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Determination of 4 EU Priority Polycyclic Aromatic Hydrocarbons in Oilseeds by GC-MS/MS Combined with d-SPE QUECHERS Technique

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ABSTRACT

In this study, a method for the simultaneous determination of 4 EU priority polycyclic aromatic hydrocarbons (PAHs): benzo[a]anthracene (BaA), chrysene (ChR), benzo[b]fluoranthene, and benzo[a]pyrene (BaP) from oilseeds by GC-MS/MS was developed and validated. Optimal sample preparation involved the use of modified QuEChERS extraction with hexane, followed by concentration of the extract and collecting it in acetonitrile, and then by two-step purifications, by freezing (-20°C) and by dispersive solid phase extraction (d-SPE QuEChERS).

The developed method was validated by evaluating the linearity on solvents (R^2=0.9947-0.9987) and sunflower seeds (R^2=0.9920-0.9980), matrix effect (≤ ±20%), recovery (81.1-109.8%), precision (RSD=0.4-11.8%) for 3 concentration levels (2, 5 and 7.5 µg/kg), LOD, LOQ, working range, linearity range, and measurement uncertainty. A high analytical sensitivity was obtained for 4 PAHs in oilseeds (LOD=0.05-0.25 µg/kg; LOQ=0.18-0.82 µg/kg), fulfilling the criteria set by the Commission Regulation (EU) No 836/2011.

Sunflower seeds (n=27) harvested from different areas of Romania were analysed using the proposed method. The 4 PAHs in the sunflower seeds ranged from not found to detectable and quantifiable concentrations (>LOQ), with maximum values of 0.56 µg/kg for BaA. BaP, considered a marker for assessing the carcinogenic risk of PAHs in food, it was not quantifiable in any sample but was detected (<LOQ) in about 26% of the analysed oilseeds.

The values determined for BaP and the sum of 4 PAHs were below the limits imposed by Commission Regulation (EU) No 835/2011 for oils and fats, of 2 ppb and 10 ppb, respectively.

Keywords: PAH, QuEChERS, sunflower seeds, method validation, Z-Sep+.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic and persistent compounds that tend to bioaccumulate in plants and crops. PAHs are formed during the incomplete combustion or pyrolysis of organic matter, found in the environment, water, and soil. These compounds contain more fused aromatic rings, based on the number of rings being classified in light PAHs (2-4 rings) and heavy PAHs (>5 rings) (Sadowska-Rociek and Surma, 2021). It is known that light PAHs are less stable and less toxic than heavy PAHs. Usually, light PAHs are found in the gaseous form and can contaminate plants in the field, whereas heavy PAHs might be adsorbed on the particulate matter, mainly in regions with high traffic (Wen et al., 2017). Low molecular weight PAHs tend to accumulate in plants, and their bioavailability is dependent on species, duration of exposure, pollution level, and the chemical properties of the PAHs.

PAHs present a potential risk for human health, being monitored by the EU and also the US EPA (Perestrelo et al., 2019). Considering the carcinogenic effects, EFSA (2008) selected 4 PAHs [benzo[a]anthracene (BaA), chrysene (ChR), benzo[b]fluoranthene (BbF), and benzo[a]pyrene (BaP)] to be indicators for the PAH's carcinogenicity. According to IARC, BaP is classified as carcinogenic to humans (group 1), and BaA, ChR, and BbF are possible carcinogens to humans (group 2B) (IARC, 2010).

Plants, including sunflowers, can accumulate PAHs from the environment, contaminated soil, and water during their growing stage. Salehi-Lisar et al. (2015) investigated the light PAHs uptake (fluorene and phenanthrene) by wheat, sunflower, and alfalfa from controlled contaminated soils, and positive correlations were found between their concentration in the

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soil and in the plant roots. PAHs are translocated from the roots to the shoots; phenanthrene tends to be translocated more than fluorene. Fluorene induces oxidative stress in plants and negatively influences the germination rate and the sunflower growth (Salehi-Lisar and Deljoo, 2015).

Sunflower seeds can be consumed directly, thus, the content of PAH should be monitored. Also, sunflower seeds are an important resource for obtaining vegetable oils, so the level of PAH contamination in seeds should be studied, as this might cause the PAH contamination of the oils.

Most studies have focused on the PAH content of vegetable oils (Molle et al., 2017; Ji et al., 2020), and a few have studied the PAH content of oilseeds (Shi et al., 2016; Wen et al., 2017). So far, the limit for BaP and 4 PAHs has not been established for oilseeds, but for oils and fats, a limit of 2 µg/kg and 10 µg/kg, respectively, was set by the Commission Regulation (EU) No 835/2011.

To determine the PAH content of foodstuffs, several methods were developed. The QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe) has been used lately to determine the PAH content of food products (Petrarca and Godoy, 2018; Singh and Agarwal, 2021). This method firstly involves the extraction of the interest compounds, followed by the clean-up of the extract through dispersive solid-phase extraction (d-SPE) using different sorbents (Perestrelo et al., 2019). This technique is easy to use, requires a reduced amount of sample and solvents, less glass materials, is less expensive than other traditional methods, and presents a high efficiency in extracting the target compounds (Perestrelo et al., 2019; Santana-Mayor et al., 2023). The QuEChERS method can be used for multiresidue analysis, being considered a green analytical chemistry method (Santana-Mayor et al., 2023).

The aim of this study was to develop and validate a d-SPE QuEChERS-GC-MS/MS method to determine the 4 EU priority PAHs in oilseeds. Using the validated method, the PAH contamination of sunflower seeds harvested in 2024 from different regions of Romania was evaluated.

MATERIAL AND METHODS

Materials

A sunflower seed sample with a fat content of 46.39% was used to establish the optimal preparation protocol and to validate the developed method.

A total of 27 sunflower seed samples originating from different counties of Romania were analysed. Samples were harvested in 2024 from the following counties: Tulcea (n=2), Vrancea (n=4), Brașov (n=1), Argeș (n=8), Dâmbovița (n=4), Teleorman (n=6), Ialomița (n=1), and Brăila (n=1).

Standards, solvents, and reagents

A standard mixture of the 4 EU priority PAHs in acetonitrile, purity 96-99.9% (DRE-A50000354AL, 10 µg/mL), and an internal standard mixture of the 4 ^{13}C-labelled PAHs (PAH-IS) in nonane (EFSA - 4 ^{13}PAH, ES-5540, 5 µg/mL) purchased from LGC-Dr Ehrenstorfer (USA), and Cambridge Isotope Laboratories, Inc (USA), were used.

To study the loss during the injection of the internal standard into GC, an injection standard consisting of a solution of 9-fluorobenzo[k]fluoranthene (FBkF) in toluene at a concentration of 100.000 µg/L, purity >99%, purchased from Biosynth Ltd (UK), was used.

The QuEChERS citrate salts (EN) used for extraction consisted of 4 g $MgSO_4$, 1 g NaCl, 0.5 g Na_2H-citrate x 1.5 H_2O, 1 g Na_3-citrate x 2 H_2O, and were purchased from Macherey-Nagel (Germany) (kit 730648.3T). The 500 mg Z-Sep$^+$ sorbent (Supel^{TM} QuE Z-sep$^+$ tube, 15 mL, kit 55486-U) used for purification was acquired from Sigma-Aldrich (Germany). Anhydrous magnesium sulphate ($MgSO_4$) was acquired from Merck (Germany). Also, QuEChERS purification kits, Supel^{TM} Que QuEChERS tubes with 300 mg Z-Sep$^+$ and 900 mg $MgSO_4$ (kit 55511-U) purchased from Sigma-Aldrich (Germany) were used.

Acetonitrile, hexane, toluene, and ultrapure water were purchased from VWR Chemicals (Belgium).

Standard solutions preparation

From the standard solution of 4 EU PAHs, a stock solution in acetonitrile of 1000 µg/L concentration was prepared. From this solution, working solutions of concentration 100 µg/L and 10 µg/L were prepared in acetonitrile. Intermediate solutions of concentration 1, 2, 3, 4, 5, 6, and 7 µg/L were prepared from the working solutions in acetonitrile.

From the standard solution of PAH-IS in nonane, a stock solution of concentration 1000 µg/L was prepared by evaporation under a nitrogen stream, and then the residue was dissolved in toluene. A working solution of concentration 100 µg/L was prepared in acetonitrile from the stock solution.

From the injection standard stock solution, solutions with concentrations of 10.000, 1.000, 100, and 10 µg/L were prepared in acetonitrile.

To prepare the calibration curve, seven calibration solutions in the concentrations of 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 µg/L were prepared in acetonitrile from the intermediate solution of 4 PAHs (1, 2, 3, 4, 5, 6 and 7 µg/L), also containing the 4 PAH-IS in the concentration of 4 µg/L, and FBkF in the concentration of 2 µg/L.

QuEChERS protocol

To establish the optimal sample preparation for PAH determination in oilseeds, the workflow presented in a previous study (Negoiță et al., 2024) and Figure 1 was followed. Based on the proposed protocol, several factors were investigated: the weight of the sample, the volume of extract to be purified, and the quantity of Z-Sep$^+$ and $MgSO_4$ used for clean-up.

Figure 1. The protocol to determine the PAH content in oilseeds by GC-MS/MS-d-SPE QuEChERS with modifications

The weight of the sample taken into the study was 2, 3, 4, and 5 g, respectively. Initially, as presented in the protocol from Figure 1 (Negoiță et al., 2024), a volume of 3.5 mL extract was purified with 500 mg Z-Sep$^+$ and 300 mg $MgSO_4$. In this study, a smaller volume of extract (2.5 mL) was purified with 300 mg Z-Sep$^+$ and 900 mg $MgSO_4$.

The values of co-extractive residues and recoveries were evaluated to study the effectiveness of the QuEChERS extraction and purification method. The co-extractive residues were determined gravimetrically by evaporation of 1 mL of final extract under a nitrogen stream, and they should be less than 0.5 mg/mL. The recovery was evaluated by spiking samples with a 4 PAHs solution, and it should be within the values set by Commission Regulation (EU) No 836/2011 of 50-120%. The sunflower seed sample used was free of PAHs and was spiked at a concentration of 2.5 µg/kg when 3.5 mL extract was used for purification and at 2 µg/kg when 2.5 mL was purified.

GC-MS/MS operation conditions

To quantify PAHs, a gas chromatograph (Trace GC Ultra) coupled with a triple quadrupole mass spectrometer (TSQ Quantum XLS) and an autosampler purchased from Thermo Fisher Scientific (USA) was used. The stationary phase consisted of a TraceGold TG-PAH capillary column (30 m × 0.25 mm I.D. x 0.10 µm) from Thermo Fisher Scientific (USA). The carrier gas used was helium (flow rate 1.5 mL/min), and the collision gas was argon.

The oven temperature program was as follows: an initial temperature of 80°C was held for 1 min; in ramp 1, the temperature increased to 210°C at a rate of 15°C/min; in ramp 2, the temperature increased to 260°C at 25°C/min; in ramp 3, the temperature increased to 305°C at 5°C/min (held for 2 min); and finally, in ramp 4, the temperature increased to 350°C at 25°C/min (held for 5 min). The transfer line and the ion source temperatures were set to 340°C and 300°C, respectively.

The analyses to determine the EU priority PAHs by GC-MS/MS in oilseeds take place within 30.67 min. The qualitative and quantitative analyses were performed in selected reaction monitoring (SRM) mode. The mass-to-charge ratios (*m/z*) for the precursor, quantifying, and qualified ions are presented in Table 1.

Table 1. Compound names, abbreviations, chemical formulas, quantifying and qualified ions for 4 PAHs, internal standards, and injection standard

Compound	Abbreviation	Chemical formula	Precursor ion, *m/z*	Quantifying ion, *m/z*	Qualified ion, *m/z*
Benzo[a]anthracene	BaA	$C_{18}H_{12}$	228	226	202
Benzo[a]anthracene $^{13}C_6$	BaA$^{13}C_6$	$C_{18}D_{12}$	234	232	208
Chrysene	ChR	$C_{18}H_{12}$	228	226	202
Chrysene $^{13}C_6$	ChR$^{13}C_6$	$C_{18}D_{12}$	234	232	208
9-fluorobenzo[k]fluoranthene	FBkF	$C_{20}H_{11}F$	270	268	249
Benzo[b]fluoranthene	BbF	$C_{20}H_{12}$	252	250	226
Benzo[b]fluoranthene $^{13}C_6$	BbF$^{13}C_6$	$C_{20}D_{12}$	258	256	232
Benzo[a]pyrene	BaP	$C_{20}H_{12}$	252	250	226
Benzo[a]pyrene $^{13}C_4$	BaP$^{13}C_4$	$C_{20}D_{12}$	256	254	228

Method validation

The following parameters were investigated to validate the developed QuEChERS method: linearity on solvents and matrix-matched samples (sunflower seeds), matrix effect, sensitivity (limit of detection - LOD, limit of quantification - LOQ), selectivity, recovery, precision, working range, linearity range, and measurement uncertainty. The methods' performance parameters were evaluated following the European Regulations 2021/808, 836/2011, Wenzl et al. (2016), ISO 8466-1:2021, Eurachem/Citac Guide, 2012, and ISO/IEC Guide 98-3:2008.

For the determination of the 4 EU PAHs from oilseeds by GC-MS/MS, the internal standard method was performed, using a

standard mixture of 4 PAH-IS with carbon atoms labelled with $^{13}C_6$ and $^{13}C_4$, respectively: BaA$^{13}C_6$, ChR$^{13}C_6$, BbF$^{13}C_6$, and BaP$^{13}C_4$. When the internal standard is used, the results are corrected automatically, without applying the recovery in the calculation.

The methods' linearity was verified on calibration curves, by the least squares method, performed on both standard solutions (0.5-3.5 µg/L) and sunflower seeds (0.5-3.5 µg/L/2.5-17.5 µg/kg). For good linearity, the acceptability criterion is that the regression coefficient (R^2) to be ≥ 0.99.

The matrix effect (%) was realized in matrix-matched calibration curves by fortifying the sunflower seeds sample with the same levels as those used for calibration curves. This parameter was evaluated by comparing the slopes of the calibration curves obtained on sunflower seed samples with those made on pure solvents.

Selectivity was demonstrated by chromatographic separation of 4 PAHs in the presence of other components from the sunflower seed samples.

For specificity, SRM detection and the use of the PAH-IS method lead to a specific analysis. The retention times of the 4 PAHs within laboratory conditions for the sunflower seed samples were almost identical to the retention times of the 4 PAH-IS. For quantification of the 4 PAHs, the ions (*m/z*) presented in Table 1 were used.

To estimate the LODs and LOQs of the 4 analytes, the calibration approach was applied, with spiking the matrix pseudo-blank (0.625, 1.25, 2.50, and 3.75 µg/kg, respectively, 0.25, 0.5, 1, and 1.5 µg/L), according to the specifications in Wenzl et al. (2016). By evaluating the linear function of the calibration curve (ISO 8466-1:2021) plotted for 0.25-1.5 µg/L, or 0.625-3.75 µg/kg, respectively, LODs and LOQs were calculated as: $LOD = (3.9 \times S_{y,a})/a$, and $LOQ = 3.3 \times LOD$, where $S_{y,a}$ is the standard deviation of the curve response and *a* is the slope of the calibration curve.

Precision was evaluated on sunflower seed samples (analyte-free) spiked at three concentration levels: 2, 5, and 7.5 µg/kg, and analysing the repeatability, reproducibility, and intermediate precision. For the repeatability analysis (intra-day), 6-8 replicates of the sample were realized. Reproducibility was determined by repeating analyses of the samples by two analysts on the same day and under the same experimental conditions. The intermediate precision (inter-day) was carried out by repeated analyses of the samples on 3 to 4 different days under the same conditions, by the same analyst. Precision was expressed as RSD (%), and the imposed criteria were to be lower than 15%, 20%, and 25% in repeatability, reproducibility, and intermediate precision conditions.

The accuracy was evaluated by recovery. The spiked samples with 2, 5, and 7.5 µg/kg were used for the recovery determination. Samples were processed and quantified based on the calibration curves obtained on standard solutions using the Xcalibur software. The imposed criterion for recovery was to range between 50 and 120% as established by Commission Regulation (EU) No 836/2011. The values of HoRRaT in repeatability and reproducibility conditions were also evaluated, which, according to Commission Regulation (EU) No 836/2011, should be <2.

Additionally, the recovery of PAH-IS to FBkF was calculated (EN 16619:2015), and it should range between 50-120% as set by Commission Regulation (EU) No 836/2011. FBkF was added to the sample extract before injection into the GC-MS/MS.

For the linearity range, to establish higher values, the sunflower seed samples were spiked with a mix working solution of native PAHs (0.5-3.5 µg/L) at different concentration levels: 2.5, 5, 7.5, 10, 12.5, 15, and 17.5 µg/kg, respectively. The ratio of the peak area of PAH and PAH-IS against the ratio of the concentration of PAH and PAH-IS from the samples studied was plotted. The correlation was assessed to be linear for a value higher than 0.99 for the correlation coefficient (R).

The working range is the concentration range between the LOQ of each PAH and the

maximum level of linearity in sunflower seed samples.

The measurement uncertainty was also determined. The uncertainty sources were identified and analyzed, and the uncertainty budget was estimated, according to the reference documents (Eurachem/Citac Guide, 2012; ISO/IEC Guide 98-3:2008). Expanded uncertainty (U_Δ) was calculated by multiplying the combined standard uncertainty (Uc) with a coverage factor (k=2) for a confidence level of 95%.

Applicability of the method

To show the applicability of the developed method, a total of 27 sunflower seed samples collected from different counties of Romania were analysed.

Statistical Analysis

All analyses were performed in duplicate, and results for recoveries and co-extractive residues were expressed as mean ± standard deviation (sd). For the validation parameters, the mean recovery, RSD in repeatability, reproducibility, and intermediate precision were calculated and expressed in percentages for 6-8 replicates. The PAH content of sunflower seeds was expressed in µg/kg.

RESULTS AND DISCUSSION

Selection of optimal sample preparation protocol

During method development, several factors should be investigated. In this study, to establish the optimal sample preparation protocol, the weight of the sample, the volume of extract, and the quantity of sorbents used were evaluated. The type of sorbent, salts, and their amount are important factors that have to be evaluated (Perestrelo et al., 2019). The co-extractive residues and recoveries determined for the spiked (2 µg/L) sunflower seeds are presented in Table 2.

Visually, the extracts from the proposed variants were clean and colourless. The values of co-extractive residues were lower than 0.5 mg/mL for all experimental variants, fulfilling the imposed criterion. Recoveries ranged from 84.5% to 106.2% when 3.5 mL of extract was purified with 500 mg Z-sep$^+$ and 300 mg $MgSO_4$ (Negoiță et al., 2024), and from 99.2% to 114.4% when 2.5 mL of extract was purified with 300 mg Z-sep$^+$ and 900 mg $MgSO_4$. All recoveries were within the limits imposed by Commission Regulation (EU) No 836/2011.

Table 2. Co-extractive residues and recoveries obtained for spiked sunflower seeds

Sample weight, g	PAH	3.5 mL extract through 500 mg Z-Sep$^+$ + 300 mg $MgSO_4$ (Negoiță et al., 2024)		2.5 mL extract through 300 mg Z-Sep$^+$ + 900 mg $MgSO_4$	
		Recovery (mean ± sd), %	Co-extractive residue (mean ± sd), mg/mL	Recovery (mean ± sd), %	Co-extractive residue (mean ± sd), mg/mL
2	BaA	84.9 ± 3.7	0.21 ± 0.02	113.2 ± 2.3	0.16 ± 0.00
	ChR	84.5 ± 14.4		104.5 ± 2.7	
	BbF	101.0 ± 0.8		101.0 ± 1.7	
	BaP	90.8 ± 0.5		108.4 ± 4.0	
3	BaA	105.1 ± 0.4	0.22 ± 0.01	114.4 ± 2.1	0.19 ± 0.00
	ChR	89.8 ± 2.6		101.7 ± 0.6	
	BbF	100.4 ± 0.6		99.3 ± 5.1	
	BaP	106.2 ± 2.3		103.4 ± 3.2	
4	BaA	95.6 ± 0.1	0.21 ± 0.01	99.2 ± 1.3	0.20 ± 0.00
	ChR	97.5 ± 1.2		103.6 ± 0.6	
	BbF	100.1 ± 2.8		97.3 ± 1.1	
	BaP	98.7 ± 3.2		100.1 ± 1.5	
5	BaA	102.4 ± 0.1	0.21 ± 0.01	107.3 ± 0.1	0.21 ± 0.00
	ChR	98.0 ± 0.2		109.8 ± 1.7	
	BbF	100.0 ± 0.3		104.1 ± 2.7	
	BaP	94.6 ± 0.5		103.6 ± 1.9	

Also, considering that a small amount of sample taken in the work (2 g) determines a low sensitivity of the method (LOD>0.30 µg/kg and LOQ>0.90 µg/kg), and a larger amount (4 g) determines a higher sensitivity of the method (LOD<0.30 µg/kg and LOQ<0.90 µg/kg) (results not shown), a weight of 4 g of sample was chosen for further experiments.

Since the sorbent 300 mg Z-sep$^+$ and 900 mg $MgSO_4$ is commercialized as a kit product, and it does not have to be weighed, it was selected for the validation procedure as the analysis time is reduced.

Method validation

Based on the developed method, the validation parameters were evaluated on the same sample of sunflower seeds used for optimization and development.

The method's linearity was evaluated based on calibration curves obtained on standard solutions and sunflower seeds. For the 4 PAHs, satisfactory linearities were obtained, with correlation coefficients R^2 ranging from 0.9947 to 0.9987 in the case of standard solutions, and from 0.9920 to 0.9980 in the case of sunflower seed samples (Table 3). Over the studied concentration ranges, the method developed on both standard solutions and oilseed samples provides analytical signals proportional to the analyte concentration, with regression coefficient values $R^2 \geq 0.99$, fulfilling the required criterion.

Table 3. Linearity (standard solutions and sunflower seeds), matrix effect (ME), and sensitivity for the 4 PAHs analysed

Calibration curves	PAH	Linearity				ME, %	Sensitivity	
		Regression equation	Slope	R^2	Standard deviation of the method, S_{x0}		LOD, µg/kg	LOQ, µg/kg
Standard solutions	BaA	y = 0.7814x - 0.0144	0.7814	0.9947	0.02130	-		
	ChR	y = 0.7135x + 0.0018	0.7135	0.9968	0.01634			
	BbF	y = 1.2693x - 0.0143	1.2693	0.9968	0.01627			
	BaP	y = 1.0681x + 0.0398	1.0681	0.9987	0.01044			
Sunflower seeds	BaA	y = 0.7797x + 0.0119	0.7797	0.9920	0.02872	-0.22	0.12	0.40
	ChR	y = 0.7116x + 0.0089	0.7116	0.9973	0.01793	-0.27	0.23	0.74
	BbF	y = 1.1551x + 0.0214	1.1551	0.9927	0.02863	-9.00	0.05	0.18
	BaP	y = 0.9987x + 0.0494	0.9987	0.9980	0.04056	-6.50	0.25	0.82

R^2 - regression coefficient, ME - matrix effect.

The matrix effect should be studied when a method is validated, showing the possible matrix interferences (Belo et al., 2017). Matrix effects can influence the PAH quantification, enhancing or suppressing the PAH content (Rutkowska et al., 2020). The matrix effect ranged between -0.22% and -9%, less than ±20% (Table 3) as set by Commission Regulation (EU) No 2021/808, indicating that the method is robust. The fact that all values were negative for the 4 EU PAHs indicates a suppression, a reduction of the analytes' signals. This could be the effect of the accumulation of less volatile substances in the GC-MS/MS system (Rutkowska et al., 2020).

Selectivity was ensured by separating the chromatographic peaks at the base, so that the response function was a linear relationship between the PAH/PAH-IS corrected area ratio, respectively, the concentration ratio, both on standard solutions (Figure 2) and on sunflower seeds (Figure 3). On standard solutions, R^2 ranged between 0.9947 and 0.9987, and for sunflower seeds it varied between 0.9920 and 0.9980. From the analysis of the results obtained, no interferences were observed that would prevent the identification of the analytes, and no peaks were recorded that interfered with the solvent used or with other compounds present in the food matrix, demonstrating that the method is selective and specific. Using the SRM detection mode and internal standards labelled with C^{13} leads to a specific analysis.

The LODs and LOQs were determined to show the method's sensitivity (Table 3).

LOD determined for BaA, ChR, BbF, and BaP were 0.12, 0.23, 0.05, and 0.25 µg/kg, respectively. For LOQ, the determined values were 0.40, 0.74, 0.18, and 0.82 µg/kg, fulfilling the required criterion of Commission Regulation (EU) No 836/2011 (LOD≤0.30 µg/kg; LOQ≤0.90 µg/kg).

Precision was evaluated by RSD (%). The RSDs ranged between 0.5-4.4%, 0.4-5.9%, and 3.0-11.8% for all 4 analytes determined in repeatability, reproducibility, and intermediate precision conditions (Table 4). RSDs were lower than the imposed criteria for the tested conditions.

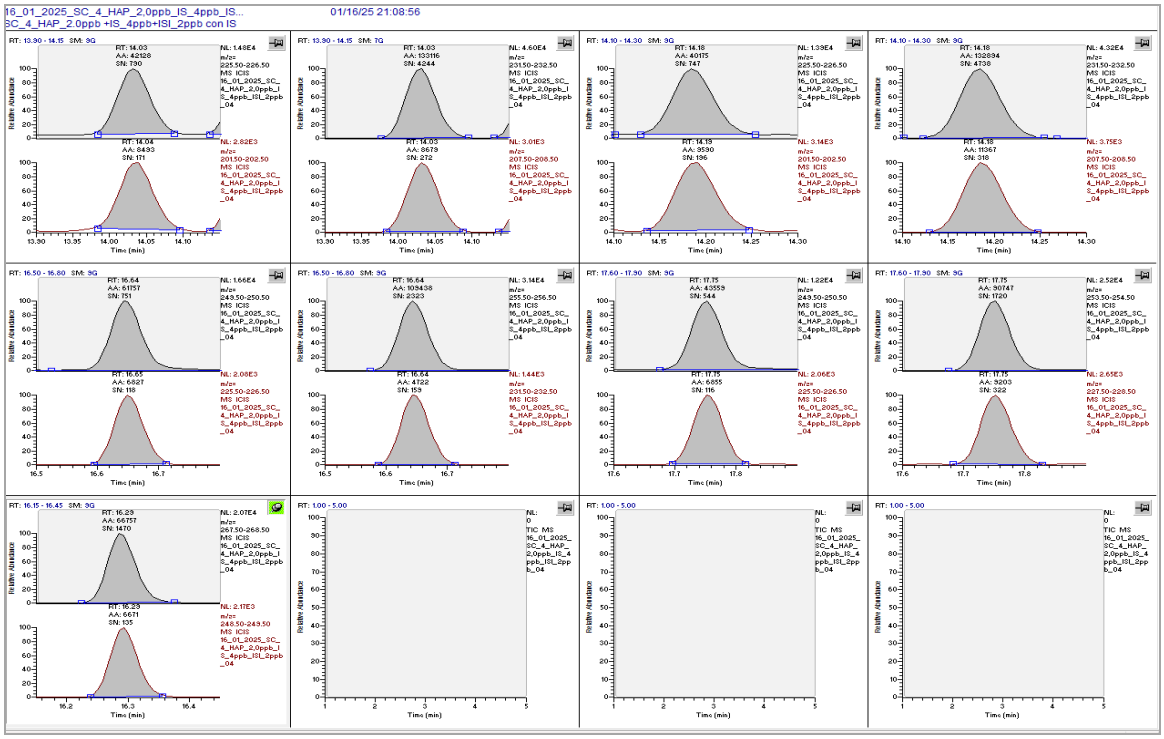

Figure 2. m/z Chromatogram of 4 PAHs (2 µg/L) in a standard mixture

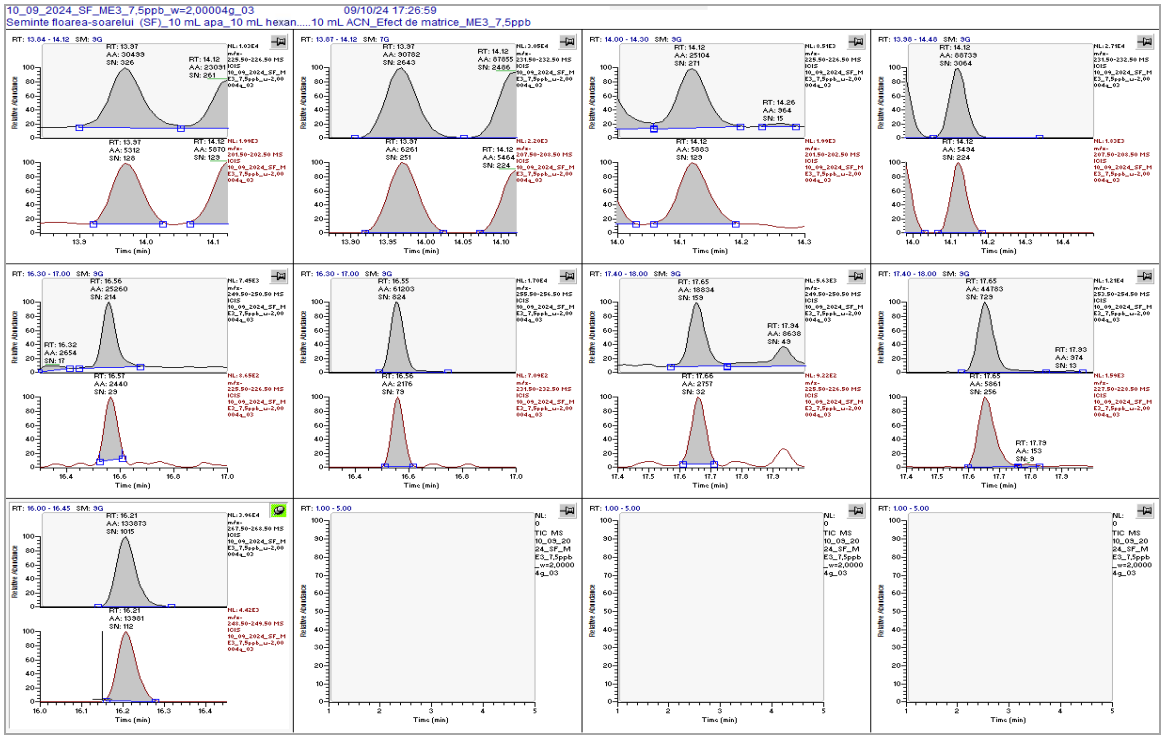

Figure 3. m/z Chromatogram of 4 PAHs (1.5 µg/L spiking level) in an extract of sunflower seeds

Recovery (%) was determined for fortified sunflower seed samples at the 2, 5, and 7.5 µg/kg levels in different conditions. The recoveries for all spiked levels and all conditions varied from 81.1 to 109.8%. Similarly, when Shi et al. (2016) fortified oilseeds at levels of 2, 5, and 10 µg/kg, the recoveries for the 4 PAHs ranged from 91.0 to 108.1%.

For HoRRaT, the values ranged between 0.01 and 0.35, lower than the imposed criterion of <2 for all spiking levels and all conditions used for validation.

The recovery of PAH-IS to FBkF ranged between 50.8% and 89.2% for all spiking levels, fulfilling the criterion to range between 50% and 120%. RSDs varied between 1.3% and 5.1%.

Table 4. Mean recovery (%), RSD (%), and HoRRaT values for the developed d-SPE QuEChERS-GC-MS/MS method

Parameter	Spiked level, µg/kg	Recovery (%) (RSD, %) (HoRRaT)			
		BaA	ChR	BbF	BaP
Repeatability	2	102.1 (3.5) (0.08)	97.4 (4.4) (0.11)	95.9 (4.0) (0.10)	97.6 (4.3) (0.10)
	5	105.8 (1.3) (0.04)	98.0 (0.5) (0.01)	93.6 (0.7) (0.02)	100.7 (2.1) (0.06)
	7.5	81.2 (1.8) (0.05)	84.1 (3.2) (0.09)	83.0 (3.3) (0.10)	82.6 (1.7) (0.05)
Reproducibility	2	103.5 (3.8) (0.10)	98.5 (5.2) (0.12)	97.0 (5.9) (0.15)	97.5 (4.0) (0.10)
	5	109.8 (2.1) (0.06)	97.6 (0.4) (0.01)	101.2 (4.1) (0.12)	98.4 (1.9) (0.05)
	7.5	81.1 (5.9) (0.05)	83.3 (3.4) (0.10)	82.4 (3.2) (0.09)	82.0 (2.7) (0.08)
Intermediate precision	2	108.5 (5.5) (0.14)	102.5 (6.7) (0.17)	103.0 (6.2) (0.16)	107.0 (8.0) (0.20)
	5	103.4 (6.9) (0.20)	98.4 (3.0) (0.09)	92.4 (4.4) (0.12)	96.4 (3.7) (0.10)
	7.5	95.3 (11.8) (0.35)	92.1 (6.7) (0.20)	89.3 (5.6) (0.17)	92.3 (7.6) (0.23)
Recovery IS/FBkF (%) (RSD_R, %)	2	66.8 (5.1)	62.1 (3.5)	53.7 (5.1)	50.8 (1.3)
	5	89.2 (3.2)	84.1 (5.0)	74.4 (3.3)	66.9 (3.4)
	7.5	69.4 (4.5)	64.6 (4.1)	55.4 (3.7)	51.8 (2.9)

The working ranges for BaA, ChR, BbF, and BaP were 0.40-16.47 µg/kg, 0.74-16.89 µg/kg, 0.18-16.46 µg/kg, and 0.82-17.64 µg/kg, respectively.

The linearity range was verified according to ISO 8466-1:2021 for oilseeds analyzed both in the validation process and for seed samples harvested from different areas of the country. The linearity ranges of the method to analyse the 4 PAHs were: 1.14-16.80 µg/kg (R^2=0.9986) for BaA (n=20), 1.35-17.32 µg/kg (R^2=0.9991) for Chr (n=21), 0.40-15.90 µg/kg (R^2=0.9915) for BbF (n=24), 1.68-18.36 µg/kg (R^2=0.9994) for BaA (n=22), where n represents the number of samples analyzed.

The uncertainty of the results for the simultaneous determination of the 4 EU PAHs in oilseeds by GC-MS/MS varied according to the estimates from the uncertainty budgets, as follows: ±20.5% for BaA, ±19.1% for ChR, ±19.5% for BbF, and ±19% for BaP.

Applicability of the method

To study the method's applicability, 27 sunflower seed samples harvested in 2024 from eight counties of Romania were analysed, and the results are presented in Table 5. When calculating the sum of 4 PAHs, if the content of one of the 4 EU PAHs was <LOQ, it was considered the content equal to LOQ.

BaA, a PAH with 4 rings, was found in only one (3.70%) of the 27 analysed samples, with a content of 0.56 µg/kg. The sample in which BaA was quantified is from Negrasi (II), Argeș County.

ChR, another PAH with 4 rings, was not found in 24 (88.89%) of the sunflower seed samples analysed. This compound had a content lower than LOQ (0.74 µg/kg) in 3 samples, of which 2 were harvested from Argeș County, Negrasi (II) and Barla localities, and one from Teleorman County, Balaci locality.

BbF, a 5-ring PAH, was not found in 19 (70.37%) sunflower seed samples analyzed. This compound had an undetectable level (<0.05 µg/kg) in one sample from Argeș County, locality Trivale. Five (18.52%) out of the 27 analyzed samples presented a BbF content lower than LOQ (0.18 µg/kg). These

samples were collected from Brașov, Argeș, and Dâmbovița Counties. Two samples (7.41%) collected from Argeș County presented a quantifiable content of BbF, with a level of 0.19 µg/kg and 0.31 µg/kg, respectively.

BaP, a 5-ring PAH, which is considered a marker for assessing the carcinogenic risk of PAHs in food products, was present in more of the sunflower seeds tested, compared to the other PAHs analysed. This compound was not found in 4 samples (14.81%), had an undetectable (Nd) level in 16 samples (59.26%), and an unquantifiable content (<0.82 µg/kg) in 7 samples (25.93%). BaP was detected in 1 sample from Tulcea, 1 from Vrancea, 2 from Argeș, 1 from Dâmbovița, 1 from Teleorman, and 1 sample from Brăila Counties.

The sum of 4 PAHs for the 27 sunflower seed samples ranged between NF to a value <1.87 µg/kg. The maximum level of the sum of 4 PAHs was found in a sample from Argeș County, Barla locality.

Table 5. PAH content in the sunflower seeds harvested in 2024 from different counties of Romania

County	Locality	PAH content (mean ± sd), µg/kg				Σ4 PAH, µg/kg
		BaA	ChR	BbF	BaP	
Tulcea	Frecatei	NF	NF	NF	<LOQ	<0.82
Tulcea	Macin	NF	NF	NF	NF	NF
Vrancea	Vulturu	NF	NF	NF	NF	NF
Vrancea	Calieni	NF	NF	NF	<LOQ	<0.82
Vrancea	Maicanesti	NF	NF	NF	NF	NF
Vrancea	Nanesti	NF	NF	NF	NF	NF
Brașov	Codlea	NF	NF	<LOQ	Nd	≤0.18
Argeș	Trivale	NF	NF	Nd	Nd	Nd
Argeș	Negrasi (I)	NF	NF	<LOQ	Nd	≤0.18
Argeș	Negrasi (II)	0.56 ± 0.06	<LOQ	0.19 ± 0.05	Nd	≤1.49
Argeș	Negrasi (III)	NF	NF	NF	Nd	Nd
Argeș	Izvoru	NF	NF	<LOQ	<LOQ	<1.00
Argeș	Barla	NF	<LOQ	0.31 ± 0.01	<LOQ	<1.87
Argeș	Caldararu	NF	NF	NF	Nd	Nd
Argeș	Costesti	NF	NF	NF	Nd	Nd
Dâmbovița	Titu	NF	NF	<LOQ	Nd	≤0.18
Dâmbovița	Niculesti	NF	NF	<LOQ	Nd	≤0.18
Dâmbovița	Morteni	NF	NF	NF	<LOQ	<0.82
Dâmbovița	Titu	NF	NF	NF	Nd	Nd
Teleorman	Putineiu	NF	NF	NF	Nd	Nd
Teleorman	Balaci	NF	<LOQ	NF	<LOQ	<1.56
Teleorman	Peretu	NF	NF	NF	Nd	Nd
Teleorman	Scurtu Mare	NF	NF	NF	Nd	Nd
Teleorman	Siliștea Mica	NF	NF	NF	Nd	Nd
Teleorman	Udeni	NF	NF	NF	Nd	Nd
Ialomița	-	NF	NF	NF	Nd	Nd
Brăila	Tudor-Vladimirescu	NF	NF	NF	<LOQ	<0.82

NF - not found; **Nd** - not detected (<LOD); **<LOQ** - unquantifiable.

Wen et al. (2017) analysed the PAH contamination of two sunflower seed samples from China and determined a BaP content of 0.2-0.4 µg/kg, while the 4 PAH content ranged between 3.1 and 5.4 µg/kg. In the study conducted by Shi et al. (2016), 17 oilseed samples were analysed, and sunflower seeds were the most contaminated. The BaP content of the two sunflower seeds analysed ranged between 0.1 and 6.3 µg/kg, while the 4 PAH content ranged from 3.1 to 37.4 µg/kg.

Three different batches (I, II, III) of sunflower seeds, harvested from the same county (Argeș) and the same locality (Negrași), presented variations in the 4 PAHs content, for each PAH it ranged from NF to 0.56 µg/kg, and for the sum varied from not detected to 1.49 µg/kg.

Considering that Commission Regulation (EU) No 835/2011 established a limit of 2 μg/kg for BaP and 10 μg/kg for the sum of 4 PAHs only for oils and fats, we evaluated the level of contamination of sunflower seeds based on these limits. None of the analysed samples exceeded the limits imposed by the EU.

CONCLUSIONS

In this study, a modified QuEChERS method for 4 EU PAHs determination in oilseed based on EN salts extraction, followed by freezing the extract, d-SPE QuEChERS purification with Z-Sep$^+$, and quantification with GM-MS/MS, was developed and validated.

Based on the proposed method, 27 sunflower seed samples harvested in 2024 from eight counties of Romania were analysed to study the PAH contamination.

Results showed that BaA and BbF were quantified in 1 and 2 samples, respectively, and ChR, BbF, and BaP were detected (<LOQ) in 3, 5, and 7 samples.

Results showed that BaP, the PAH considered the most carcinogenic, was detected (<LOQ) in 7 of the 27 sunflower seeds harvested in 2024 from Romania.

The content of BaP (NF - <0.82 μg/kg) and the sum of 4 PAHs (NF - <1.87 μg/kg) in the analyzed sunflower seeds were below the level proposed by the EU, demonstrating that oilseeds cultivated and harvested from different areas of the country, used in the manufacture of vegetable oils are safe from the point of view of these chemical contaminants.

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