Journal of Chromatography A, 1218 (2011) 23–31

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Development and optimisation of a dopant assisted liquid chromatographic-atmospheric pressure photo ionisation-tandem mass spectrometric method for the determination of 15 + 1 EU priority PAHs in edible oils

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ARTICLE INFO

Article history:
Received 29 March 2010
Received in revised form
28 September 2010
Accepted 4 October 2010
Available online 29 October 2010

Keywords:
15 + 1 EU priority PAHs
APPI-MS/MS
Edible oil
Dopant
DACC

ABSTRACT

European food legislation defines a set of 16 polycyclic aromatic hydrocarbons (PAHs) as of high concern for human health. The EU set contains structurally very similar PAHs with ring numbers between 4 and 6, and so raises some separation aspects and problems, which were not experienced with traditionally analysed PAHs. Many of the currently applied gas chromatographic mass spectrometric (GC–MS) methods suffer from separation problems, while high performance liquid chromatography with fluorescence detection (HPLC-FLD) is neither capable of detecting the whole set of EU priority PAHs nor does it (compared to GC–MS) allow structural identification. In addition HPLC-FLD shows limitations with difficult matrices due to interferences. The aim of this paper is to fill this gap by describing a liquid chromatographic dopant assisted atmospheric pressure photo ionisation tandem mass spectrometric (LC-DA-APPI-MS/MS) method for the determination of 15 + 1 EU priority PAHs in edible oil, which complies with the requirements set by European food legislation. Measurements were performed in positive ion mode. Anisole at a flow rate of 30 μl/min was used as dopant. Sample preparation was performed offline by donor–acceptor complex chromatography (DACC). Compared to HPLC-FLD methods the presented method enables the determination of all 15 + 1 EU priority PAHs at the low μg/kg concentration range including less fluorescence active compounds like benzo[*j*]fluoranthene and indeno[1,2,3-*cd*]pyrene. By analysing four reference materials it could be demonstrated that this method provides accurate results and is sufficiently sensitive for food control purposes. Statistically significant differences between the reference values and the measured analyte contents were not found. The method performs well also for very complex samples. Repeatability relative standard deviations (RSDr) of the determination of the target PAHs in olive oil were for most analytes below 5%. The limit of detection (LOD) of the method met the requirement set by EU legislation (0.3 μg/kg).

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that are ubiquitous and persistent environmental contaminants. Several studies identified food as an important contributor to the exposure of humans to PAHs and so their presence in food is a matter of concern [1]. PAH contamination of food can be caused by pollution, e.g. exposure to contaminated water, contaminated soil, or polluted air, and some processes such as drying, roasting, smoking or frying [2–5]. Consequently the contamination of food by PAHs has to be monitored on a continuous and reliable basis.

In 2002 the European Commission's Scientific Committee on Food (SCF) assessed the toxicity of 33 PAHs, confirmed a subset of eight PAHs from the 16 PAHs prioritised by US Environmental Protection Agency (US-EPA), and identified seven additional PAHs as of major concern for human health [6]. An additional hazardous compound (benzo[*c*]fluorene) was identified in 2005 by the Joint FAO/WHO Experts Committee on Food Additives (JECFA) [7]. In EU it was decided to merge for the purpose of monitoring of the levels of PAHs in food the set of priority PAHs identified by SCF in 2002 with that of JECFA and to create thereby the 15 + 1 EU priority PAHs (EU PAHs).

These 15 + 1 EU priority PAHs should be monitored in food to enable long-term exposure assessments and to verify the validity of the use of benzo[*a*]pyrene (BaP) as a marker for the "total-PAH content" of food. As a consequence to these findings new European legislation came into force, aiming to harmonise the maximum

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doi:10.1016/j.chroma.2010.10.015

Table 1
Selected method parameters and method performance parameters of the LC-DA-APPI-MS/MS method.

Substance	k	α	Parent ion [m/z]	Collision energy [eV]	Quantifier ion [m/z]	Qualifier ion [m/z]	Ion ratio	Linearity	Intermediate precision (n = 18)		Method LOD [μg/kg]
								Mandel test	Retention time [%RSD]	Peak area of quantifier ion [%RSD]	
BcL	1.5		216	50	213	189	0.24	Y	2.1	5.8	0.28
CPP	2.3	1.50	226	50	224	222	0.03	Y	2.2	12.6	0.28
BaA	2.7	1.15	228	41	225	202	0.17	Y	2.2	4.9	0.19
CHR	3.4	1.23	228	41	226	202	0.16	Y	2.1	7.9	0.32
5MC	3.6	1.08	242	46	239	226	0.27	Y	2.1	5.7	0.24
BjF	4.5	1.23	252	48	250	226	0.11	Y	2.2	9.0	0.34
BbF	5.1	1.14	252	48	250	226	0.12	Y	1.9	8.2	0.34
BkF	6.4	1.26	252	48	250	226	0.10	Y	1.5	9.8	0.32
BaP	7.1	1.10	252	51	250	226	0.15	Y	1.2	7.9	0.24
DlP	7.7	1.09	302	46	300	298	0.01	Y	1.1	7.6	0.34
BgP	9.2	1.19	276	80	274	272	0.33	Y	0.6	8.5	0.36
DhA	9.4	1.03	278	46	276	274	0.46	Y	0.8	9.8	0.34
IcP	9.9	1.05	276	80	274	272	0.57	Y	0.5	8.3	0.30
DeP	10.4	1.04	302	51	300	298	0.47	Y	0.6	8.0	0.27
DiP	12.5	1.21	302	53	300	298	0.43	Y	0.3	11.1	0.35
DhP	13.2	1.05	302	53	300	298	0.44	Y	0.4	11.3	0.24

limit for BaP in certain types of food and to stimulate the monitoring of the new set of PAHs [8,9]. The sets of PAHs that are prioritised by EPA, and EU are depicted together with their chemical structures in Table 1 of the electronic supplement.

Various methods have been published in the last decades for the analysis of PAHs in several matrices. However, they cover mostly the set of PAHs prioritised by the US-EPA (EPA-PAHs) which comprises compounds with ring numbers between 2 and 6. Only a limited number of papers deal with the analysis of the EU-PAHs [10–15], which are structurally more similar to each other than the EPA PAHs, containing only 4–6 rings.

Analytical methods applied for the determination of PAHs are mostly based on gas (GC) or liquid chromatographic (HPLC) techniques and different sample preparation steps. Hampikyan and Colak reviewed recently the topic of PAHs in food [16]. Moret and Conte reviewed more specifically the occurrence of PAHs in fats and edible oils, and analytical methodology for their determination in these matrices [17]. Mass spectrometry is widely used in combination with GC, while fluorescence detection (FLD) is used for LC methods. GC–MS analysis is hampered by the low volatility of the high molecular weight PAHs (dibenzopyrenes) and low chromatographic resolution of some PAHs on traditionally applied capillary columns. Also interferences with non-target PAHs (e.g. triphenylene and chrysene) [18] can occur. In contrast to this, HPLC-FLD does not suffer from problems caused by the low volatility of PAHs, but from low fluorescence activity of some target analytes, e.g. cyclopenta[*cd*]pyrene, that impedes the analysis at the low μg/kg content range. Therefore new methods need to be developed that are able to overcome the shortcomings of the traditionally applied techniques. HPLC with mass spectrometric detection is a promising option, but PAHs are very stable molecules and hardly ionisable with conventional atmospheric pressure ionisation techniques like electrospray (ESI) or atmospheric pressure chemical ionisation (APCI). Takino et al. investigated the determination of PAHs by LC–ESI-MS [19]. In direct ESI they could not detect any signals even after flow injection of a 10 μg/ml solution of PAHs. However infusing silver ions post-column into the mobile phase resulted in a dramatic increase of the signal intensities, making the method suitable for the determination of PAHs in river water at the pg/ml level. Lien et al. reported recently on the application of tropylium ions as post column derivatisation reagent for the determination of 10 PAHs by LC–ESI-MS/MS and compared the results to direct LC-APPI-MS/MS [20]. They achieved with both techniques on-column detection limits in the range between 0.16 ng and 0.84 ng, depending of the individual analyte. Robb et al. [21] developed recently atmospheric pressure photo ionisation (APPI) as a novel ionisation technique for liquid chromatography–mass spectrometry (LC–MS). APPI permits the analysis of some unpolar and low-molecular mass compounds that cannot be ionised by ESI. The ionisation efficiency of APPI compared to APCI and ESI was recently reviewed by March et al. for different classes of compounds [22]. Compared to ESI, APPI also allows the application of higher mobile phase flow rates of up to ~2 ml/min and so shortening of analysis time. The atmospheric pressure photo ionisation process was reviewed by different authors [23,24]. It is initiated at high temperature by photons emitted from a vacuum UV (VUV) lamp. The Kr-lamp which was applied for this study emits photons of 10.03 eV and 10.64 eV. Compounds, that have lower ionisation energies (IE) than the energy of the photons are ionised. Conventional mobile phase constituents such as acetonitrile or methanol remain unionised due to their much higher IE [25]. In direct (without dopant) positive ion APPI the analyte is ionised by the photons, and the initial reaction is the formation of a molecular radical ion $[M]^+$ [26]. However, Syage found that the molecular radical ion could further abstract hydrogen from protic substances leading to the formation of protonated molecular ions $[M+H]^+$ [26]. In

negative ion APPI the ionisation is initiated by thermal electrons, which are then captured by molecules with high electron affinity like oxygen. The resulting superoxide ion can further react with other molecules [27]. The thermal electrons are released in photo ionisation reactions or by photoelectron emission from metallic surfaces.

Short et al. [28] demonstrated in direct photo ionisation experiments for benzo[*a*]pyrene, indeno[1,2,3-*cd*]pyrene and benz[*a*]anthracene that different VUV lamps, emitting photons with different photon energies, provide unique advantages for each lamp under different conditions. However direct photo ionisation of PAHs was found quite inefficient [29]. Robb and Blades concluded from the literature data that "thermospray may actually have been responsible for many results previously attributed to direct APPI" [24].

To further enhance the ionisation of the analytes, a dopant is used in combination with the APPI method (dopant assisted APPI, DA-APPI). The dopant is usually infused post-column, by means of a tee, into the eluent of the HPLC column. Photo ionisation initiates the formation of dopant radical cations that react further with the analyte via several routes. If the ionisation energy (IE) of the analyte is lower than that of the dopant charge exchange will occur [29]. Other possible ionisation mechanisms comprise proton transfer between the dopant radical cation and the analyte, or of protonated solvent clusters and the analyte, which takes place if the proton affinity of the analyte is higher than that of the dopant radical cation or of the protonated solvent cluster, respectively [27,30].

Moriwaki et al. investigated the effect of dopant composition, and found that toluene and anisole gave highest peak areas for some lower molecular weight EPA-PAHs [31]. Similar was studied by Itoh et al., who found a mixture of toluene and anisole in the ratio of 1:1 most suitable for the analysis of PAHs [29].

However, it was found that the signal abundances in dopant assisted APPI are sensitive to eluent flow rates [32]. Kauppila et al. gained highest signal intensities at a flow rate of ~200 μl/min [32]. The effect of eluent flow rate was reasoned with the faster depletion of dopant radical ions at higher flow rates. The authors also concluded that both the decrease of signal intensity and the maximum applicable eluent flow rate depend on the ion source geometry [33].

The effects of solvent flow, dopant flow and lamp current on DA-APPI of acridine and 9-methylanthracene were targeted by Robb and Blades [30]. They demonstrated that the ionisation efficiency via proton transfer depends for the respective analyte besides operational parameters very much on its gas phase basicity and solvation energy. According to them the decrease of ionisation efficiency caused by increased solvent flow rates is especially pronounced for substances with low gas phase basicities and low solvation energies. Depending on the properties of the analyte, the nature of the dopant and the composition of the mobile phase ionisation via both charge exchange and proton transfer can take place [34].

Atmospheric pressure photo ionisation was applied in many fields. Raffaelli and Saba reviewed the application of DA-APPI in different disciplines such as pharmaceutical chemistry, environmental chemistry, or synthetic organic chemistry [25].

This paper deals with the analysis of a set of PAHs prioritised in EU in edible oils. Most publications on the application of APPI for the determination of PAHs focus on EPA PAHs, or on a subset thereof. The public literature database suffers still from a lack of information on both ionisation efficiencies and performance characteristics of liquid chromatographic mass spectrometric methods for the determination of the 15 + 1 EU-PAHs in food.

Compared to the 16 EPA PAHs, the higher degree of structural similarity of EU PAHs provides challenges also from the chromatographical point of view. So apart from mass spectrometric detection the separation of these substances also needs special attention.

To our best knowledge, no study has been published yet on this topic. Therefore this paper reports on the development and optimisation of a liquid chromatographic dopant assisted atmospheric pressure photo ionisation tandem mass spectrometric (LC-DA-APPI-MS/MS) method for the determination of the 15 + 1 EU PAHs in food, which is in agreement with provisions given in European food legislation [35]. The focus of the present paper is put on the optimisation of both separation and mass spectrometric detection, as well as on the demonstration of the applicability of the method for real food samples in particularly contaminated edible oil matrices.

2. Experimental

2.1. Materials

Acetonitrile (ACN), methanol (MeOH), ethyl acetate (EtOAc), iso-propanol (iPrOH) (all gradient grade), toluene (TOL) (>99.9%) and anisole (ANI) (>99%) were purchased from Merck (Darmstadt, Germany). HPLC grade water was produced with a Millipore Milli-Q system (Millipore, Brussels, Belgium). Xylene (>99%) and 2,4-difluoroanisole (>99%) were purchased from VWR International (Leuven, Belgium).

A mixed PAH standard solution (PAH-Mix 183, 10 mg/l in cyclohexane) containing the 15 + 1 EU PAHs at equal concentrations was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). BCR 459 (blank coconut oil) and BCR 458 (coconut oil doped with 6 PAHs) are certified reference materials (CRMs) and were obtained from the Institute for Reference Materials and Measurements (Geel, Belgium). Test samples containing the 15 + 1 EU PAHs in both olive oil (FP635) and olive pomace oil (FP630), were previously subject of inter-laboratory comparisons organised by FAPAS® and were purchased from The Food and Environment Research Agency (York, UK).

Commercial olive oil was obtained for calibration purposes from a local retail store.

ChromSpher PI 80 mm × 3 mm × 5 μm (column length × internal diameter × particle size) and ChromSpher PAH 250 mm × 2.1 mm × 5 μm columns were purchased from Varian N.V. (St.-Katelijne-Waver, Belgium).

2.2. Instrumentation

Liquid chromatographic tandem mass spectrometric (LC–MS/MS) experiments were carried out on an Accela high speed LC system coupled to a Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, Zellik, Belgium). The system was controlled by the Xcalibur control software. A Finnigan Ion Max APCI/APPI combination probe was applied as interface between the LC and the MS. The Ion Max combination probe was equipped with a PhotoMate VUV light source. A Shimadzu LC-20AD pump (Shimadzu Benelux N.V., Deurne, Belgium) was integrated in the system for the post-column infusion of the dopant. A Harvard 11 Plus syringe pump (Harvard Apparatus, Holliston, USA) was used for flow injection of standard solutions for the optimisation of MS parameters.

Sample preparation by donor–acceptor complex chromatography (DACC) was carried out on an Agilent 1100 HPLC system (Agilent N.V., Diegem, Belgium) equipped with both isocratic and quaternary pumps, column diversion (DV) and redirection (V1) valves, diode array and fluorescence detectors as well as with an Agilent 1200 fraction collector. For statistical evaluation and linearity check, the Validata v3.02.52 Excel macro package was used (Wegscheider-Rohrer-Neuböck, Leoben, Austria).

Univariate optimisation of MS parameters like sheath, auxiliary and collision gas pressure, tube lens and skimmer offset, lenses and

collision energy were carried out automatically by the auto-tune function of Xcalibur® control software in flow injection analysis and infusion of the intermediate stock standard solution. Each experiment was performed in three replicates. The respective optima were determined for each substance individually. According to the auto optimisation the following parameters were set and kept constant during further method development and measurement phases: sheath gas pressure was set to 20 [expressed as arbitrary unit, ARB], auxiliary gas pressure was five ARB, collision pressure was 1.5 ARB, while the ion sweep gas pressure was set to zero. Tube lens values were 65 V for BcL and 120 V for the other substances, while skimmer offset was set to 0 V.

The optimum collision energy was different for each analyte. They are listed together with parent, daughter and qualifier ions in Table 1. The most abundant ion transitions were selected for analyte quantification, while for each analyte a second transition was recorded for peak qualification. For almost all analytes the transition from the molecular cation $[M]^+$ to the $[M-2]^+$ ion showed highest signal intensities. The $[M-2]^+$ cations were more abundant than the $[M-26]^+$ or $[M-28]^+$ ions, which were measured by Cai et al. [36], e.g. for BaP the relative difference between the abundances of the $[M-2]^+$ ion and $[M-28]^+$ ion was more than 17%.

Unit resolution was applied, and the scan time was set to 0.7 s.

Further critical parameters like vapour and capillary temperature as well as kind of dopant and dopant flow rate were optimised individually. Full chromatographic runs were conducted for this purpose, because these parameters are influenced by the eluent flow and eluent composition. The respective experiments are described in detail later in the text.

2.3. Sample preparation

A 100 ng/ml intermediate stock standard solution containing 15 + 1 EU PAHs was prepared gravimetrically on a five digit analytical balance (Sartorius N.V., Vilvoorde, Belgium) by dilution of PAH-Mix 183 in iPrOH. This solution was further used for the preparation of matrix matched calibration standards.

In order to cover the European maximum level for BaP in fats and oils, matrix matched calibration solutions were prepared at 6 concentration levels equidistantly distributed over the concentration range between 0.2 and 7.5 ng/ml. Therefore 1050 μl of blank olive oil and the appropriate amount of intermediate stock standard solution in iPrOH (100 ng/ml) were mixed. Finally, an appropriate amount of iPrOH was added to keep the edible oil to iPrOH ratio constant (70% edible oil to 30% iPrOH). The addition of iPrOH to the edible oil samples was necessary to reduce viscosity, which facilitated injection into the HPLC system. Three independent replicates were prepared at every concentration level. The certified reference materials and FAPAS® test samples were prepared by pipetting 1050 μl of the respective sample into HPLC autosampler vials, which were then diluted with 450 μl iPrOH. All preparation steps were controlled gravimetrically.

Sample preparation of the matrix matched calibration standards and edible oil samples was performed on an HPLC system equipped with the ChromSpher PI DACC column. Loading of samples and elution of the edible oil matrix were carried out in forward flow direction at an iPrOH flow rate of 1 ml/min. A total volume of 1 ml was injected in five consecutive injections of 200 μl each with delay times between injections of 3 min. The elution of the oil matrix was monitored with a diode array detector (DAD) at 242 nm and 254 nm. After 40 min of loading and sample clean-up time the DV was switched to back-flush flow in order to elute PAHs fast and in a narrow band from the DACC column. The quaternary HPLC pump was employed for the elution of PAHs. The eluent was a mixture of 70% EtOAc in ACN, supplied at a flow rate of 2.5 ml/min. The high amount of EtOAc was necessary to elute the strongest retained PAHs, DiP and DhP, from the DACC column. The column temperature was kept at 30 °C. The elution of PAHs was monitored by FLD at emission wavelengths of 380 nm, 420 nm, 450 nm and 508 nm, respectively, while 270 nm was applied as excitation wavelength. The fraction eluting between 40.1 and 41.75 min was collected and evaporated at 40 °C under a gentle stream of nitrogen until ~1 ml remained. This volume was transferred into a 1.5 ml conical shaped HPLC vial, and was further evaporated to dryness. The residue was reconstituted in 100 μl 80% ACN/H_2O mixture and 25 μl was injected into the LC–MS/MS system.

2.4. Sample analysis

A ChromSpher PAH 250 mm × 2.1 mm, 5 μm column equipped with a 10 mm × 2.1 mm C18 guard column was used for the separation of the target compounds. Additionally, two 0.5 μm mechanical in-line filters (Vici AG, Schenkon, Switzerland) were built in prior to the check valve and the guard column to prevent clogging of the system.

A chromatographic method based on a mobile phase gradient composed of ACN, H_2O and EtOAc, which is used in combination with fluorescence detection [37], served as basis for the development of the new method.

The target chromatographic performance characteristics, which were set as goal for method development for at least the analytes of the same molecular mass comprised: retention factors between 1 and 10, resolution factors (Rs) higher than 1.5 and selectivity factors (α) above 1.05.

3. Results

3.1. Method optimisation

The effect of the mode of operation of the applied ion source on the ionisation of PAHs was investigated. Three different modes of operation were possible: APCI alone, APPI alone, and a combined APCI/APPI operation mode. The effect of the operation mode was studied on the signal abundances gained with a 100 ng/ml standard solution in ACN at a mobile phase flow rate of 100 μl/min. Applying APCI alone showed unsatisfactory low signal intensities for all of the target analytes, which can be reasoned by the low proton affinity of the target substances. The experiments in the combined APCI/APPI operation mode and APPI alone mode were conducted in one sequence, applying only different levels of discharge currents (between 0 and 22 μA) on the corona needle. The Krypton VUV lamp was turned on during the whole sequence. Measurements were performed in positive and negative ion mode. A discharge current of 0 μA represents the APPI alone mode, while discharge currents above 0 μA result in the combined APCI/APPI operation mode. The signal intensities of the target analytes in APPI alone were used as a reference for comparison of the results obtained under different operation modes.

All target analytes showed decreased signal intensities when positive polarity was applied. The signal intensities of the ions with smaller mass-to-charge (m/z) ratios (from BcL to 5MC) dropped with discharge currents in the range between 1 and 7 μA by about 20% while at higher currents the drop was 10–15%. The higher m/z ratios showed a rapid decrease in signal intensity in the 1–7 μA range, approximately 35–40%, which increased to intensity loss of 60% at the upper end of the investigated discharge current range. In negative mode very similar tendencies were observed. However the loss of signal intensities in the discharge current range between 1 and 7 μA was for all m/z ratios only about 10%, which increased

to approximately 60% at 22 μA. The only exception was 5MC that showed in the combined APCI/APPI operation mode at a discharge current of 12 μA about 10% higher signal intensities than in the APPI alone mode.

For all other analytes the APPI alone operation mode gave superior results. The ionisation efficiency gained in APPI alone positive ion operation mode outreached the one gained in negative ion mode by far. Hence APPI alone operation in positive ion mode was applied in further experiments.

3.1.1. Mobile phase solvents

All commonly used reversed phase solvents such as H_2O (IE = 12.1 eV), ACN (IE = 12.2 eV) or MeOH (IE = 10.84 eV) have ionisation energies (IE) above the energies of the photons emitted from the Kr lamp (10 and 10.6 eV). Therefore ionisation of solvent molecules does not occur. Consequently low baseline noise and good signal to noise ratios should be achieved. Although the ACN based method works well with FLD detection, unsatisfactory poor signal intensities were observed when standards in ACN were injected into the LC-APPI-MS/MS system. This ion suppression effect could be due to the high proton affinity and high photoabsorption cross-section of acetonitrile, which lowers the number of available photons for the ionisation [38]. Also isomerisation of solvent molecules can occur and produce ions with lower IEs. The consequent easier ionisation of solvent molecules could result in the same phenomena [39].

Therefore, modification of the mobile phase composition was necessary. Methanol was chosen as replacement for acetonitrile. The higher ionisation potential (IP) value of methanol should provide low background levels, which should be advantageous with regard to the achievable signal-to-noise ratios. However, methanol shows weaker eluent strength in reversed phase chromatography than acetonitrile. Elution strength nomograms were applied for adjusting the mobile phase gradient to keep retention times almost constant. It has to be mentioned that the application of methanol changed chromatographic selectivity and so, compared to acetonitrile, the elution order of two analytes. With methanol BgP was eluted prior to DhA.

Also flow rates had to be adjusted to meet the limitations given by the APPI-MS/MS instrument. However, to elute the strongest retained analytes (DiP and DhP) within an acceptable time frame, the eluent strength and the flow rate were set to the upper limit of the applicable range. Flow rates between 300 μl/min and 700 μl/min were found to provide acceptable sensitivity. Hence a flow rate of 700 μl/min was applied for successive experiments in order to achieve fast separation. The mobile phase gradient program started with a 1 min isocratic period with an initial eluent composition of 8% water in methanol. Then a linear gradient was applied to 100% methanol in 8 min. Afterwards a linear ethyl acetate gradient was programmed to reach 65% ethyl-acetate in methanol within 7 min followed by a 2 min isocratic period with the same eluent composition in order to complete elution of DiP and DhP. Next the gradient was directed back to initial composition and the column was flushed and equilibrated for 5 min before the next injection. The injection volume was 25 μl and the column temperature was set to 30 °C.

Chromatographic separation is critical for isomeric groups of analytes with nearly identical mass spectra. These are in particular BaA/CHR; BjF/BbF/BkF/BaP; BgP/IcP and DlP/DeP/DiP/DhP. However, all of these critical groups of analytes were separated with the described LC method with resolution values higher than 1.5. Overlapping of peaks occurred only for analytes that could be distinguished mass spectrometrically, e.g. BgP/DhA (Rs = 0.6) IcP/DeP (Rs = 0.9) and CHR/5MC (Rs = 0.6). The obtained retention factors (k) and selectivity values (α) are compiled in Table 1. The targeted retention factor values were achieved for all compounds except the strongly retained DiP and DhP. For these two substances higher values had to be accepted.

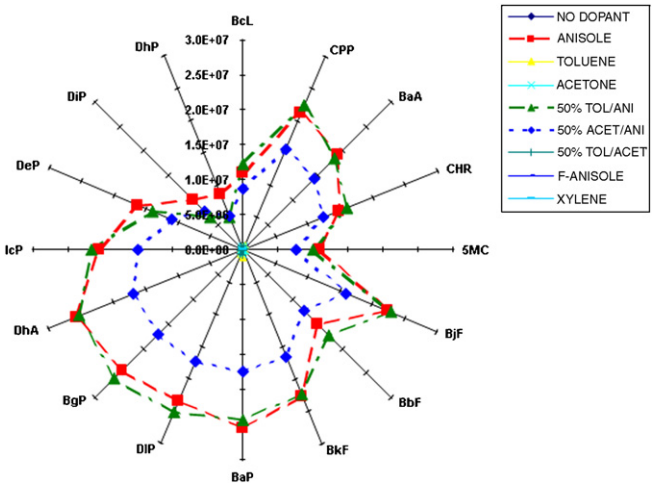

Fig. 1. Signal intensities gained for the 15 + 1 Eu priority PAHs in dependence of dopant quality (TOL: toluene, ANI: anisole, ACET: acetone, F-anisole: 2,4-difluoroanisole).

Once the optimum program of the mobile phase gradient was identified, recording of analyte signals was split into five time windows in order to keep the number of recorded masses per time interval at a minimum, and to increase the sensitivity for the individual compounds. The switching times were 3.75 min, 6.4 min, 11.8 min and 15 min, respectively. The ion transitions used for identification and quantification purposes are listed together with the applied collision energies in Table 1.

3.1.2. Dopant quality and quantity

The ionisation efficiency of many photoactive substances may be increased in APPI-MS/MS by the use of a readily ionisable dopant that acts as an intermediate in the ionisation reaction [40]. The dopant "conserves" the energy of the photons that is otherwise easily lost by collision with gas-phase constituents of the atmospheric pressure interface, and transfers it either directly, or via solvent molecules to the analyte [32]. For chromatographic reasons the dopant is added post-column. Itoh et al. [29] and Smith et al. [41] showed for some PAHs, that neither toluene (IE = 8.83 eV) nor acetone (IE = 9.71 eV) provides enough ionisation power, however, the presence of anisole (IE = 8.20 eV) in toluene dopant has proven to be effective, especially for heavier (EPA-) PAHs. The experiments with anisole, toluene and acetone (both individually and mixed) were repeated with the EU priority PAHs and extended with m-xylene and 2,4-difluoroanisole as other potential dopants. A constant dopant flow of 30 μl/min was supplied with the external LC-20AD pump.

Fig. 1 shows the abundances of the different analytes for different dopant compositions. Toluene, acetone, xylene and 2,4-difluoroanisole neither alone nor in mixture gave enough sensitivity for determination of the analytes at low concentration level. The respective data points are located on the scale of Fig. 1 almost in the centre and one cannot be distinguished from another.

Acetone with its high proton affinity (PA) can be easily protonated and should undergo proton transfer reactions. However the performance of the pure substance was weak for PAHs, which indicates that proton transfer is not the main ionisation mechanism for the target analytes. The ionisation efficiency of the target PAHs increased drastically when 50% anisole was added to acetone.

Anisole however gave the best results in this study. As acetone, it possesses a high PA, but the IE is much lower than that of acetone or toluene. Hence it can produce radical cations which can react in

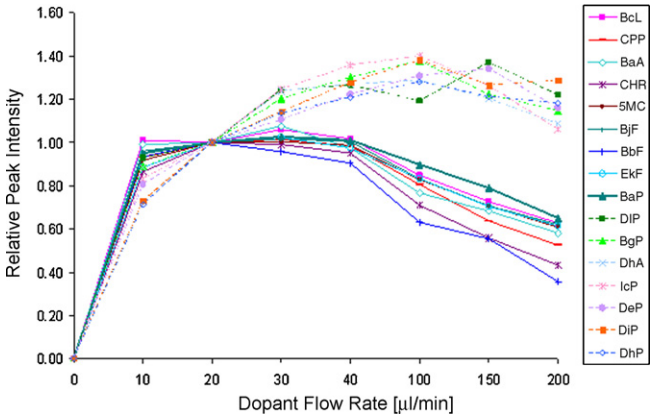

Fig. 2. Effect of dopant flow rate on the peak intensities for the 15 + 1 EU priority PAHs. Peak intensities are represented relative to the peak intensities observed at 20 μl/min.

charge exchange reaction with the target PAHs, and because of its high PA, anisole will not be neutralised by proton transfer and so it remains available for longer time in the ion source. In accordance with the observations made by Itoh et al. [29], results obtained with anisole containing 50% toluene were almost identical with the results gained with pure anisole.

However, pure anisole was finally chosen as dopant because it provided highest signal intensities for most of the regulated PAHs.

Lower ionisation efficiencies were observed for the late eluting dibenzopyrenes, independently of the kind of applied dopant, which can be attributed to the high proportion of ethyl acetate in the mobile phase and its ion suppression effect.

Apart from the dopant quality, also the effect of the dopant flow rate was subject of investigation. For that purpose anisole was introduced post-column into the eluent flow with flow rates between 0 and 200 μl/min and peak intensities were determined for every compound.

Fig. 2 shows normalised signal intensities of the target analytes at different flow rates. The signals at a dopant flow rate of 20 μl/min were applied for normalisation. As can be seen, the ionisation efficiency was affected by the dopant flow rate differently for each analyte. Substances eluting later than BaP, with higher m/z values and ring numbers, such as DlP, BgP, DhA, IcP, DeP, DiP and DhP gave enhanced signal intensities at higher dopant flow rates, and reached almost stable signal intensities at flow rates above 40 μl/min. Remarkably, this signal enhancement occurred more or less simultaneously with the introduction of EtOAc in the mobile phase. Since EtOAc possesses a slight ion suppressor effect, increasing the dopant flow compensated the ion suppression. Opposite to this, decreased signal intensities were observed for BaP and earlier eluting compounds at increased dopant flow rates. Consequently a gradient of the flow rate of the dopant could be applied for improving the ionisation efficiency of late eluting compounds. However and for reasons of simplicity a constant dopant flow rate of 30 μl/min was applied in further experiments. This dopant flow provided high sensitivity for early as well as late eluting PAHs.

3.1.3. Vaporiser and capillary temperature

The effect of vaporiser temperature and inlet capillary temperature on the ionisation of PAHs was investigated in the temperature range between 400 °C and 550 °C and 200–350 °C, respectively.

In general, vaporiser temperatures in range of 400–450 °C gave higher signal intensity values than higher vaporiser temperatures. The highest signal abundances were recorded at the lower limits of the investigated temperature ranges. Raising the vaporiser temperature to above 500 °C caused decrease of the signals. The reason for this effect could not be elucidated. Compared to that, the inlet capillary temperature did not have such a pronounced effect on the measured signal. Only slight differences were observed across the investigated temperature range. However highest peak intensities were gained at 350 °C. The exceptions were BcL and 5MC for which the signal abundances were higher at 300 °C than at 350 °C.

Since a gradient of these temperatures cannot be programmed the vaporiser temperature and the capillary temperature were kept constant at 400 °C and 350 °C, respectively.

3.2. Evaluation of method performance

To demonstrate the applicability of the developed method for the analysis of edible oil samples, method performance characteristics were determined using matrix matched calibration solutions, certified reference materials (CRM458) and quality control samples (FP635 and FP630). Guidance on minimum method performance requirements was taken from Commission Regulation (EC) No. 333/2007 and Commission Decision 2002/657/EC [35,42]. Two ion transitions (quantifier and qualifier) as well as their ratio served for identification of the individual compounds.

Intermediate precision values were calculated from the analysis of matrix matched calibration standards (in total $n = 18$) which were analysed in three sequences distributed over a period of one month. Homogeneity of variances, allowed pooling of precision data for different concentration levels and average relative precision values were calculated for the whole working range both for retention times and measured signal intensities. It shall be noted that the signal intensities were not corrected with internal standards. The respective values are given in Table 1. The precision of analysis methods for the official control of BaP in food is compliant with EU legislation, if it does not exceed a Horrat value of 2. The achieved intermediate precision values were far below this threshold.

A blank oil matrix was analysed to determine background levels. Matrix matched standards were applied for instrument calibration. Internal standards were intentionally not used in order to avoid compensation of matrix effects, which provides some kind of worst case scenario. The individual calibration levels were 0.2 ng/ml, 0.5 ng/ml, 1 ng/ml, 2.5 ng/ml, 5 ng/ml and 7.5 ng/ml, respectively. Linearity was checked over the whole calibration range. Each calibration level was prepared and injected in triplicate. Linearity was confirmed for all analytes by Mandel's fitting test (see Table 1). Fig. 3 depicts the chromatogram of a 1 ng/ml matrix matched calibration solution (equal to 50% of the legal limit for BaP content in fats and edible oils) demonstrating separation efficiency, and sufficient sensitivity of the method to apply it in food control.

The limit of detection was determined according to DIN 32645 [43]. European legislation defines for BaP a maximum tolerated value for LOD of 0.3 μg/kg. The LOD of the current method was 0.24 μg/kg BaP in the certified blank oil, which corresponds to an absolute amount of 6.3 pg BaP on column.

The trueness of the method was investigated by the analysis of a certified reference material (CRM 458) that is certified for five EU PAHs, thus giving information on trueness of the method for these PAHs. Eq. (1) was applied for evaluation of the results [44]. The measurement uncertainty was estimated as a first approximation from intermediate precision data. A statistical significant difference of the measurement result and certified value cannot be postulated at the 95% confidence level if Eq. (1) is fulfilled. Statistical evaluation of the measurement results did not indicate any bias. Details are given in Table 2.

$$2 \geq |Y| = \frac{X_m - X_{CRM}}{\sqrt{u_m^2 + u_{CRM}^2}} \quad (1)$$

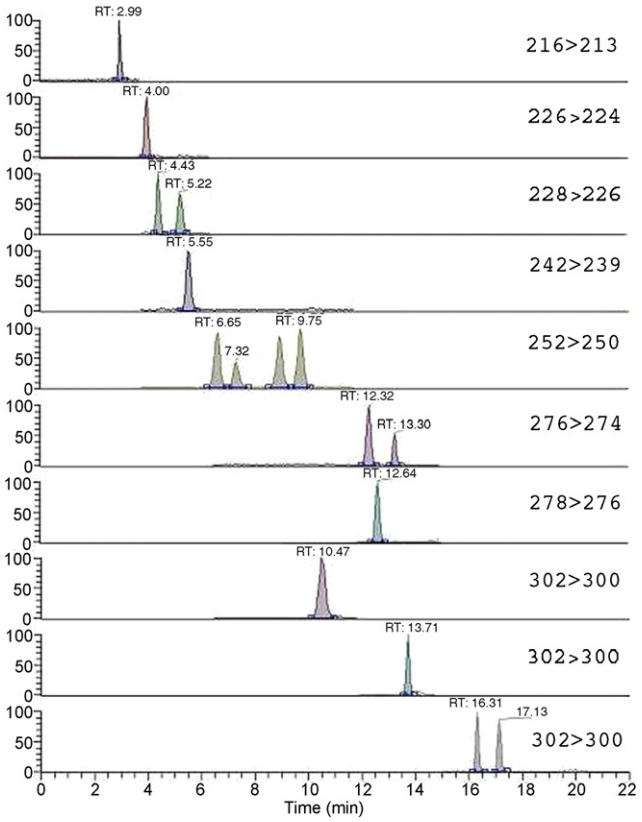

Fig. 3. Chromatogram of a 1 ng/ml matrix matched calibration solution demonstrating separation efficiency and sensitivity of the method. The concentrations of the PAH corresponded to half of the maximum level defined for BaP in legislation (t_R = 9.75 min, m/z 252 → 250). Retention order is BcL, CPP, BaA, CHR, 5MC, BjF, BbF, BkF, BaP, DlP, BgP, DhA, IcP, DeP, DiP, DhP. Mass transitions of the recorded base peak ions are given at the right.

X_m: average measured value; X_{CRM}: certified value; u_m: standard uncertainty of the measurement; u_{CRM}: standard uncertainty of the certified value.

In order to evaluate the method performance also for the substances not included in CRM 458, a quality control sample containing the 15 + 1 EU priority PAHs at low concentration levels (FP635) was analysed in triplicate. A typical chromatogram for this sample is presented in Fig. S1 of the electronic supplement, and a summary of measurement results is given in Table 3. Reference values (here expressed as assigned values, X), target standard deviation values (σ_p) as well as accepted concentration ranges were taken from the proficiency test report. Target standard deviations represent fitness for purpose criteria and were derived from the modified Horwitz-equation [45]. Analysis results considered in the proficiency test as satisfactorily have to be within the concentration range given by $X \pm 2 \times \sigma_p$. All analysis results obtained by LC-DA-APPI-MS/MS for sample FP635 met the concentration range for satisfactory performance. The only exception was 5MC, which was at a level below the LOD of the method. However, no value was assigned for this substance in the proficiency test.

Table 2
Results gained for the certified reference material BCR458.

Substance	X_{CRM} [μg/kg]	u_{CRM} [μg/kg]	X_m [μg/kg]	u_m [μg/kg]	$\|Y\|$	Acceptance
CHR	4.9	0.2	4.6	0.42	0.6	Yes
BkF	1.87	0.08	1.92	0.22	0.2	Yes
BaP	0.93	0.04	0.89	0.08	0.4	Yes
BgP	0.97	0.03	0.93	0.09	0.4	Yes
IcP	1.0	0.03	0.94	0.09	0.6	Yes

X_{CRM}: certified value, u_{CRM}: standard uncertainty of the certified value, X_m: average measured value, u_m: standard uncertainty of the measured value, $|Y|$ result according to Eq. (1).

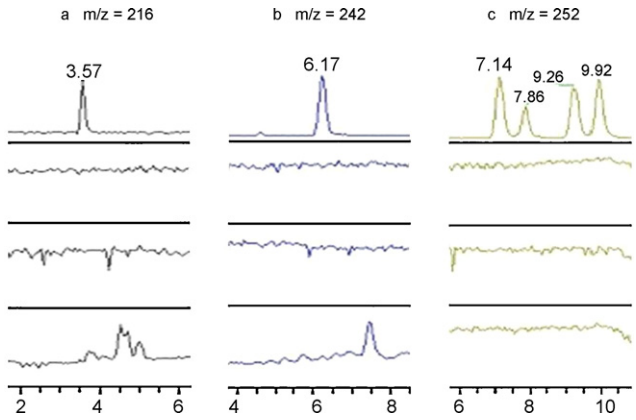

Fig. 4. Study of matrix effects in selected ion monitoring mode (SIM) for m/z = 216 (BcL), m/z = 242 (5MC) and m/z = 252 (BjF, BbF, BkF, BaP). Chromatograms (from the top) represent 10 ng/ml PAH standard solution in solvent (to indicate the retention times of the respective analytes), solvent blank, blank (unspiked) olive oil, and blank (unspiked) olive pomace oil extracts.

The rather large difference between the assigned and measured value for BcL could be reasoned by losses during solvent evaporation. The application of a suitable internal standard could serve to remediate this problem.

3.3. Matrix effects and performance for difficult matrices

Ion suppression effects might severely affect LC–MS measurements. Strategies for the elucidation of matrix effects were described by Antignac et al. [46]. Ion suppression and matrix effects were evaluated by infusion of a 10 ng/ml standard solution containing 15 + 1 EU PAHs into the effluent of the analytical column. A syringe pump was applied for the constant supply of the standard solution and a T-piece for connection to the system. The experiments were performed in MS SIM mode, measuring only the parent ions listed in Table 1.

Fig. 4 gives an overview on the chromatographic profiles obtained with a PAH standard solution in solvent, the pure solvent, an olive oil extract and an olive pomace oil extract (from top to bottom). The latter two samples were commercial samples and purchased in local supermarkets. Matrix effects would be indicated compared to the pure solvent by either elevated or decreased baselines, or additional (also negative) peaks in close vicinity of the standard peaks. For 14 out of 16 analytes the baseline profile was as flat as that of the pure solvent, which proved the absence of matrix effects. For illustration, the profile for ion m/z = 252 representing four compounds (BjF, BbF, BkF, and BaP) was extracted from the recorded chromatograms (see Fig. 4c). Fig. 4a and b presents the troublesome m/z = 216 (BcL), and m/z = 242 (5MC) traces, where the olive oil matrix showed positive peaks (m/z = 216), or increased noise and higher baseline (m/z = 242) at or close to the retention times of the target compounds. However, despite the retention times of the negative peaks and the analyte peaks were not identical, interferences cannot be excluded.

To demonstrate the method's applicability for difficult oil samples, sample FP630 was analysed. This sample consists of highly contaminated olive pomace oil, and was used in a proficiency test (FAPAS®). Fluorescence detection was not suitable for such a highly contaminated sample due to the high number and intensity of interfering compounds. A typical FLD chromatogram of this olive pomace oil sample, overlaid with 10 ng/ml PAH standard solution

Table 3
Results of the determination of PAHs in the olive oil quality control sample (FP635).

Substance	X_a [μg/kg]	σ_p [μg/kg]	Accepted concentration range [μg/kg]	X_m	SD [μg/kg]	Acceptance
BcL	0.87	0.19	0.49–1.25	0.54	0.02	Yes
CPP	0.54	0.12	0.30–0.78	0.62	0.03	Yes
BaA	3.17	0.70	1.77–4.57	2.71	0.10	Yes
CHR	9.52	2.09	5.34–13.70	9.63	0.40	Yes
5MC	n.a.	n.a.	n.a.	<LOD	–	–
BjF	1.22	0.27	0.68–1.76	1.18	0.06	Yes
BbF	2.40	0.53	1.34–3.46	2.27	0.19	Yes
BkF	0.98	0.22	0.55–1.41	0.71	0.04	Yes
BaP	2.06	0.45	1.15–2.97	1.91	0.03	Yes
DlP	1.47	0.32	0.82–2.12	1.03	0.03	Yes
BgP	1.54	0.34	0.86–2.22	1.39	0.06	Yes
DhA	2.15	0.47	1.20–3.10	2.03	0.02	Yes
IcP	1.61	0.35	0.90–2.32	1.38	0.08	Yes
DeP	1.52	0.34	0.85–2.19	1.79	0.04	Yes
DiP	1.21	0.27	0.68–1.74	0.98	0.01	Yes
DhP	1.50	0.33	0.84–2.16	1.22	0.09	Yes

Assigned value (X_a), target standard deviation (σ_p) and acceptance range is taken from the FAPAS® report. X_m: average of measured values, SD: standard deviation of measured values

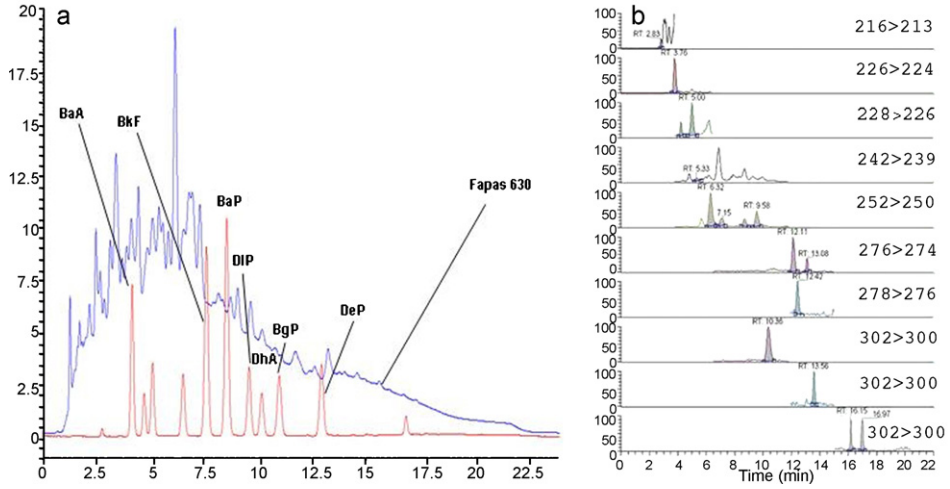

Fig. 5. Chromatograms recorded for a highly contaminated olive pomace oil (FP630) sample with (a) HPLC-FLD at ex: 270 nm and em: 420 nm and (b) HPLC-DA-APPI-MS/MS after DACC extraction. Mass transitions of the recorded base peak ions are given at the right. To illustrate the magnitude of matrix effects, the FLD chromatogram was overlaid with the chromatogram of a 50 ng/ml standard solution in solvent. Retention order in (b) is BcL, CPP, BaA, CHR (5MC), BjF, BbF, BkF, BaP, DlP, BgP, DhA, IcP, DeP, DiP, DhP.

in solvent, is shown in Fig. 5a. As can be seen neither identification nor quantification was possible. Opposite to this both identification and quantification are possible with the presented LC-DA-APPI-MS/MS method. Fig. 5b contains the chromatograms of the different ion transitions recorded for sample FP630. Table 2 of the electronic supplement summarises the results of the measurements. The overestimation of BcL might be caused by matrix effects of this complex sample. Results of all other compounds, for which content values were assigned, were within the range considered satisfactory. Hence the performance of the LC-DA-APPI-MS/MS method was superior to the performance of HPLC-FLD and the applicability of the former method for difficult samples was proven.

4. Summary

In this paper a liquid chromatographic dopant assisted atmospheric pressure photo ionisation tandem mass spectrometric (LC-DA-APPI-MS/MS) method is described for the first time for the determination of EU priority PAHs in edible oils. The performance characteristics of the method complied with provisions given in EU legislation.

The method is based on an automated simple, but efficient sample preparation by donor–acceptor complex chromatography followed by LC-DA-APPI-MS/MS. The method was optimised with respect to mobile phase composition, mobile phase flow, kind and quantity of applied dopant, and interface temperatures.

It was demonstrated that methanol is an appropriate eluent and anisole is an essential dopant constituent for the efficient ionisation of the target analytes. Mobile phase and dopant flow rates of 700 μl/min and 30 μl/min gave best results for the majority of analytes. Moderately high inlet and capillary temperature values were found appropriate for the analysis.

The presented method enables, contrary to fluorescence detection, the determination of all 15 + 1 EU priority PAHs including the fluorescence inactive cyclopenta[*cd*]pyrene within one single run at the low μg/kg concentration range. Enhanced sensitivity was demonstrated also for the low fluorescent compounds benzo[*j*]fluoranthene and indeno[1,2,3-*cd*]pyrene. Fluorescence detection provides lower limits of detection for the other target compounds. However it might be prone to interferences especially when analysing complex matrices.

It was demonstrated with certified reference materials and samples used in interlaboratory comparisons that the described

LC-DA-APPI-MS/MS analysis method is sensitive and accurate even in case of difficult, highly contaminated samples. The method has potential for further automatisation, by online coupling of sample preparation and analysis and so to reduce further the required man power and also possible sources of error. The application of isotope labelled internal standards could be beneficial for further improving the accuracy of analysis results. The presented LC-DA-APPI-MS/MS analysis method has the potential of becoming a good complementary alternative of gas chromatographic mass spectrometric methods for the determination of 15 + 1 EU priority PAHs in edible oil.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.015.

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