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Optimizing separation conditions of 19 polycyclic aromatic hydrocarbons by cyclodextrin-modified capillary electrophoresis and applications to edible oils

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ABSTRACT

For the first time, the separation of 19 polycyclic aromatic hydrocarbons (PAHs) listed as priority pollutants in environmental and food samples by the United States Environmental Protection Agency (US-EPA) and the European Food Safety Authority was developed in cyclodextrin (CD)-modified capillary zone electrophoresis with laser-induced fluorescence detection (excitation wavelength: 325 nm). The use of a dual CD system, involving a mixture of one neutral CD and one anionic CD, enabled to reach unique selectivity. As solutes were separated based on their differential partitioning between the two CDs, the CD relative concentrations were investigated to optimize selectivity. Separation of 19 PAHs with enhanced resolutions as compared with previous studies on the 16 US-EPA PAHs and efficiencies superior to 1.5×10^5 were achieved in 15 min using 10 mM sulfobutyl ether-β-CD and 20 mM methyl-β-CD. The use of an internal standard (umbelliferone) with appropriate electrolyte and sample compositions, rinse sequences and sample vial material resulted in a significant improvement in method repeatability. Typical RSD variations for 6 successive experiments were between 0.8% and 1.7% for peak migration times and between 1.2% and 4.9% for normalized corrected peak areas. LOQs in the low μg/L range were obtained. For the first time in capillary electrophoresis, applications to real vegetable oil extracts were successfully carried out using the separation method developed here.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) form a large group of about 10,000 compounds with two or more fused aromatic rings. Their origin is both anthropogenic (incomplete combustion of organic matter such as oil, wood or fossil fuels due to human activity) [1,2] and natural (e.g. forest fires and volcanoes). However, PAHs are known to have carcinogenic and mutagenic effects caused by the binding of their metabolites to DNA [3]. The exposure of humans to these compounds creates health risks, especially with food contaminated by PAHs coming from environment or production practices (smoking, heating, and drying) [4–7].

Some decades ago, the United States Environmental Protection Agency (US-EPA) established a list of priority pollutants: 'the 16 US-EPA PAHs' [8]. Moreover in 2002, the Scientific Committee on Food of the European Commission (today replaced by the European Food Safety Authority (EFSA)) published another list of 15 priority PAHs for monitoring the contamination of food products, including some compounds of the US-EPA list [9]. Later, in 2008, a 16th compound (benzo(c)fluorene, BcFLR) was officially included into the EU priority PAHs by the EFSA [10]. This new list is commonly called '15+1 EU priority PAHs', so that it can be distinguished from the 16 US-EPA

Abbreviations: ACP, acenaphthene; ACY, acenaphthylene; ANT, anthracene; BaA, benzo(a)anthracene; BbFA, benzo(b)fluoranthene; BjFA, benzo(j)fluoranthene; BkFA, benzo(k)fluoranthene; BcFLR, benzo(c)fluorene; BghiP, benzo(ghi)perylene; BaP, benzo(a)pyrene; CZE, capillary zone electrophoresis; CHR, chrysene; CD, cyclodextrin; CPcdP, cyclopenta(c-d)pyrene; DS, degree of substitution; DBahA, dibenzo(a,h)anthracene; DBaeP, dibenzo(a,e)pyrene; DBahP, dibenzo(a,h)pyrene; DBaiP, dibenzo(a,i)pyrene; DBalP, dibenzo(a,l)pyrene; EFSA, European Food Safety Authority; FA, fluoranthene; FLR, fluorene; IP, indeno(1,2,3-cd)pyrene; LIF, laser-induced fluorescence; MeOH, methanol; MCH, 5-methylchrysene; Me-β-CD, methyl-β-CD; NPH, naphthalene; PHE, phenanthrene; PAH, polycyclic aromatic hydrocarbons; Pyr, pyrene; SBE-β-CD, sulfobutyl ether-β-cyclodextrin; US-EPA, United States Environmental Protection Agency

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PAHs. Eight PAHs known to be mutagenic or carcinogenic are in common between the two priority lists, resulting in 24 PAHs under regulations (see Supplementary Data).

To determine complex mixtures of PAHs at low concentrations in food and environmental samples, reliable analytical methods are needed [11,12]. Analytical procedures to quantify PAHs are mostly based on liquid chromatography coupled to diode array detector [13–15] or fluorescence detector [15–18] and gas chromatography coupled to mass spectrometry [19–22]. Capillary electrophoresis (CE) with its high separation efficiencies, low reagent and sample consumption, speed of analysis, and easier transfer to chip format, is an interesting alternative to previous chromatographic methods [23–25]. Capillary zone electrophoresis (CZE), the most classical form of CE, however, is not suited for the analysis of such neutral and hydrophobic compounds, but micellar electrokinetic chromatography (MEKC) is well adapted [26–28]. In this case, micelles formed from surfactants allow both the separation of PAHs and the increase in their solubility in the aqueous electrolyte. However, the addition of a micellar phase alone usually does not provide enough selectivity to separate a large number of PAHs, since they are too strongly associated with the micelles. Usually, the addition of modifiers, such as organic solvents or cyclodextrins (CDs), to the buffer is necessary [29–35].

In MEKC, the addition of CDs is the most successful strategy to improve method selectivity. Thanks to their ability to form host–guest inclusion complexes with hydrophobic compounds, the partitioning of components between the micellar and aqueous/CD phases is modified [36,37]. However, CD-modified MEKC suffers from a lack of selectivity evidenced by long analysis times and co-migrations of similar PAHs [38].

Electrochromatography (CEC) can also be employed for PAH separations but it appears in the literature that PAHs are often used as model compounds to characterize the CEC performance of the stationary phases: packed- or monolithic-based ones [39–41]. Applications to complex PAH mixtures and to real samples are still expected although monolithic stationary phases already feature high efficiency and/or high selectivity [42–44].

An excellent alternative to these approaches is the use of a dual CD system in CZE [45,46]. Enhanced PAH separations using mixtures of neutral and anionic CDs have been demonstrated in capillary [47–49] and, more recently, in microfluidic chip formats [50]. In this approach, PAHs are separated based on their different complexation constants between the neutral CD, which moves at the speed of the electroosmotic flow (EOF) and the negatively charged CD, which moves more slowly. The unique selectivity offered by the dual sulfobutyl ether-β-CD (SBE-β-CD)/methyl-β-CD (Me-β-CD) system for the analysis of the 16 US-EPA priority PAHs in contaminated soils was demonstrated [48]. However, several groups of PAHs were not fully baseline resolved (e.g. fluorene (FLR)/anthracene (ANT), chrysene (CHR)/phenanthrene (PHE)/benzo(a)anthracene (BaA), and benzo(b)fluoranthene (BbFA)/indeno(1,2,3-cd)pyrene (IP)/pyrene (Pyr)). Given these limitations, 1 year later the same group tried to introduce one more neutral CD: native γ- and α-CDs were tested [49]. Surprisingly, better PAH separation was obtained with the α-CD, which usually is not expected to interact with the biggest PAHs because of its smaller cavity size. Finally, the overall electrophoretic separation of the 16 US-EPA PAHs was enhanced by adding 4 mM α-CD to the background electrolyte (BGE), although acenaphthene (ACP)/naphthalene (NPH)/FLR and BbFA/IP were still not fully baseline resolved. Moreover, 'microprecipitation, i.e. spikes' was observed in the electropherogram and benzo(ghi)perylene (BghiP), the most hydrophobic compound, 'appeared to interact strongly with SBE-β-CD and/or the capillary wall, and in some cases it did not appear in the electropherogram', proving solubility and therefore repeatability problems [49]. Therefore, the separation of the 16 US-EPA PAHs still needed to be improved. Moreover, until now, no publication dealing with the determination of the 15+1 EU priority PAHs using CE has been published. This paper presents, to our best knowledge, the first development of CD-modified CZE with laser-induced fluorescence (LIF) detection for separation and sensitive analysis of the two lists of priority PAHs: the 16 US-EPA PAHs and the 15+1 EU priority PAHs, and its application to edible oil extracts.

2. Materials and methods

2.1. Chemicals

Benzo(a)pyrene (>99.6%, BaP), BaA (>99.5%), CHR (>99.6%), BbFA (>99.5%), IP (>99.5%), benzo(j)fluoranthene (>98.5%, BjFA), 5-methylchrysene (>99.5%, MCH), dibenzo(a,l)pyrene (>99.4%, DBalP), dibenzo(a,i)pyrene (>99.9%, DBaiP), dibenzo(a,h)pyrene (>99.0%, DBahP), dibenzo(a,e)pyrene (>99.0%, DBaeP) at 10 mg/L in acetonitrile, cyclopenta(c-d)pyrene (>99.5%, CPcdP) at 100 mg/L in acetonitrile, BcFLR (>98.2%) at 10 mg/L in cyclohexane and a standard mixture of the 16 US-EPA PAHs at 10 mg/L in acetonitrile were purchased from CIL Cluzeau (Sainte-Foy-La-Grande, France). ACP (>99.0%), acenaphthylene (>99.0%, ACY), ANT (>98.0%), benzo(k)fluoranthene (>98.0%, BkFA), BghiP (>98.0%), dibenzo(ah)anthracene (>97.0%, DBahA), fluoranthene (>98.0%, FA), FLR (>98.0%), PHE (>98.0%), NPH (>99.0%), Pyr (>98.0%), and umbelliferone (≥98.0%) were supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Me-β-CD with an average degree of substitution (DS) of 12.6 (average molecular weight of 1310 g/mol), urea for electrophoresis (≥99.99%) and sodium tetraborate decahydrate (≥99.5%) were from Sigma-Aldrich. SBE-β-CD with an average DS of 6.2 (average molecular weight of 2115 g/mol) was supplied by Cydex Pharmaceuticals (Lawrence, KS, USA). Methanol (MeOH), ethanol, cyclohexane, ethyl acetate, and acetonitrile (ACN) (analytical grade) were provided by VWR (Fontenay-sous-Bois, France). Ultra-pure water was delivered by a Direct-Q3 UV system (Millipore, Molsheim, France).

The final composition of the BGE was 10 mM sodium borate buffer (pH 9.2), 600 mM urea, 10 mM SBE-β-CD, 20 mM Me-β-CD in 90:10 (v/v) water–MeOH mixture. Three stock solutions of 10 mM sodium tetraborate decahydrate with 2.5 M urea, 100 mM SBE-β-CD and 100 mM Me-β-CD were prepared every week by dissolving the appropriate amounts in ultra-pure water and stored at 4 °C. The final BGE was obtained by adding 10% MeOH (v/v) and appropriate amounts of the three previous stock solutions to reach the final concentrations. All BGEs were daily prepared and filtered through 0.20 μm cellulose acetate membrane (VWR). Stock PAH mixture solutions were prepared at 1 mg/L in ACN by mixing appropriate volumes of the standard mixture of the 16 US-EPA PAHs and individual PAH standard solutions, and then stored at 4 °C. PAH standard mixtures were prepared each day by diluting the stock mixture solution in a 30:70 (v/v) MeOH–BGE mixture to the desired concentration. Stock solution of umbelliferone used as internal standard (IS) was prepared at 1.6 g/L in ethanol and diluted to 1.6 mg/L with ultra-pure water.

2.2. Apparatus and software

All CE experiments were carried out with an Agilent Technologies HP 3D system (Massy, France) hyphenated with LIF detection. Fluorescence excitation radiation was obtained from the 325 nm, 15 mW output of a HeCd laser (Model 3056-M-A02, Melles Griot, Voisins-Le-Bretonneux, France) coupled to a Zetalif Evolution LIF detector (Picometrics, Toulouse, France). All

radiations below 350 nm were discarded by the filter. A rise time of 0.05 s and a photomultiplier voltage of 750 V were selected. Separations were performed using 50 μm id × 49 cm (detection at 33.5 cm) bare fused-silica Polymicro capillaries purchased from Photonlines (Marly-Le-Roi, France). HP 3D ChemStation software controlled the instrument and allowed data acquisition.

2.3. Electrophoretic procedures

Before the first use, capillaries were conditioned by successive flushing with 1 M NaOH, 0.1 M NaOH, ultra-pure water and finally BGE, each under 935 mbar for 10 min (about 20 capillary volumes). Every day, the capillary was flushed with water followed by BGE, each under 935 mbar for 10 min. Between each run, different rinse sequences using water and organic solvents such as ACN and MeOH were tested to achieve good repeatabilities. At the end of the day, capillaries were rinsed by flushing successively with water, ACN, MeOH and water each under 935 mbar for 5 min for water (about 10 capillary volumes) and 15 min for organic solvents (about 80 capillary volumes for ACN and 50 capillary volumes for MeOH). Injections were performed hydrodynamically under 18 mbar for 5 s (about 0.3% of the capillary volume with a viscosity of 1.1 cP at 25 °C for a water/MeOH (9/1) mixture). Separations were run at 25 °C. Inlet BGE was changed between each run whereas outlet BGE was daily renewed.

2.4. Determination of electroosmotic mobility

No fluorescent neutral markers could be found because all the compounds tested were always delayed due to an interaction with the anionic CD. Electroosmotic mobilities were therefore calculated directly from electropherograms as previously described by Szolar et al. [47]. As was already mentioned in this earlier study, MeOH associated with the anionic CD, slowing down its migration in comparison with water. This is why, the first two peaks recorded on each electropherogram were attributed to water and MeOH, respectively. Consequently, the migration time of the first negative peak appearing on each electropherogram was taken as the electroosmotic migration time.

2.5. Real samples preparation

Samples of edible oils (sunflower oil and Isio 4® oil, which is a mixture of rapeseed, sunflower, grape seed, and high content oleic acid sunflower oils) were provided by Lesieur (Coudekerque, France). Oil extractions were performed with cartridges packed with 25 mg of molecularly imprinted polymers (Affinilute MIP PAH®, 3 mL, Biotage, Uppsala, Sweden). The procedure published in Biotage application note was followed [51]. About 10 g of oil was exactly weighed into a 10 mL volumetric flask and diluted to volume with cyclohexane. Cartridges were conditioned with 1 mL of cyclohexane and 2 mL of diluted oil samples were loaded onto the cartridge. After being dried completely under vacuum, the cartridge was washed with 2 × 1 mL of cyclohexane to remove fat content (e.g. lipids, triglycerides, fatty acids). Finally, after a drying step, elution was carried out with 3 mL of ethyl acetate. The eluate was evaporated to dryness under a nitrogen stream and reconstituted in first ACN, next MeOH, and finally BGE to reach a final composition for ACN–MeOH–BGE of 1:3:6 (v/v/v) before CE analysis. Blank oil extracts were spiked only with IS at 100 μg/L. Oil extracts were also spiked with 16 PAHs at 100 μg/L (except for MCH, BaP, and DBaeP, 150 μg/L, ANT, FA, and BghiP, 200 μg/L, and IP, 400 μg/L).

3. Results and discussion

As already discussed, the CE separation of the 16 US-EPA PAHs still needed to be improved (not all baseline resolved and solubility problems related to their high hydrophobicity) and the 15+1 EU priority PAHs for food applications were not studied in previous published works [48,49]. This is why, in this work, separation conditions previously published were revisited to improve the selectivity and the repeatability of the method, especially taking into account that additional PAHs with higher hydrophobicity values were also targeted.

3.1. Effect of buffer concentration on analysis time

When using LIF detection with excitation wavelength at 325 nm, only 16 out of the 24 compounds could be detected at a concentration of 100 μg/L (11 PAHs out of the 16 US-EPA PAHs and 5 PAHs out of the 8 PAHs belonging only to the '15+1 EU priority PAHs' of food interest). From other 8 PAHs, BjFA, ACP, and PHE could be detected at higher concentrations whereas NPH, ACY, CPcdP and FLR did not give measurable fluorescence signal even at 10 mg/L. Finally, BcFLR was only available in cyclohexane and thus could not be added to the working standard mixture of other PAHs containing ACN without creating a microemulsion. For this reason, this compound was studied separately. In the first work by Brown et al. [48], the procedure for the BGE preparation did not allow to understand whether the quoted 50 mM referred to the concentration of used sodium tetraborate decahydrate in the BGE or to the actual concentration of borate species. This parameter was therefore investigated for the analysis of the 16 US-EPA PAHs standard mixture. Upon decreasing the buffer concentration, ionic strength also decreases causing an increase in the electroosmotic mobility and hence a speed up of the PAH migration. Moreover, at lower ionic strength, it becomes possible to increase the applied separation voltage without generating detrimental heating effect, speeding up the analysis even more. Separations were performed with borate concentration ranging from 80 mM down to 10 mM, which allowed an increase in applied voltage from 15 to 18 kV, and led to halving the analysis time (from 15.6 min at 80 mM to 8.0 min at 10 mM), while maintaining resolution. Experimentally, simultaneous increases in EOF and complexed PAH electrophoretic mobilities of about 20% and 8% (for the last migrating peak, BghiP) were respectively obtained. These observed variations in electroosmotic and electrophoretic mobilities can be easily explained from theory by the decrease in ionic strength. Consequently, a borate concentration of 10 mM was thus chosen for the following experiments.

3.2. Effect of several experimental parameters on peak area repeatability

In previously published work [49], the separation of the 16 US-EPA PAHs suffered from solubility problems clearly evidenced by the presence of spikes and peak tailing on electropherograms. Moreover, the last peak, BghiP, 'appeared to interact strongly with SBE-β-CD and/or the capillary wall, and in some cases it did not appear in the electropherogram', revealing notable repeatability problems. In our study, as additional PAHs of food interest were more hydrophobic than the 16 US-EPA PAHs, solubility issues were also expected (see Supplementary Data for log *P* values). Similarly to conditions applied by Jiménez et al. [38], urea was added to the BGE in order to increase the solubility of these lipophilic PAHs in the aqueous media. Among the tested concentrations (between 400 mM and 1 M), an urea concentration of 600 mM was selected since it appeared to provide sufficient solubility of PAHs in the BGE.

First tests were carried out on the 11 US-EPA PAHs that can be detected at 100 μg/L using the 325 nm laser, to evaluate the method repeatability for the analysis of the less hydrophobic studied PAHs (log *P* between 4.5 and 6.7) in the initial conditions. The BGE was composed of 10 mM sodium borate buffer (pH 9.2), 600 mM urea, 10 mM SBE-β-CD and 20 mM Me-β-CD. PAH standard mixtures were obtained by dilution of the stock solution in 5/95 (v/v) MeOH–BGE mixture to a final concentration of 100 μg/L each and they were stored in a polypropylene vial for analysis. Once PAH standard mixtures were prepared, analysis was immediately performed. To correctly evaluate the repeatability of the method, 6 successive assays were performed using a default rinsing sequence between each run, which consisted in only one flush of BGE for 5 min. The RSD for peak migration times gave satisfactory values from 0.8% to 1.7%, whereas for corrected peak areas, unacceptable values between 7% and 19% were obtained. Moreover, corrected peak areas did not vary randomly, but a constant fall from 12% to 32% was observed along the 6 repeated analyses. This lack of repeatability could be explained by PAH adsorption either to capillary silica walls or to polypropylene vials.

At this point, MeOH/BGE ratios between 5% and 15% (v/v) were investigated to solubilize PAHs in samples. The increase in organic solvent to 10% allowed an improvement in the corrected peak area RSDs, which fell between 9% and 13%, according to PAHs, for 6 consecutive analyses. However, when 15% MeOH was added no further improvement was noticed. Although RSDs were lower, a continuous decrease in corrected peak areas from 11% to 24% still occurred. Consequently, for further studies of the 11 US-EPA PAHs, standard PAH solutions were diluted in 1/9 (v/v) methanol–BGE mixtures. However, when the 5 PAHs from the '15+1 EU priority PAHs' were additionally studied, 30% of MeOH was required in the sample because of the high hydrophobicity of the 4 dibenzopyrenes.

The continuous decrease in corrected peak areas was first attributed to adsorption of the hydrophobic PAHs to the inner surface of the polypropylene vials. Therefore, polypropylene vials were replaced by glass ones, leading to RSDs much lower for corrected peak areas, between 4% and 8%. Nevertheless, a continuous (although lower, from 8% to 17%) decrease in corrected peak areas over the 6 assays demonstrated that solubility problems were not fully solved yet.

Repeatability was also suspected to depend strongly on PAH solubility in the BGE. This is why an organic solvent is generally added to improve solubilization and therefore to reduce PAH adsorption to capillary walls. MeOH and ACN are the two most commonly used organic solvents in CE [52,53]. However, as CDs are less soluble in ACN than in MeOH, the latter solvent was tested. Separations were performed with BGE containing 10%, 15%, and 20% (v/v) methanol. Finally, 10% MeOH was found to be the best compromise between peak area repeatability and analysis time. Indeed, RSD values were between 3% and 7% with a random variation of peak area values over the sequence of 6 analyses, proving clearly that method repeatability was improved. Additional assays were carried out by adding ethanol to the BGE instead of MeOH, but this led to higher RSDs for corrected peak areas (20–25% with a mean decrease of 40%). Thus, addition of 10% methanol in BGE was finally retained.

In addition to this, several rinsing sequences were evaluated between two consecutive runs. Under initial conditions, capillary was only rinsed with 11 capillary volumes of BGE. More complex sequences were therefore evaluated by successively flushing varying volumes of water, organic solvent (ACN or MeOH), water and BGE. Finally, the best rinsing conditions were met by successively percolating water for 5 min, MeOH for 10 min, water for 5 min and BGE for 15 min, each under 935 mbar, since lower RSD values in corrected peak areas (3–6%) were obtained.

Finally, an IS, umbelliferone, was used to further improve repeatability. Hence, the responses measured for each analyte *i* on the electropherograms were the normalized corrected peak areas $(A_i/t_{Mi})/(A_{IS}/t_{MIS})$. As a result, corresponding RSDs went down to between 1% and 5%, with area values showing random variation for the 11 US-EPA PAHs.

3.3. Effect of cyclodextrin relative concentrations on selectivity

Considering that PAHs are separated based on their differential partitioning between neutral and anionic CDs, the selectivity of the method was expected to depend strongly on the two CD concentrations. The CD concentrations may also alter EOF; the neutral CD was expected to reduce EOF due to an increase in BGE viscosity while the anionic CD should also cause a significant decrease in EOF as a consequence of an increase in ionic strength.

Initial experiments were carried out at a fixed Me-β-CD concentration of 20 mM with SBE-β-CD (DS 6.2) varying from 5 to 20 mM. This relatively high concentration of neutral CD was necessary to maintain a good solubility of PAHs in sample and BGE. A minimum anionic CD concentration of 5 mM was selected to provide a sufficient selectivity while 20 mM was the maximum concentration allowing the detection of slowest migrating peaks. For each BGE composition, Joule effect was evaluated by plotting current vs applied voltage. Separation voltage was set to the highest value consistent with the linear range of the current vs applied voltage plot. The corresponding electropherograms of the 16 PAHs, which are fluorescent at a concentration of 100 μg/L using the LIF at 325 nm, in the presence of umbelliferone (IS), are presented in Fig. 1 and the corresponding figures of merit gathered in Table 1. The migration order of PAHs did not follow a general trend with respect to hydrophobicity alone (see Supplementary Data for log *P* values) or molecular structure alone, and must be related to both. Electrophoretic behavior of PAHs only depended on the pattern of distribution between the two CDs, with first emerging PAHs having more affinity for the neutral CD, and last emerging ones favoring complexation with the anionic CD.

The general pattern of improving separation with increasing anionic CD concentration from 0 to 25 mM mentioned by Szolar et al. [47] was not observed here. As shown in Table 1, this trend was only verified for first migrating compounds (e.g. the DBahP/DBahA pair), whereas resolutions between last migrating compounds began to go down with increasing SBE-β-CD concentration (e.g. R_s (BghiP/DBaeP) was 8.4 at 5 mM and 5.0 at 20 mM). Indeed, for compounds having high affinity with the anionic CD, an addition of this CD prevented the differential partitioning required to achieve a good separation, since these compounds tend to spend a majority of their time in the anionic CD cavity.

For SBE-β-CD concentrations varying from 5 to 20 mM, electroosmotic mobility significantly decreased from 48.3 to 32.6×10^{-5} cm² V^{-1} s^{-1} (Table 1) representing a loss of about 33%, mainly due to an increase in the BGE ionic strength.

Absolute values of electrophoretic mobilities of early migrating PAHs, such as DBahA and DBahP, generally increased as a function of SBE-β-CD concentration (Fig. 2A), suggesting that complexation phenomenon prevailed over the increase in ionic strength. On the contrary, absolute values of electrophoretic mobilities of late migrating PAHs increased first until becoming constant or even decreasing. Indeed, for increasing amounts of anionic CD, PAHs tended to be more incorporated in the hydrophobic CD cavity until ionic strength became predominant over complexation phenomenon, thus decreasing absolute values of electrophoretic mobilities. This was the case for DBaeP, BghiP, DBalP, IP, BbFA, Pyr, BaP, FA, BkFA and MCH. In the extreme, for the compound exhibiting the highest electrophoretic mobility (in absolute value), DBaeP, the electrophoretic mobility (in absolute value) decreased with the

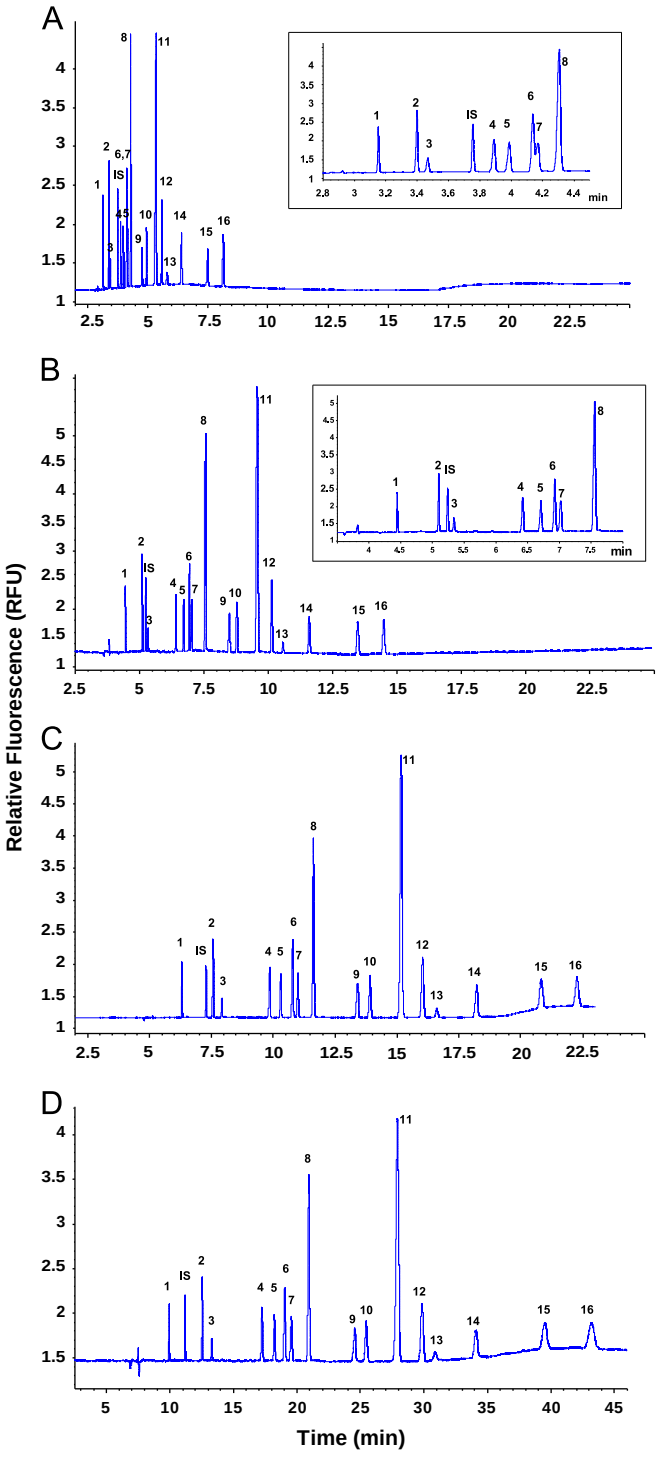

Fig. 1. Influence of the SBE-β-CD concentration in the BGE on the electropherograms of a standard mixture of 16 PAHs. Bare fused-silica capillary, 50 μm I.D. × 49 cm (LIF detection at 33.5 cm). BGE: 10 mM sodium borate buffer (pH 9.2), 600 mM urea, 20 mM Me-β-CD with SBE-β-CD concentrations of (A) 5 mM (V=20 kV); (B) 10 mM (V=18 kV); (C) 15 mM (V=15 kV); and (D) 20 mM (V=12 kV) in 9:1 (v/v) water–MeOH mixture. Temperature, 25 °C. Hydrodynamic injection, 5 s, 20 mbar. LIF detection, λ_{exc}=325 nm and emission through 350 nm long-pass filter; PMT, 750 V; rise time, 0.05 s. PAH concentration, 100 μg/L each in ACN–MeOH–BGE 6:34:60, (v/v/v). Umbelliferone concentration, 30 μg/L. Identification: 1, DBahP; 2, DBahA; 3, ANT; 4, CHR; 5, BaA; 6, DBaiP; 7, MCH; 8, BkFA; 9, FA; 10, BaP; 11, Pyr; 12, BbFA; 13, IP; 14, DBalP; 15, BghiP; 16, DBaeP; IS: umbelliferone.

anionic CD concentration even from 5 mM. Over the concentration range studied, DBaeP was either totally incorporated in anionic CD cavities, or ionic strength effects prevailed over complexation phenomena in all conditions tested. As expected, the number of theoretical plates decreased upon increasing SBE-β-CD concentration, which can be explained by diffusion phenomenon since migration times were increased. A SBE-β-CD concentration of 10 mM was retained for further optimization because it corresponded to the best compromise between resolution ($R_s > 1.8$) and analysis time (14.5 min), see Table 1.

Subsequent experiments were performed by varying the neutral CD concentration while maintaining SBE-β-CD concentration at 10 mM in order to better understand the influence of the neutral CD concentration on separation selectivity. The maximum Me-β-CD concentration was set at 40 mM to limit the increase in viscosity, whereas 5 mM was the bottom limit, to ensure enough solubility of PAHs. The resulting electropherograms of the 16 PAH standard mixture recorded at a constant voltage of 18 kV are shown in Fig. 3. The increase in BGE viscosity between 5 and 40 mM Me-β-CD induced a decrease in the electroosmotic mobility from 45.8 to 38.2 × 10^{-5} cm^2 V^{-1} s^{-1}.

Upon varying the Me-β-CD concentration, several reversals in PAH migration order were observed, as opposed to the situation with SBE-β-CD concentration variations, for which PAHs always migrated in the same order. This emphasizes the higher impact the neutral CD has on selectivity. As expected, because of a competition effect, the higher the neutral CD concentration in the BGE, the lower the PAH electrophoretic mobility (in absolute value), as shown in Fig. 2B. Moreover, the increase in BGE viscosity upon increasing Me-β-CD concentration reinforced this effect. More accurately, a higher decrease in electrophoretic mobility (in absolute value) occurred between 5 and 40 mM Me-β-CD for the early emerging peaks than for the late emerging ones, e.g. decreases by about 85% and 76% were noted for DBahP and DBaeP, respectively. This behavior is consistent with the fact that an increase in neutral CD concentration had a higher impact on the PAHs with more affinity for this type of CD than on those with less affinity for it. Eventually, the decrease in electrophoretic mobility (in absolute value) for PAHs mostly associated with the anionic CD is probably explained by viscosity effect.

Eventually, the CD composition allowing the best compromise between separation selectivity, efficiency and analysis time was 20 mM Me-β-CD and 10 mM SBE-β-CD. Under these optimized conditions, in order to provide a deeper insight into method selectivity, three new PAHs (BjFA, ACP and PHE) that could not be detected at the 100 μg/L level were added to the studied standard mixture at 1 mg/L for ACP and PHE and 200 μg/L for BjFA. ACP and PHE were fully baseline resolved from other PAHs whereas BjFA co-migrated with Pyr. As BcFLR was provided in cyclohexane, this compound was injected separately from the mixture in the presence of umbelliferone (IS) and analyzed in the same electrophoretic conditions. By comparing its normalized migration time (t_{MBcFLR}/t_{MIS}) with normalized migration times of other PAHs, BcFLR was found to be separated from all other PAHs and to migrate between BkFA and FA (normalized migration times: t_{MBcFLR}/t_{MIS}=1.60 ± 0.015, t_{MBkFA}/t_{MIS}=1.47 ± 0.01, t_{MFA}/t_{MIS}=1.66 ± 0.013 and estimated resolutions: R_s (BcFLR/BkFA)=7.7 ± 0.2 and R_s (FA/BcFLR)=2.0 ± 0.2). Finally, 19 PAHs were baseline resolved in less than 15 min with efficiencies greater than 1.5 × 10^5 and resolutions greater than 1.5 for all pairs, except for BjFA and Pyr, which still co-migrated. The resulting electropherogram is presented in Fig. 4 with its corresponding electrophoretic figures of merit given in Table 2. A considerable improvement of method selectivity and repeatability was therefore achieved in comparison with previously published work [48,49]. For a signal to noise ratio of 10, the limits of quantitation of the method were estimated, from 5 repetitions, between 6.0 and > 1000 μg/L depending on the PAH (Table 2).

Table 1
Electroosmotic mobilities, analysis times, theoretical plates (N) and resolutions (R_s) as a function of SBE-β-CD concentration from the electropherograms of a 16 PAH standard mixture shown in Fig. 1.

	[SBE-β-CD] (mM)							
	5		10		15		20	
Electroosmotic mobility (10^{-5} cm² V^{-1} s^{-1})	48.3		42.0		38.3		32.6	
Analysis time (min)	8.1		14.5		22.1		45.8	
	N[a]	R_s	N[a]	R_s	N[a]	R_s	N[a]	R_s
DBahP	6.83		5.97		4.77		3.52	
DBahA	4.47	14.0	4.31	24.3	3.40	28.4	2.59	32.1
ANT	2.79	2.8	3.24	5.8	2.53	6.2	2.22	8.0
CHR	2.51	14.9	2.76	23.8	2.33	26.7	1.72	28.9
BaA	2.64	3.1	2.75	4.8	2.30	5.4	1.70	5.9
DBaiP	3.18	5.0	3.19	5.4	2.42	5.5	1.71	3.8
MCH	2.40	0.9	2.75	1.8	2.17	2.3	1.50	3.3
BkFA	2.40	4.1	2.57	6.0	2.10	6.4	1.49	7.1
FA	1.48	10.9	2.22	15.4	1.89	15.7	1.15	15.0
BaP	2.10	3.8	2.50	4.1	1.92	4.1	1.21	2.9
Pyr	1.17	7.3	2.00	9.6	1.64	9.0	0.91	8.1
BbFA	1.94	4.2	2.23	5.4	1.76	5.7	1.06	5.4
IP	1.87	4.1	2.16	3.9	1.72	3.7	1.03	2.9
DBalP	1.98	10.7	2.07	9.9	1.58	9.3	0.90	8.2
BghiP	1.35	15.7	1.86	14.3	1.40	12.8	0.59	10.6
DBaeP	2.13	8.4	1.85	6.7	1.36	6.2	0.46	5.0

[a] Number of theoretical plates (10^5) calculated using the equation: $N = 5.54 \times (t_m/w_{1/2})^2$ with $w_{1/2}$, the peak width at half height.

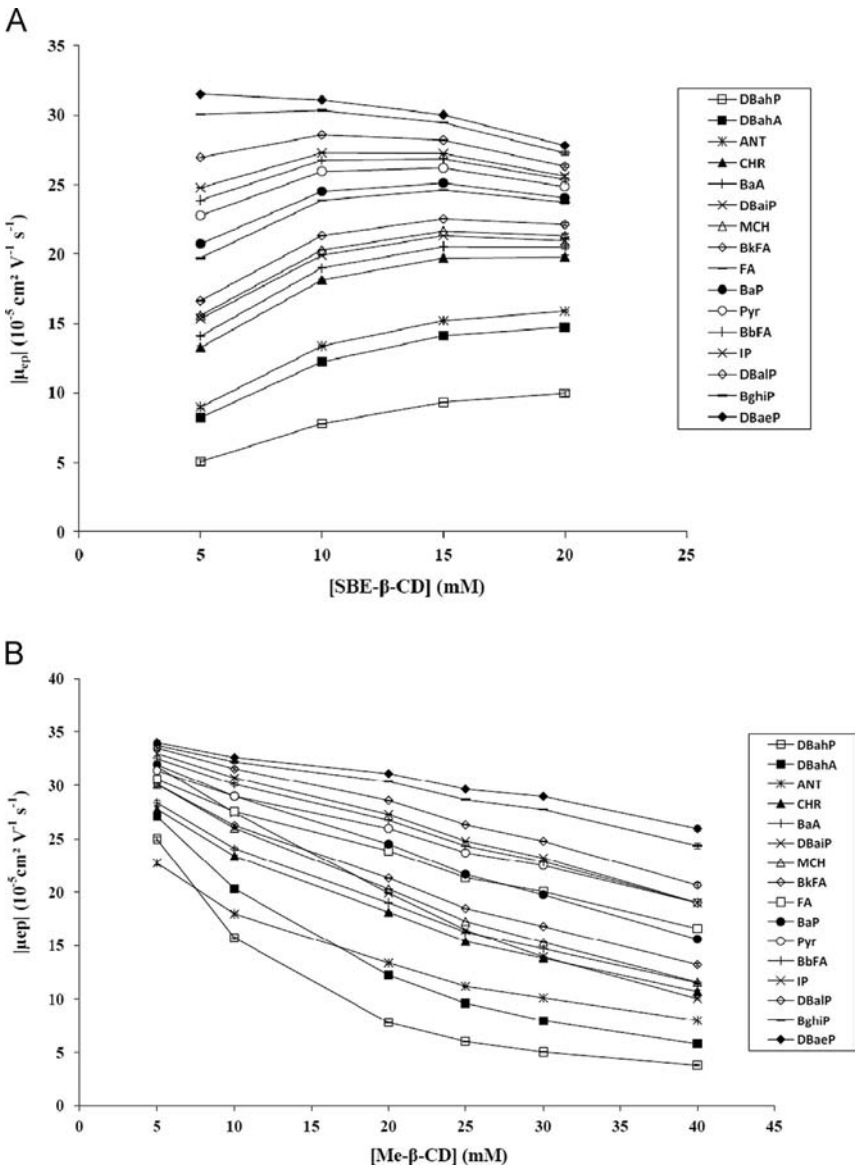

Fig. 2. Variation of the electrophoretic mobility (in absolute value) of 16 PAHs as a function of (A) SBE-β-CD concentration (experimental conditions: see Fig. 1); (B) Me-β-CD concentration (experimental conditions: see Fig. 3).

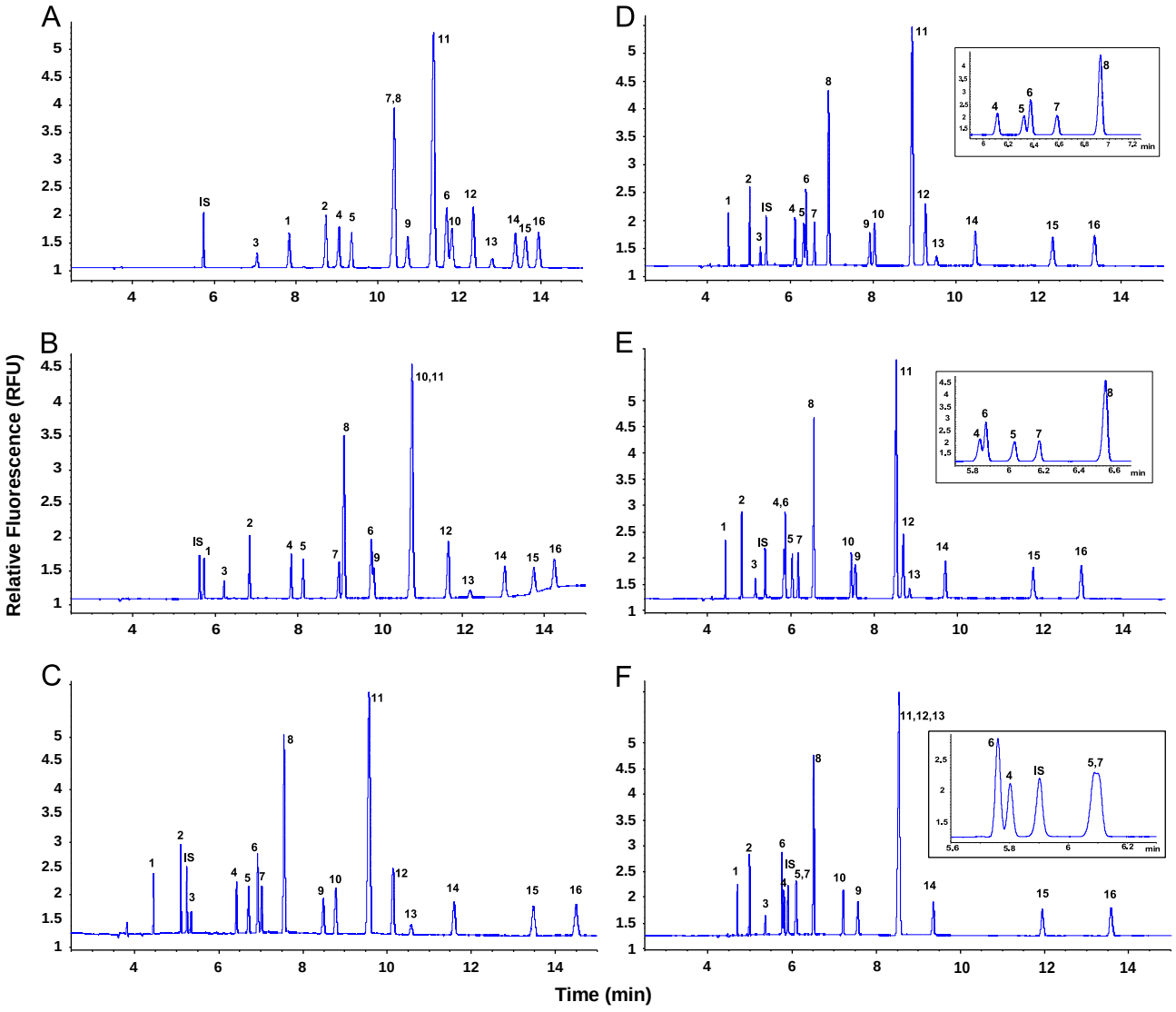

Fig. 3. Influence of the Me-β-CD concentration in the BGE on the electropherograms of a standard mixture of 16 PAHs. BGE: 10 mM sodium borate buffer (pH 9.2), 600 mM urea, 10 mM SBE-β-CD with Me-β-CD concentrations of (A) 5 mM; (B) 10 mM; (C) 20 mM; (D) 25 mM; (E) 30 mM; and (F) 40 mM in 9:1 (v/v) water–MeOH mixture. Applied voltage, 18 kV. Other conditions and identification: see Fig. 1.

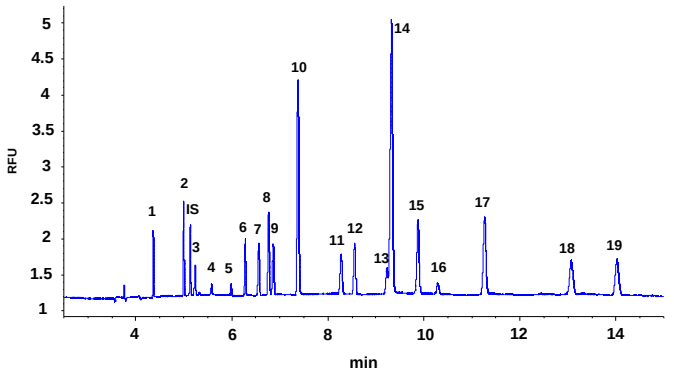

Fig.4. Electropherogram of a standard mixture of 19 PAHs. BGE: 10 mM sodium borate buffer (pH 9.2), 600 mM urea, 10 mM SBE-β-CD, and 20 mM Me-β-CD in 9:1 (v/v) water–MeOH mixture. Temperature, 25 °C. Applied voltage, 18 kV. PAH concentration, 100 μg/L (except for ACP and PHE, 1 mg/L, and BjFA, 200 μg/L) in ACN–MeOH–BGE 6:34:60, (v/v/v). Other conditions: see Fig. 1. Identification: 1, DBahP; 2, DBahA; 3, ANT; 4, ACP; 5, PHE; 6, CHR; 7, BaA; 8, DBaiP; 9, MCH; 10, BkFA; 11, FA; 12, BaP; 13, BjFA; 14, Pyr; 15, BbFA; 16, IP; 17, DBalP; 18, BghiP; 19, DBaeP; IS: umbelliferone.

3.4. Application to real samples

The developed CD-CZE method was applied to real samples, two edible oils: a sunflower oil and an Isio 4® oil, which is a mixture of rapeseed, sunflower, grape seed, and high content oleic acid sunflower oils. Each oil was first extracted with a commercial molecularly imprinted polymer dedicated to PAH analysis. The obtained extract was next evaporated to dryness and reconstituted in ACN–MeOH–BGE (1:3:6, v/v/v) before CE analysis. Oil extracts were first spiked only with IS at 100 μg/L and next with 16 PAHs at 100 μg/L (except for MCH, BaP, and DBaeP, 150 μg/L, ANT, FA, and BghiP, 200 μg/L, and IP, 400 μg/L), listed by US-EPA and/or EFSA as priority pollutants in environmental and food samples and representing the full migration window of studied PAHs. The spiked levels were selected in order to insure a signal to noise ratio of at least 30 for all PAHs, allowing an estimation of potential matrix effect.

Fig. 5 presents the resulting typical electropherograms obtained for a standard mixture (A), a sunflower oil extract (B), and an Isio 4® oil extract (C). When both oil extracts were spiked with IS at 100 μg/L (Fig. 5-B (1) and 5-C (1)), the resulting

Table 2
Migration times (t_{Mi}), theoretical plates, limits of quantitation (LOQ) and relative standard deviations (RSD) for migration times and normalized corrected areas for the CD-CZE analysis of a 19 PAH standard mixture (conditions, see Fig. 4).

PAH	t_{Mi} (min)	Theoretical plates (10^5)	LOQ[a] (μg/L)	RSD for migration times ($n=6$) (%)	RSD for normalized corrected areas ($n=6$) (%)
DBahP	4.4	5.37	20	0.8	2.2
DBahA	5.0	4.32	15	0.9	2.0
IS	5.1	3.91	20	0.8	2.3
ANT	5.2	3.16	50	0.8	1.2
ACP	5.6	1.64	> 1000	0.9	2.1
PHE	6.0	2.41	> 1000	0.9	2.2
CHR	6.3	2.91	25	0.9	2.1
BaA	6.6	2.65	30	1.0	3.8
DBaiP	6.8	2.76	20	1.1	4.1
MCH	6.9	2.34	30	1.0	3.7
BkFA	7.4	2.42	10	1.0	2.2
FA	8.3	1.85	40	1.1	3.4
BaP	8.6	2.43	30	1.2	2.1
BjFA	9.2	2.95	125	1.3	3.3
Pyr	9.3	1.56	10	1.3	3.3
BbFA	9.9	2.08	20	1.3	2.9
IP	10.3	1.95	> 100	1.4	4.9
DBalP	11.3	1.89	20	1.5	3.5
BghiP	13.1	1.52	45	1.6	4.3
DBaeP	14.0	1.72	40	1.7	3.9

[a] LOQs were estimated at 100 μg/L (except for ACP and PHE, 1 mg/L, and BjFA, 200 μg/L) for a signal to noise ratio of 10.

electropherograms are quite clean with a flat baseline. Only a small peak appears, for both oil extracts, corresponding to normalized migration time of Pyr. The Isio 4® extract was spiked with 6 μg/L of Pyr, which corresponded to the estimated Pyr concentration, and the resulting electropherogram shows (Fig. 5-C (3)) a unique peak with an increased area value by a factor of about 2, confirming the presence of Pyr in the oil extract.

When both oil extracts were spiked with IS plus 16 PAHs, the resulting electropherograms (Fig. 5-B (2) and 5-C (2)) show that the separation was maintained from a qualitative point of view. As Isio 4® is made from a mixture of rapeseed, sunflower, grape seed, and high content oleic acid sunflower oils, it seems that the developed CE method is thus reliable for different kinds of edible oil extracts.

From a quantitative point of view, Table 3 presents the average normalized corrected areas and normalized heights for CE analyses ($n=3$) of PAHs in standard mixture, spiked sunflower oil extract, and spiked Isio 4® oil extract. After comparing the average results obtained for each PAH in each extract with the ones obtained with the standard mixture (homogeneity of the variance plus Student test with a 5% risk), it was observed that the normalized corrected areas were not significantly different for all PAHs except DBahP, DBaiP, and DBalP in sunflower oil extract and DBalP and BghiP in Isio 4® oil extract. For these compounds, belonging to the most hydrophobic tested PAHs, a matrix effect was measured inducing an under-estimation of the PAH amount between 7% and 21%. Comparing normalized heights obtained with standard mixture and oil extracts, they were not significantly different for all PAHs except DBahP, DBaiP, IP, DBalP, BghiP, and DBaeP in sunflower extract and DBahP, DBaiP, and DBalP in Isio 4® extract. Once more, a difference appeared for the most hydrophobic tested PAHs, inducing a decrease in LOQs for the determination of these compounds in real samples. This matrix effect observed for the most hydrophobic PAHs would necessitate for the validation step either calibration in oil samples or standard addition method. The full validation study is currently being performed in the laboratory and will be presented in another paper. Nevertheless, using the CE method developed here, PAHs in different spiked edible oil extracts were successfully separated.

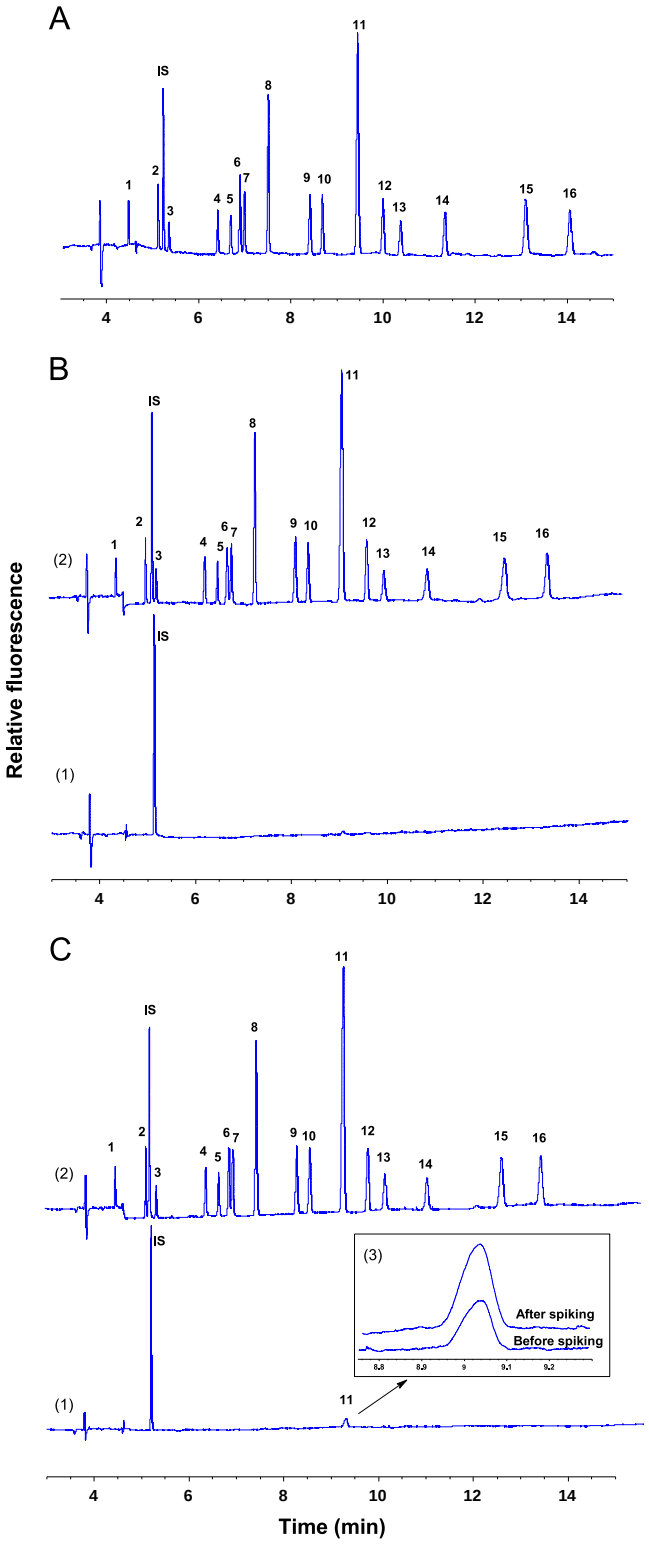

Fig. 5. CE analysis of (A) standard mixture, (B) sunflower oil extract, and (C) Isio 4® oil extract, (1) spiked with IS at 100 μg/L, (2) spiked with IS at 100 μg/L plus 16 PAHs and (3) superimposition of blank oil and the same oil spiked with IS at 100 μg/L and Pyr at 6 μg/L. CE conditions and PAH identification: see Figs. 4 and 1, respectively. PAH concentration, 100 μg/L (except for MCH, BaP, and DBaeP, 150 μg/L, ANT, FA, and BghiP, 200 μg/L, and IP, 400 μg/L) in ACN–MeOH–BGE 1:3:6 (v/v/v).

Table 3
Normalized corrected areas and normalized heights for CE analyses ($n=3$) of standard mixture, spiked sunflower oil extract, and spiked Isio 4® oil extract. Experimental conditions: see Fig. 5.

PAH	Standard mixture		Sunflower oil		Isio 4® oil	
	Normalized corrected areas	Normalized heights	Normalized corrected areas	Normalized heights	Normalized corrected areas	Normalized heights
DBahP	0.23 ± 0.02[a]	0.24 ± 0.01	0.19 ± 0.02	0.19 ± 0.01	0.21 ± 0.02	0.21 ± 0.01
DBahA	0.37 ± 0.04	0.38 ± 0.04	0.34 ± 0.02	0.34 ± 0.01	0.37 ± 0.02	0.37 ± 0.01
ANT	0.20 ± 0.02	0.17 ± 0.01	0.19 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.16 ± 0.01
CHR	0.29 ± 0.02	0.24 ± 0.01	0.28 ± 0.02	0.24 ± 0.01	0.29 ± 0.01	0.26 ± 0.01
BaA	0.26 ± 0.02	0.22 ± 0.02	0.25 ± 0.01	0.22 ± 0.01	0.25 ± 0.01	0.22 ± 0.01
DBaiP	0.46 ± 0.01	0.41 ± 0.01	0.40 ± 0.03	0.29 ± 0.01	0.43 ± 0.02	0.36 ± 0.01
MCH	0.41 ± 0.04	0.35 ± 0.03	0.40 ± 0.02	0.32 ± 0.01	0.42 ± 0.01	0.35 ± 0.01
BkFA	1.10 ± 0.06	0.93 ± 0.07	1.05 ± 0.02	0.90 ± 0.02	1.09 ± 0.03	0.95 ± 0.02
FA	0.45 ± 0.04	0.33 ± 0.04	0.45 ± 0.02	0.35 ± 0.01	0.46 ± 0.01	0.37 ± 0.02
BaP	0.42 ± 0.03	0.34 ± 0.03	0.39 ± 0.01	0.31 ± 0.01	0.41 ± 0.03	0.34 ± 0.01
Pyr	1.75 ± 0.13	1.20 ± 0.10	1.79 ± 0.10	1.27 ± 0.05	1.81 ± 0.03	1.34 ± 0.10
BbFA	0.41 ± 0.03	0.32 ± 0.01	0.39 ± 0.02	0.31 ± 0.01	0.41 ± 0.01	0.33 ± 0.01
IP	0.26 ± 0.03	0.20 ± 0.02	0.24 ± 0.02	0.16 ± 0.01	0.24 ± 0.01	0.18 ± 0.01
DBalP	0.33 ± 0.03	0.24 ± 0.02	0.26 ± 0.02	0.16 ± 0.01	0.23 ± 0.01	0.17 ± 0.01
BghiP	0.42 ± 0.01	0.30 ± 0.01	0.38 ± 0.03	0.22 ± 0.01	0.39 ± 0.02	0.27 ± 0.01
DBaeP	0.37 ± 0.03	0.27 ± 0.01	0.32 ± 0.02	0.22 ± 0.01	0.33 ± 0.01	0.26 ± 0.01

[a] Average value ± standard deviation.

3.5. Concluding remarks

A highly selective CD-CZE method suitable for the analysis of PAHs was developed. The use of a buffer containing 10 mM SBE-β-CD and 20 mM Me-β-CD allowed the simultaneous separation of 19 PAHs of environmental and food concerns listed by international regulatory institutions in less than 15 min with RSD varying from 0.8% to 1.7% and from 1.2% to 4.9% for migration times and normalized corrected peak areas, respectively. Efficiencies greater than 10^5 and resolutions greater than 1.5 were obtained, except for the pair BjFA/Pyr. Applications to oil extracts demonstrated the reliability of the method with real samples. Heavy PAH isomers such as the 4 dibenzopyrenes, which are generally difficult to separate using chromatographic methods, were easily resolved with good repeatabilities, proving the capability of CE methods for the analysis of neutral and hydrophobic compounds. As two PAHs were still co-migrating, an experimental design will be used to further optimize the method resolution.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.11.062.

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