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HIGHLIGHTS

- PARAFAC a usefull tool coupled with excitation-emission fluorescence spectroscopy
- EEM-PARAFAC, a cheap, green and usefull tool to determine PAHS in smoked tuna
- EEM-PARAFAC succeeds in identifying unequivocally and quantifying three PAHs
- Using a reduced orthogonal design to obtain mixtures for calibration standards

Unequivocal identification and quantification of PAHs content in ternary synthetic mixtures and in smoked tuna by means of excitation-emission fluorescence spectroscopy coupled with PARAFAC

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Abstract

It is well known that several polycyclic aromatic hydrocarbons (PAHs), products of incomplete pyrolysis of organic material, have proved to be extremely toxic to humans. Food can be contaminated by these compounds in many different ways and diet represents nowadays the major source of exposure to PAHs for non-smokers population. In the present study, three of the most important carcinogenic PAHs in foods, according to the legislation currently in force, i.e. benzo[a]pyrene (BaP), benzo[a]anthracene (BaA) and chrysene (Chry), were firstly arranged in ternary mixtures, following an experimental design. Then, an organic extraction from a commercial smoked tuna, potentially affected by PAHs, was performed to investigate the presence of the three compounds under study. A spectrofluorimetric method based on the second order calibration of excitation-emission fluorescence matrices (EEMs) and parallel factor analysis (PARAFAC) decomposition was proposed in this work as analytical approach for PAHs detection. Both in the ternary mixtures and in the food matrix (smoked tuna), PAHs were unequivocally identified and quantified with decision limit (CCα) and capability of detection (CCβ) equal to 0.11 μg L^{-1} and 0.21 μg L^{-1} for BaP, 0.27 μg L^{-1} and 0.53 μg L^{-1} for BaA and 0.18 μg L^{-1} and 0.35 μg L^{-1} for Chry, respectively, when the probabilities of false positive (α) and false negative (β) were fixed at 0.05. In the investigated smoked tuna, detectable levels of BaP were found, whereas BaA and Chry were absent.

Keywords

Polycyclic aromatic hydrocarbons; excitation-emission matrix fluorescence spectroscopy; parallel factor analysis; smoked tuna; unequivocal identification.

Abbreviations

PAHs, Polycyclic aromatic hydrocarbons; BaP, benzo[a]pyrene; BaA, benzo[a]anthracene; Chry, chrysene; PARAFAC, parallel factor analysis; EEM, excitation–emission fluorescence matrix; CCα, decision limit; CCβ, capability of detection; DMSO, dimethylsulfoxide ; DMF, N, N-dimethylformamide; EPA, Environmental Protection Agency; IARC, International Agency for Research on Cancer; SCF, Scientific Committee on Food; EFSA, European Food Safety Authority; AOAC, Association of Official Analytical Chemists; SPE, solid phase extraction; SFE, supercritical fluid extraction; ASE, accelerated solvent extraction; MAE, microwave assisted extraction; SPME, solid-phase microextraction; GPC, gel permeation chromatography; HPLC, high performance liquid chromatography; GC-MS, Gas Chromatography–Mass Spectrometry; HRGC-MS, high resolution GC-MS.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds composed by multiple aromatics rings which are widespread in the environment as products of incomplete combustion or pyrolysis of organic material, such as coal, petroleum, wood and natural gas [1,2]. 16 PAHs have been classified for decades as priority environmental pollutants by the European Union (EU) and the US Environmental Protection Agency (EPA) [3].

Food can be contaminated by PAHs that are present in water, air, soil or packaging materials, as well as those that are formed during food processing or certain home cooking practices, e.g. smoking, barbecuing, grilling, roasting, toasting, heating, drying, baking, frying and ohmic-infrared cooking [4]. Thus, the major source of PAHs exposure, in non-cigarette smokers and non-occupationally exposed workers, is diet, responsible for more than 90% of the total exposure to PAHs of population in many countries of the world [5].

A substantial number of studies showed that PAHs are extremely toxic to lung, breast, stomach, esophagus and pancreas [6,7]. Several of these compounds, in fact, have been classified by the International Agency for Research on Cancer (IARC) as probably carcinogenic to humans (Group 2A) or possibly carcinogenic to humans (Group 2B) and benzo[a]pyrene (BaP) directly as carcinogenic to humans (Group 1) [8].

In 2002, the Scientific Committee on Food (SCF) [9] stated that benzo[a]pyrene (BaP) might be used as a marker of the occurrence of the carcinogenic PAHs in food products [10], but in 2008 a report of the European Food Safety Authority (EFSA), showed that BaP is not always detectable, providing a negative response in about 30% of all the testing samples, even if others PAHs, above all chrysene, were found [11]. Thus, the EFSA 'Panel on Contaminants in the Food Chain' highlighted that benzo[a]pyrene (BaP), chrysene (Chry), benzo[a]anthracene (BaA) and benzo[b]fluoranthene (PAH4) plus four additional PAHs, i.e. benzo[k]fluoranthene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene (PAH8), are currently the only possible indicators for the carcinogenic potency of PAHs in food. This led to the Regulation UE 835/2011, which sets the maximum level of BaP in smoked meat and smoked fish products to 5 µg kg^{-1} together with the additional limit of the sum of PAH4 (30 µg kg^{-1}) [12].

Smoked foods present significant variations in PAH amounts, even within the same kind of food. This is due to the variability of smoke composition, which is greatly affected by process environment oxidizing power, combustion temperature, type of generator, smoking time, fuel used, presence of smoking flavour agents and fat content in the food [4]. Traditional smoking techniques, in which the smoke produced by the incomplete combustion of the wood comes into direct contact with the product, can lead to a high contamination by PAHs; for this reason, commercial alternatives are used nowadays, like immerging food items in smoke extracted solution (the so called "liquid smoke") [13].

The determination of PAHs in foods requires a preliminary extraction followed by multistep clean-up procedures [14], to isolate these compounds, occurring at the level of μg kg^{-1} or ng kg^{-1}, from all the interferers present in a complex fat and protein containing food matrix. Standard methods for PAHs detection in food have been published by Association of Official Analytical Chemists (AOAC) [15].

Grimmer and Böhnke's procedure [16], involving saponification, extraction with cyclohexane, liquid-liquid partition of cyclohexane extract with N, N-dimethylformamide (DMF), followed by silica gel column chromatography is still widely used. Its most common modifications concern change of extraction and partition solvents (e.g. dimethylsulfoxide, specific solvent for PAHs which allows to separated them from triglycerides, instead of DMF [17] and use of pre-packed cartridges for solid phase extraction (SPE) [18], which guarantee time and solvent savings as well as better reproducibility, compared to chromatographic columns. In addition to liquid–liquid extraction, column chromatography and SPE, other techniques like supercritical fluid extraction (SFE) [19], microwave assisted extraction (MAE) [20], solid-phase microextraction (SPME) [21]. Furthermore, concerning the analytical methods, Gas Chromatography–Mass Spectrometry (GC–MS) [22], high resolution GC-MS (HRGC–MS) [23] and high performance liquid chromatography (HPLC) fluorescence detection (FLD) or Diode Array Detector (DAD) [24,25] are nowadays the most applied techniques for qualitative–quantitative analysis of PAHs in food.

In the present work, molecular fluorescence spectroscopy has been used for the determination of BaP, BaA and Chry in a commercial smoked tuna. A search with keywords "EEM" and (PAHs OR "polycyclic aromatic hydrocarbons") in Scopus gave 276 papers since 1980, none of them dealt with the determination of PAHs by using excitation-emission fluorescence in smoked food products. This technique was selected as analytical technique because highly sensitive, easy to use, fast, non-destructive and low-cost. The interpretation of fluorescence spectral data of the three compounds is complex

due to overlapping signals of other molecules or ions, since fluorescence is a non-separative technique; for this reason, the excitation-emission matrices (EEM) need to be analyzed by means of three ways techniques in order to extract useful information from a huge amount of data. PARAFAC decomposition of trilinear data provides unique profiles estimations of the different fluorophores, when an appropriate number of factors is chosen to fit the model. This means that it is possible to identify unequivocally each factor with each analyte, thus separating the signals of each fluorophore. Moreover, thanks to the second order property of PARAFAC, it is also possible to quantify in presence of uncalibrated interferers. In this context, ternary mixtures of BaP, BaA and Chry were prepared and analysed with fluorescence spectroscopy coupled with PARAFAC as chemometric tool. Finally, the procedure was used to determination and identification of these PAHs in smoked tuna.

2. Materials and methods

2.1. Chemical compounds and reagents

Benzo[a]pyrene (CAS no. 50-32-8; 96% purity) was purchased from Alfa Aesar Gmbh (Karlsruhe, Germany), benzo[a]anthracene (CAS no. 56-55-3; 99% purity) from Acros Organics (Geel, Belgium) and chrysene (CAS no. 218-01-9; 95% purity) from Merck KGaA (Darmstadt, Germany), *n*-Hexane (CAS no. 110-54-3; for liquid chromatography LiChrosolv®) was supplied by Merck KGaA (Darmstadt, Germany) and dimethyl sulfoxide (CAS no. 67-68-5; 99% purity) by VWR International S.A.S. (Fontenay-sous-Bois, France). (Chemical compounds in table S1 in supplementary material). Deionised water was obtained by using the Milli-Q gradient A10 water purification system from Millipore (Bedford, MA, USA). Sep-Pak Silica Plus Long cartridge (690 mg Sorbent per Cartridge, 55-105 µm Particle Size) and Sep-Pak Plus Short C-18 cartridges (360 mg Sorbent per Cartridge, 55-105 µm Particle Size) were supplied by Waters (Milford, MA, USA).

2.2. Standard solutions of selected PAHs and food matrix

Stock solutions of each polycyclic aromatic hydrocarbon were individually prepared in *n*-hexane at a concentration of 100 mg L^{-1}. Intermediate solutions for each analyte at 100 µg L^{-1} were prepared by dilution with *n*-hexane. Then, final solutions for the analysis were prepared daily by further dilution with *n*-hexane from the intermediate solutions. All these solutions were stored at low temperature (4°C) in amber flasks. Smoked tuna was obtained from a local supermarket in the area of Burgos, Spain.

2.3. Instruments

For the lyophilization of smoked tuna, a freeze-drier FreeZone 12 L Console Freeze-Dry System with drying chamber, Labconco was used.

Ultrasound-assisted extraction (UAE) of lyophilized smoked tuna was carried out in an ultrasonic bath (BandelinSonorex RK52, Berlin, Germany) with 35 kHz frequency, maximum power of 240 watts, and internal dimension of 150×140×100 mm.

Sample pre-concentration was performed in a rotary evaporator at pressure of 335 mbar (ILMVAC, Ilmenau, Germany).

The excitation-emission fluorescence measurements were performed at room temperature on a PerkinElmer LS 50B Luminescence Spectrometer (Waltham, MA, USA) equipped with a xenon discharge lamp. A standard cell holder and a 10 mm quartz SUPRASIL® cell with cell volume of 3.5 mL by PerkinElmer (Waltham, MA, USA) were used.

EEMs were recorded in the following ranges: emission wavelengths from 340 to 500.5 nm (each 0.5 nm) and excitation ones from 230 to 305 nm (each 5 nm). Excitation and emission monochromator slit widths were both set to 10 nm and the scan speed was 1500 nm min^{-1}.

2.4. Software

Fluorescent signals were recorded by the FL WinLab software (PerkinElmer). PARAFAC models were developed with the PLS Toolbox 6.0.1 [26] for use with MATLAB (version R2018b.lnk). The least squares regressions were built and validated by STATGRAPHICS Centurion XVII [27]. Decision limit, CCα, and capability of detection, CCβ, were calculated by using the DETARCHI program [28].

2.5. Calibration models

Calibration was carried out in *n*-hexane, by fixing the distribution of concentrations for each one of the three PAHs (BaA, BaP and Chry) in the range of 0–10 μg L^{-1} and each analyte was at 9 levels of concentration. In addition to the 9 different concentrations, 3 determinations of the blank (*n*-hexane) were performed.

2.6. Ternary mixtures

Mixtures of BaP, BaA and Chry were developed in *n*-hexane following a five-level experimental design [29], shown in Table 1.

For this kind of orthogonal design, 25 experiments are necessary and up to 24 possible factors can be included: in this case 3 factors have been used, representing the three compounds used to arrange the mixtures.

The five levels are indicated by -2, -1, 0, 1 and 2 correspond to the five different concentrations of the PAHs in the mixtures: 0, 1, 3, 5 and 7 μg L^{-1}. Level 0 (the central one) is chosen as the 'repeater' level: this is the level at which experiment 1 is performed for all the factors. In this way, the design ensures that each level is represented five times over the 25 experiments, so that each individual compound is measured at each of the five concentration levels five times, an important prerequisite for a balanced mixture design.

The experimental design was performed twice: firstly, to obtain the model, and then, to

evaluate the model prediction. In addition to the 25 experiments, in both cases, 6 blanks were measured.

2.7. Commercial smoked tuna preparation

The commercial smoked tuna, after being weighed, was finely homogenized by using a kitchen blender and freeze-dried two days long. After lyophilization, the dried sample was weighed again and then divided into 5 grams portions, which were placed in well-sealed glass bottles and stored in dark at 4°C before extraction and analysis.

To determine the amount of BaP, BaA and Chry in smoked tuna, two of these 5 g portions were extracted as such, while the other two were spiked with the intermediate solutions of the selected three PAHs (300 µL), before the extraction procedure.

2.8. Commercial smoked tuna extraction and clean-up

In order to isolate the PAHs fraction from the smoked tuna, an extraction and a multistage clean-up, with a slight modification of the procedure by Garcia-Falcon et al. [30] was performed. *N*-hexane was chosen as extraction solvent because it is one of the most used solvent for the extraction of organic compounds (including PAHs) from different matrices [31]. In addition, it does not interfere in the fluorimetric determination of BaP, BaA and Chry, since it does not have a fluorescence signal in the range of excitation and emission wavelength selected (or at least in the major part of it). This procedure was preferred to the others in the literature because it does not involve saponification as first step. This is fundamental for a work with fluorescence analysis, since an alkaline hydrolysis with KOH or NaOH would contaminate the extracts, whose fluorescent signals would be enhanced in intensity and distorted by the presence of these metals, thus hindering their analysis [32,33].

By using an ultrasonic bath, 5 g (dry mass) of the lyophilized smoked tuna was extracted with 25 mL, 15 mL and 10 mL of *n*-hexane for 1 hour each. The combined extracts (about

50 mL) were centrifuged at 7500 rpm for 10 min and the supernatant was decanted and concentrated to 5 mL in rotary evaporator.

In the first clean-up step, the concentrated extract (5 mL) was passed through a normal phase Sep-Pak Silica Plus cartridge and eluted with 10 mL of *n*-hexane to avoid losses. Then, the eluate (15 mL) was subjected to three consecutive liquid-liquid extractions with *n*-hexane and DMSO, this one previously equilibrated with *n*-hexane. 15 mL, 10 mL and 5 mL of the saturated DMSO were used respectively and solvents partition was allowed, until the emulsion layer, originated after agitation, disappeared (about 2 hours).

The collected DMSO extracts (30 mL) were washed with 10 mL of *n*-hexane, diluted with 75 mL MILLI-Q water and then passed through an inverse phase Sep-Pak C18 Plus cartridge previously activated with acetonitrile (5 mL) followed by deionised water (10 mL). The eluates were discarded, while the cartridge containing the PAHs of interest was dried and then eluted with *n*-hexane (5 mL).

The obtained hexane solution was concentrated to dryness and the residue was furtherly dissolved in fresh *n*-hexane with different amounts depending on the saturation of the fluorescence signal, for the quantification of BaP, BaA and Chry in the analysed samples.

3. Theory

3.1. PARAFAC and Excitation–Emission Matrix

Parallel Factor Analysis (PARAFAC) is a chemometric tool, able to model n-way data. In the case of three-way data, given a data tensor **X** with size I × J × K, the proper PARAFAC model can be written by using a second-order model as in the Equation (1) [34].

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} , \qquad i = 1, 2,\ldots, I;\ \ j = 1, 2,\ldots, J;\ \ k = 1, 2, \ldots, K \qquad (1)$$

where x_{ijk} is the element in the position i, j, k of the three-way array **X**, F is the number of factors and e_{ijk} is the residual of the fitting model.

A PARAFAC model structured as in Equation (1) is a trilinear model, because it is linear in each of the three profiles. PARAFAC decomposes tensor **X**=(x_{ijk}) into three loading matrices **A**, **B** and **C**, whose columns are the vectors $\mathbf{a}_f$ (a_{1f}…a_{If}), $\mathbf{b}_f$ (b_{1f}…b_{Jf}) and $\mathbf{c}_f$ (c_{1f}..c_{Kf}). The *f*-th factor of the PARAFAC model is defined by these three vectors [34].

Signals coming from the EEMs can be arranged into a three-way data tensor **X**=(x_{ijk}) and then a PARAFAC decomposition can be applied to these data [35].

In this sense, Equation (1) expresses the fluorescence intensity x_{ijk} in a sample *i*-th which contains F fluorophores, at an emission wavelength λ_j, when excited at a wavelength λ_k. Therefore, the vectors $\mathbf{a}_f$, $\mathbf{b}_f$ and $\mathbf{c}_f$ are named as the sample, emission and excitation profiles of the *f*-th fluorophore, respectively. The sample profile refers to the amount of each fluorophore in each sample, while the excitation and emission profiles correspond to the excitation and emission spectra of each fluorophore.

If the experimental data tensor is compatible with the structure in Equation (1), data are trilinear. In a trilinear model, profiles are the same in all the samples and differ only in the proportion involved in each of them, that is to say that the emission and excitation spectra of the analyte are the same in all the samples. Thus, except for a scale factor, the estimates values must coincide with the sample profiles and the emission and excitation spectra of the F fluorophore in the sample. The degree of trilinearity of a data tensor is measured by the core consistency diagnostic (CORCONDIA) index [34], which should be close to 100%. If the data are trilinear and the appropriate number of factors has been chosen to fit the model, the PARAFAC decomposition provides unique profile estimations.

Additionally, in the case of a chemical analysis, the availability of the excitation-emission spectra of the target analyte in a reference sample allows checking the trilinearity of the

data, by comparing the profiles obtained with the PARAFAC decomposition and those experimentally obtained. Besides, this information is used together with the knowledge about the sample profile [36].

Therefore, it is possible to identify and quantify the analyte of interest in the presence of unknown interferents because the interferent(s) appear as new factor(s) without affecting the rest. The uniqueness property can be used for the unequivocal identification of analytes by means of the excitation and emission spectra. This advantage is known as the "second-order property" [37].

4. Results and discussion

The experimental spectra were obtained from the EEMs of the standards solutions prepared as described in Section 2.2 measured in *n*-hexane.

4.1. Calibration models

After preparation and analysis of the three calibration lines, as described in section 2.5, the EEMs recorded for all the samples were arranged in three-way data tensor. The tensors for the three PAHs ($\underline{\mathbf{X}}_1$, $\underline{\mathbf{X}}_2$ and $\underline{\mathbf{X}}_3$) have the same dimension, 12x322x16, the first dimension of the tensor represents the number of samples, while the second and the third correspond to the number of emission and excitation wavelengths recorded, respectively. PARAFAC decomposition was applied to these tensors with the non-negativity constraints in three modes, because both the excitation and emission spectra must always be positive as well as the sample profile. Rajkó et al. in reference [38] showed that unique solution can be preserved under mild assumptions, such as the nonnegativity restriction. In this work, it was used to avoid non-sense minimal negative values that used to appear in the spectral profiles.

PARAFAC models with two factors were performed for each compound, showing a CORCONDIA of 100% in all three cases; while the decomposition model explains variance was 99.73%, 99.26% and 98.27% for BaP, Chry and BaA, respectively. The first

factor of the models was the analyte of interest, i.e. BaP, Chry or BaA and the second factor was an impurity from the solvent *n*-hexane in all three models (Figures S.2, S.3 and S.4, contains the sample, emission and excitation profiles from this model for the three PAHs).

After PARAFAC decompositions, a Least Square regression between the sample loadings and the true concentration was built for all the three analytes. Sample number 8 of BaP (6 μg L^{-1}) was removed because its standardized residual was greater than 2.5 in absolute value. In all cases, the regression models were significant (p-values < 10^{-4}) and there was not lack of fit (p-values > 0.05) when the confidence level were fixed at 95%. Table 2 shows the parameters of the calibration lines estimated for each analyte.

The decision limit (CCα), for a probability of false positive, α, fixed at 0.05 and the capability of detection (CCβ), when the probabilities of false positive and false negative, β, are equal to 0.05, were calculated for all the three analytes. The lowest values for CCα and CCβ were obtained for BaP, while the highest for BaA (see row 6 and 7 in Table 2).

4.2. Ternary mixture

The 31 samples (25 different mixtures plus 6 blanks) obtained performing the experimental design previously described, were arranged in a three-way data tensor $\underline{\mathbf{X}}_4$ (31x322x16) and a PARAFAC decomposition was applied to analyse the data. A PARAFAC model with four factors was chosen, in which the first factor was BaP, the second one was BaA, the third Chry and the last one was the solvent *n*-hexane. Non-negativity constraints in three modes were applied also in this case.

The PARAFAC model built with the tensor $\underline{\mathbf{X}}_4$ was trilinear, since the CORCONDIA index was equal to 99%, with an explained variance of 99.89%. In Fig. 1 the emission (a) and excitation (b) profiles of this PARAFAC model are shown, while in Fig. 2 the three-dimensional plot (EEM of the mixture n°19 in table 1) is reported.

In the present work, the identification of each analyte was performed by means of the correlation between its emission and excitation reference spectra and the emission and excitation loadings estimated from the corresponding PARAFAC mode.

The correlation coefficients for the emission and excitation profiles were 0.992 and 0.995 for BaP, 0.980 and 0.963 for BaA and 0.996 and 0.991 for Chry, respectively.

A least square regression between the sample loadings and the true concentration of each compound in the mixtures was performed for the three PAHs. Then, the regressions 'predicted concentration' versus 'true concentration' [39]. Table 3 shows the parameters of this regression line. The procedure is unbiased, for all the analytes because the p-values are greater than 0.05 in both cases.

4.3. Validation and prediction of ternary mixtures' model

To validate the model built with the five-levels experimental design, the 31 analysis were performed again, but, in this case, they were preceded by the measurements of the single compounds in different concentrations to carry out three new calibration lines, in the range of the five levels. The concentration for each PAHs are shown in table 4.

A new three-way array $\underline{\mathbf{X}}_5$ (52x322x16) was built with the composition of 31 samples in table 1 plus the first 21 samples in table 4. Then, a PARAFAC decomposition with four factors (with non-negativity constraints) was applied to $\underline{\mathbf{X}}_5$, obtaining a CORCONDIA index of 99%, with explained variance equal to 99.84%. Internal validation was carried out with the sample loadings of the PARAFAC model. Least square regressions between the sample loadings and the true concentration were performed using the first 37 loadings of the model that constitute the training set. The last 15 sample loadings (corresponding to the second half of the experimental design from table 1) were used as test set. This accuracy lines in prediction was validated as in the previous case, being the mean of

relative errors in absolute value in prediction for BaP, BaA and Chry 6.8%, 3.6% and 2.5%.

4.4 Smoked tuna extraction

As already described in section 2.7, two portions of 5 g lyophilized smoked tuna were extracted as such (named extract A and B) and others two (extract C and D) were added with 300 µL of BaP, BaA and Chry intermediate solutions.

After the extraction procedure, the four extracts were dissolved in different amount of *n*-hexane to be analysed, based on the different intensity of each fluorescence signals: extracts B and C were dissolved in 30 mL of n-hexane, extract A in 20 mL and D in 10 mL. Then, all of them were diluted three times, thus analysing each extract in four different dilutions.

As in the case of the validation of ternary mixtures (section 4.3), new calibration lines of BaP, BaA and Chry were built and analysed in the same day of the extracts measurements.

The data for this study was built by combining the fluorescence spectra of the extracts A, B, C and D (16 samples) with those of the new calibration models again according to table 4 (26 samples) and of the 31 experiments that made up the ternary mixtures design. So, the three-way data tensor $\underline{\mathbf{X}}_6$ (73x317x15). It can be seen from the dimension of the tensor $\underline{\mathbf{X}}_6$ that the emission and excitation ranges have been reduced by some wavelength, to avoid signal saturation problems. The PARAFAC decomposition of this resulting tensor needed five factors and showed a CORCONDIA index equal to 88% and explained variance equal to 99.82%. Fig. 3 shows sample (3a), emission (3b) and excitation (3c) profiles.

Again, for the identification of the PAHs, the correlation between the PARAFAC spectral loadings and the reference spectra have been used. Fig. 4 shows the comparison

between the emission reference spectra (in red) and the emission loadings of the PARAFAC obtained after smoked tuna extraction (in blue). It must be highlighted that the values have been normalized in this figure to compare them. The correlation coefficients for the emission and excitation profiles were 0.975 and 0.982 for BaP, 0.993 and 0.996 for BaA and 0.997 and 0.993 for Chry, respectively.

Once the decomposition has been done by using the loadings corresponding to the sample profile, it is possible to conclude that only BaP is present in the smoked tuna investigated (see samples from number 58 to number 65, blue diamonds in Fig. 3 a). The average concentration of BaP in extracts A and B, bearing in mind the dilution factors of each measurement, was 5.42 μg kg^{-1}, calculated for kg of wet smoked tuna (before lyophilization), since the wet form, not the dry one, is what you eat. This BaP concentration is just in accordance with the maximum allowed by law; however, it must be specified that the amount of BaP calculated by PARAFAC, before the modifications related to the dilution factors and to the difference between wet weight and dry weight, was found to be 0.1 μg L^{-1}, which is just at the limit of CCα (see table 2).

The concentrations of BaA and Chry, in both extracts A and B, were not significantly different from zero. Meanwhile, analysing the two extracts C and D obtained with the standard addition method, the average amount (n = 8) of each compound in both extracts resulted to be 2.23 μg L^{-1}, 1.21 μg L^{-1}, 1.94 μg L^{-1} for BaP, BaA and Chry, respectively. Their respective 95% confidence intervals, expressed in μg L^{-1} are [1.84, 2.62], [0.71, 1.72], and [1.68, 2.19]. The three PAHs were recovered with a percentage of 72.3 (BaP), 40.5 (BaA) and 64.6 (Chry).

5. Conclusions

An analytical method based on EEM fluorescence spectroscopy associated with PARAFAC to detect polycyclic aromatic hydrocarbons (PAHs) has been set up and tuned by a five-level experimental design. This procedure has been performed for the first time

to detect three cancerogenic PAHs in a food matrix (smoked tuna fish). Ternary mixtures of BaP, BaA and Chry were prepared and analysed by the EEM/PARAFAC method for evaluating its feasibility in detecting and quantifying the three PAHs. Thanks to the 'second order property' of PARAFAC, when mixtures were analized, BaP, Chry and BaA were unequivocally identified by means of the correlation between the pure spectra and the PARAFAC excitation and emission spectral loadings, being these correlations coefficients: 0.992 and 0.995 for BaP, 0.980 and 0.963 for BaA and 0.996 and 0.991 for Chry. Moreover, the accurate procedure for the three PAHs has been assured, being their capability of detection 0.21, 0.53 and 0.35 μg L^{-1} for BaP, BaA and Chry respectively in the case of individual calibration and 0.39, 0.25 and 0.29 μg L^{-1} for BaP, BaA and Chry in the ternary mixtures (in all case the probabilities of false positive and false negative were fixed at 0.05). In smoked tuna, only BaP was identified and quantified in a concentration just within the limit allowed by legislation; BaA and Chry were not detected.

Sample CRediT author statement:

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Declaration of competing interest:

There are no conflicts of interest to declare.

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Figures Captions

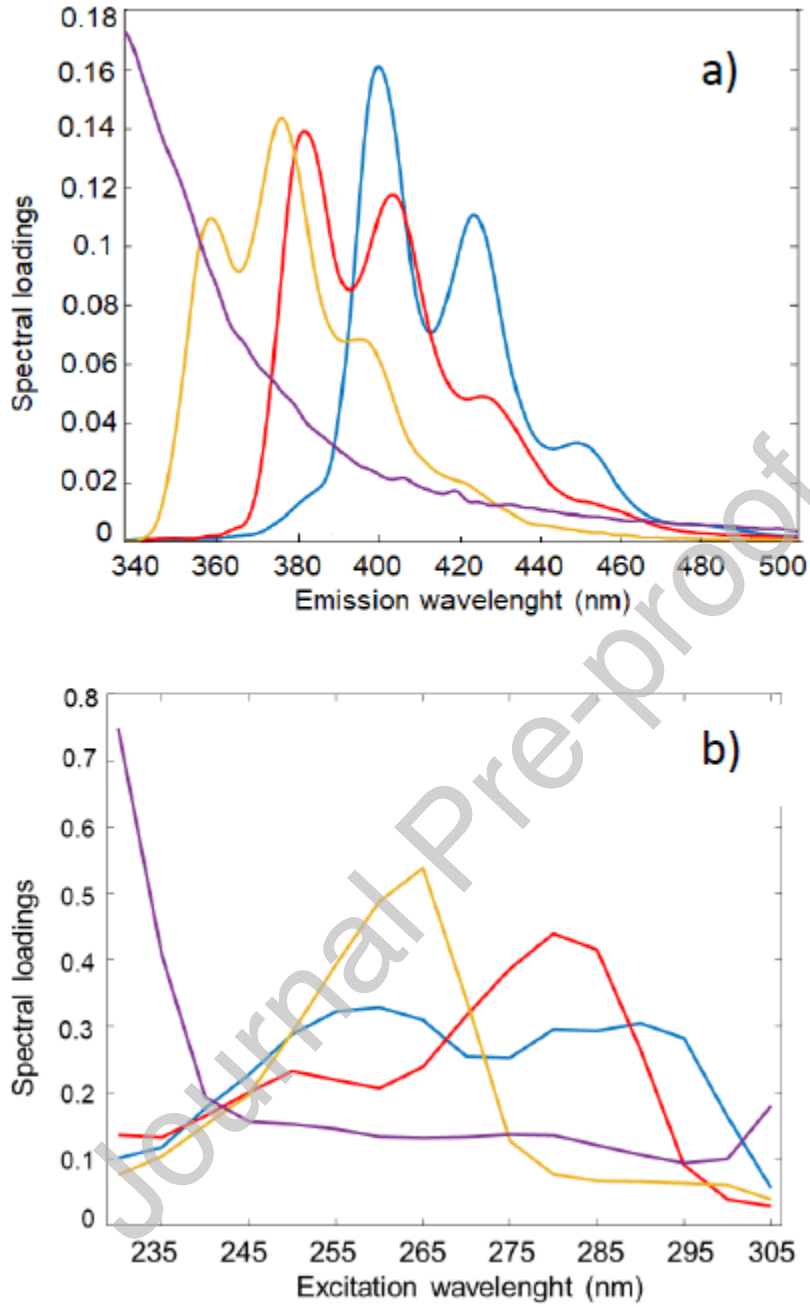

Fig. 1. Emission (a) and excitation (b) profiles of the four-factors PARAFAC model (section 4.2): BaP is in blue lines, BaA in red, Chry in yellow and solvent in purple.

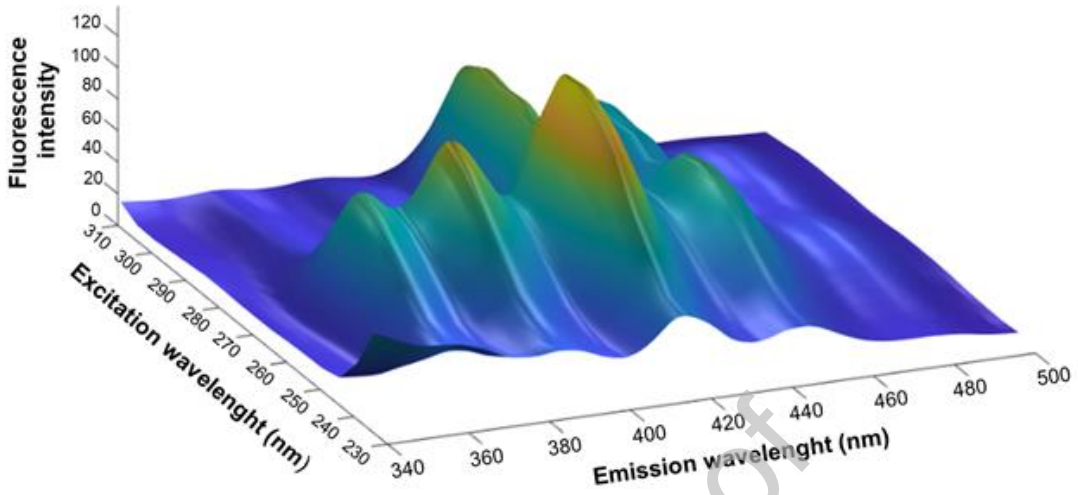

Fig. 2. Three-Dimensional plot of mixture n°19 (in table 1).

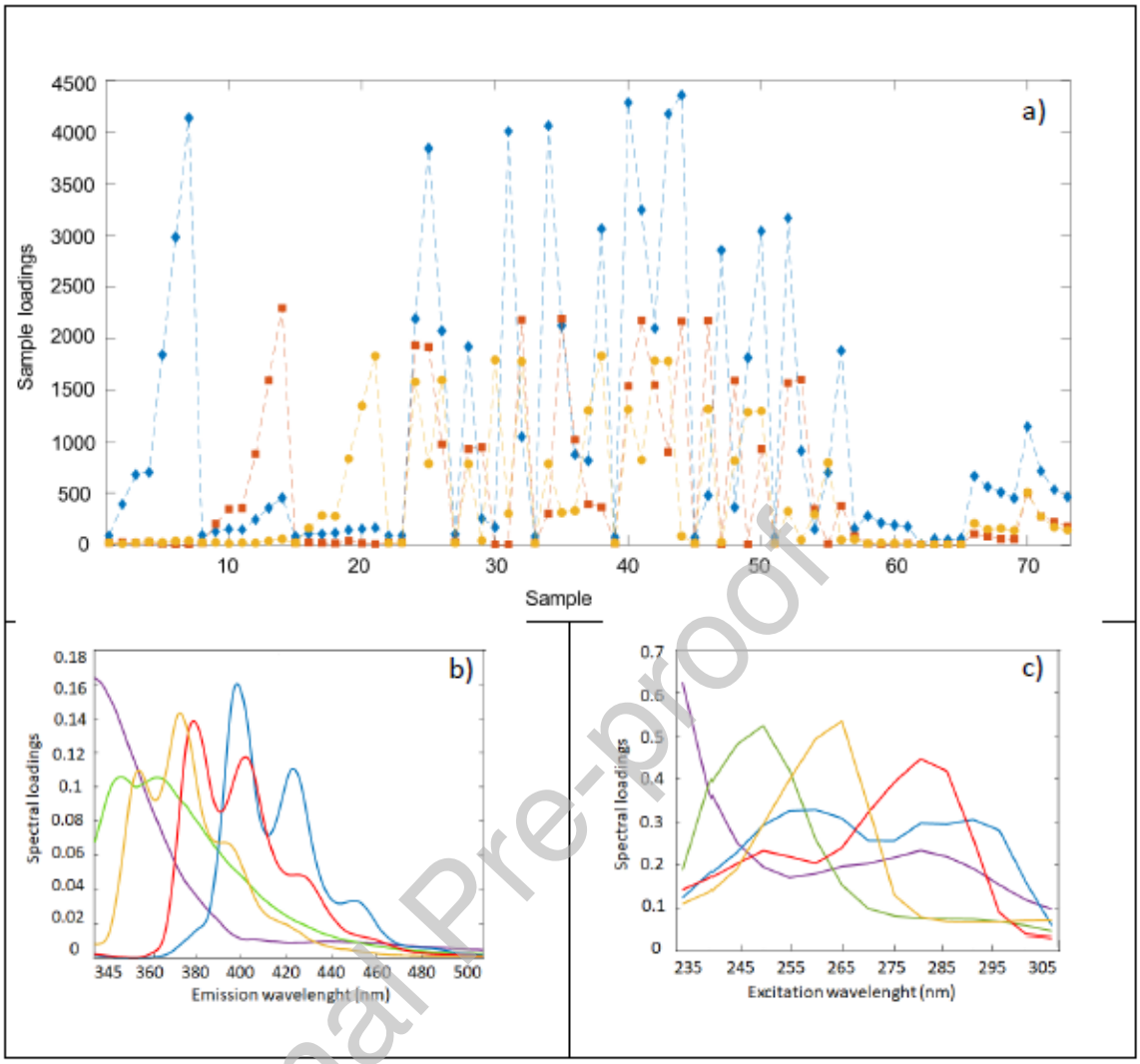

Fig.3. a) Sample profile of the five-factors PARAFAC model (section 4.4), showing only the 3th, 4t^{h} and 5th factors: BaP in blue diamonds, BaA in red squares and Chry in yellow circles. The composition of this matrix involves the first 26 standars in table 4, sample number 27 to 58 in table 1 (25 + 6 blanks) and last 16 samples belonging to the four smoke tuna extracts. b) Emission and c) excitation profiles of PARAFAC model: BaP in blue, BaA in red, Chry in yellow, solvent in purple and the blank of the procedure in green.

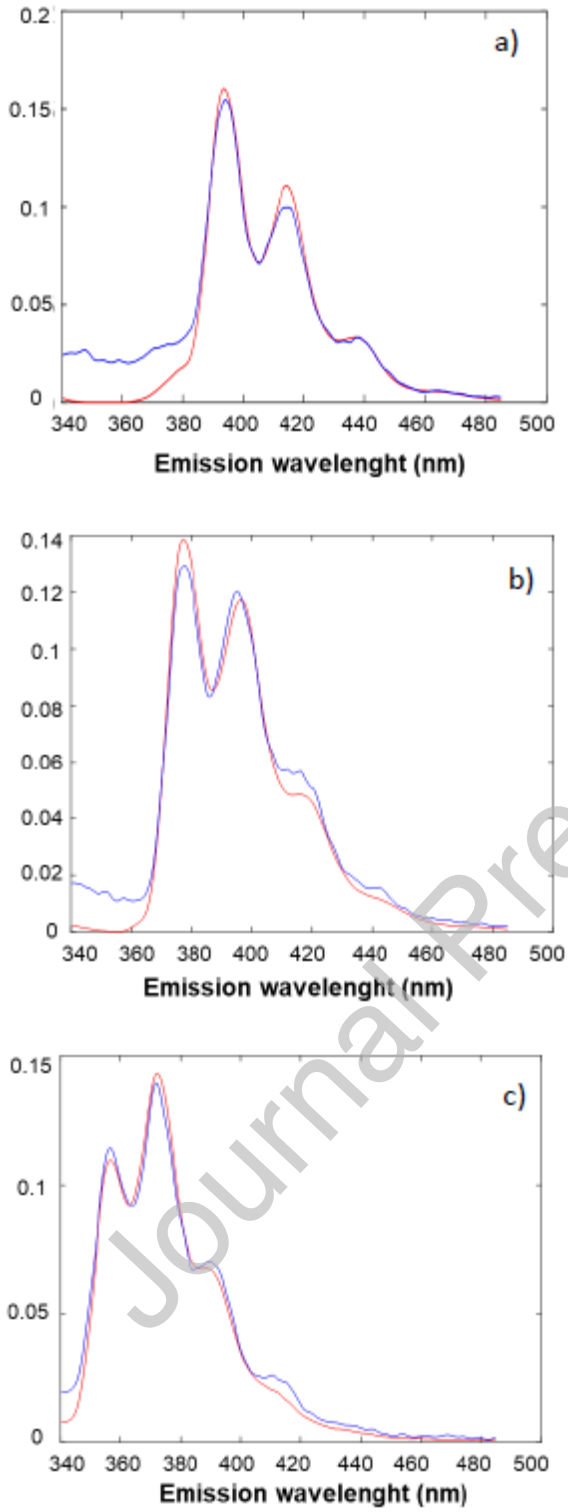

Fig.4. Comparison between the emission reference spectra (in red) and the emission loadings of the PARAFAC model obtained after smoked tuna extraction (in blue) for: BaP (left panel), BaA (middle panel) and Chry (right panel).

TABLES

Table 1
Composition of the experimental design, expressed by levels (second to fourth column) and by concentrations (three last columns).

Sample	Levels			Concentration (μg L^{-1})		
	BaA	BaP	Chry	BaA	BaP	Chry
1	0	0	0	3	3	3
2	0	-2	-2	3	0	0
3	-2	-2	2	0	0	7
4	-2	2	-1	0	7	1
5	2	-1	2	7	1	7
6	-1	2	0	1	7	3
7	2	0	-1	7	3	1
8	0	-1	-1	3	1	1
9	-1	-1	1	1	1	5
10	-1	1	2	1	5	7
11	1	2	1	5	7	5
12	2	1	0	7	5	3
13	1	0	2	5	3	7
14	0	2	2	3	7	7
15	2	2	-2	7	7	0
16	2	-2	1	7	0	5
17	-2	1	-2	0	5	0
18	1	-2	0	5	0	3
19	-2	0	1	0	3	5
20	0	1	1	3	5	5
21	1	1	-1	5	5	1
22	1	-1	-2	5	1	0
23	-1	-2	-1	1	0	1
24	-2	-1	0	0	1	3
25	-1	0	-2	1	3	0

Table-2
Parameters of the regression line "sample loading versus true concentration" of BaP, BaA and Chry. Decision limit (CCα) and capability of detection (CCβ) when the probabilities of false positive (α) and false negative (β) were fixed at 0.05

	BaP	**BaA**	**Chry**
Slope, b_1	534.8	295.1	238.9
Intercept, b_0	70.9	19.5	114.3
P-value (significance of regression model)	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
P-value (lack of fit)	0.05	0.15	0.09
Correlation coefficient, ρ	0.999	0.999	0.999
Residual standard deviation, s_{yx}	29.10	40.30	21.66
Number of outlier samples	1	-	-
Decision limit, CCα (µg L^{-1})	0.11	0.27	0.18
Capability of detection, CCβ (µg L^{-1})	0.21	0.53	0.35

Table 3. Parameters of the regression line "predicted concentration versus true concentration" of BaP, BaA and Chry (Ternary mixtures). Decision limit (CCα) and capability of detection (CCβ) when the probabilities of false positive (α) and false negative (β) were fixed at 0.05

	BaP	**BaA**	**Chry**
Slope, b_1	1.00	1.00	1.00
Intercept, b_0	$4.8\ 10^{-7}$	-2.510^{-8}	$-8.7\ 10^{-8}$
P-value (significance of regression model)	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
P-value (lack of fit)	0.19	0.11	0.99
Correlation coefficient, ρ	0.999	0.999	0.999
Residual standard deviation, s_{yx}	0.11	0.07	0.08
P-value (H_0: slope =1)	0.999	0.999	0.999
P-value (H_0:intercept = 0)	0.999	0.999	0.999
Decision limit, CCα (µg L^{-1})	0.20	0.13	0.15
Capability of detection, CCβ (µg L^{-1})	0.39	0.25	0.29

Table 4
Distribution of concentrations for the three PAHs under study used to perform the calibration

Sample	BaA (μg L^{-1})	BaP (μg L^{-1})	Chry (μg L^{-1})
1	0	0	0
2	0.5	0	0
3	1	0	0
4	1	0	0
5	3	0	0
6	5	0	0
7	7	0	0
8	0	0	0
9	0	0.5	0
10	0	1	0
11	0	1	0
12	0	3	0
13	0	5	0
14	0	7	0
15	0	0	0
16	0	0	0.5
17	0	0	1
18	0	0	1
19	0	0	3
20	0	0	5
21	0	0	7
22	0	0	0
23	6	3	6
24	6	6	3
25	3	3	6
26	0	0	0

Declaration of interests

☒ 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.