohol	0.995	0.9998	0.02	0.07	43
Interval 2					
Eugenol	0.995	0.997	0.02	0.06	104
Lylal	0.98	0.9990	0.4	1.2	57
Interval 3					
Citronellol	0.94	0.996	0.07	0.2	133
Citral	0.998	0.9991	0.013	0.04	117
Interval 4					
Farnesol	0.98	0.998	0.14	0.4	178
Amyl cinnamal	0.997	0.994	0.02	0.05	203
Alpha-isomethyl ionone	0.997	0.990	0.012	0.04	237

R²: regression coefficient; r: correlation coefficient between the spectral loading and the measured spectrum; LOD: limit of detection; LOQ: limit of quantification; EF: Enrichment factor.

*LOD and LOQ were calculated based on the standard deviation of the regression curve.

chromatographic coelution and retention time shifts of the selected PAS. Regarding the qualitative analysis, Fig. 3 shows a representation of the original elution time profiles and the chromatographic loadings obtained of the best PARAFAC2 model for each interval. As it can be seen, the obtained chromatographic loadings are very alike to the original chromatographic (raw) data in all cases. In intervals 3 and 4, the optimum number of components obtained by the model is exactly the same as the number of analytes (2 and 3 components, respectively). However, in intervals 1 and 2 it was observed that with an additional component a more coherent model was obtained (loadings are more similar to the real profiles for both, chromatographic and spectral modes, residuals do not show strange contributions, and statistical quality parameters are still good). These unexpected contributions were assigned to an unknown absorbing interference in interval 1, and to a residual influence coming from the mixture of standards in interval 2.

Moreover, selected models showed good values of the statistical quality parameters (Table 1, Step 1, Calibration). Exception was the relatively high lack of fit value in interval 4 (12%), probably due to the influence of the baseline, which is modelled in this case together with one of the analytes. The addition of an extra component was not adequate, as it divided the contribution of a single analyte into two different components. Thus, this problem was noted down and saved for further consideration in the following steps.

Table 2 shows the quality parameters of the calibration models obtained by regressing the score matrix and the nominal concentrations of the calibration dataset. Satisfactory results were obtained for all the PAS, showing coefficients of determination (R²) above 0.94 and spectral correlation coefficients (r) higher than 0.97 in all cases. LODs for the four analytes in the selected intervals which determination was possible univariately were an order of magnitude higher with multivariate methods than the ones obtained by univariate methods, but it should be considered that the methodology to calculate the LOD in [33] was different.

In this step, enrichment factors (EF) of the studied analytes in the procedure were also calculated, in order to obtain a measure of the concentration achieved. EF is defined as the ratio between the analyte concentration in the organic phase after the extraction and the initial concentration of the analytes within the water sample. Obtained values ranged from 16 to 237 (see Table 2), which is in concordance with the values given in [33]. It should be remarked that the high EF of alpha-isomethyl ionone, taken into account sample and extractant volumes (10 mL and 50 μL respectively), could insinuate some matrix effect in the extraction of this analyte.

All these results confirm the ability of PARAFAC2 to correctly recover the chromatographic and spectral profiles corresponding to the selected PAS that enables to perform their accurate quantification.

4.2. Validation of PARAFAC2 predictive ability

In this step, the calibration and validation datasets were modelled together in order to test the predictive ability of PARAFAC2 with similar samples to the ones used in the calibration step. Quality parameters of the best fitting models are exposed in Table 1 (Step 2, Validation of PARAFAC2 predictive ability). Suitable values of LOF (1–3%), EV (99.90–99.98%) and CC (91–100%) were obtained for all the intervals. As expected, the number of components in this step was exactly the same as in the previous one (calibration) for most of the intervals. For interval 4, however, 2 additional components demonstrated to explain the behaviour of the baseline drift better and, not as in the previous step, in components not corresponding to any analyte, as it can be seen in Fig. 4. The addition of these extra components lead to a decrease in the LOF (from 8% in a 3-component model to 1% in a 5-component model) meanwhile the core-consistency in the selected model (99%) was indicative of no-overfitting. The reason why in this case it is possible to model the baseline, and not when only the calibration standards were considered, can be either the higher number of samples considered or the fact that validation standards provide more proper information about the drift of the baseline, or both.

The statistical errors of prediction of each analyte in the validation standards are shown in Table 3. As it can be observed, good predictive ability was achieved for all the analytes, with relative errors (RE) below 15% in all cases except for the low levels of coumarin (15%) and lylal (18%). Nevertheless, it has to be noticed that the low level was very close to the LOQ for coumarin and slightly lower than the LOQ for lylal. Therefore, errors can be considered acceptable and demonstrated the accurate quantification of the PAS by PARAFAC2 in similar matrix samples.

4.3. Quantitation of PAS in real samples

In the third step, calibration standards and real samples were modelled together with the purpose of using PARAFAC2 to predict the concentration of the analytes in real samples, where unknown interferences may be possibly present. In this case, only the real samples that were suspicious of containing at least one of the six analytes under study were considered (EP and ET2 for interval 1, EP, ET2, ET3 and ET4 for interval 2, BM, EC, EP, ET1, ET2, ET3, ET4

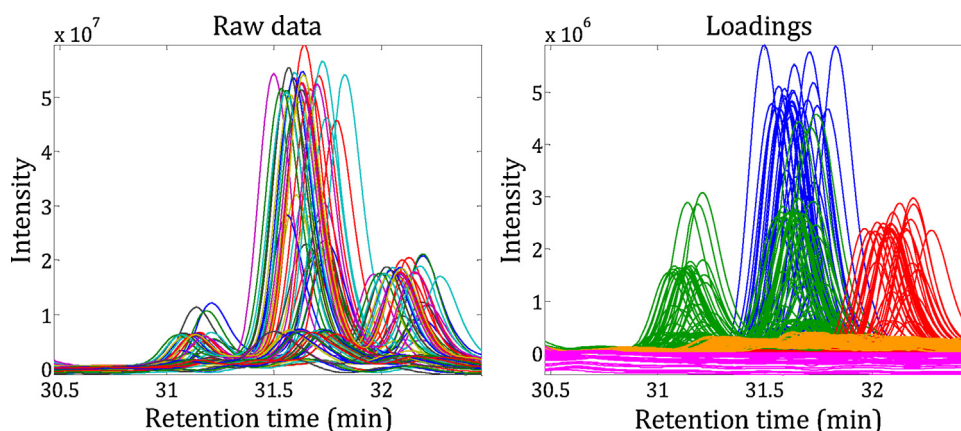


Fig. 4. Raw elution profile data for interval 4 and loadings obtained after applying PARAFAC2 model with 5 components to the calibration and validation samples together (step 2). The first PARAFAC2 component is presented in blue, the second in green, the third in red, the fourth in orange and the fifth in magenta. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Statistical errors of prediction of the selected PAS in the validation samples.

	High level		Low level	
	RMSEP ($\mu\text{g/mL}$)	REP (%)	RMSEP ($\mu\text{g/mL}$)	REP (%)
Interval 1				
Hydroxycitronellal	9.33	7	6.36	11
Coumarin	0.0560	7	0.0153	15
Cinnamyl alcohol	0.0373	5	0.00793	11
Interval 2				
Eugenol	0.0507	6	0.0141	14
Lylal	1.20	14	0.192	18
Interval 3				
Citronellol	0.0801	10	0.0595 ^a	13 ^a
Citral	0.0795	10	0.00532	7
Interval 4				
Farnesol	0.221	11	0.0399	10
Amyl cinnamal	0.0533	7	0.00382	5
Alpha-isomethyl ionone	0.0428	6	0.00360	5

RMSEP: root mean square error of prediction; REP: relative error of prediction.

^a Errors calculated at the medium level for citronellol (0.457 $\mu\text{g/mL}$) because the low concentration value (0.08 $\mu\text{g/mL}$) was under the LOD.

for interval 3 and EP for interval 4). In anyway, the models were later applied to the non-suspicious samples as well to check if the analytes could be determined in them.

In interval 2 and 4, no real samples with a measurable content of the analytes of interest were found. Therefore, only results of intervals 1 and 3 are presented here. Table 1 (Step 3, Quantitation in real samples) shows the quality parameters of the best obtained models in these intervals. As it can be seen, suitable values of LOF (2–3%), EV (99.89–99.96%) and CC (98%) were obtained and, in both intervals, a higher number of components than the appropriate in the previous steps were required due to the presence of interferences. In interval 1, 5 components were obtained, whereas in interval 2, 4 components were modelled, so that 1 additional component and 2 additional components were required to model the interferences in each interval, respectively. Table 4 shows obtained PAS concentrations in both intervals.

In interval 1, analytes were found in only two samples (EP and ET2). In ET4, chromatographic peaks that might correspond to the analytes were visually detected, but they were not adequately determined probably due to the high presence of interferences that decreased their detection levels. All real cosmetic samples considered for analysis in interval 3 showed quantifiable amounts of the analytes. Indeed, except for EC, citronellol was present in all the samples. In a previous work, citronellol has been found in most of

similar samples as well [9], but in our case, the analyzed samples show lower concentration possible due to the fact that samples with lower perfume content have been used here. In aqueous samples (SPW and BBW), no analytes were found in neither of these intervals.

The determination of citronellol in some of the samples presents relatively high RSDs comparing to other method validation values. These are due to the difficulty in the analysis. It has to be taken into account that there are two main types of sources of error in the determination: experimental error and data treatment error; and in real samples data treatment error is increased due to the complexity of the sample (very complex matrix, interferences. ...).

An indicative of the good prediction capability of the multi-way model is that similar concentrations to the ones obtained by univariate calibration are found for the same analytes (the four which determination was possible univariately) [33]. Concentration results obtained by both methods are in good concordance. The main difference is found in ET1 and ET4 samples, where multi-way analysis is able to determine low concentrations of citronellol (in ET1 below LOQ), while this analyte was found under the LOD in univariate analysis. This could be due to the different capability of detection of both approaches.

4.4. Validation of the method performance

In a final step, PARAFAC2 was applied to the calibration and recovery datasets together in order to validate the performance of the method in real spiked samples. Recovery studies were made for the baby bath water (BBW1) and for the *eau de cologne* (EC) samples. BBW1 was selected because it did not show appreciable interferences at any of the intervals. EC contained quantifiable amounts of citral, so the quantity of citral present in the sample was taken into account to calculate the recoveries.

The quality parameters as well as the number of components obtained for the optimum models are also summarized in Table 1 (Step 4, Validation of the method performance). As before, all quality parameters of the models showed good values, LOF (%) ≤ 5 ; EV (%) ≥ 99.8 and CC (%) ≥ 84 . Regarding the number of components, the same as in the calibration (Section 4.1) and validation steps (Section 4.2) were found for intervals 1 and 3. The number of components for interval 2 and 4, however, differed from the previous results. In interval 2 the probable reason is a shift in the chromatogram retention times. The peak of isoeugenol (peak 5 in Fig. 2a) shifted to lower retention times in the recovery samples and its contribution was now significant. Therefore, an additional component was necessary to explain this extra variability. This shift

Table 4

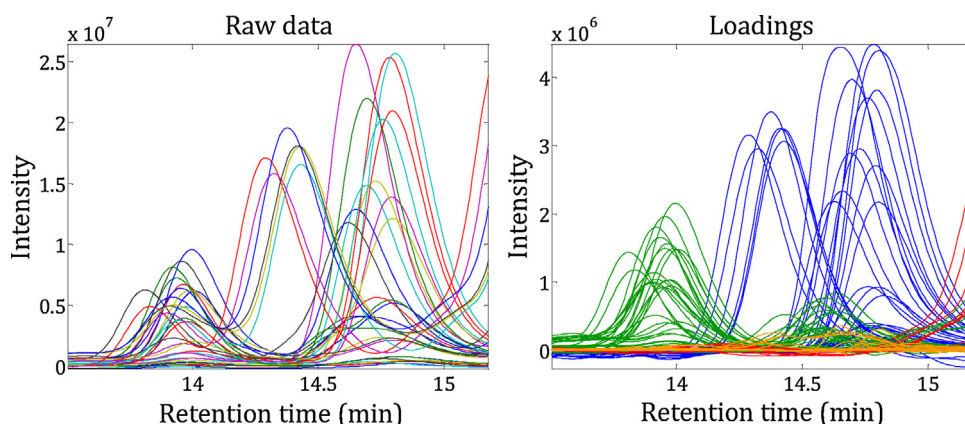
Concentrations (n=3, mg/kg) and RSDs (%) of PAS in real samples in intervals 1 and 3.

	Interval 1						Interval 3			
	Hydroxy.		Coumarin		Cin. al.		Citronellol		Citral	
	C	RSD	C	RSD	C	RSD	C	RSD	C	RSD
EC	–	–	–	–	–	–	n.d.	n.d.	(91 ± 4) · 10	4
EP	<LOQ	<LOQ	11 ± 1	9	<LOQ	<LOQ	(5 ± 2) · 10 ³	30	n.d.	n.d.
BM	–	–	–	–	–	–	96 ± 3	3	9 ± 1	9
ET1	–	–	–	–	–	–	(7 ± 1) · 10	17	<LOQ	<LOQ
ET2	<LOQ	<LOQ	(23 ± 1) · 10	5	n.d.	n.d.	(25 ± 4) · 10	17	(22 ± 3) · 10	14
ET3	–	–	–	–	–	–	(13 ± 3) · 10 ²	21	n.d.	n.d.
ET4	–	–	–	–	–	–	(125 ± 5) · 10	4	4.1 ± 0.3	7

Hydroxy.: Hydroxycitronellal; Cin. al.: Cinnamyl alcohol.

C: concentration (mg/kg); RSD: relative standard deviation (%).

n.d.: not detected; <LOQ: below limit of quantification; –: not modelled; ±: confidence interval.

**Fig. 5.** Raw elution profile data for interval 2 and loadings obtained after applying a PARAFAC2 model with 4 components to the calibration and recovery samples (step 4). The first PARAFAC2 component is presented in blue, the second in green, the third in red and the fourth in orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

can be attributable either to column resolution loss between early data (calibration and validation) and late data (recovery) or to time passage (6 months). Fig. 5 shows the raw data, where the shift in the retention times of the analytes can be appreciated, and the loadings obtained when a PARAFAC2 model with 4 components is considered for interval 2. In interval 4, and due to the discrepancy in the number of components between the different considered combinations of datasets, a PARAFAC2 model including calibration, validation and recovery datasets was built, and the 5-component model was considered the best; lack of fit was very acceptable (4%), core consistency was suitable (97%) and spectral loadings had chemical meaning. As in the step 2 where calibration and validation datasets are modelled together (see Fig. 4) and regarding chromatographic loadings, three components of the model showed a high correlation with the three compounds of that interval and the other two acceptably explained the behaviour of the baseline drift.

The concentrations for the samples in the recovery set at each interval were calculated and the obtained values were compared to the added concentrations (recovery values) as shown in Table 5. The recovery values were acceptable for all the PAS, ranging from 82 to 119% in all cases (approximately ± 20% of accuracy), except for eugenol (129%) and farnesol (158%) in EC where higher recoveries than expected were obtained. The complex sample matrix could be experimentally affecting the analysis of these two analytes in that particular sample at some extent. Indeed, experimentally, a difficulty in the formation of the drop was observed when that sample was analyzed, but it did not impede the analysis. Anyway recovery values were considered tolerable. Moreover, in all real spiked samples, RSD (%) values were satisfactory (≤ 11%), which is indicative

Table 5

Recovery values obtained for real spiked samples (described in Section 2.3.4).

	Recovery in BBW sample (n = 3)		Recovery in EC sample (n = 3)	
	ER (%)	RSD (%)	ER (%)	RSD (%)
Interval 1				
Hydroxycitronellal	103	8	111	6
Coumarin	83	7	95	4
Cinnamyl alcohol	100	6	104	6
Interval 2				
Eugenol	119	3	129	4
Lylal	96	11	98	7
Interval 3				
Citronellol	117	11	106	6
Citral	100	2	102	3
Interval 4^a				
Farnesol	110	4	158	9
Amyl cinnamal	93	3	100	4
Alpha-isomethyl ionone	82	2	100	8

ER: Extraction recovery; RSD: relative standard deviation.

^a In interval 4 the model was built including calibration, validation and recovery datasets.

not only of the high precision of the method but also of the good model performance.

5. Conclusions

This work explores the feasibility of using multi-way analysis techniques for the extension of a previous proposed USAEME-

SFOD-HPLC-DAD method [33] for determining hydroxycitronellal, coumarin, lylal, eugenol, citronellol and farnesol when the other 18 regulated PAS are present. Due to co-elution problems in the chromatograms, these analytes could not be adequately determined by univariate methods.

In the first step of the work, PARAFAC2 has been used to decompose the data into different contributions, some of them directly related to the chemical compounds. Besides, the relative concentration of each analyte in each calibration standard, given in the score matrix, has been used to construct satisfactory regression curves with the real concentration values. In some cases, these have been even built in the presence of interferences.

Then, validation of the obtained PARAFAC2 models has been made using standards and real spiked samples. In both cases satisfactory results have been obtained. In addition, PARAFAC2 models have been satisfactorily used for the determination of the analytes in several real samples, even in the presence of interferences not previously modelled in the calibration dataset.

Thanks to PARAFAC2 the problem of retention time shifts between chromatographic measurements has not been restrictive. Furthermore, the baseline drift has been successfully modelled by adding new components to the models when necessary. However, a higher number of samples with proper information about the drift of the baseline were required for this purpose.

In summary, determination of 6 PAS (hydroxycitronellal, coumarin, lylal, eugenol, citronellol and farnesol) when the other 18 PAS were present has been possible despite co-elution profiles, overlapping spectra, unknown interfering compounds, retention time shifts and baseline drifts thanks to the use of PARAFAC2. Therefore, it has been demonstrated, that combining univariate and multivariate approaches, it is possible to determine the 24 regulated PAS.

Conflict of interest

The authors have declared no conflict of interest.

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