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Development and validation of GC/MS method for simultaneous determination of 16 polycyclic aromatic hydrocarbons (PAHs) in pork meat matrix

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ABSTRACT

The major processes for introducing polycyclic aromatic hydrocarbons (PAHs) in food are smoking and grilling of different products. But in addition, PAHs can permeate in the food chain due to their high lipophilicity and ability to be accumulated in specific tissue, through contaminated animal feed. Further, when some parts of these animals are marketed as food, the accumulated PAHs can go to the human organism. Some of them are classified as highly toxic, carcinogenic and mutagenic for animal and human organisms so they are under consideration of International and European legislation. This work reports development and validation of simple and fast GC/MS method for 16 PAHs determination. Comparison of two methods for sample preparation in pork meat matrix standard extraction/saponification procedure and modified QuEChERS method is also done. In addition, this paper report the calibration step of instrument and a recovery study for 16 PAHs in model pork meat, using modified QuEChERS procedure for sample pretreatment. The calibration step with accessible and suitable for use in real laboratory conditions internal standard (chrysene D₁₂) is done in the range 10–100 ppb using toluene as solvent. The obtained results show very good linearity ($R^2 = 0.99$ to 1.00). For the recovery study six model samples were spiked with 16 PAHs and they all are subjected to QuEChERS procedure. The recovery is calculated and the obtained data (71–120%) is in a good correlation with requirements of international legislation. Finally, LOD values for all 16 investigated compounds of modified GC/MS method and for the instrument were determined.

KEYWORDS

polycyclic aromatic hydrocarbons (PAHs), GC/MS analysis, QuEChERS procedure for sample preparation

INTRODUCTION

The group of polycyclic aromatic hydrocarbons (PAHs) is largely distributed in the nature [1]. They contain two or more fused aromatic rings. They are formed as a result of many natural and anthropogenic processes. The two main sources are pyrogens (incomplete combustion of coal, oil, gas, wood, waste or other organic substances) and petrogenic pathways [2–4]. The major processes for introducing PAHs in food are smoking and grilling of different products. A lot of other sources of contamination especially of plants further used as a row materials for production of different good as well as for direct food of animals or humans are described in the scientific literature among which contaminated soil [5–7], particles from air [8, 9], contamination of different oils during process of their production [10] and many other [11–14]. PAHs can be also largely distributed in a seafood directly used for cooking [15–17]. Finally, PAHs can permeate in the food chain due to their high lipophilicity and ability to be

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accumulated in specific tissue, through contaminated animal feed. Further, when some parts of these animals are marketed as food, the accumulated PAHs can go to the human organism [18]. Moreover, part of these substances, those with 4–6 fused rings are described and classified as carcinogenic and mutagenic both for human and animal organisms and they are of a special apprehension [19–21].

Due to the high toxicity of some of PAHs and harmful effect on human health, they are under consideration of International and European legislation [22, 23]. In present days there are methods for determination of PAHs in one or several matrices using different kind of instrumental technics. The most common are HPLC combined with fluorescent detection and gas chromatography with mass spectrometric detection [24–29]. There is scarce data in the scientific literature for methods for determining of all 16 PAHs in specific matrices and often they are related to the specific apparatus or method for sample preparation [30–32].

Process of sample preparation is very important part of every analytical procedure. This process is different depending on the fat content in the matrix. Related to the PAHs determination often it is a long complicated procedure needed by specific apparatus or reagents [33–35].

In our previous work we tried to find an appropriate internal standard to replace used in EN 16619:2015 method ^{13}C labeled internal standard benzo[a]pyrene $^{13}\text{C}_4$ and to make some modifications in the method for determination of only benzo[a]pyrene in two matrices wheat flour and smoked pork leg. The main purpose of this investigation was to adapt an internal standard that would make the method applicable in real laboratory conditions for analysis, since one of the components of the final cost of the analysis is the price of the standards used. Used in the document EN 16619:2015 benzo[a]pyrene $^{13}\text{C}_4$ as an internal standard is relatively expensive, which of course will also affect the final cost of the analyzes. Finally we succeed to modified methods conditions in order to use cheaper and more available Chrysene D_{12} as an internal standard and to cover the requirements of European legislation according to recovery rates for benzo[a]pyrene [36]. Further, we continued our work on the modification of procedure and herein we report the development and validation of GC/MS method suitable for determination of all 16 PAHs under International and European legislation, in smoked pork meat matrix using Chrysene D_{12} as an internal standard. In addition, we compare two methods for sample preparation – a standard one (Soxhlet extraction, saponification and clean up) and modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method for smoked pork meat samples. The GC conditions were also been developed in order to improve the sensitivity and selectivity of the chromatographic system.

MATERIALS AND METHODS

Chrysene D_{12} purchased by Supelco is prepared with concentration of 658 ng/g in toluene.

CRM PAH 16 Compounds mix 500 ug/mL for calibration and determination is purchased by Reagecon

Diagnostics Ltd (Shannon, Ireland). KOH is purchased by Sigma–Aldrich (Deisenhofen, Germany).

QuEChERS extract pouch Bond Elut 5982-6650 is purchased by Agilent Technologies.

SPE test tube Bond Elut 5982-5056 is purchased by Agilent Technologies (Sofia, Bulgaria). SPE test tube 0.5 g silica gel (Sigma Aldrich, Deisenhofen, Germany) + 0.5 g sodium sulfate (Sigma Aldrich, Deisenhofen, Germany) is prepared in laboratory.

Chilled vacuum smoked pork meat is used for this investigation. All used solvents were analytical grade and they are used without any additional treatment.

Standard solution preparation

All calibration solutions (concentration range in final calibration solutions) in the working range 10–100 ng/g are prepared gravimetrically in toluene. For all 16 aimed PAHs and chrysene D_{12} initial stock solutions with four concentrations 21.7; 41.6; 101.2 and 201.2 [ng/g] for PAHs and around 150 [ng/g] for Chrysene D_{12} are used. Starting from stock solutions, calibration solutions with four concentration 10.8; 20.6; 51.2; 101.5 [ng/g] for PAHs and 70 [ng/g] for Chrysene D_{12} are prepared by mixing with internal standard solution.

Chromatographic conditions

GC conditions. GC column SLB-5 MS (Supelco, Merck KGaA, Darmstadt, Germany) 30 m' 0.32 mm i. d.' 0.25 μm film thickness. Column oven temp.: 70 °C; Injection temp.: 300 °C; Injection mode: Splitless; Sampling time: 0.50 min; Flow control mode: Linear velocity; Inlet pressure: 52.3 kPa; Total flow: 78 mL/min; Column flow: 3 mL/min; Purge flow: 15 mL/min; High pressure injection: ON (150 kPa for 0.50 min); Oven temperature program: 70 °C (hold 2 min); ramp 1 (8–300 °C); ramp 2 (20–340 °C, hold 10 min).

MS conditions. Ion source temp.: 220 °C; Interface temp.: 300 °C; Solvent cut time: 3 min; MS mode: SIM; Monitored ions are presented in Table 1.

Standard extraction and clean up procedure for sample preparation

Step 1. Soxhlet extraction

Five grams of smoked pork meat is mixed with 5 g of sodium polyacrylate and 5 g sand. To the obtained mixture 200 μL internal standard chrysene D_{12} are added and sample is homogenized in the mortar. Further, the obtained mixture is subjected to the Soxhlet extraction in 100 mL of hexane for 7 h. Further, the solvent is evaporated under vacuum.

Step 2. Saponification

Four milliliter 60% KOH in water and 8 mL of ethanol are added to the raw extract from step 1. The obtained solution is shaken 10 min on vortex. The mixture is put in 50 mL tube and it is extracted with 3×20 mL hexane. The organic layers are collected and concentrated under vacuum.

Table 1. Retention time, target, and referenced monitored ions for aim compounds

No	Name	t_{Rmin}	Target ion m/z	Ref. ion 1 m/z	Ref ion 2 m/z
1	Naphthalene	5.90	128	127	129
2	Acenaphthylene	10.23	152	151	76
3	Acenaphthene	10.75	153	154	152
4	Fluorene	12.25	166	165	82
5	Phenanthrene	14.97	178	176	89
6	Anthracene	15.12	178	176	89
7	Fluoranthene	18.42	202	101	200
8	Pyrene	19.01	202	101	200
9	Benz[a]anthracene	22.57	228	226	114
10	Chrysene	22.66	228	226	229
11	Benzo[b]fluoranthene	25.49	252	126	250
12	Benzo[k]fluoranthene	25.56	252	126	250
13	Benzo[a]pyrene	26.26	252	126	250
14	Indeno[1,2,3-cd]pyrene	28.82	276	138	277
15	Dibenzo[a,h]anthracene	28.93	278	139	279
16	Benzo[g,h,i]perylene	29.33	276	138	137
17	Chrysene D ₁₂	22.56	240	238	236

Table 2. The characteristics of linearity for studied substances in investigated range 10.83–101.53 [ng/g]

Name	Regression equation	R^2
Naphthalene	$2.0054x + 0.1162$	0.99
Acenaphthylene	$1.9917x + 0.0253$	0.99
Acenaphthene	$1.2985x + 0.0110$	0.99
Fluorene	$1.1542x - 0.0019$	0.99
Phenanthrene	$1.0961x - 0.0430$	0.99
Anthracene	$1.4800x + 0.0295$	0.99
Fluoranthene	$1.1625x + 0.0054$	1.00
Pyrene	$1.2531x + 0.0122$	0.99
Benz[a]anthracene	$0.4639x + 0.0563$	0.99
Chrysene	$1.5014x + 0.0300$	1.00
Benzo[b]fluoranthene	$0.7430x - 0.0349$	0.99
Benzo[k]fluoranthene	$1.6465x - 0.0382$	0.99
Benzo[a]pyrene	$0.7632x + 0.0089$	0.99
Indeno[1,2,3-cd]pyrene	$0.9999x + 0.0030$	0.99
Dibenz[a,h]anthracene	$0.9160x - 0.0085$	0.99
Benzo[ghi]perylene	$1.1544x + 0.0066$	0.99

Step 3. Solid phase extraction (SPE)

The obtained raw concentrate from step 2 is mixed with 200 μ L of toluene and the volume of sample is reduced to 1 mL under nitrogen atmosphere. The obtained residue is put on SPE column containing 0.5 g silica gel and 0.5 g sodium sulfate. The aim compounds are eluted gravitationally with 10 mL of hexane. The obtained sample is concentrated under vacuum to 0.5 mL final volume and injected to GC/MS system.

QuEChERS procedure for sample preparation

Five grams homogenized sample is placed in 50 mL polypropylene tube. Internal standard chrysene D₁₂ solution 0.9 mL is added. Further, 0.084 g spiking standard solution of all 16 PAHs with concentration 5,000 ng/g is added. The tube is shake vigorously for 1 min on a vortex. After 8 mL

acetonitrile is added and the tube is again shaken vigorously for 1 min on a vortex. One QuEChERS extract pouch (Agilent 5982-6650) is added, sample is shaken 1 min and centrifuge at 5,000 rpm for 5 min. Six milliliter from the obtained extract is transferred to 15 mL dispersive SPE test tube (Agilent 5982-5056), shaken for 1 min and centrifuged at 5,000 rpm for 5 min. 1 μ L of obtained extract is injected in chromatographic system.

RESULTS AND DISCUSSION

Herein, we reported the development of GC conditions and calibrating step of GC/MS instrument as well as a recovery study for 16 PAHs under control of international legislation in smoked pork meat sample using both standard (extraction and clean up) method and modified QuEChERS procedure for sample pretreatment.

Taking into account our preliminary investigations on the topic described in Givechev et al. 2018, herein an internal standard chrysene D₁₂ is used [36]. We also modified the chromatographic conditions according to those used in Givechev et al. 2018 in order to improve sensitivity and

Figure 1. Identification chromatogram in SIM mode of standard solution of all 16 aimed PAHs with concentration 50 ppb

Figure 2. A. Chromatogram of aimed compounds after Soxhlet extraction and saponification as sample preparation procedure; B, C and D are zoomed parts of chromatogram between 4.0 and 6.4 min in order to determine compounds with closed t_R in the interval; E and F are zoomed parts of chromatogram between 15.7 and 23.3 min in order to determine compounds with closed t_R in the interval; G and H are zoomed parts of chromatogram between 31.0 and 40.0 min in order to determine compounds with closed t_R in the interval

selectivity of the chromatographic system [36]. The obtained chromatogram in SIM mode of standard solution of all 16 aimed PAHs with concentration 50 [ng/g] at new optimized chromatographic conditions described above in materials and methods section is presented on Fig. 1 and their identification retention times in Table 1.

For the calibration step a set of calibration standards in concentration range from 10 ng/g to 100 ng/g were prepared gravimetrically. Then an internal standard procedure using chrysene D₁₂ for calibration is realized and calibration curves were created using 4 points with concentrations 10, 20, 50, and 100 ng/g. The results of calibration step show very good linearity R^2 from 0.99 to 1.00 for all 16 studied PAHs in the working range 10–100 ng/g that fit with the legislation (Table 2).

The process of sample pretreatment play key role in the successful separation and analysis of aim component. In addition the approach used for sample preparation is different according to the fat content of sample and fat content samples needs to pass more complicate procedure in order to remove fat content. Next step of our work was to investigate different procedures for sample pretreatment. The matrix of smoked pork was especially selected as a high

fat content matrix. Three samples of smoked pork meat were subjected to a standard extraction and clean up procedure as it is described in the material and methods section. The whole procedure was realized in three stages and it took around 16 h (separated in 2 days). The obtained samples are further injected to the GC/MS system, using chromatographic conditions from our preliminary investigations and resulting chromatogram is presented on Fig. 2 [36].

It can be clearly seen from the obtained chromatogram (Fig. 2) that there are undesired compounds from the matrix which pass through the sample preparation procedure together with aimed compounds. Moreover, some of them interfere determination of several of target compounds (naphthalene, acenaphthylene, benzo[k]fluoranthene) which are completely covered by matrix component or reduce the recovery rates. On the other hand the used GC conditions have shown low sensitivity.

In order to optimize the process of sample preparation according to the time of process and purity of obtained for analysis samples as well as to realize our aim, to determine simultaneously all 16 aimed PAHs, a modified QuEChERS method for sample preparation and modified chromatographic

Figure 3. Chromatograms of aimed compounds after QuEChERS procedure for sample preparation procedure: A. Reference compounds at 50 ppb concentration, B. Spiked sample with 50 ppb concentration of aimed compounds, C. Matrix without spiking

conditions described in material and methods section was used. Some other authors also report for QuEChERS procedures for sample preparation. According to Surma et al. in 2014 QuEChERS method for application for PAHs determination in food of animal origin has not been reported as yet [37]. Authors experimented different stationary and mobile phases in order to achieve the best results according to linearity, selectivity, recovery, repeatability, the limit of detection (LOD) and limit of quantification (LOQ). Their experiments reveal that ethylacetate as solvent as well as primary-secondary amines (PSA) and C18 as sorbents are the best choice for extraction of studied samples of animal origin. One year later Khorshid et al. apply QuEChERS method for determination of PAHs in fish. The used QuEChERS method is followed by additional cleanup procedure of solid phase extraction [15]. In 2018 Al-Thaiban et al. reported for development of QuEChERS method for determination of PAHs in smoked meat products for Qatar [38]. The described method include several steps taking around 1 h of sample preparation, several solvents are used as well as centrifugation procedure and heating for concentration of sample. In our case the procedure takes only about 15 min and usual laboratory equipment is used. Moreover only acetonitrile is used like a solvent. The key modification in the chromatographic conditions was changing in the column dimensions and injection conditions. Dimensions of the new column provided more sample loading and both with injection

corrections more sensitivity of the instrument. The obtained chromatogram after QuEChERS method purification is presented on Fig. 3.

Table 3. Comparison between two sample preparation procedures

Standard procedure	Modified QuEChERS method
3 steps procedure	1 step procedure
many steps (large possibility of losses of aimed PAHs)	small possibility of losses of PAHs
time consuming procedure	time-saving procedure
very unclear matrix, because of using of very strong nonpolar solvents (hexane, toluene) able to extract many undesired compounds of matrix	there are no extracted interfered compounds
Coextraction of large amounts of fats	Coextraction of insignificant amounts of fats
Need for saponification of fats in order to be removed from the aim extract	No needs of saponification step
High costs of time and reagents	Significantly lower costs of time and reagents
Low recovery	High recovery fitting in the legislation
Low sensitivity because of presence of high levels of matrix impurities	High sensitivity because of lack of interfered compounds

Table 4. Recovery data for all 16 PAHs

No	Name	Recovery %	RSD% [<i>n</i> = 6]	No	Name	Recovery %	RSD% [<i>n</i> = 6]
1	naphthalene	81	7.6	9	benzo[a]anthracene	100	4.0
2	acenaphthylene	89	5.6	10	chrysene	91	1.9
3	acenaphthene	92	4.6	11	benzo[b]fluoranthene	90	4.4
4	fluorene	97	5.3	12	benzo[k]fluoranthene	104	4.3
5	phenanthrene	89	2.8	13	benzo[a]pyrene	120	4.3
6	anthracene	86	4.3	14	indeno[1,2,3-cd]pyrene	71	5.6
7	fluoranthene	91	2.9	15	dibenzo[a,h]anthracene	120	5.9
8	pyrene	91	1.7	16	benzo[g,h,i]perylene	104	4.9

Table 5. LOD values of instrument (LODi) and of method (LODm) for all 16 investigated PAHs

No	Substance	LODi, ng/g	LODm, ug/kg
1	Naphthalene	0.45	0.90
2	Acenaphthylene	0.22	0.45
3	Acenaphthene	0.29	0.58
4	Fluorene	0.25	0.50
5	Phenanthrene	0.14	0.29
6	Anthracene	0.17	0.35
7	Fluoranthene	0.13	0.27
8	Pyrene	0.18	0.35
9	Benz[a]anthracene	1.92	3.85
10	Chrysene	1.12	2.24
11	Benzo[b]Fluoranthene	2.29	4.57
12	Benzo[k]Fluoranthene	2.35	4.69
13	Benzo[a]pyrene	1.66	3.33
14	Indeno[1,2,3-cd]pyrene	2.87	5.74
15	Dibenz[a,h]anthracene	2.52	5.05
16	Benzo[ghi]perylene	2.80	5.60

The corresponding LOQ values can be calculated using the relationship between LOQ and LOD values, $LOQ = 3 \cdot LOD$.

As it can be clearly seen from the chromatograms on Fig. 3 all 16 investigated PAHs are well separated and detected.

Some comparison and short conclusions for two used methods for sample preparation are summarized in Table 3.

Further, the recovery study was realized on six replicates of 5 g of smoked pork meat using modified QuEChERS method for sample preparation. All 6 samples are moved through whole QuEChERS procedure. Then the recovery were calculated and it is summarized in Table 4.

The obtained data shows recovery from 71% to 120% which is in a very good correlation with requirements of legislation where accepted recovery is between 50% and 120% [39].

Finally, LOD values of modified GC/MS method and for the instrument were determined and they are presented in Table 5.

CONCLUSIONS

A fast and easy to realize GC/MS method for determination of 16 PAHs under the control of International and European legislation was created and validate. Two different methods

for sample preparation were used and compared according to their rapidity and suitability for aim compounds determination. The created modified QuEChERS method is realized in a single step. It is easy and fast for realization and it led to obtaining of pure analytical results for aimed compounds fitting with requirements of the International legislation.

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