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Determination of nitrated polycyclic aromatic hydrocarbons and their precursors in biotic matrices

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Abstract

Analytical method for the determination of ultra-trace levels of nitro-PAHs in various biotic matrices has been developed. Soxhlet extraction and/or solvent extraction enhanced by sonication were used for isolation of target analytes; GPC followed by SPE were employed for purification of crude extracts. GC–MS/NCI technique was utilised for identification/quantitation of target analytes. Performance characteristics of implemented method were obtained through thorough in-house validation procedure. The main sources of uncertainties were critically evaluated, possible strategies of their elimination/minimisation were considered and consequently employed. Examination of real-life samples of various foodstuffs (complete human diet, mate tea, pumpkin seed oil, parsley, sausages) was performed in this study.

Keywords: Biotic matrices; Polynuclear aromatic hydrocarbons

1. Introduction

Traces of nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) can be found in most of environmental compartments. Nitro-PAHs are formed either during incomplete combustion processes (pyrosynthesis) or as a result of a wide range of reactions taking place in atmosphere. In the latter case, the corresponding parent compounds, polycyclic aromatic hydrocarbons (PAHs), mostly adsorbed on air particulates, react with nitrogen oxides forming various nitro derivatives [1–3].

Although nitro-PAHs typically occur in terrestrial ecosystem in much lower concentrations compared to PAHs, they are supposed to pose a significant risk for human health because of their distinctly adverse biological activities demonstrated in biota. Many of them have been proven to have mutagenic and/or carcinogenic effects directly or after biotransformation to hydroxynitro derivatives [4,5].

PAHs, very often together with nitro-PAHs, represent a group of organic contaminants commonly determined in pilot studies and/or monitoring programs focused on the atmospheric pollution. While quite comprehensive data illustrating the relationship between the levels of atmospheric PAHs and contamination of terrestrial vegetation (including food crops) due to an imission burden are available, only a scarce information on this aspect has been reported for nitro-PAHs. Also the incidence of nitro-PAHs in food chains has not been systematically investigated yet and only few references concerned with limited range of foodstuffs are available. Consequently, the

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human dietary exposure and related risk assessment is unclear and the legislation limits have not been established yet [6–13].

Although various methods for routine analysis of food-borne PAHs are available, none of them is applicable for simultaneous analysis of nitro-PAHs. The main reason is the lack of sufficient detection sensitivity enabling to detect the concentrations of nitro-PAHs that are typically about one or two orders of magnitude lower than PAHs levels. In addition, due to the presence of polar substituents, nitro-PAHs, compared to parent compounds, exhibit rather different chromatographic behaviour in the course of clean-up and GC/HPLC separation. Another problem encountered in HPLC is due to a distinct detection mechanism: fluorescence detection, which is common in analysis of PAHs, can be utilised only after reduction of nitro-PAHs to amino-derivatives (both off-line and on-line procedures were employed for this purpose) [14].

In GC analysis, mass spectrometric detector operated in electron impact ionisation mode (GC–MS/EI) is commonly used for PAHs determination. However, as long as a special pre-concentration of ultra trace nitro-PAHs is not accomplished, sufficient detection sensitivity might not be achieved. Taking the advantage of negative chemical ionisation (GC–MS/NCI) may bring both the required specificity and sensitivity for nitro-PAHs detection. Unfortunately, it is not possible to determine PAHs in this ionisation mode, so two separate GC runs are needed for the analysis of these two related groups of compounds. The use of conventional GC detector such as ECD was also reported in some studies [13]. However, due to its limited selectivity, rather poor precision of results cannot be avoided. Coupling capillary GC with thermal energy analyzer (GC–TEA) may provide both selectivity and sensitivity needed in nitro-PAHs determination. Unfortunately, GC–TEA system is owned only by a few laboratories [6].

Regarding the sample preparation step, various extraction/isolation methods were applied. Practically all reported methods involve some kind of extraction procedure that is realised most often by organic solvent in Soxhlet apparatus, microwave extractor or simply in a glass flask by sonication. Alternatively, supercritical fluid extraction employing carbon dioxide as an extraction medium or dialysis utilising polyethylene membrane were described. Following clean-up steps typically involve gel permeation chromatography, adsorption chromatography, liquid–liquid extraction or solid-phase extraction and in some cases additional clean-up by HPLC [14–18].

It should be noted that none of the published studies reported performance characteristics of applied method and their long-term stability. Therefore, the aim of the presented study was to develop a comprehensively characterised analytical procedure enabling reliable examination of various types of biotic matrices for the presence of even ultra-trace (sub-ppb) concentrations of target analytes, i.e., at levels that typically occur in real-life samples. Validation was carried out according to the standard protocol. Attention was paid to measurement/estimation of standard uncertainties in order to adopt measures compensating/eliminating respective critical aspects.

2. Experimental

2.1. Standards

Individual standard solutions of 1-nitronaphthalene (1-nNap) and 2-nitronaphthalene (2-nNap), both 100 μg/ml in toluene, were obtained from AccuStandard (USA); nitro-PAHs standard mixture SRM 1587 containing 2-nitrofluorene (2-nFln), 9-nitroanthracene (9-nAnt), 3-nitrofluorantene (3-nFlt), 1-nitropyrene (1-nPyr), 7-nitrobenzo(*a*)anthracene (7-nBaA), 6-nitrochrysene (6-nChr) and 6-nitrobenzo(*a*)pyrene (6-nBaP) in concentrations ranging from 3.9 to 7.6 μg/ml was purchased from NBS (USA), standard solutions of deuterated nitro-PAHs 2-nitrofluorene(d_9) (2-nFln d9), 9-nitroanthracene(d_9) (9-nAnt d9), 1-nitropyrene(d_9) (1-nPyr d9), 6-nitrochrysene(d_{11}) (6-nChr d11), each 50 μg/ml in deuterated toluene, were obtained from Cambridge Isotope Laboratories (UK), 2-methyl-1-nitronaphthalene (2-Me-1-nNap) crystalline (99%) from Aldrich (Germany). Working standard solutions were prepared in toluene and stored in refrigerator at 4 °C.

2.2. Samples

Several kinds of biotic matrices largely differing in content of moisture and lipids (potential co-extracts) were used in experiments. Low fat plant matrices were represented by fresh parsley (harvested in a garden located close to urban area) and mate tea (obtained from retail market). Foods of animal origin with high fat content were represented by sausage (smoked, homemade), while pumpkin seed oil (obtained from retail market) represented plant lipids. Mixed total diet samples were prepared as a freeze-dried powder.

2.3. Instrumentation, materials

2.3.1. Clean-up system

2.3.1.1. Gel permeation chromatography (GPC)

Automated GPC System (ASPEC XL, Gilson, France), was equipped either by (i) stainless steel column, 500×8 mm, packed with BioBeads S-X3, particle size 200–400 mesh (BioRad Laboratories, USA), or by (ii) rigid PL gel column, 600 mm×7.5 mm; particle size 50 μm (Pl Laboratories, UK)

2.3.1.2. Solid phase extraction (SPE)

Vacuum separator Dorcus (Tessek, Czech Republic) was used for handling SPE cartridges: (i) LiChrolut CN 500 mg (Merck, Germany); (ii) LiChrolut EN 200 mg (Merck, Germany)

2.3.2. Identification and quantification

2.3.2.1. Gas chromatography–mass spectrometry (GC–MS)

Gas chromatograph HP 6890 coupled with mass spectrometric detector HP 5973 equipped with chemical ionisation module, Autosampler HP 7683 ALS, HP ChemStation software (Hewlett-Packard, USA) and capillary columns (i) DB-5 MS 60 m× 0.25 mm×0.25 μm (J&W Scientific, USA); (ii) DB-XLB 30 m×0.25 mm×0.1 μm (J&W Scientific, USA)

2.4. Analytical method

Procedure flow chart is shown in Fig. 1.

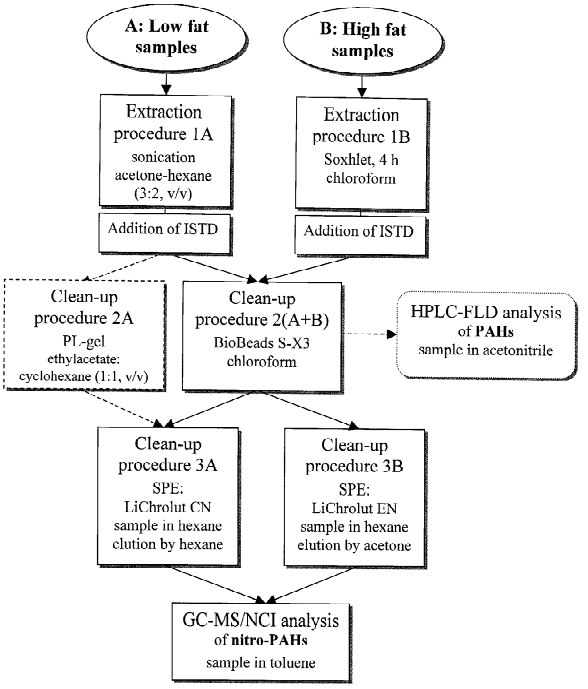


Fig. 1. Procedure flow chart.

2.4.1. Extraction

2.4.1.1. Procedure 1A

Plant matrices, with low lipid and high moisture content (like parsley) were deep-frozen (−25 °C) prior to handling to enable homogenisation. After taking from freezer the raw material was immediately disintegrated in laboratory homogeniser. Approximately 50 g of this sample were taken and desiccated with anhydrous sodium sulphate. Extraction was carried out by three 150–200 ml portions of acetone–hexane (3:2, v/v) mixture. Sonication was used to enhance extraction efficiency of each 20 min extraction step. In case of dry raw material (such as mate tea) the sample (5–10 g) was directly extracted without any special preparation.

2.4.1.2. Procedure 1B

Samples with higher lipid content (>5%) were prior to the extraction disintegrated in laboratory homogeniser (e.g., diet) or ground with sea sand (e.g., sausage) and desiccated, as any traces of water would rapidly lower the extraction efficiency. Low

water content samples (sausages) were mixed with anhydrous sodium sulphate (c.a. twice the weight of sample). Removing of water from the samples with higher moisture content (diet) should be preferably realised by freeze-drying. The amount of sample taken for the extraction was approximately 15 g. The extraction is performed using Soxhlet apparatus with 350 ml of chloroform for 4 h with ca. eight cycles/h.

2.4.2. Clean-up

2.4.2.1. Procedure 2A+2B

Extracts obtained by either isolation procedure (A or B) were at first evaporated using rotary vacuum evaporator and transferred in chloroform into volumetric flasks (5–25 ml) according to their lipid content. The maximum amount of lipids injected onto GPC column was limited by the column capacity that was 300 mg. Internal standard (solution of deuterated nitro-PAHs) was added to achieve a concentration of 1 ng/ml. Before injection on GPC the sample had to be centrifuged (15 min at 3500 rpm) and/or filtered through membrane filter (maximally 5 μm) to remove all the insoluble particles and to achieve a clear solution that could be injected onto GPC column. One ml of sample was then fractionated by GPC on a BioBeads S-X3 column with chloroform as a mobile phase; 8.5 ml of eluate were collected (fraction 13.5–22 ml). PAHs are eluted in similar fraction as nitro-PAHs. Alternatively, rigid PL-gel column can be used for the vegetable and tea samples and run in ethylacetate–cyclohexane (1:1, v/v), but this is not suitable for PAH analysis (due to excessively large elution volume).

2.4.2.2. Procedure 3A

SPE employing LiChrolut CN cartridge (cyano phase) was used to remove minor matrix components from vegetable samples left in GPC fraction (procedure 2A+2B, see above). After evaporation and dissolving the residue in 0.5 ml of hexane, the sample was loaded onto a 500-mg cartridge. The analytes were eluted with another 12 ml of hexane. Finally, the eluate was evaporated and the residue dissolved in 200 μl of toluene.

2.4.2.3. Procedure 3B

SPE employing LiChrolut EN cartridge was used to remove residual lipids not removed by GPC. Respective fraction (procedure 2A+2B, see above) was evaporated, the residue dissolved in 0.5 ml of hexane and loaded onto a 200-mg cartridge. After its rinsing with another 10 ml of hexane, the target analytes were eluted with 5 ml of acetone. Finally, the eluate was evaporated and dissolved in 200 μl of toluene.

2.4.3. GC–MS/NCI separation and identification/quantitation

2.4.3.1. GC separation

Two types of GC systems operated under following conditions were used for separation of sample components.

GC System 1: DB-5 MS column; helium as carrier gas at average linear velocity 27 cm/s (constant flow mode). Oven temperature was programmed starting at 90 °C, held for 2 min, then raised by 4 °C/min to 130 °C followed by 8 °C/min to 300 °C, the final temperature was held for 10 min. The total analysis time was 44 min.

GC System 2: DB-XLB column; helium as carrier gas at average linear velocity 37 cm/s (constant flow mode). Oven temperature was programmed starting at 90 °C, held for 2 min, then raised by 8 °C/min to 130 °C followed by 15 °C/min to 300 °C, the final temperature was held for 4.5 min. The total analysis time was 23 min.

One μl of sample was injected into glass double tapered liner heated to 300 °C in pulsed splitless mode (207 kPa (30 p.s.i.) for 0.5 min), with splitless period held for 3 min.

The GC–MS interface was heated to 300 °C.

2.4.3.2. MS/NCI identification/quantitation

MSD was operated in negative chemical ionisation mode (NCI) using methane as a reaction gas at 40% flow-rate. Ion source was heated to 150 °C and electron multiplier voltage was set 500 V higher than the value adjusted by autotune procedure (typically 1700–1900 V). Information on m/z used for data acquisition is summarised in Table 1.

Table 1
Ions (m/z) monitored during MS data acquisition (GC System 2)

Group no./ time window (min)	Analyte	Retention time (min)	m/z[a] (amu)
1	1-nNap	10.8	**173**, 174
10–13	2-Me-1-nNap	11.0	**187**
	2-nNap	11.3	**173**, 174
2	2-nFln	14.9	**211**
13–15.8	2-nFln (d9)	14.9	**220**
	9-nAnt	14.9	**223**
	9-nAnt (d9)	14.9	**232**
3	3-nFlt	17.5	**247**, 248
15.8–17.9	1-nPyr	17.7	**247**, 248
	1-nPyr (d9)	17.7	**256**
4	7-nBaA	18.6	**273**, 274
17.9–20.5	6-nChr	19.2	**273**, 274
	6-nChr (d11)	19.2	**284**
5	6-nBaP	21.6	**297**, 298
20.5–22.8			

[a] Molecular ions (M^-) used for quantitation shown in bold.

3. Results and discussion

Prior to the development and optimisation of the overall analytical procedure, the stability of analytes and their potential losses during critical operations involved in individual sample preparation steps were investigated.

3.1. Light stability

It is a well-known fact that not only a formation of nitro-PAHs, but also their residence time in atmosphere are influenced by the light intensity. Therefore, the photostability of nitro-PAHs was tested. The solutions of standards in toluene were placed into GC vials and stored under various light/temperature conditions: (i) transparent vial and amber vial were both exposed to daylight at ambient temperature; (ii) another vial was stored in dark at 24 °C; and (iii) reference vial was kept in freezer at −15 °C. Concentration trends checked during 12 days experiment are shown in Figs. 2 and 3.

The highest rate of photodegradation could be seen for 9-nAnt and 7-nBaA, with both compounds completely degraded within 24 h exposition to daylight in transparent vials. 1-nPyr and 6-nChr were also shown to be very unstable compared to other analytes. All the deuterated equivalents displayed the same photostability as the parent compounds.

Far better light stability of nitro-PAHs was obtained when the solutions were placed in amber vials. Only negligible degradation took place in solutions stored in dark at ambient temperature.

Therefore any exposure to light should be avoided during standards/sample handling. Amber glassware and protective aluminium foil should be used for this purpose.

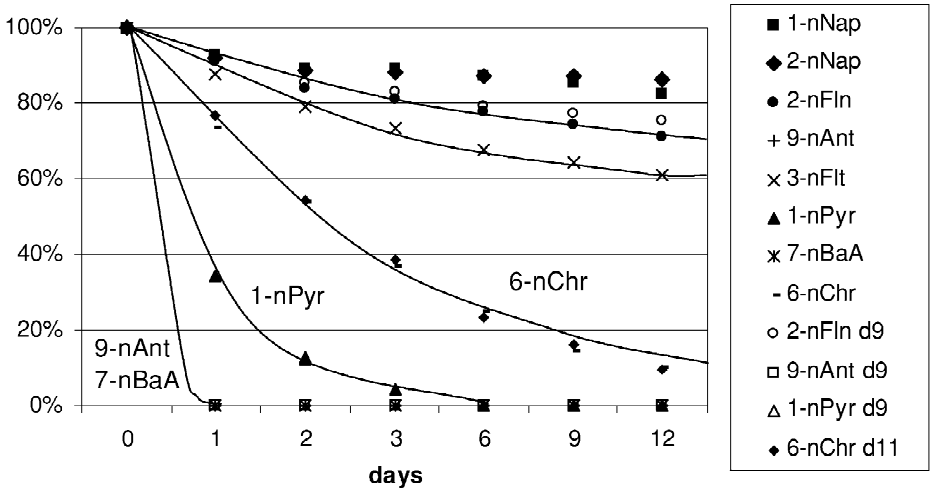


Fig. 2. Photostability of nitro-PAHs in toluene (conc. 10 ng/ml each); transparent vial, exposed to daylight, average temperature 18 °C.

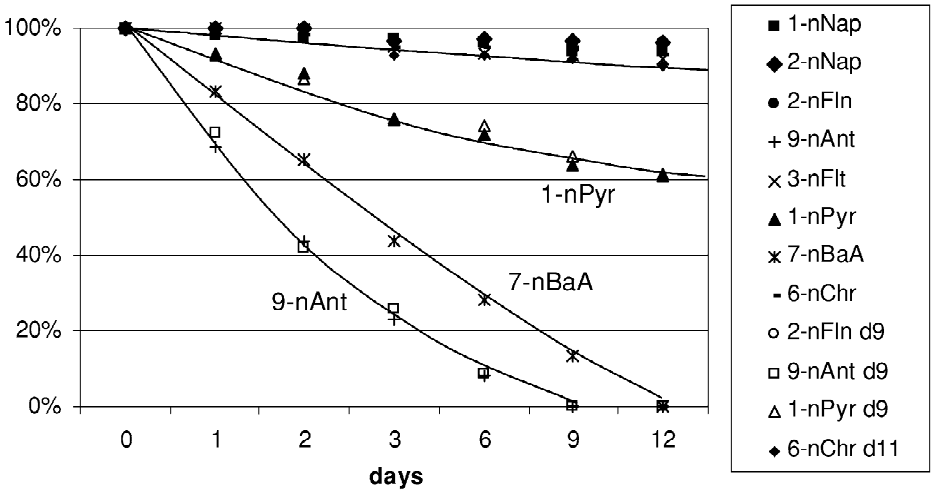


Fig. 3. Photostability of nitro-PAHs in toluene (conc. 10 ng/ml each); amber vial, exposed to daylight, average temperature 18 °C.

The breakdown of nitro-PAHs under experimental conditions could be roughly characterised by the first-order kinetic. Calculated rate constants are summarised in Table 2. However, the breakdown of nitro-PAHs is a complex process that might be accelerated by some organic compounds and even by other nitro-PAHs [19], so the breakdown rates in real samples could not be expected to be fully equal to values determined in pure solvent.

3.2. Volatilisation during sample handling

Evaporation of different solvents using rotary vacuum evaporator (RVO) and/or a gentle stream of nitrogen is an unavoidable operation realised during the sample preparation steps. Extent of potential losses of volatile analytes that could occur under particular experimental conditions should be specified in order to take relevant preventive measures.

Table 2
Rate constants (day^{-1}) for photodegradation of nitro-PAHs in toluene (for conditions and raw data see Figs. 1 and 2)

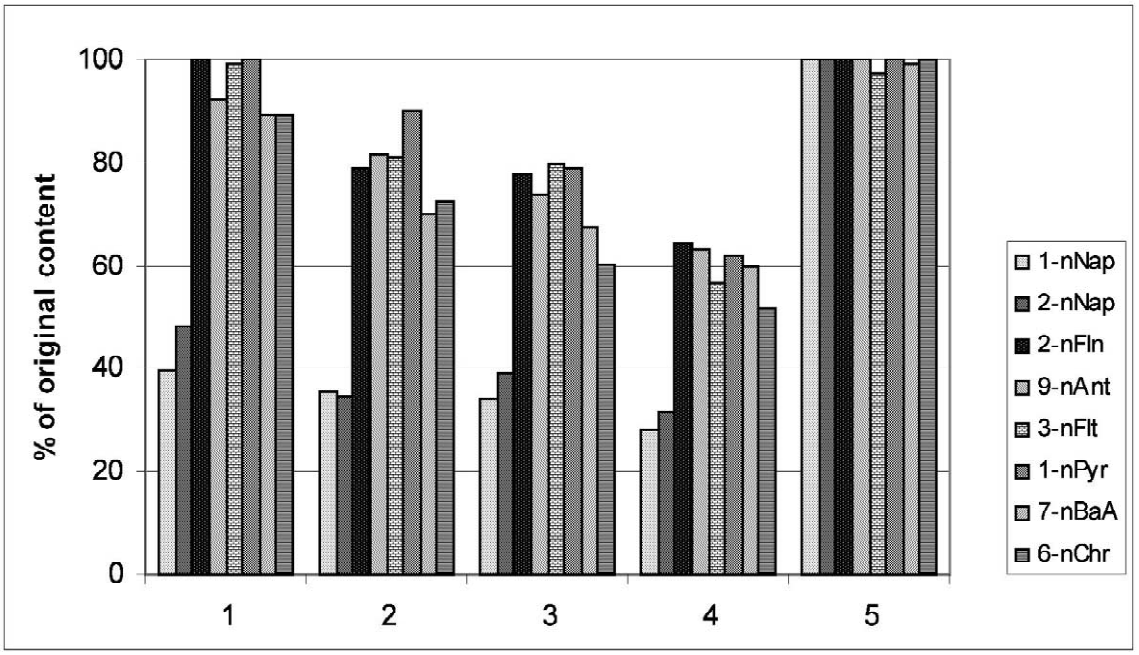
Nitro-pah	Transparent vial	Amber vial
1-nNap	2×10^{-2}	5×10^{-3}
2-nNap	2×10^{-2}	5×10^{-3}
2-nFln	4×10^{-2}	5×10^{-3}
9-nAnt	>4	0.4
3-nFlt	5×10^{-2}	1×10^{-2}
1-nPyr	1.2	4×10^{-2}
7-nBaA	>4	2×10^{-1}
6-nChr	3×10^{-1}	5×10^{-3}

Several procedures were applied for evaporation of nitro-PAHs dissolved either in chloroform or in hexane–acetone mixture (3:2, v/v) solvents used in the course of sample processing prior to GC analysis. Significant losses of target analytes were encountered (see Fig. 4) especially for low-molecular mass analytes such as nNaps. With the use of nitrogen stream, the losses were even higher (see Fig. 4) (nNaps were completely removed within 2 min blowing off the residual solvent to dryness). To prevent losses due to volatilisation, the addition of a ''keeper''-solvent with higher boiling point (e.g., toluene) is recommended before evaporation (see column 5 in Figs. 4 and 5). Some of the high-boiling coextracts (e.g., lipids) can also act as keepers during evaporation of real samples. The use of nitrogen for blowing off the residual solvent is not recommendable for the target analytes, even with the use of keeper.

3.3. Optimisation of individual analytical steps

3.3.1. Extraction

Hexane–acetone (3:2, v/v) solvent mixture used for extraction of nitro-PAHs from desiccated plant



Evaporation procedure

1 evaporation just to dryness

2 evaporation to dryness + 2min

3 evaporation to dryness + 5min

4 evaporation to dryness + 15min

5 100 μl of toluene (keeper) added, + evaporation next to dryness

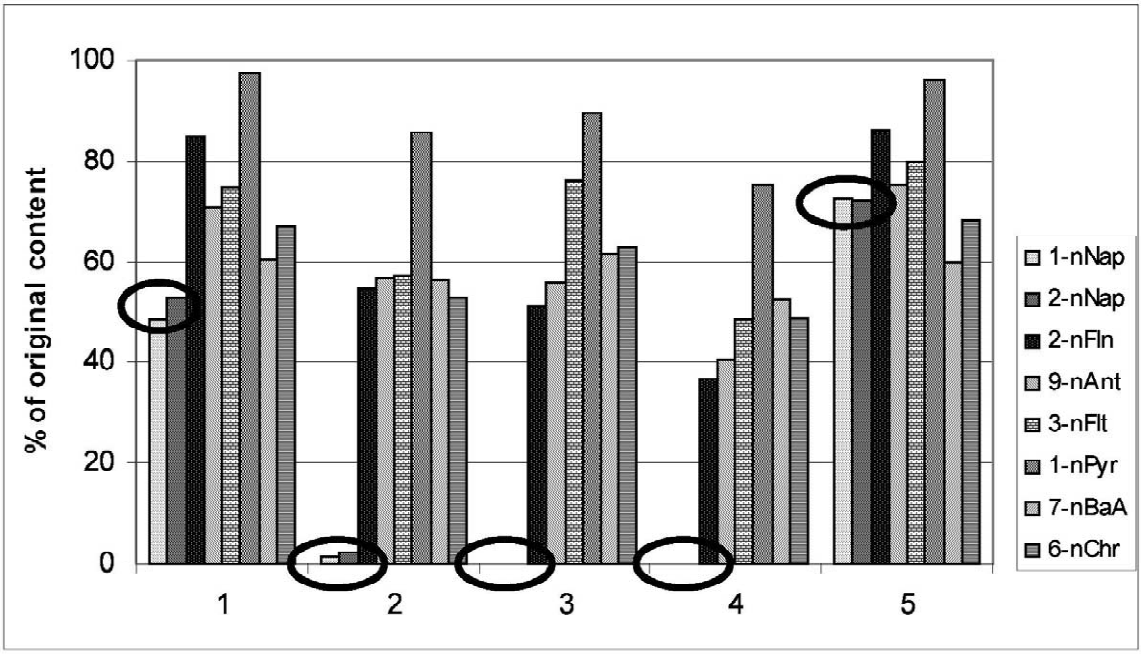
Fig. 4. Recoveries of nitro-PAHs under various conditions of RVO evaporation: 50 ml of hexane:acetone mixture (3:2, v/v) containing 5 ng of nitro-PAHs (each). Evaporation procedure (1) evaporation just to dryness; (2) evaporation to dryness + 2 min; (3) evaporation to dryness + 5 min; (4) evaporation to dryness + 15 min; (5) 100 μl of toluene (keeper) added + evaporation next to dryness.

matrices with low lipid content proved in our earlier experiments to be efficient also for the isolation of parent PAHs. Because of their relatively hydrophobic nature, nitro-PAHs are, similarly to PAHs, associated mainly with lipidic portion of biotic matrices. Intensive extraction procedure such as that employing Soxhlet apparatus enabled good penetration of solvent into fatty sample and consequently obtaining high, well repeatable recoveries.

In all types of extraction experiments, spiking of respective matrix by target analytes (labeled ones whenever possible) was the only, although not ideal, way for checking the recovery of this step. Unfortunately, relevant reference material with certified concentrations of target analytes is not available on the market at present.

3.3.2. Clean-up

Efficient removing of co-extracted matrix components represents a basic prerequisite for sufficiently accurate determination of ultra-trace concentrations of nitro-PAHs. Gel permeation chromatography was chosen as a general purification procedure enabling the separation of a major portion of plant pigments (chlorophylls, carotenoids, etc.) and/or lipids that are typically contained in crude extracts of biotic matrices. One of the limiting factors in determination of very low (sub ppb) levels of target analytes is the maximum amount of a sample that could be loaded onto the column used within a clean-up step. Although the use of high capacity GPC column (those with I.D. about 25 mm are typically used) is a conceivable solution to increase



Evaporation by nitrogen

1 evaporation just to dryness

2 evaporation to dryness + 2min

3 evaporation to dryness + 5min

4 evaporation to dryness + 15min

5 100 μl of toluene (keeper) added, + evaporation next to dryness

Fig. 5. Recoveries of nitro-PAHs during partial RVO evaporation and subsequent blowing off by a gentle stream of nitrogen: 50 ml of hexane–acetone mixture (3:2, v/v) containing 5 ng of nitro-PAHs (each). Evaporation by nitrogen: (1) evaporation just to dryness; (2) evaporation to dryness + 2 min; (3) evaporation to dryness + 5 min; (4) evaporation to dryness + 15 min; (5) 100 μl of toluene (keeper) added, + evaporation next to dryness.

sample capacity, we preferred employing of a relatively ''narrow-bore'' GPC column (with I.D. 8 mm only) for purification of extracts because of following reasons: (i) lowered ''reagent blanks'' thank to a relatively low volume analytes fraction (which may be a crucial problem in ultra-trace analysis); (ii) diminished evaporation time and hence a reduced risk of losses of analytes; (iii) decreased elution volumes and, consequently, reduced expenses related to solvents cost (including expenses needed for their storage and discarding); (iv) lower cost of GPC column.

Several types of GPC systems (either soft or rigid gel, mobile phases with different polarities) were tested. In Fig. 6 there is illustrated separation of nitro-PAHs from matrix components–green pigments (chlorophylls and their breakdown products) and plant waxes contained in parsley extract in system consisting of Bio-Beads S-X3 column and chloroform as a mobile phase. Similarly, separation of analytes from bulk fats contained in crude sausage extract could be obtained in this system. The maximum amount of lipids that could be separated was 300 mg.

It should be noted that under experimental conditions the elution of nitro-PAHs occurs earlier than that of PAHs (see Fig. 6). Therefore, more matrix coextracts are contained in fraction containing these target analytes.

Considering the workplace hazard posed by the

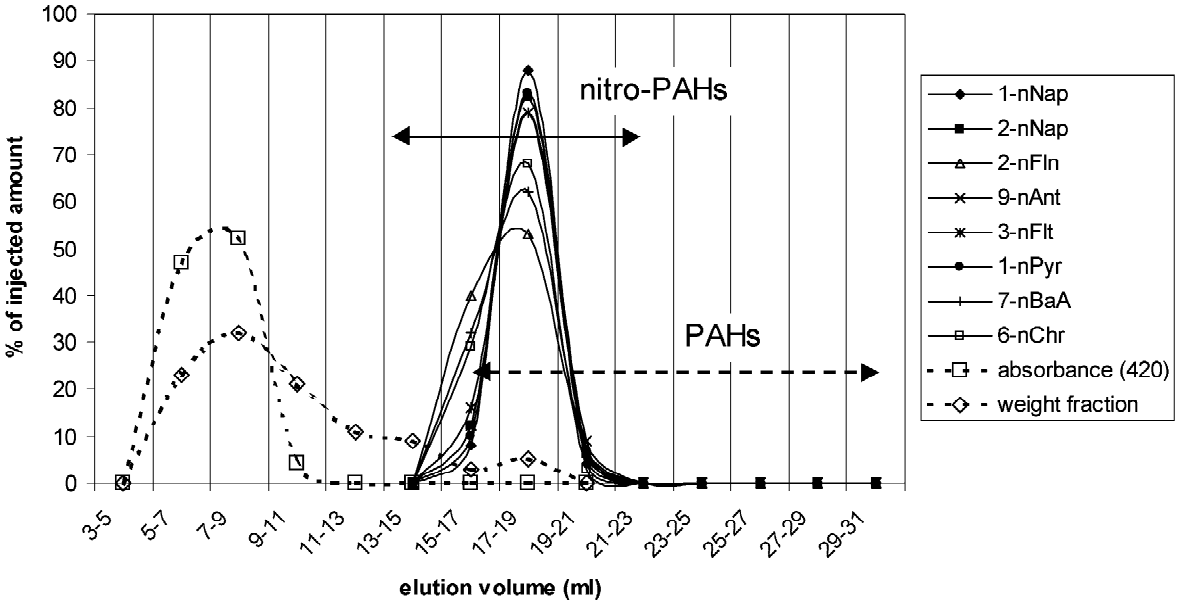


Fig. 6. GPC fractionation of spiked parsley extract on BioBeads S-X3, mobile-phase chloroform; 1 ml of injected crude extract was equivalent to 10 g of fresh matrix; elution of matrix components was monitored spectrophotometrically (absorbance at 420 nm corresponds mainly to chlorophylls); weight fractions were determined in separate experiment. Elution ranges corresponding to elution of particular group of analytes are shown by arrows.

chlorinated solvent, ethylacetate–cyclohexane (1:1, v/v) solvent mixture was examined as an alternative mobile phase in next experiments. Increased elution volumes of all the sample components (namely of PAHs) resulted from the use of this less polar mobile phase (lower polarity factor compared to chloroform) with the same BioBeads S-X3 column. The excessive elution volumes limit the use of this system in PAH/nitro-PAH analysis. In Fig. 7 there is shown the elution pattern of parsley extract (the same sample as that used for experiment shown in Fig. 6 was fractionated) obtained in system consisting of rigid ''high resolution'' Pl gel and ethylacetate–cyclohexane mobile phase. However, because of lower sample capacity (approximately 100 mg of lipids can be fractionated) of this kind of GPC column, its use is not suitable for purification of samples with higher fat content. On the other hand, better robustness of rigid gel separation compared to that realised by soft Bio-Beads S-X3 might favour its choice for purification of plant samples.

While sample fraction obtained by GPC can be, after transfer into suitable solvent, examined for PAHs content, determination of nitro-PAHs occurring in examined matrices typically at sub-ppb levels is not possible. Under GC–MS conditions identified as relevant to their quantitation, many matrix components are still contained in GPC fraction (see some tailing of residual matrix into fraction of analytes documented in Figs. 6 and 7) yielded the same ions as target analytes. High chemical noise obscured namely quantitation of nitro-PAHs eluted at higher retention times (see Fig. 8). Therefore, additional clean-up step had to be incorporated into our procedure. As a complementary strategy to GPC in terms of separation principle, adsorption chromatography was applied for this purpose. In the first phase, small silica columns (5 g of sorbent) were prepared for clean-up of GPC fraction. Although most of interfering compounds could be removed by this procedure (sample loaded in hexane; hexane–dichloromethane mixture used for elution) with good recoveries of analytes, this approach could not be used for routine analyses not only because of its labour intensive preparation of purification columns, but mainly due to rather poor repeatability of this

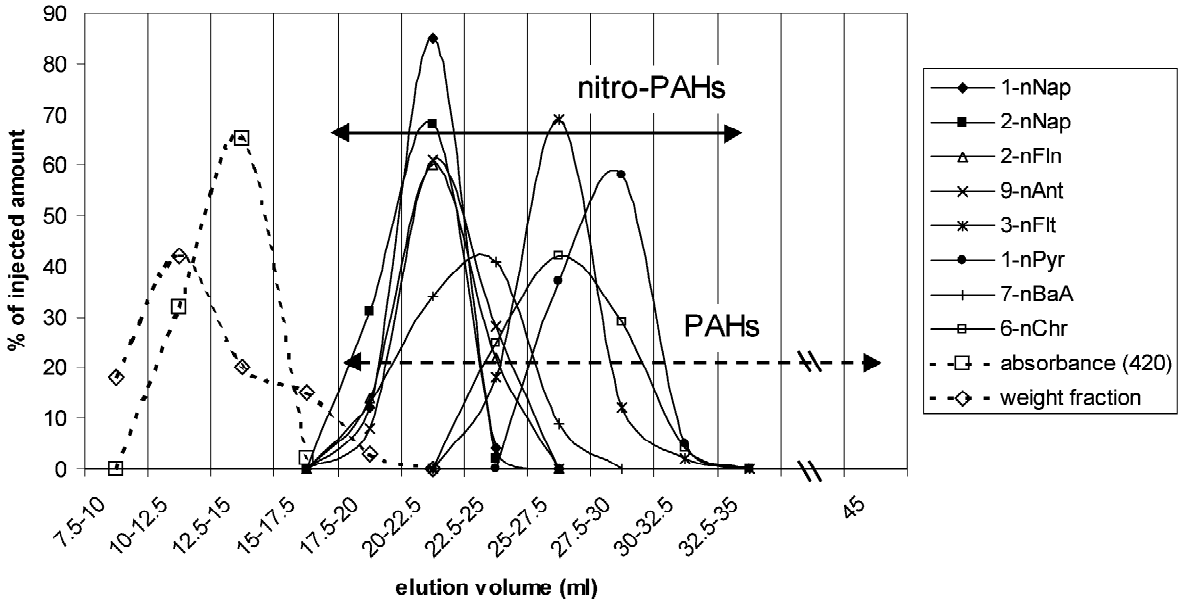


Fig. 7. GPC fractionation of spiked parsley extract on PL-gel, ethylacetate–cyclohexane (1:1, v/v) mobile phase; 1 ml of injected crude extract was equivalent to 10 g of fresh matrix; elution of matrix components was monitored spectrophotometrically (absorbance at 420 nm corresponds mainly to chlorophylls); weight fractions were determined in separate experiment. Elution ranges corresponding to elution of particular group of analytes are shown by arrows.

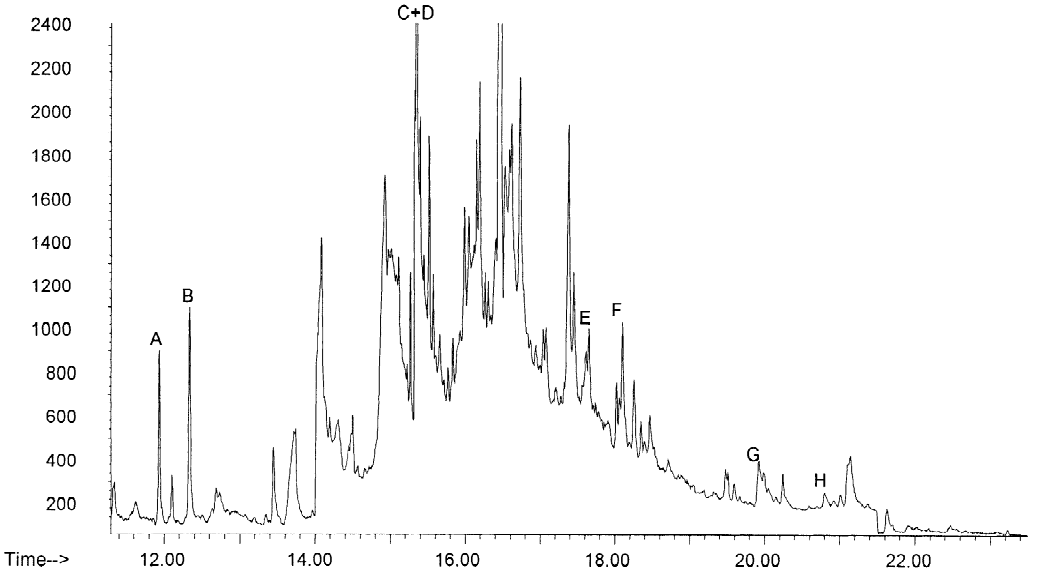


Fig. 8. GC–MS/NCI analysis of fresh parsley extract purified by GPC on PL gel; injected amount was equivalent to 75 mg of fresh matrix. Chromatogram in total ion count view (for ions see Section 2.4). (A) 1-nNap, (B) 2-nNap, (C) 2-nFln, (D) 9-nAnt, (E) 3-nFlt, (F) 1-nPyr, (G) 7-nBaA, (H) 6-nChr.

step (changing activity of silica in dependence on moisture) that would contribute to the increase of the combined uncertainty of the whole procedure.

Based on a large series of experiments in which several combinations of SPE cartridges/solvents were tested, CN–silica cartridges (500 mg) were identified as the most suitable tool enabling efficient clean-up of plant extracts (see Fig. 9). While residual waxes and pigments contained in GPC fraction are trapped on this sorbent (sample loaded in hexane), complete elution of (not retained) nitro-PAHs can be accomplished by hexane. For removing of residual lipids from GPC fraction obtained by purification of fatty samples, LiChrolut EN (200 mg) cartridges were found as the most suitable. In this particular case, nitro-PAHs are efficiently adsorbed from hexane solution loaded onto the cartridge, while lipids possessing low affinity to this sorbent are not retained. Acetone or dichloromethane is then used for eluting the analytes from cartridge. The uncertainty of this step was relatively low (high and repeatable recoveries) provided that automated handling of SPE cartridges is carried out.

3.3.3. Identification/quantification

High-resolution gas chromatographic separation combined with mass spectrometric detection represents obviously the method of choice for identification/quantification of nitro-PAHs. However, using a low resolution MSD operated in electron impact ionisation mode, we failed to detect ''natural'' (sub ppb) levels of nitro-PAHs in sample extracts prepared in the way described in Section 2.4.

Fortunately, significantly better detection sensitivity was obtained by negative chemical ionisation MS technique. On the other hand, in spite of improved detectability, some other problems (mainly those connected with GC separation) had to be solved until relevant performance characteristics were achieved.

In the first set of experiments DB-5MS capillary column (60 m×0.25 mm×0.25 μm) commonly used for separation of complex mixtures of organic contaminants was used to get efficient resolution of target analytes from matrix components. However, due to a relatively low volatility of nitro-PAHs with more than three aromatic rings, their residence time on the column was too long and some band broaden-

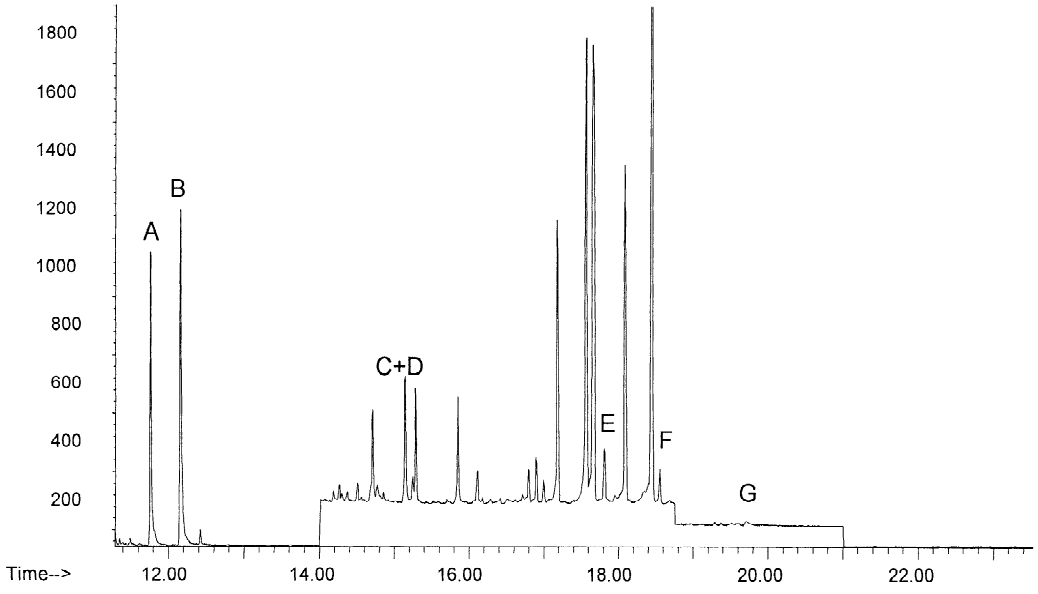


Fig. 9. GC–MS/NCI analysis of fresh parsley extract purified by GPC on PL-gel in the first step, followed by SPE (CN silica cartridge) in the next one; injected amount of sample was equivalent to 75 mg of fresh matrix. Chromatogram in total ion count view (for ions see Section 2.4). (A) 1-nNap, (B) 2-nNap, (C) 2-nFln, (D) 9-nAnt, (E) 3-nFlt, (F) 1-nPyr, (G) 7-nBaA, (H) 6-nChr.

Table 3
Limits of detection (pg per injection, $S/N=3$) of nitro-PAHs determined in various GC systems (1 μl of standard solution in toluene injected): parameters of GC systems are specified in Section 2.4.3

Nitro-PAH	EI	NCI	
	GC System 1 DB-5MS	GC System 1 DB-5MS	GC System 2 DB-XLB
1-nNap	3.0	0.2	0.1
2-nNap	3.0	0.2	0.1
2-nFln	5.5	0.4	0.3
9-nAnt	7.5	0.7	0.3
3-nFlt	10.0	1.0	0.4
1-nPyr	9.0	1.0	0.4
7-nBaA	7.0	2.8	0.3
6-nChr	15.0	4.0	0.3

ing in time (i.e., decreasing the height of peaks) could be observed for the late eluting analytes. In addition, contrary to relatively inert PAHs, some tailing that can be attributed to the interaction of polar nitro group with active sites in GC system could be observed.

Considering column dimensions, i.e., parameters influencing efficiency/resolution and taking into account the demand for long-term thermal stability of separation system, a new type of capillary, DB-XLB (30 m×0.25 mm×0.1 μm) was chosen for optimisation of GC run. Half-length of column together with a thinner film of stationary phase (a proprietary second-generation arylene technology) allowed under faster temperature programming not only a significant reduction of analysis time but also a remarkable improvement of detectability namely for later eluting analytes. This was mainly due to increased signal to noise ratio of narrowed peaks. Although LODs obtained in this way were relatively low, they were still insufficient for quantification of common real-life levels of nitro-PAHs in food samples. It was decided that for further enhancement of analytes signal, MS detection parameters should be appropriately adjusted. Lowered LODs shown in Table 3 were attained by setting electron multiplier voltage (EMV) by 500 V above the value obtained by autotune procedure. Significantly improved signal to noise ratio resulted from the change of this parameter.

3.3.4. Calibration

Phenomena known as ‘‘matrix-induced detector response enhancement’’ were obviously responsible for recoveries largely exceeding 100% that were determined in spiked samples when standards in pure solvent were used for calibration. As shown in Fig. 10, significantly higher peaks were obtained for 2-nNap injected in sample extract in which some matrix components are unavoidably left even after thorough two-step purification. While these co-extracts protect analytes during injection and enhance their transfer onto separation column, adsorption (sometimes even breakdown) on active sites present in injector occurs when analytes are injected only in

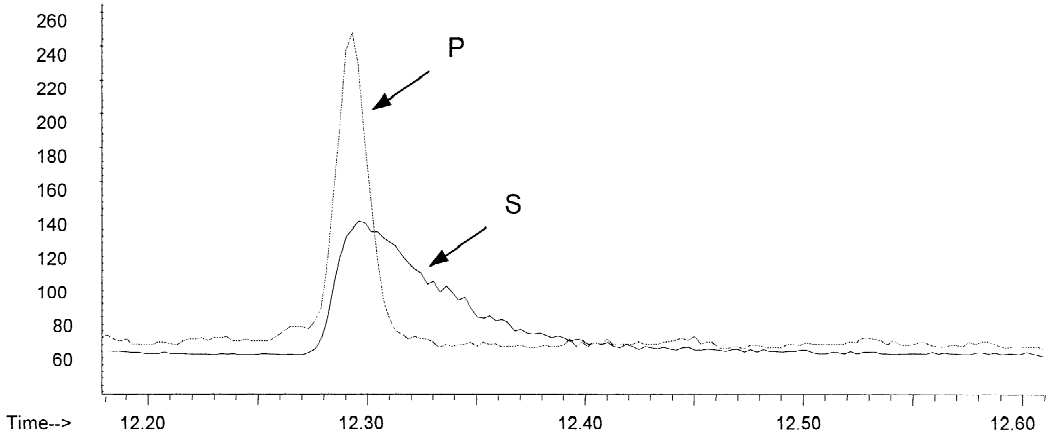


Fig. 10. Illustration of matrix-induced enhancement response effect: 1 pg of 2-nNap injected in pure solvent (S) and in purified sample of pumpkin seed oil (P). Concentration of 2-nNap in oil was equivalent to 1 μg/kg. Chromatogram shows extracted m/z 173.

pure solvent. Generally, the use of deuterated nitro-PAH analogues represents the best quantitation strategy leading to best attainable accuracy of results. Unfortunately, for nNaps, contaminants that were shown to be most abundant in examined samples, no labeled standards are commercially available. Although preparation of matrix-matched standards is a theoretical alternative, in particular case it was not practicable, as the spectrum of analysed matrices is quite wide and each of them may contain traces of nitro-PAHs. Therefore, the compensation for this kind of matrix effect had to be carried out by 2-methyl-1-nitronaphthalene (2-Me-1-nNap) that is eluted from DB-XLB column between target nNaps. Unfortunately, this internal standard could not be used for the whole analytical procedure as it has different behaviour during clean-up. As shown in Fig. 11, monitoring of M^- ions enabled a selective detection of both target analytes and their deuterated analogues.

It should be also noted that the use of soft ionisation technique, in particular case represented by NCI, does not provide unambiguous confirmation of analyte identity because of a lack of more intensive fragmentation ions in mass spectrum. Commonly, retention time might be conceived as relevant supplementary information for identification purpose. However, poor repeatability of retention times namely for nitronaphthalenes was observed. In Fig. 12 there are illustrated shifting retention times that were obtained in two consecutive injections of parsley samples. On the other hand, no change of their values occurred for standard mixture. According to these reasons retention times related to respective internal standard (2-Me-1-nNap or deuterated analogues) are more practical.

While the record shown in Fig. 12 was obtained in relatively clean chromatographic system (new injection liner and separation capillary), severe problems were encountered for later injected samples within long sequences.

Severe tailing of analytes and, consequently, their impaired detectability was encountered after repeated injections of matrix samples. As shown in Fig. 13, the peak of 2-nNap completely disappeared in noise after 200 injections. Minimising of the amount of co-injected matrix together with regular cleaning of the inlet part of GC system are obviously important prerequisites for a long-term stability of required performance characteristics. Some improvement of such situation could also be obtained by rinsing the column with solvent.

Similarly, contamination of NCI ion source has to be kept under control—its periodical disassembly and cleaning should always be a part of QA/QC program.

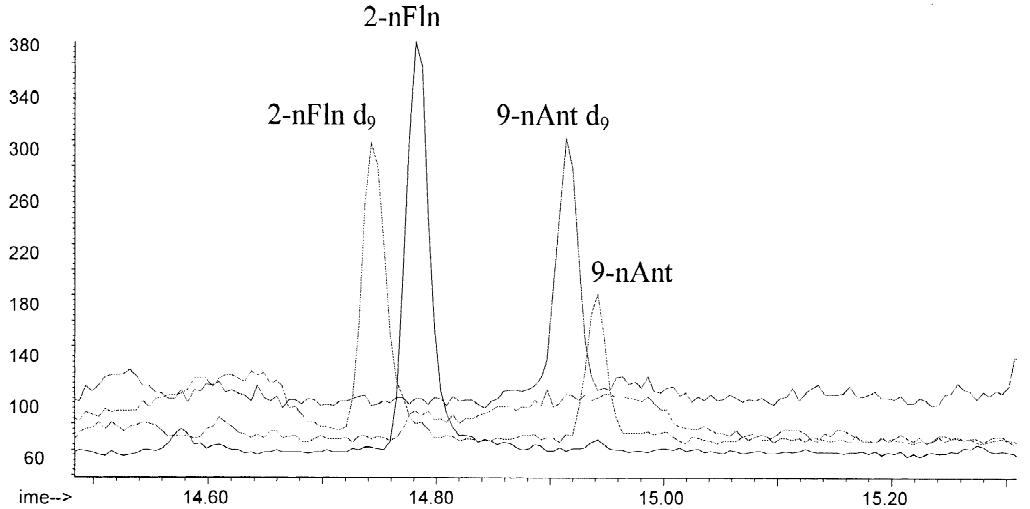


Fig. 11. The use of deuterated nitro-PAHs as internal standards in purified extract of freeze-dried diet sample. Concentrations of analytes were equivalent to 0.1–0.2 μg/kg. Chromatogram shows extracted m/z 211, 220, 223 and 232.

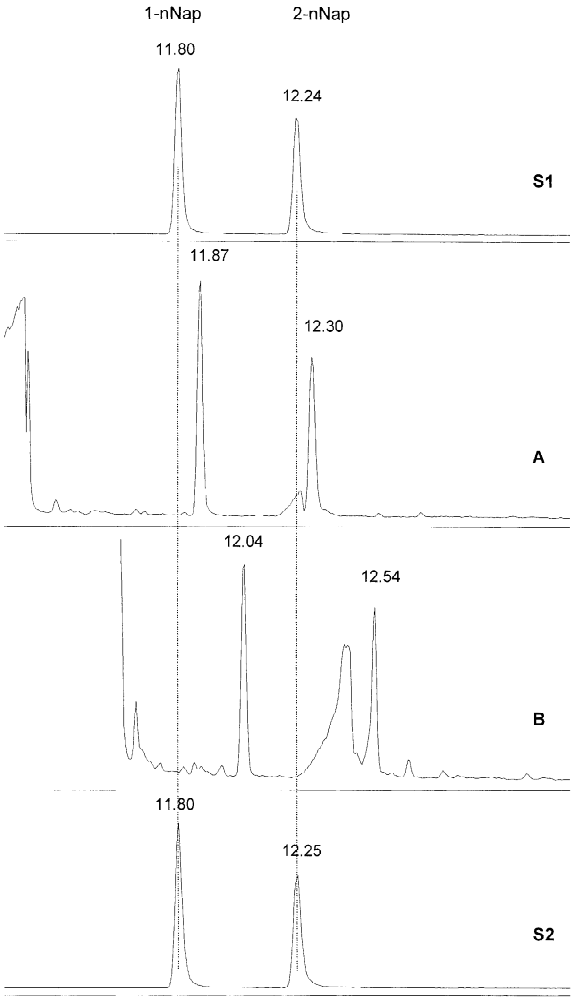


Fig. 12. Illustration of retention time shift of 1- and 2-nNap within injection sequence: (1) standard solution (S1); (2) parsley extract (A); (3) parsley extract (B); (4) standard solution (S2).

3.4. Validation

Performance characteristics of developed method have been determined for several types of matrices through their repeated analyses; the results are summarised in Tables 4–7.

Limits of detection (Table 4) were determined as the concentration of analyte in real sample eliciting the signal 3 times higher than the corresponding baseline noise level ($S/N=3$).

Repeatability is expressed as a relative standard deviation (RSD) calculated for the whole procedure. Relative standard deviations of nitro-PAHs analysis of diet samples at ''natural'' concentrations, i.e., below 0.1 μg/kg (see Table 5), indicate rather poor repeatability compared with ''classic'' general model of precision (Horwitz function) [20] and these results can be regarded as semi-quantitative only. On the other hand, determination of the same levels of nitro-PAHs in parsley can be carried out with a relatively satisfactory precision. This is because of the relatively low lipid content in this matrix, what allows loading of higher aliquots of sample onto GPC column in purification step.

Limit of quantitation (LOQ) for diet samples and plant materials (represented in particular case by parsley) can be estimated at about 1.0 and 0.3 μg/kg, respectively. It should be noted that concentrations of nitro-PAHs in most of diet samples are well below this LOQ. Hence samples spiked with nitro-PAHs standards were used to determine precision (repeatability) of developed procedure at concentration levels well above LOQ (see Table 6).

Average recoveries of analytical procedure at ppb levels were tested using spiked samples (see Table 7). Considering the repeatability of results as well as the uncertainty of spiking procedure, recoveries about 70% were recorded for nitro-PAHs in diet samples—but mostly at concentration levels exceeding those found in real samples. On the other hand, satisfactory recoveries (70–85%) were obtained for parsley samples at spiking levels 0.5–1 μg/kg. Unavoidably the uncertainties of nitro-PAHs measurements at levels typical for real-life samples are significantly higher than the values estimated for their parent compounds.

In Table 8 some characteristic features of PAHs and nitro-PAHs analyses are shown for comparison.

4. Conclusions

Determination of nitro-PAHs in biotic samples by common techniques available in routine laboratories concerned with analysis of food contaminants might pose an extremely complicated task because of typically sub-ppb levels of target analytes. Matrix-dependent sample preparation approach has to be applied. Multiple clean-up procedure is necessary to achieve adequate detection parameters of GC–MS/

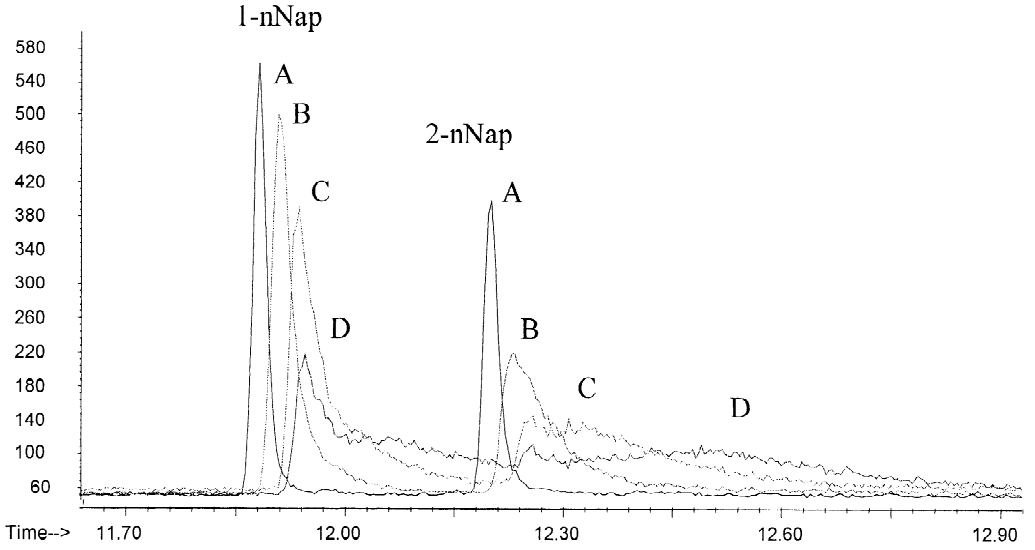


Fig. 13. Worsening of peak shapes during a long-term use of a chromatographic column when analysing matrix samples with high content of coextracts. Chromatogram shows extracted m/z 173. (A) Clean system, (B) after 50 injections, (C) after 100 injections, (D) after 200 injections.

NCI analysis. To attain reasonable accuracy of measurement, several critical aspects have to be taken into account and respective measures adopted:

- Losses of analytes due to photodegradation and/or volatilisation that may occur during sample handling prior to GC analysis have to be prevented
- Successive shift of GC retention times of analytes during repeated injections of real samples may be encountered while no distinct changes occur for analytes injected in pure solvent. Since typically only one abundant ion is available in MS/NCI spectrum (hence confirmation based on spectral information is limited), standard addition is needed for unambiguous confirmation of peak identity.
- Manual setting of optimal MSD parameters is needed to attain sufficient detection sensitivity.
- Matrix-induced detector response enhancement may lead to severe overestimation of results as far as standards in pure solvent are used for external calibration. Improved accuracy of measurements can be obtained by the use of isotope dilution technique. As long as deuterated standards are not

Table 4
Limits of detection (μg/kg, $S/N=3$) of nitro-PAHs in examined matrices attainable in GC System 2 under optimal conditions, i.e., clean injector liner and new separation capillary

Nitro-PAH	Mate tea	Pumpkin oil	Parsley	Freeze-dried diet
1-nNap	0.01	0.2	0.05	0.05
2-nNap	0.01	0.2	0.05	0.05
2-nFln	0.02	0.4	0.05	0.05
9-nAnt	0.1	0.3	0.05	0.1
3-nFlt	0.05	0.4	0.1	0.1
1-nPyr	0.07	0.4	0.1	0.1
7-nBaA	0.07	n.d.	0.05	0.1
6-nChr	0.04	n.d.	0.1	0.1

n.d., not determined.

Table 5
Repeatability of nitro-PAHs analysis in ''real'' samples

Nitro-PAH	Freeze-dried diet (μg/kg)[a]	RSD (%)	Parsley (μg/kg)[b]	RSD (%)
1-nNap	0.05[c]	87	0.12	19
2-nNap	0.05[c]	68	0.11	20
2-nFln	0.05[c]	75	0.05	38
1-nPyr	0.1[c]	52	–	–
9-nAnt	–	–	0.07	32
3-nFlt	–	–	0.1	14

[a] Mean value calculated from eight replicate samples.
[b] Mean value calculated from five replicate samples.
[c] Values at LOD.

Table 6
Repeatability of nitro-PAHs analysis, spiked samples with original levels of analytes below LOD (spiking level 0.5–1 μg/kg)

Nitro-PAH	Measured values			Theoretical values RSD (%)[a] "Horwitz"
	Mate tea RSD (%) ($n=4$)	Parsley RSD (%) ($n=3$)	Freeze-dried diet RSD (%) ($n=10$)	
1-nNap	14	12	14	32
2-nNap	12	15	15	32
2-nFln	2	2	16	32
9-nAnt	23	2	17	32
3-nFlt	6	7	16	32
1-nPyr	5	10	25	32
7-nBaA	15	2	14	32
6-nChr	12	2	23	32

[a] Repeatability was estimated from the Horwitz function [20] as the one-half of the reproducibility.

Table 7
Recoveries of nitro-PAHs analysis, spiked samples with original levels of analytes below LOD (spiking level 0.5–1 μg/kg)

Nitro-PAH	Recovery (%)		
	Mate tea	Parsley	Freeze-dried diet
1-nNap	63	75	61
2-nNap	64	79	64
2-nFln	60	78	81
9-nAnt	64	77	72
3-nFlt	56	70	71
1-nPyr	69	76	71
7-nBaA	73	85	80
6-nChr	65	74	76

available, the use of internal standard possessing similar structure to that of analytes may provide compensation of injection problems. The use of matrix-matched standards is not a conceivable solution.

Table 8
Comparison of characteristics of analyses of PAHs and nitro-PAHs in food

Group of compounds	Typical levels occurring in food (μg/kg)	Typical recoveries of analytes (%)	Typical uncertainties of results expressed as RSD (%)
PAHs	10^{1}–10^{2}	70–95	10^{0}–10^{1}
nitro-PAHs	$<10^{-2}$–10^{0}	55–85	10^{1}–10^{2}

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