Microchemical Journal 183 (2022) 108072

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Non-separative determination of isomeric polycyclic aromatic hydrocarbons by electrospray Ag(I) cationization mass spectrometry and multivariate calibration

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ARTICLE INFO

Keywords:
Flow injection analysis
Mass spectrometry
Ag (I) cationization
Polycyclic aromatic hydrocarbons
PLS models
Experimental design

ABSTRACT

A new approach for the determination of isomeric polycyclic aromatic hydrocarbons using a stand-alone mass spectrometry method is proposed. The aim of the work is to study quantitative possibilities of multivariate calibration and electrospray Ag(I) cationization mass spectrometry for the non-separative determination of polycyclic aromatic hydrocarbons isomers. The method is based on flow injection analysis, electrospray ionization and tandem mass spectrometry (FIA-ESI-MS/MS). No chromatographic column was included into the instrumental configuration and the analysis time was 1.7 min. Seven polycyclic aromatic hydrocarbons were selected as test compounds and the ionization was achieved by forming complexes with Ag (I). Individual quantification of all the isomers was carried out by using PLS multivariate calibration and experimental design for calibration modeling. The PLS models were used to predict the concentration of the analytes in a set of external validation samples and satisfactory results were obtained. RMSE, expressed as a relative value, were found to be between 23 and 34 %. Results obtained with multivariate analysis were compared with those corresponding to univariate calibration to show its high potential.

1. Introduction

The development of fast analytical methods is currently a concern of great interest. Within this strategy, the direct introduction of the compounds of interest into a mass spectrometer has demonstrated its huge potential for analyte identification and quantification [1–3]. However, the use of these non-separative methodologies presents several disadvantages, such as matrix effects due to the absence of a previous separation step and the presence of isobaric interferences that could hamper analyte quantification [2,4]. Besides, the determination of isomeric compounds using the aforementioned methods represents a remarkable challenge because of their identical chemical composition [4,5]. Individual determination can be achieved if the compounds produce unique fragmentation spectra. However, in many cases, this situation is not possible. The use of multivariate calibration techniques adds a new tool for the individual quantification of these kind of compounds [4].

Electrospray ionization (ESI) is a sensitive MS ionization technique for ionic, easily ionizable or protonated species. It has been coupled to different separation techniques, such as liquid chromatography [6,7] or capillary electrophoresis [8], and as a stand-alone technique with different types of analysers [9,10]. However, neutral and non-polar compounds are less sensitive to this atmospheric pressure ionization technique [11]. Different alternatives have been proposed to try to solve this problem, as the use of ionization promoters [10,12] or by forming complexes with different organic and inorganic cations, such as tropylium cation [13] or Ag (I) [14,15]. Ag (I) was found to cationize many classes of compounds, yielding Ag^+ complexes with minimal interferences from secondary reactions [14], and it has been applied to the identification and characterization of steroids [16] or aromatic compounds [14,17], among others.

In this work, an evaluation of the possibilities of the use of multivariate calibration and electrospray Ag (I) cationization mass spectrometry was performed to individually quantify non-polar isomers. Specifically, a set of polycyclic aromatic compounds were taken under consideration. Polycyclic aromatic hydrocarbons (PAHs) are a class of compounds comprised of at least two fused aromatic rings which have

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https://doi.org/10.1016/j.microc.2022.108072
Received 31 July 2022; Received in revised form 23 September 2022; Accepted 12 October 2022
Available online 17 October 2022

been listed by the International Agency for Research on Cancer (IARC) as possible carcinogens [18]. Due to their toxicity, plenty of methods have been proposed for PAHs determination, mainly gas chromatography coupled to mass spectrometry (GC–MS) and liquid chromatography with fluorescence detection [18–20]. These methods imply a chromatographic separation step, which involves long times of analysis. Some examples can be found in the literature where non-separative methods were proposed [14,21–23]. However, in those methods quantification was not performed [14], isomers were not considered [21,22] or use laboratory-specific instrumentation [23].

To the best of our knowledge, this approach has not been previously used for the determination of isomers using stand-alone mass spectrometric methodologies. If the hypothesis proposed in this work proved to be satisfactory, it would expand the number of compounds that could be determined using these non-separative methodologies.

2. Material and methods

2.1. Chemicals and standard solutions

Methanol (MeOH), acetonitrile (ACN), phenanthrene (Phe), anthracene (Anth), fluoranthene (Fluo), pyrene (Pyr), benzo[*k*]fluoranthene (BkF), benzo[*b*]fluoranthene (BbF) and benzo[*a*]pyrene (BaP) were purchased from Sigma Aldrich (Steinheim, Germanmy). Stock solutions of Phe, Anth and Fluo were prepared in methanol; meanwhile, Pyr, BkF, BbF and BaP stock solutions were prepared in acetonitrile. All of them were prepared at a concentration of 400 mg/L and stored in the dark at 4 °C.

$AgNO_3$ were supplied by Sigma Aldrich. A stock solution was prepared in MeOH, at a concentration of 400 mg/L. Working solution was prepared daily at a concentration of 0.2 mM in methanol.

2.2. FIA-ESI-MS/MS analysis

The instrumental setup used consisted of a 1200 series LC chromatograph equipped with a binary pump, a membrane degasser, an autosampler, two six port valves and a 6410 LC/MS triple quadrupole (QqQ) mass spectrometer, all from Agilent Technologies (Waldbronn, Germany). No chromatographic column was included into the instrumental configuration. Methanol was used as carrier phase and the flow rate was maintained at 0.4 mL/min. 0.2 mM silver nitrate solution was introduced into the carrier phase at a flow rate of 0.1 μL/min by using a *T*-connector and an isocratic pump. The injection volume was 10 μL. The triple quadrupole mass spectrometer was equipped with an electrospray ionization (ESI) source. The QqQ nebulizer pressure and voltage were set at 50 psi and + 4000 V, respectively. Nitrogen was used as the drying (12 L/min, 350 °C) and as collision gas. The run time was 0.4 min and the analysis time (time between injections) was 1.7 min.

2.3. Optimization of the mass spectrometric conditions

Analyte fragmentation studies were performed using product ion scan analysis mode. Three different precursor ions per analyte were considered: $[PAH]^{+\cdot}$, $[Ag + PAH]^{+}$ and $[Ag + 2(PAH)]^{+}$. Precursor ion formation was optimized evaluating different fragmentor values (cone voltages). Different collision energies (from 1 to 225 eV) were also evaluated.

For calibration modelling, the multiple reaction monitoring (MRM) analysis mode was used. Transitions were selected based on the results obtained from the fragmentation evaluation: all the *m/z* ratios with abundance intensities higher than 10 % were selected. A total of 110 MRM transitions were used for multivariate calibration. All the measurements were performed at unit mass resolution and dwell time was fixed at 1 ms.

Table 1
Concentration data (mg/L) of the evaluated compounds in the calibration (sample nos. 1–25) and validation steps (samples nos. 26–35).

	Phe	Anth	Fluo	Pyr	BkF	BbF	BaP
no.	Calibration Standards in MeOH						
1	1.0	0.40	0.40	0.40	0.40	0.40	0.40
2	1.0	0.0	0.0	0.80	0.20	0.80	0.40
3	0.0	0.0	0.80	0.20	0.80	0.40	0.20
4	0.0	0.80	0.20	0.80	0.40	0.20	0.20
5	2.0	0.20	0.80	0.40	0.20	0.20	0.60
6	0.50	0.80	0.40	0.20	0.20	0.60	0.80
7	2.0	0.40	0.20	0.20	0.60	0.80	0.60
8	1.0	0.20	0.20	0.60	0.80	0.60	0.40
9	0.50	0.20	0.60	0.80	0.60	0.40	0.80
10	0.50	0.60	0.80	0.60	0.40	0.80	0.80
11	1.5	0.80	0.60	0.40	0.80	0.80	0.0
12	2.0	0.60	0.40	0.80	0.80	0.0	0.60
13	1.5	0.40	0.80	0.80	0.0	0.60	0.0
14	1.0	0.80	0.80	0.0	0.60	0.0	0.40
15	2.0	0.80	0.0	0.60	0.0	0.40	0.60
16	2.0	0.0	0.60	0.0	0.40	0.60	0.60
17	0.0	0.60	0.0	0.40	0.60	0.60	0.20
18	1.5	0.0	0.40	0.60	0.60	0.20	0.0
19	0.0	0.40	0.60	0.60	0.20	0.0	0.20
20	1.0	0.60	0.60	0.20	0.0	0.20	0.40
21	1.5	0.60	0.20	0.0	0.20	0.40	0.0
22	1.5	0.20	0.0	0.20	0.40	0.0	0.0
23	0.50	0.0	0.20	0.40	0.0	0.0	0.80
24	0.0	0.20	0.40	0.0	0.0	0.80	0.20
25	0.50	0.40	0.0	0.0	0.80	0.20	0.80
	External Validation Standards in MeOH						
26	1.0	0.0	0.20	0.60	0.20	0.40	0.0
27	0.50	0.80	0.0	0.40	0.60	0.20	0.40
28	0.0	0.40	0.60	0.0	0.0	0.20	0.20
29	1.5	0.20	0.80	0.20	0.80	0.60	0.60
30	0.50	0.0	0.40	0.80	0.60	0.80	0.0
31	2.0	0.80	0.20	0.60	0.40	0.0	0.20
32	2.0	0.40	0.80	0.80	0.20	0.80	0.60
33	1.5	0.60	0.0	0.40	0.0	0.40	0.80
34	1.0	0.20	0.60	0.0	0.80	0.60	0.80
35	0.0	0.60	0.40	0.20	0.40	0.0	0.40

2.4. Calibration standards, data acquisition and model construction

Calibration standards in methanol were designed using a calibration design [24] at five uniformly distributed concentrations. Calibration ranges were from 0 to 0.8 mg/L for all the compounds, except Phe, with a calibration range between 0 and 2 mg/L (Table 1). Thus, the calibration set comprises 25 standards with uncorrelated concentrations. The complete design was obtained by using a cyclic generator (-2, 1, 2, 1, −2), a repeater of 0, and a difference vector (0 2 3 1) [24]. Ten standards in methanol were also prepared for external validation of the models (Table 1). None of the external validation samples have the same combination of concentrations as the calibration samples. In addition, the selection of the concentrations in these samples has been carried out to cover the largest possible number of situations, including samples with all the compounds and others where some of them are missing.

Data acquisition was performed using the MassHunter software (version B.07.01) from Agilent Technologies. An in-house script was used to obtain the signal intensity for each MRM transition monitored. Multivariate analysis was performed by partial least square calibration (PLS) using The Unscrambler® statistical package (CAMO Software) [25]. The NIPALS (Nonlinear Iterative Partial Least Squares) algorithm was used. Independent variables in the PLS1 were the intensities of all the MRM transitions detected during data acquisition. Dependent variables were the concentrations used in the models.

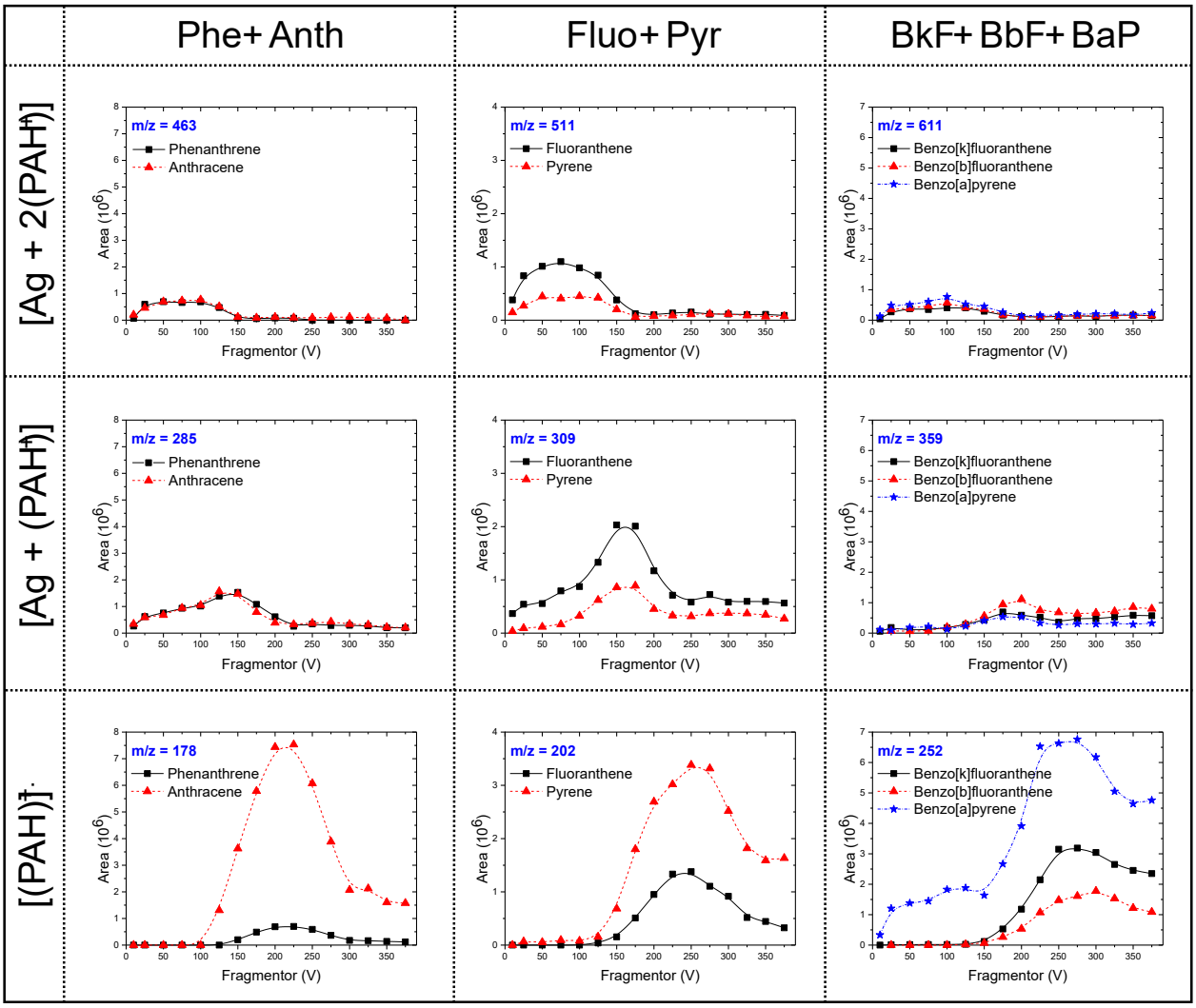

Fig. 1. Ag-PAH complexes and radical molecular ion intensities of the evaluated compounds at different fragmentor energies (10–375 V).

3. Results

3.1. Mass spectrometry optimization

When Ag^+ cationization was used in the ESI source, three different ions were formed per compound: $[Ag + 2(PAH)]^+$, $[Ag + PAH]^+$ and $[PAH]^+$ [14]. It has been observed that in-source collision-induced dissociation was produced with fragmentation of the $[Ag + 2(PAH)]^+$ complex to the monomer complex $[Ag + PAH]^+$ which fragmented further to yield the radical molecular ion $[PAH]^+$ [14]. None of these species were formed if Ag^+ was not added prior to MS detection. Thus, fragmentor energy (also called cone voltage) was first optimized to get the maximum intensity for each ion.

Fragmentor energy was optimized in the 10–375 V range. Fig. 1 shows the results for the compounds evaluated. It was observed that as the energy increased, the predominant ion was the molecular one. Slight differences among isomers were also observed. For example, while the most abundant ion for Phe was the monomer $[Ag + Phe]^+$ at a fragmentor energy of 150 V, the most abundant ion for its isomer, Anth, was the molecular ion $[Anth]^{+\cdot}$ at an energy of 225 V. Similar behaviour was also observed for Fluo/Pyr, being the most intense ions $[Ag + Fluo]^+$ at 175 V and $[Pyr]^{+\cdot}$ at 250 V, respectively. For BkF/BbF/BaP, the most intense ion in every case was the radical molecular ion at 275 V or 300 V. These differences have already been described and used for qualitative purposes but not for quantitative ones [14]. Optimum fragmentor

Table 2
MRM transitions selected for multivariate calibration.

Compounds	Precursor ion (Fragmentor)	Product ion (CE)
Phe and Anth	178 (225)	178 (1 and 10), 176 (40), 151 (50)
	285 (150)	285 (1)
	285 (125)	285 (1), 178 (20), 152 (75), 151 (75), 150 (100)
	463 (50)	463 (1), 285 (20), 178 (40)
	463 (100)	463 (1), 285 (10), 178 (30), 152 (100)
Fluo and Pyr	202 (250)	202 (1 and 5), 201 (40), 200 (50)
	309 (150)	309 (2)
	309 (175)	309 (2), 202 (15), 200 (100)
	511 (75)	511 (1 and 2), 446 (2 and 3), 309 (15), 278 (10), 276 (2), 202 (40)
BbF, BkF and BaP	252 (275)	252 (1 and 3), 250 (50), 248 (75), 224 (75)
	252 (300)	252 (4), 250 (50), 248 (75)
	359 (175)	359 (1 and 5), 252 (10 and 15), 250 (75), 224 (100), 222 (150)
	359 (200)	359 (3), 252 (20), 250 (75)
	611 (100)	611 (2 and 5), 359 (15 and 20), 252 (30, 50 and 75), 250 (150)

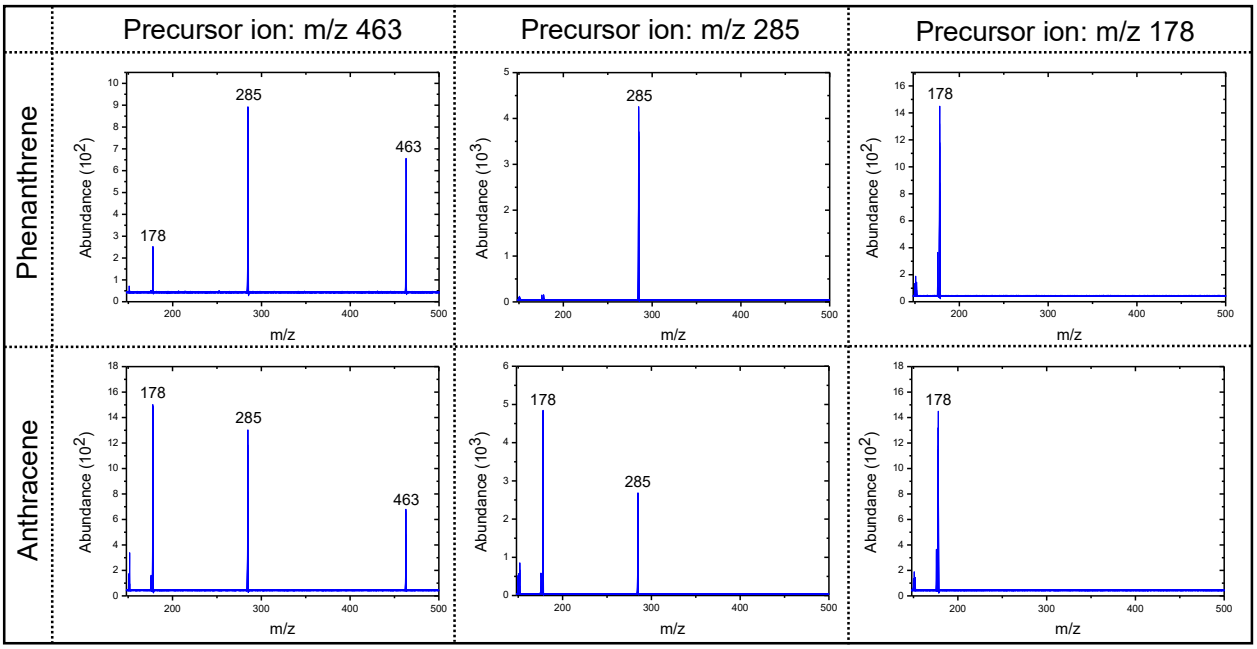

Fig. 2. Product ion spectra of phenanthrene and anthracene at the different collision energies (1–225 eV) evaluated. Abundance was calculated as the summation of intensities obtained for each *m/z* ratio at all the CE evaluated.

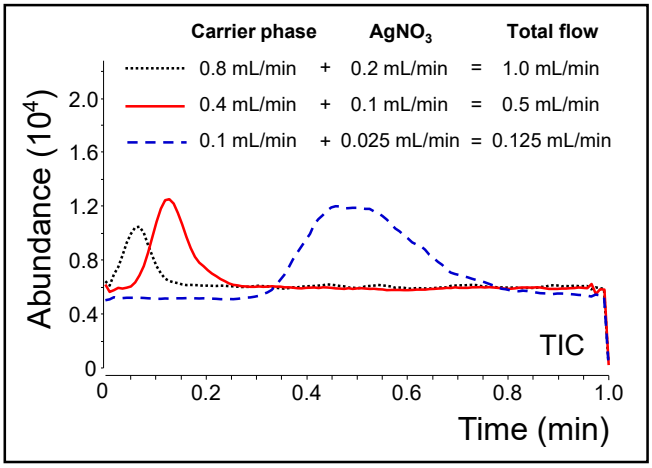

Fig. 3. Total ion chromatogram (TIC) obtained at the different carrier phase and $AgNO_3$ solution flow rates evaluated.

energies were set for the three ions observed for each compound (Table 2).

To select suitable MRM transitions for isomers quantification, spectra obtained using product ion scan acquisition mode were recorded. Standard solutions in methanol of each compound were used at a concentration of 10 mg/L. Three different precursor ions were selected per compound ($[Ag + 2(PAH)]^+$, $[Ag + PAH]^+$ and $[PAH]^{+\cdot}$). Different collision energies (CE, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 150, 225 eV) were evaluated.

Fig. 2 shows the results obtained for Phe and Anth. Abundance was calculated as the summation of intensities obtained for each *m/z* ratio at all the CE evaluated. As can be seen, a specific transition was observed for one of the compounds (285 → 178, *m/z*). The monomer complex of Anth easily fragmented to the radical molecular ion. This fragmentation was observed from a collision energy of 1 eV. However, Phe did not present this behaviour as no fragmentation of the monomer was observed. Similar behaviour was observed for Fluo/Pyr, but in this case the fragmentation of the monomer to the molecular ion (309 → 202, *m/z*) was observed for both compounds, being much more intense for Pyr. For BkF/BbF/BaP, no specific transitions were found, but differences among intensities were observed. The results obtained here would allow the individual quantification of the different isomers considered. To achieve this, 110 MRM transitions were selected (all the *m/z* ratios with abundance intensities higher than 10 %). Table 2 shows the selected transitions.

3.2. Optimization of the FIA conditions

To quantify the different isomers, the compounds were directly injected into the mass spectrometer. MeOH was selected as the carrier phase. $AgNO_3$ was used as ionization promoter and was introduced into the carrier phase by using a *T*-connector and an isocratic pump. The concentration of the $AgNO_3$ solution (in methanol) was fixed at a concentration of 0.2 mM [15]. Different carrier phase and $AgNO_3$ solution flow rates were evaluated: from 0.1 to 0.8 mL/min and from 0.025 to 0.2 mL/min, respectively. In every case, a 4:1 ratio (v:v) was maintained. Fig. 3 shows the total ion chromatogram (TIC) obtained for the different flow rates evaluated. The area of the analytical signal raised when the total flow rate decreased (from 1.0 to 0.125 mL/min). Reproducibility, measured as TIC area (RSD, %), was worse when the highest flow rate was used (14.3 % for 1.0 mL/min). This value improved when the carrier phase and $AgNO_3$ flow rates were decreased (6.1 % and 3.4 % for a total flow of 0.5 mL/min and 0.125 mL/min, respectively). However, when the total flow rate was fixed at 0.125 mL/min, the analysis time was increased. As a compromise situation, a flow rate for the carrier phase of 0.4 mL/min and 0.1 mL/min for the $AgNO_3$ solution (total flow rate, 0.5 mL/min) were selected.

3.3. Quantification of individual PAH isomers

With the aim of exploring the analytical possibilities of the proposed method for the individual quantification of the different isomeric compounds, multivariate calibration models using Partial Least Squares (PLS1) were built for each compound (described in section 2.4.). Cross validation (leave one out) was used to select the optimum number of PLS factors and to test the predictive capacity of the model, leaving out one sample and predicting it with the model generated by the others. This

Table 3
Characteristics of the PLS1 calibration models: number of optimum PLS factors and transitions after applying Martens uncertainty criterion and average relative predictive error (E %) in the cross validation (CV) and the external validation steps. The bias values in the external validation step are shown. E (%) using univariate calibration in the external validation step is also included.

Compound	Multivariate Calibration					Univariate calibration
	PLS factors	Number of transitions	E_{CV} (%)	$E_{External\ validation}$ (%)	bias (mg/L)	$E_{External\ validation}$ (%)
Phenanthrene	2	4	32	34	−0.091	
Anthracene	3	8	26	28	0.014	30
Fluoranthene	2	4	29	25	−0.054	
Pyrene	3	7	21	29	−0.053	
Benzo[*k*]fluoranthene	3	7	31	32	−0.057	
Benzo[*b*]fluoranthene	3	5	28	33	0.011	
Benzo[*a*]pyrene	2	5	31	26	−0.008	

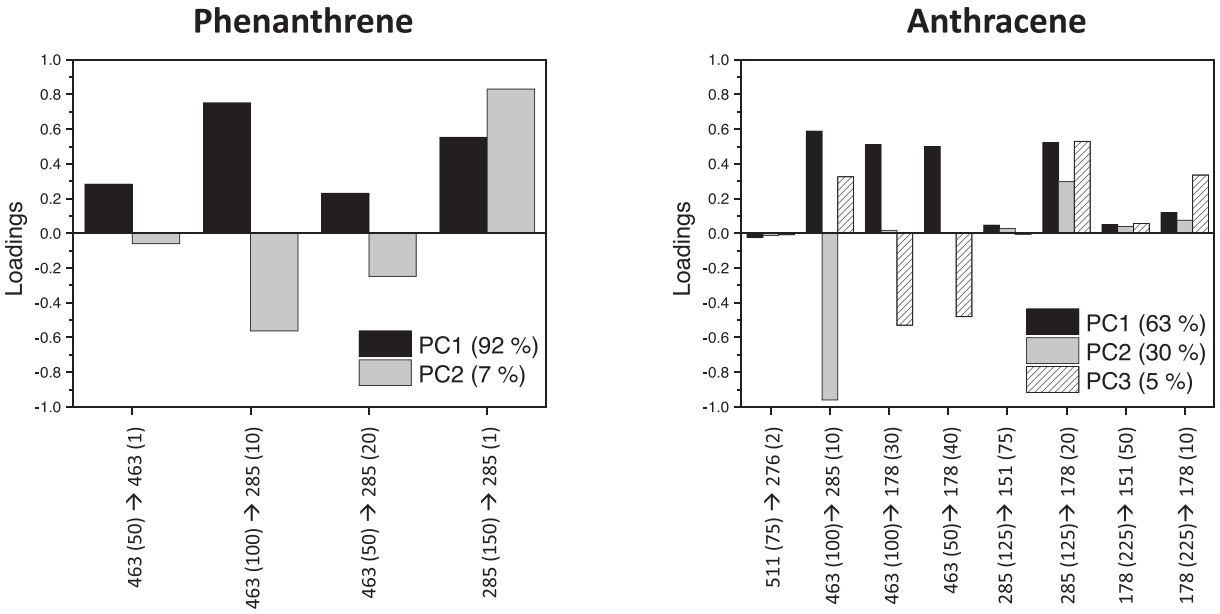

Fig. 4. Loads of the first two and three PLS factors for phenanthrene and anthracene, respectively, against the MRM transitions used in each model.

was repeated until all the samples had formed part of the prediction group. The Martens uncertainty criterion (included in the The Unscrambler®, version 10.0.1, statistical package) was used as the MRM variable selection technique. This eliminates from the model all the variables whose regression coefficients have uncertainty values greater than their absolute value.

The optimum number of PLS factors and the number of transitions selected for each model are shown in Table 3. The number of PLS factors ranged from 2 (Phe, Fluo and BaP) to 3 (Anth, Pyr, BkF and BbF) and the number of transitions were found between 4 (Phe and Fluo) and 8 (Anth). As an example of the models' performance, Fig. 4 depicts a plot of the PLS-factors loads against the transitions in the models of Phe and Anth. The first two PLS factors of the Phe model explained 99 % of the variance in the cross-validation data set (92 % and 7 %, respectively) and the first three components of Anth explained 98 % (63 %, 30 % and 5 %, respectively). For Phe, all the transitions contributed positively to the model in the first PLS component. Only the transition 285 → 285 at a fragmentor of 150 V and a CE of 1 eV contributed positively to the model in the second PLS component. This behaviour could be related to the observed higher stability of the monomer complex $[Ag + Phe]^+$, which did not fragment to the radical ion (Fig. 2). For Anth, an important contribution to the model corresponded to the transition 285 → 178 at a fragmentor of 125 V and a CE of 20. This could be related to the easy fragmentation of the monomer complex to the radical ion previously reported (Fig. 2). Ng et al. [14] have related this behaviour to the fact that the formation of $[PAH]^{+\cdot}$ is favoured for PAHs with lower ionization potential (IP). In this case, the IP of anthracene is 7.44 eV, while the IP of phenanthrene is 7.89 eV.

Repeatability was evaluated by comparing the predicted concentration of a set of 5 spiked samples at the intermediate concentration level studied (1.0 mg/L for phenanthrene and 0.40 mg/L for all other compounds). The relative standard deviation (RSD) ranged between 16 and 25 %. Multivariate detection limits (MDL) were obtained following a strategy [26–27] based on the prediction uncertainty provided by The Unscrambler® [25]. The detection limit was 0.2 mg/L for all analytes except for phenanthrene which was 0.5 mg/L.

Table 3 also shows the root mean square error (RMSE, %) in the cross-validation step and the external validation step, expressed as a relative value considering the average of the added concentration for each compound. The error was calculated following the equation:

$$E\ (\%) = \frac{\sqrt{\sum_{i=1}^{I}(c_i - \hat{c}_i)^2}}{\bar{c}} \times 100$$

where c_i is the reference value, $\hat{c}_i$ is the predicted concentration, I is the number of samples and $\bar{c}$ is the average of the compounds concentrations (reference values) [4]. Errors were found to be between 21 and 32 % in the cross-validation step and between 25 and 34 % in the external validation step. Similar error values have been previously reported when other non-separative methodologies were used [4,28]. The values of bias for each analyte in the external validation step are also shown in

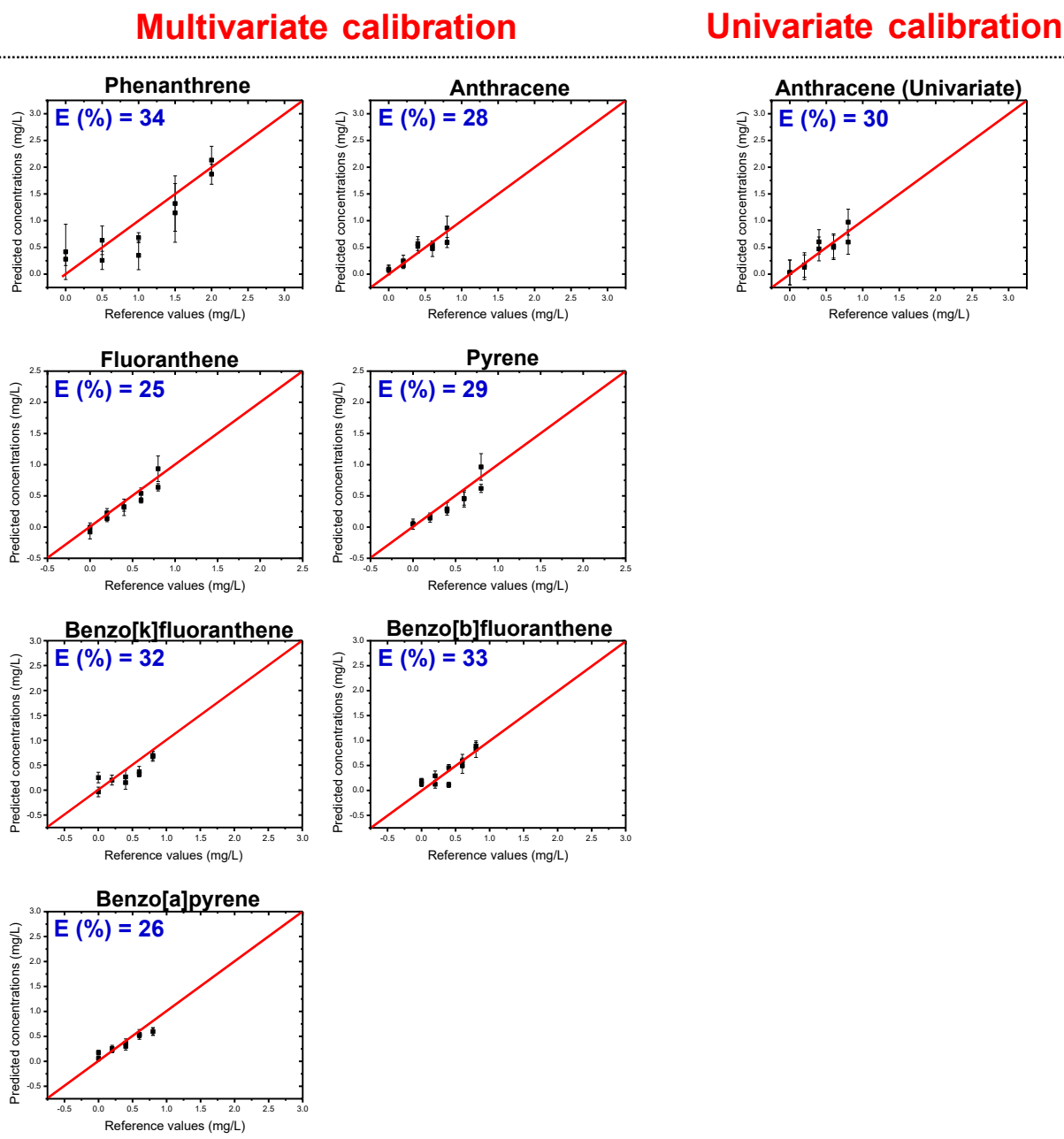

Fig. 5. Correlation plots of predicted vs added concentrations (reference values) for the evaluated compounds in the validation step when the models with the selected variables were used to predict an external set of samples. Results obtained with the univariate calibration model are also included.

Table 3. They ranged between −0.091 and 0.014 mg/L for phenanthrene and anthracene respectively. These values are considered to be sufficiently good, and no trend is observed.

To evaluate the potential of the proposed methodology, univariate calibration was also used. In this case, it was only possible to quantify Anth using its specific transition (285 → 178, CE 20). The other compounds do not present any specific transition. Results are also shown in Table 3. When using univariate calibration, error for Anth was 30 %. This value was similar to that obtained with the PLS models (28 %).

Fig. 5 represents the concentrations of the studied compounds in the external validation samples set obtained with the proposed methodology (predicted concentrations, y axis, versus de reference values, x axis). Results obtained with the univariate calibration models are also included and confidence intervals are shown for both univariate and multivariate calibration. For multivariate one, this interval is based on the variance of the concentration predicted by the model [25,27]. Results show that when multivariate calibration is used, it is possible the semi-quantification of all the compounds. When univariate calibration is used, it was only possible to quantify Anth. These results demonstrate the high potential of using multivariate calibration and electrospray Ag (I) cationization mass spectrometry for the individual quantification of neutral and non-polar isomeric compounds, expanding the number of compounds that could be determined using stand-alone mass spectrometry methodologies.

The main contribution of this methodology compared to others described in the literature is the development of a method without chromatographic separation that allows the semi-quantification of PAHs isomers. The use of the methodology leads a significant reduction in the analysis time (1.7 min between injections) which implies a saving of time and money.

The proposed method can be used for the analysis of biological samples such as saliva and urine. This would require the optimization

and validation of the sample preparation step. The method could be used for rapid analysis of many samples in a very short time. Subsequently, if the concentration obtained was abnormal (for example, a very high value), the sample could be analyzed with a reference method to confirm the results obtained. These methods require more time since they usually involve separation of the analytes. However, this will only occur in a limited number of samples since the vast majority can be discarded with the rapid method proposed in this work.

4. Conclusions

The use of a non-separative method based on FIA-ESI-MS/MS using Ag(I) as ionization promoter and the application of multivariate calibration techniques (PLS) has allowed the individual semi-quantification of the studied PAHs isomers. The planning of the concentrations of the mixtures with experimental design for calibration enabled to generate models capable of predicting the concentration of the compounds in samples with any combination of the analytes. While the use of univariate calibration does not allow the individual quantification of isomers (except Anth), the use of multivariate calibration permitted the quantification of species that even do not present specific fragmentation patterns. The differences in intensity observed in the set of transitions recorded and the application of chemometric techniques have made it possible to quantify isomeric compounds under complex conditions in which there has been no chromatographic separation.

The formation of complexes with Ag (I) has turned out to be a suitable option for the ionization of the PAHs studied. Run time was 0.4 min and the time between injections was 1.7 min, being possible to analyze around 35 samples per hour. These results open a door to the quantification of complex mixtures (isomers with isobaric interferences) with fast, non-separative methods, based on the direct introduction of the sample in the mass spectrometer. In addition, these methods reduce the cost of the analysis to be carried out in much less time. To do this, it is necessary to use well-established chemometric techniques that are easily applicable with currently available software.

CRediT authorship contribution statement

Ana María Casas-Ferreira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Miguel del Nogal Sánchez:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Encarnación Rodríguez-Gonzalo:** Conceptualization, Methodology, Writing – review & editing, Supervision. **José Luis Pérez Pavón:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Acknowledgments

This work was supported by the Spanish Ministry of Science and Innovation (Project PID2021-127459NB-100) and the Junta de Castilla y León (Project SA111P20).

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