ELSEVIER

Journal of Chromatography A, 855 (1999) 501–514

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Separation and identification of polar degradation products of benzo[*a*]pyrene with ozone by atmospheric pressure chemical ionization–mass spectrometry after optimized column chromatographic clean-up

T. Letzel[a], E. Rosenberg[b], R. Wissiack[b], M. Grasserbauer[b], R. Niessner[a,*]

[a]*Institute of Hydrochemistry, Technical University of Munich, Marchioninistrasse 17, D-81377 Munich, Germany*
[b]*Institute of Analytical Chemistry, Vienna University of Technology, Getreidemarkt 9, A-1060 Vienna, Austria*

Received 11 March 1999; received in revised form 4 June 1999; accepted 4 June 1999

Abstract

The environmental relevance of oxidized degradation products of polycyclic aromatic hydrocarbons (PAHs) increases due to enhanced combustion of organic matter and fossil fuels. For PAHs consisting of more than three condensed aromatic rings, soot aerosols are the main carrier, on the surface of which they can react with trace gases like ozone. In this study the clean-up procedure and analysis of ozonized benzo[*a*]pyrene (B[a]P) was optimized. B[a]P and its degradation products were preseparated into three fractions. Different reversed-phase materials were evaluated for high-performance liquid chromatographic separation. Among these, a phenyl-modified silica material proved best-suited and the chromatographic separation was optimized on this material. For the detection of separated degradation products, liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) was used. With this method, 29 components could be characterized. Besides the three known main degradation products (B[a]P-1,6-dione, B[a]P-3,6-dione, B[a]P-6,12-dione, B[a]P-4,5-dione and 4-oxa-benzo[*d,e,f*]chrysene-5-one (B[def]C-lactone), were identified for the first time with the help of reference substances. B[def]C-lactone is known as a substance with a mutagenic potential similar to B[a]P. Several other compounds could be tentatively identified.

Keywords: Benzo[*a*]pyrene; Polynuclear aromatic aromatics; Ozone

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are an ubiquitous class of chemicals originating from incomplete combustion of organic matter and fossil fuels. The emissions are mostly anthropogenic, e.g. from diesel engines [1], domestic heating [2] and other sources (like tobacco smoke [3]). The primary compartment of PAHs is the troposphere, where soot, mainly, acts as a carrier for PAHs consisting of more than three condensed aromatic rings. During their transport in the troposphere, the PAHs are exposed to reactive trace gases like ozone and NO_x or may undergo photolysis by UV-radiation [4]. Knowledge of the degradation pathways of PAHs and of their oxidized derivatives in the atmosphere is important, because fine aerosols are easily deposited in the

*Corresponding author. Tel.: +49-89-7095-7981; fax: +49-89-7095-7999.

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00716-5

alveolar region of the lung [5]. There, the oxidized PAH derivatives show a higher resorption potential than unreacted PAHs because hydrophilic molecules are more easily resorbed in the alveolus [6] than hydrophobic molecules, which remain on the surface of the alveolus.

Many PAHs have to be considered carcinogenic since they give a positive response to carcinogen tests like the Ames test [7,8] and exhibit high DNA binding probabilities [9]. A prominent example is benzo[*a*]pyrene (B[a]P), a PAH consisting of five condensed aromatic rings. Several studies investigated the mutagenic effect of DNA adducts with B[a]P metabolites [10,11] and the mutagenicity was corroborated with human cells [12]. In a recent investigation, it was shown that the metabolic pathway of B[a]P in the body has several possibilities [13]. Besides oxidation to B[a]P-7,8-dihydrodiol-9,10-epoxide, the so-called *ultimate carcinogen*, there are other biochemical pathways with B[a]P-diones. The primary metabolites 1,6-, 3,6- and 6,12-B[a]P-dione are well known as the main products of B[a]P degradation in the atmosphere by reaction with oxidative gases [14] or photolysis [15]. The hygienic relevance of B[a]P and the fact that B[a]P is preferably bound to particles [16] and occurs in the atmosphere in comparatively high concentrations, up to 6.5 ng/m^3 [17], are the reasons why B[a]P was chosen as a representative PAH in this study.

B[a]P-diones were first identified as B[a]P degradation products by thin-layer chromatography and chemical ionization–mass spectrometry (CI–MS) [14]. Later, other B[a]P oxidation products were identified by gas chromatography with mass spectrometric detection (GC–MS) [18,19]. For the separation of thermally labile or polar trace substances, however, high-performance liquid chromatography (HPLC) is a more suitable technique, in which normal-phase (NP) as well as reversed-phase (RP) column materials can be applied.

In the past, HPLC separation of oxidized PAHs has been performed on octadecyl (C_{18}) modified silica RP material, but large product fractions could not be separated [20]. This work presents a comprehensive optimization of the column chromatographic clean-up of PAH metabolites on a NP column, followed by RP-HPLC. Different stationary phases [21] [octyl- (C_8), ethyl- (C_2), cyanoalkyl-(CN) or phenyl (Ph)-modified silica] have been evaluated to determine the optimum separation conditions. The isolated chromatographic peaks were identified by MS with an atmospheric pressure chemical ionization (APCI) interface, which turned out to be the most sensitive detection method for these B[a]P degradation products [20] known to date. The application of a new generation LC–APCI–MS system allowed controlled ion fragmentation, to support the identification of unknown substances.

2. Experimental

2.1. Chemicals

Analytical-grade dichloromethane (DCM), *n*-hexane, methanol (MeOH) and toluene were obtained from Merck (Darmstadt, Germany). High-purity water was taken from a Milli-Q water system (Millipore, Eschborn, Germany). The reference substances B[a]P-*cis*-4,5-dihydrodiol (B[a]P-4,5-dihydrodiol), B[a]P-*cis*-7,8-dihydrodiol (B[a]P-7,8-dihydrodiol), B[a]P-r-7,t-8-dihydrodiol-t-9,10-epoxide (B[a]P-diolepoxide), B[a]P-1,6-dione, B[a]P-3,6-dione, B[a]P-4,5-dione, B[a]P-6,12-dione, B[a]P-7,10-dione, 4-hydroxy-B[a]P (B[a]P-4-ol), 8-hydroxy-B[a]P (B[a]P-8-ol) and B[a]P-r-7,t-8,c-9,c-10-tetrahydrotetrol (B[a]P-tetrol) were purchased from the Midwest Research Institute (Kansas City, KS, USA). 3-Hydroxy-B[a]P (B[a]P-3-ol) and 4-oxa-benzo[*d,e,f*]chrysene-5-one (B[def]C-lactone) were obtained from the PAH-Institute Dr. Schmidt (Greifenberg, Germany). Benzanthrone was supplied by Aldrich (Milwaukee, WI, USA) and pure B[a]P (>98%) by Sigma (St. Louis, MO, USA). Ozone was generated by photodissociation of gaseous oxygen (99.95%, Messer Griesheim, Krefeld, Germany) using UV-radiation from a Hg-line source [22].

2.2. Sample generation and preparation

Kiln-fired glass fiber filters (∅, 3.7 cm, GF/C Whatman, Springfield Mill, UK) were covered with 1 mg of B[a]P dissolved in *n*-hexane (1 mg B[a]P/ml hexane) and evaporated to dryness with nitrogen. Subsequently, the filters were exposed in a filter-

holder to oxygen containing 2 ppm O_3 for 1 h. Before exposure, and after 30 min, the filters were moistened with water (t_1=0 min; t_2=30 min). After ozonization, the filters were extracted for 15 min in an ultrasonic bath (Sonorex, Berlin, Germany) with 3 ml of DCM–MeOH–toluene (1:1:1, v/v/v). The filters were removed and the extract was evaporated to dryness in a nitrogen stream. The residue was dissolved in 1 ml of toluene (complete dissolution of all compounds) and the solution was applied to a silica column (length, 120 mm; ∅, 5 mm; filled with 0.8 g of silica (J.T. Baker, Deventer, The Netherlands). The elution was carried out either with 5 ml each of toluene, DCM and MeOH, as described previously [18], or, as a result of the optimization procedure, with 5 ml of toluene, 15 ml of DCM and 5 ml of MeOH. In the previously published method, the MeOH fraction was used for measurements. In the optimized method, both the DCM fraction and the MeOH fraction were used for HPLC separation.

2.3. HPLC-instrumentation and columns

The chromatographic system employed for performance tests of the different columns and for the development of the separation methods was from Shimadzu (Kyoto, Japan) and was equipped with two HPLC pumps (LC6A), a column oven (CTO-10A) set at 26°C, a controller (SCL-6A), a diode array UV–Vis detector (SPD-M6A) and an injector from Rheodyne (Cotati, USA) with a 20-μl loop.

The apparatus used for liquid chromatographic separation with mass spectrometric detection of the PAH metabolites was a HP series 1100 HPLC–MS system (Hewlett-Packard, Waldbronn, Germany and Palo Alto, CA, USA) consisting of a HP G1311A quaternary pump, a HP G1316A column thermostated at 26°C, a HP G1322A degasser unit, a HP G1313A autosampler and a HP G1315A diode array UV–Vis detector coupled in series with a HP G1946A mass-selective detector, equipped with a G1947A APCI interface.

In both systems, diode array detection was performed at 254 nm (4 nm bandwidth). The parameters for the APCI interface were optimized as described elsewhere [23]. The measurements were performed in the positive ionization mode with a 90, 120, 160 or 190 V fragmentor voltage, a 450°C vaporizer temperature, a 350°C drying gas temperature, a 5-l/min drying gas flow, a 50 p.s.i.g. drying gas pressure, a 6-μA corona current and a 4000-V capillary voltage.

Using methanol–water as the mobile phase, the chromatographic separation was optimized stepwise, starting with isocratic elution and developing different gradient programs. The separation conditions are given with the corresponding chromatograms.

In this study, five analytical columns with different stationary phases (CS, Langerwehe, Germany) were evaluated; their characteristics are given in Table 1.

3. Results and discussion

3.1. Optimization of the clean-up step

The reaction of moistened B[a]P deposited on filters in a filter-holder with ozone mimics the atmospheric degradation of B[a]P in the dark at high humidity. The moistening with water gave a higher yield of radical reactions caused by OH radicals compared to the reaction in the absence of water [24]. In all experiments, a large fraction of B[a]P remained unreacted on the filters. After extraction of the filters, the extract is transferred to a silica clean-up column. The elution from the silica column with toluene separates the non reacted B[a]P from the mixture of oxygenated compounds that remains on the column. The subsequent elution with DCM leads to the clearly visible formation of two separate orange bands in the column. These bands are caused by the resolution of the different B[a]P-diones on the clean-up column. This is a significant improvement over the clean-up procedure described in the literature (elution with DCM and MeOH), in which the two substance bands appear as one and coelute completely in the ‘MeOH fraction’ [18]. Using the optimized method, the first orange band is eluted with DCM, while the second band is still retained on the silica column. The elution of this and all further compounds is achieved using MeOH. A third group of components was retained in a pink band at the top of the column; it was only recovered in low yields and thus could not be identified.

Fig. 1 shows chromatograms of the different

Table 1
Characterization of the column materials evaluated for chromatographic separation of the oxidized degradation products of B[a]P

Column type	Nucleosil 120-3C_{18}	Nucleosil 120-3C_8	Multospher 120 RP2-3	Nucleosil 120-7CN	Nucleosil 120-7C6H5
Length (mm)	125[a]	125[a]	125	250	250
Inner diameter spherical (mm)	4	4	4	4	4
Stationary phase	Octadecyl -$(CH_2)_{17}$–CH_3	Octyl -$(CH_2)_7$–CH_3	Ethyl -CH_2–CH_3	Cyanopropyl -$(CH_2)_3$–CN	Phenyl -$(CH_2)_3$–C_6H_6
Particle diameter (μm)	3; spherical	3; spherical	3; spherical	7; spherical	7; spherical
Pore volume (ml/g)	0.65	0.65	0.70	0.65	0.65
Surface (m^2/g)	200	200	185	200	200
Endcapped	No	No	No	No	No

[a] With pre-column (same material, 17×4 mm).

fractions obtained by either the previously published (Fig. 1a) or by the optimized (Fig. 1b and c) clean-up procedure. The DCM fraction contains the less polar substances, the degradation product BaP-6,12-dione (peak no. 2) and apolar unreacted B[a]P (peak no. 1). The methanol fraction contains the more polar substances and the two degradation products B[a]P-3,6-dione (peak no. 3) and B[a]P-1,6-dione (peak no. 4). This efficient pre-separation on a silica pre-column reduces the complexity of the obtained fractions in the subsequent HPLC analysis. All samples investigated in this study have been processed using this optimized clean-up procedure.

3.2. Development and optimization of the liquid chromatographic separation

Previously described separations of the oxidized degradation products of B[a]P made use of HPLC columns with octadecyl-silica stationary phases [18,25,26]. These methods proved to be useful for the baseline separation of the main degradation products B[a]P-1,6-dione, B[a]P-3,6-dione and B[a]P-6,12-dione [18]. However, they fail to separate the more polar degradation products. Since no further improvement in the chromatographic separation could be reached by variation of the solvent gradient, different stationary phase materials were evaluated.

With a C_8 stationary phase, better separation of the less polar compounds contained in the DCM fraction was achieved under isocratic conditions (Fig. 1b), while more polar compounds of the MeOH fraction (Fig. 1c) showed a similar elution behavior as on the C_{18} column. Application of a solvent gradient with a higher water content at the beginning of the run did not lead to an improvement.

With a C_2-silane modified silica stationary phase material, the chromatographic separation deteriorated (Fig. 2a).

Since the variation of the chain length of common alkylsilane stationary phases did not improve the separation, cyanoalkylsilane- and phenylsilane-modified silica phases were tested. These materials are semi-polar and can be used in either normal- or reversed-phase mode. With the CN-phase, the separation could be improved (Fig. 2b), but no suitable solvent gradient for a satisfactory separation within an acceptable time was found.

The best results were obtained with the phenylsilane stationary phase. Under isocratic conditions, B[a]P-diones elute 2 min later than on the CN column, and the polar substances could be separated to some extent (Fig. 2c). An optimized methanol–water solvent gradient was developed, resulting in a satisfactory separation for both the DCM and the MeOH clean-up fractions. The optimized chromatogram for the MeOH fraction is shown in Fig. 3. For the DCM fraction, a similar improvement in the separation was obtained; for conciseness, however, only the chromatograms of the MeOH fraction are shown. Thus, all further experiments were performed with the phenylsilane column.

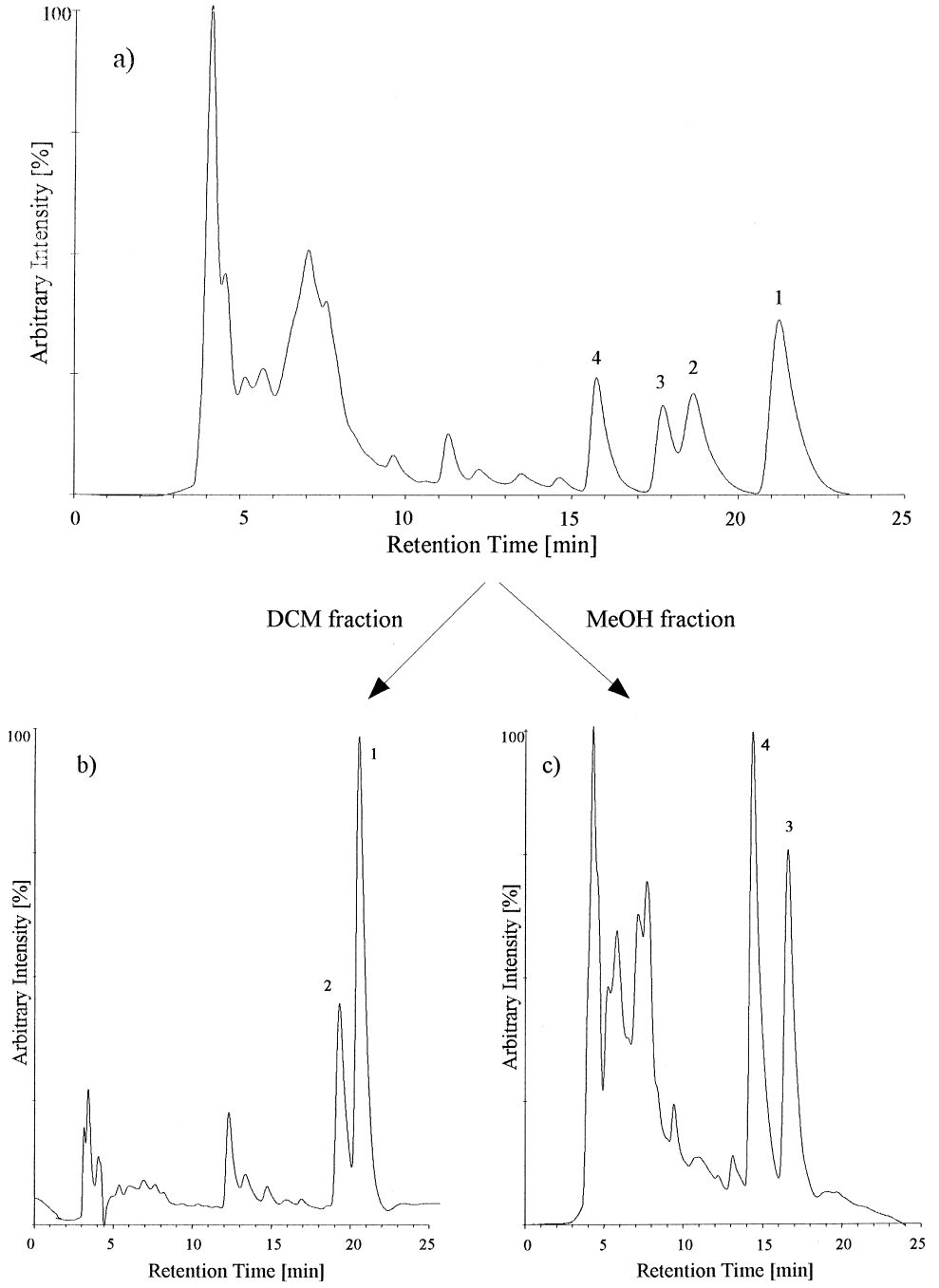

Fig. 1. HPLC chromatograms obtained from the different fractions of the conventional and the optimized clean-up procedure: (a) MeOH eluate of the conventional clean-up procedure, (b) DCM fraction and (c) MeOH-fraction of the optimized clean-up procedure. Chromatographic conditions: Nucleosil 120-3C_8 column, 125×4 mm, 3 μm; isocratic separation with MeOH–H_2O (80:20, v/v) at 1 ml/min. Detection wavelength, 254 nm. UV-detectable peaks that were also identified by their mass spectra are labeled as follows: (1) B[a]P, (2) B[a]P-6,12-dione, (3) B[a]P-3,6-dione and (4) B[a]P-1,6-dione.

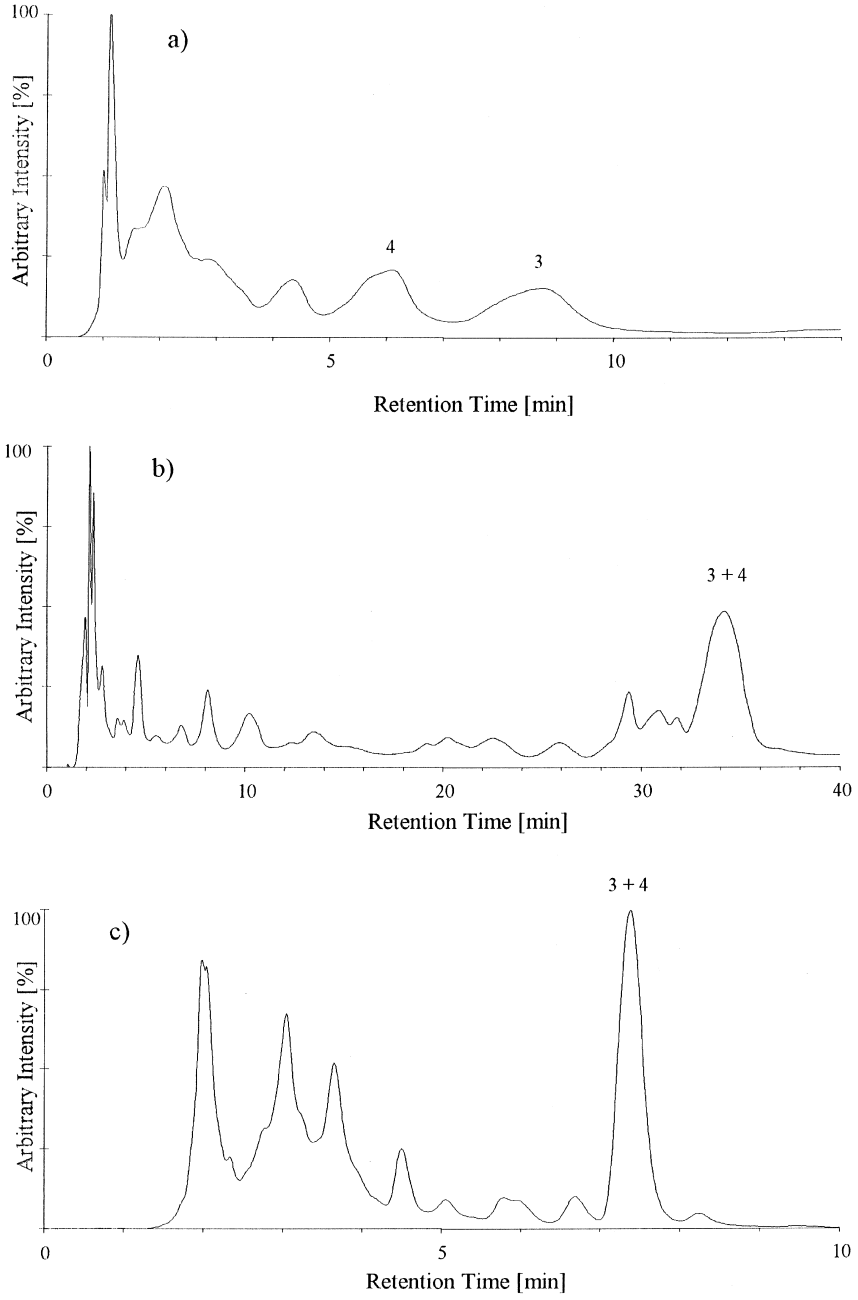

Fig. 2. (a) RP-HPLC separation of the MeOH-fraction from the improved clean-up procedure. Chromatographic conditions: Multospher 120 RP2-3 column, 125×4 mm, 3 μm; isocratic separation with MeOH–H_2O (40:60, v/v) at 1 ml/min. Detection wavelength, 254 nm. UV-detectable peaks that were also identified by their mass spectra are labeled (3) B[a]P-3,6-dione and (4) B[a]P-1,6-dione; (b) Optimization of the RP-HPLC separation of the MeOH-fraction from the improved clean-up procedure. Chromatographic conditions: Nucleosil 120-7CN column, 250×4 mm, 7 μm; gradient separation with 1 ml/min MeOH–H_2O (30:70, v/v, for 12 min, in 5 min to MeOH–H_2O, 40:60, v/v, hold for 22 min); (c) RP-HPLC separation of the MeOH fraction using the improved clean-up procedure. Chromatographic conditions: Nucleosil 120-7C6H5 column, 250×4 mm, 7 μm; isocratic separation with MeOH–H_2O (80:20, v/v) at 1 ml/min.

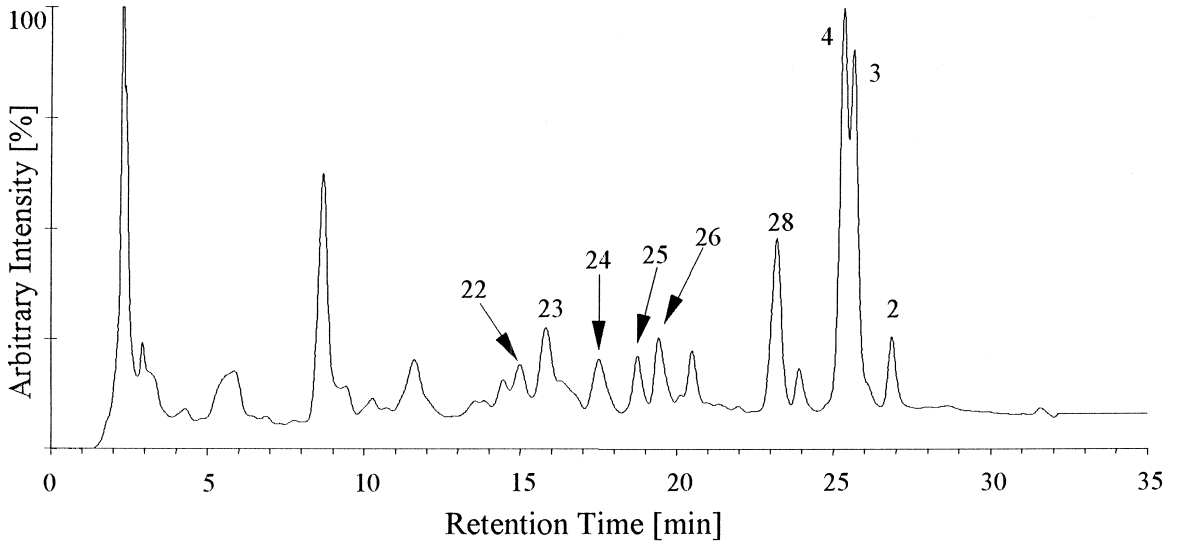

Fig. 3. Diode array detection (DAD) chromatogram of the separation of the MeOH fraction using the improved clean-up procedure. Chromatographic conditions: Nucleosil 120-7C6H5 column, 250×4 mm, 7 μm; gradient separation with 1 ml/min (MeOH–H_2O, 50:50, v/v, for 2 min, to MeOH–H_2O, 65:35 v/v, in 10 min, to MeOH–H_2O, 80:20, v/v, in 11 min, hold for 12 min, then back to initial conditions and equilibration for 5 min). Detection wavelength, 254 nm. UV-detectable peaks are assigned in Table 4.

3.3. Identification of degradation products

3.3.1. Use of reference substances

For most chromatographic peaks, a tentative identification had to be based on the interpretation of the fragmentation pattern observed in the APCI mass spectra.

For the identification of five degradation products, however, reference substances could be used. Additionally, B[a]P derivatives carrying various functional groups were used to characterize the chromatographic properties of the applied Ph column and solvent gradient. Table 2 lists the reference substances used, along with their retention times (RTs) under optimized chromatographic conditions for the phenylsilane column, molecular mass and spectroscopic data.

The retention of these compounds on the Ph column depends primarily on the polarity of the B[a]P metabolite, which is determined by the number and kind of functional groups (decreasing retention time with increasing number and polarity of the functional groups).

The B[a]P derivatives span a retention time window ranging from 9.6 min for the B[a]P-tetrol up to 25.2 min for B[a]P-6,12-dione. The order of elution for the hydroxy-B[a]P derivatives is as expected for a RP separation: the B[a]P derivative with four OH-groups is the least retained, eluting at 9.6 min, the derivative containing three oxygen atoms in the molecule (two OH-groups and one epoxy-group) elutes at 11.2 min, the B[a]P-dihydrodiol at about 14 min and the derivative with a single OH-group elutes at 19 min. The five B[a]P-diones elute in the retention time window between 22 and 25 min. This elution order nicely demonstrates the increasing retention with decreasing polarity in the order $B[a]P(OH)_4 < B[a]P(OH)_3 < B[a]P(OH)_2 < B[a]P(OH) < B[a]P(=O)_2$.

In addition, the retention time is dependent on the size of the aromatic system (increasing retention time with increasing size), which is due to π-electron interactions between the stationary phase and the analytes. The importance of this effect for B[a]P-dihydrodiols will be discussed below.

3.3.2. Detection and identification of PAH metabolites by APCI–MS

An essential tool for the identification of the investigated compounds was, in addition to the

Table 2
Molecular mass, chromatographic, mass spectroscopic (m/z 100–450) and UV spectral data (220–500 nm) for the available reference substances of B[a]P degradation products

Substance	Retention time (min)	Molecular mass (g/ml)	MS ion peaks, m/z [intensity (%)]	UV absorption peaks, nm [intensity (%)]
B[a]P-tetrol	9.6	320	299 (10), 283 (50), 271 (20), 259 (100), 257 (60), 255 (45)	248 (90), 267(45), 280 (90), 338 (70), 348 (100)
B[a]P-diolepoxide	11.2	302	303 (10), 299 (60), 285 (80), 259 (100), 257 (80), 255 (30), 243 (20)	249 (100), 270 (35), 280 (55), 330 (40), 345 (55)
B[a]P-4,5-dihydrodiol	13.7	286	285 (5), 283 (30), 269 (100), 255(10), 241 (10)	266 (90), 274 (100), 299 (15), 312 (15), 326 (15)
B[a]P-7,8-dihydrodiol	14.3	286	285 (5), 283 (15), 269 (100), 255 (10)	258 (100), 285 (45), 295 (55), 351 (80), 367 (100)
Benzanthrone	18.7	230	231 (100)	255 (100), 262 (100)
B[a]P-8-ol	19.0	268	269 (100)	280 (100), 370 (40), 385 (40)
B[a]P-3-ol	19.2	268	269 (100)	232 (70), 261 (100), 296 (70), 308 (75), 380 (60)
B[a]P-4-ol	19.4	268	269 (100)	270 (100), 291 (50), 301 (50), 376 (30)
B[a]P-4,5-dione	22.1	282	283 (100), 255 (15)	274 (100), 336 (20)
B[def]C-lactone	23.5	270	271 (100), 228 (10), 227, (50), 226 (10)	226 (50), 268 (100), 353 (20), 393 (15)
B[a]P-7,10-dione	23.6	282	283 (100), 255 (20)	245 (100), 268 (65), 278 (65), 343 (75)
B[a]P-1,6-dione	23.7	282	283 (100), 255 (5)	222 (100), 253 (60), 442 (40), 446 (40)
B[a]P-3,6-dione	24.0	282	283 (100), 255 (5)	247 (100), 291 (35), 345 (35), 486 (45)
B[a]P-6,12-dione	25.2	282	283 (100), 255 (5)	228 (100), 291 (75), 301 (65), 357 (30), 370 (35)

retention times and the UV spectra, the acquisition of APCI–mass spectra. In order to obtain the most information from the mass spectra, fragmentation was investigated under different conditions.

3.3.2.1. Polarity of the detected ions

Most compounds produce a detectable molecular ion in negative-ion detection mode, but even at higher fragmentor voltages, there is no increase in information due to the absence of further characteristic fragment ions.

For the detection of organic acids, negative-ion detection is known to be suitable, but since no acids were used as standards in this work, positive-ion detection proved to be more versatile. For the hydroxyl or carbonyl derivatives, better results were obtained in the positive-ion mode.

3.3.3. Influence of fragmentor voltage

At low fragmentor voltage (e.g. 70 or 90 V), the compounds exhibited mainly the (quasi-) molecular ion, at a higher voltage (e.g. 120 V), a mixture of both protonated molecular and fragment ions was observed, and at a high fragmentor voltage (e.g. 160 or 190 V), the protonated molecule completely disappeared while a number of protonated fragment ions occurred. Table 2 therefore lists the protonated molecules that were obtained with a fragmentor voltage of 120 V, which usually includes both protonated molecular and some significant protonated fragment ions. Since these spectra have the highest information content, the data reported in Tables 3 and 4 for the analysis of the DCM and MeOH fractions, respectively, were acquired under the same conditions. The identification of the un-

Table 3
Assigned substances (sorted by retention time) in the DCM fraction with UV spectra (220–500 nm) where available, MS spectra (m/z 100–450) and (tentative) identifications

Peak no.	Retention time (min)	MS ion peaks, m/z [intensity (%)]	UV absorption peaks, nm [intensity (%)]	(Tentative) identification
5	8.5	219 (10), 177 (10), 163 (40), 149 (100), 121 (20)		Hydrophenanthrenone-lactone
6	11.2	219 (100)		
7	13.3	225 (60), 197 (100)		Ketone
8	16.0	285 (20), 283 (20), 271 (100), 255 (80), 241 (20)	272 (100), 305 (20), 333 (20)	B[a]P-diol
9	18.4	279 (20), 205 (40),163 (80), 149 (100), 121 (10)		
10	18.5	279 (20), 205 (40),163 (80), 149 (100), 121 (10)		
11	21.0	299 (100), 271 (20), 231 (20)	253 (100), 321 (40), 417 (60), 425 (60)	Hydroxy-B[a]P-dione
12	22.2	283 (100), 255 (15)	274 (100), 336 (20)	B[a]P-4,5-dione[a]
13	22.5	271 (60), 228 (20), 227 (100), 226 (20)		Lactone
14	23.4	271 (50), 228 (20), 227 (100), 226 (30)		B[def]C-lactone[a]
1	24.2	253 (100)	266 (98), 287 (85), 297 (100), 365 (45), 385 (50)	B[a]P[a]
2	25.0	283 (100), 255 (5)	228 (100), 291 (75), 301 (65), 357 (30), 370 (35)	B[a]P-6,12-dione[a]
15	27.1	411 (30), 369 (10), 341 (100), 297 (20), 283 (50), 253 (40)		
16	30.2	391 (100), 338 (10)		

[a] Identified using a reference material.

known substances is based on the evaluation of all available spectra of the compounds obtained under different conditions (e.g. different fragmentor voltages).

3.3.4. Identification of compounds in the DCM fraction

In Table 3, the chromatographically separated substances from the DCM fraction are listed with their RTs, MS and UV spectroscopic data. Besides unreacted B[a]P (RT 24 min), 16 other degradation products were detected. Chromatographic peaks that were also found in blank samples or whose intensity was too low to obtain reliable MS or UV spectra are not considered. The assignment of possible structures to the analyte peaks follows their order of elution.

The first eluting substance recognized as a degradation product of B[a]P with ozone is peak no. 5. This peak and peak no. 6 both show a putative (quasi-)molecular ion with m/z 219 ($[M+H]^+$). While substance 6 shows m/z 219 as the main peak without further fragmentation, substance 5 exhibits more protonated fragments. Besides the parent ion at m/z 219 with 10% normalized intensity, the substance shows protonated fragment ions with m/z 177 $[M+H-42]^+$, m/z 163 $[M+H-56]^+$, the base peak at m/z 149 $[M+H-70]^+$, and m/z 121 $[M+H-98]^+$. This fragmentation indicates a substance with the formula $C_{13}H_{14}O_3$, which has a 'ring and double bond equivalent' (RDB) of seven. It might be a polycyclic, unsaturated lactone (loss of m/z 56, corresponding to C_2O_2), where the carbonyl group has a CH_2-group in the α-position (loss of m/z 42, corresponding to C_2H_2O). The other prominent

Table 4
Assigned substances (sorted by retention time) in the MeOH fraction with UV spectra (220–500 nm) where available, MS spectra (*m/z* 100–450) and (tentative) identifications

Peak no.	Retention time (min)	MS ion peaks, *m/z* [intensity (%)]	UV absorption peaks, nm [intensity (%)]	(Tentative) identification
17	5.0	213 (100), 171 (25)		
18	8.7	319 (100), 301 (10), 299 (15) 289 (40), 283 (40)		Carboxylic acid
19	9.2	301 (55), 287 (100), 273 (85)		
20	11.2	319 (100), 301 (40), 287 (55), 259 (45), 231 (35)		Carboxylic acid
21	14.5	285 (70), 283 (50), 255 (40), 241 (100), 239 (20), 227 (10)	275 (100), 322 (20)	B[a]P-diol
22	15.8	285 (75), 283 (40), 255 (100), 241 (85), 239 (40), 227 (10)	266 (95), 274 (100), 314 (25), 318 (20)	B[a]P-diol
23	16.8	285 (70), 283 (30), 255 (100), 241 (70), 239 (20), 227 (5)	249 (85), 266 (95), 274 (100), 328 (20)	B[a]P-diol
24	17.4	285 (100), 283 (5), 255 (10), 241 (15)	233 (80), 252 (98), 276 (100), 409 (30)	B[a]P-diol
25	18.3	285 (55), 275 (100), 247 (60)	253 (100), 259 (98), 367 (40)	
26	19.1	313 (70), 285 (25), 275 (100), 259 (25)	247 (85), 264 (100), 332 (40), 345 (50)	
27	19.8	317 (60), 313 (100), 299 (25), 283 (30), 273 (75)		
28	21.8	299 (100), 271 (25), 243 (5)	253 (100), 321 (40), 417 (60), 425 (60)	Hydroxy-B[a]P-dione
4	23.7	283 (100), 255 (5)	222 (100), 253 (60), 442 (40), 446 (40)	B[a]P-1,6-dione[a]
3	24.0	283 (100), 255 (5)	247 (100), 291 (35), 345 (35), 486 (45)	B[a]P-3,6-dione[a]
2	25.2	283 (100), 255 (5)	228 (100), 291 (75), 301 (65), 357 (30), 370 (35)	B[a]P-6,12-dione[a]
29	26.8	341 (100), 283 (40)		
30	30.5	391 (5), 338 (100), 283 (25)		

[a] Identified using a reference material.

fragments may be explained by the loss of $CH_2(CO)_2$, *m/z* 70, and $C_4H_2O_3$, *m/z* 98, probably in the form of a cyclic lactone or acid anhydride ring. A structure compatible with the above interpretation of the fragmentation pattern is shown in Fig. 4a (heptahydrophenanthrene-2-one-1,10-lactone or isomers). What still remains unclear is how the aromatic ring system of B[a]P can be hydrated in the course of the degradation reaction and under oxidative conditions. Compared with the available reference substances, this substance elutes much earlier.

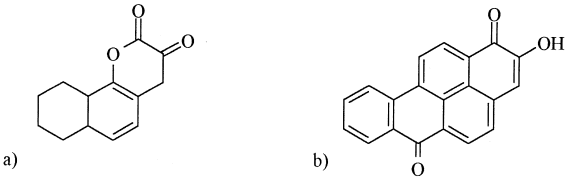

Fig. 4. Possible structures of two B[a]P degradation products that were tentatively identified by LC–APCI–MS, i.e., an α-carbonyl-lactone (4a) and a hydroxy-dione (4b); in both cases, other isomers are equally probable.

A possible explanation is the loss of its aromatic structure in a large part of the molecule. With the loss of aromaticity, the interaction of π-electrons of the analytes with those from the phenylic stationary phase is lost and the elution becomes significantly faster. Since there are no standards available to verify this hypothesis, the identification is only tentative.

Peak 7 is interpreted as a ketone of a lower PAH derivative. The protonated molecule m/z 225 ($[M+H]^+$) shows a loss of m/z 28 (CO) to the ion m/z 197 $[M+H-28]^+$. Another possibility is the loss of C_2H_4 with m/z 28 of a hydrated molecule. However, the polarity of this molecule is higher than that of benzanthrone, which elutes about 5 min later.

On the other hand, it elutes earlier than substance 8, which has been identified as one of the B[a]P-diol isomers. While the available standards of B[a]P-diol isomers eluted between 13.5 and 14.5 min, the unknown compound no. 8, which, due to its characteristic mass spectrum, is believed to be a B[a]P-diol, elutes at about 16 min (the same holds true for peaks 22, 23 and 24 in the MeOH fraction). Due to the stronger retention, compared to the B[a]P-dihydrodiols, the unidentified compounds are believed to be fully aromatic systems, e.g., hydrochinones. Molecules with a given carbon skeleton and a smaller aromatic electron system have a lower interaction with the phenylic stationary phase than the analogous fully aromatic molecules. The stronger retention is caused by a stronger π–π electron interaction and results in the higher RTs of these diols.

Peaks 9 and 10 both exhibited the same fragmentation pattern, which equals the mass spectrum of phthalic acid dibutylester. Phthalic acid dibutylester is a plasticizer, which might have come from the polyethylene water bottle that was used for filter moistening. However, the peaks were not found in moistened blank samples, which indicates that they are B[a]P oxidation products. The actual origin and identity of these peaks will be a subject of future investigations.

Compound 11 has the base peak at m/z 299 $[M+H]^+$, and a mass spectrum similar to that for peak 28. All spectra of these compounds, obtained under different conditions, showed the protonated molecule at m/z 299 $[M+H]^+$, with prominent protonated fragments at m/z 271 $[M+H-28]^+$, m/z 243 $[M+H-56]^+$ and m/z 215 $[M+H-84]^+$, which might be explained by the loss of one, two and three CO groups, respectively, from the molecule. There is also a protonated fragment at m/z 270 $[M+H-29]^+$, which is a typical loss for phenolic groups. Fig. 4b shows the suggested structure for one of the isomers of the unknown compound, which is a 2-hydroxy-B[a]P-1,6-dione ($C_{20}H_{10}O_3$, M=298 g/mol, RDB=16). All other isomers are equally probable. The RT of this molecule is in accordance with the expected RT, i.e. earlier than the B[a]P-diones but later than the the hydroxy-B[a]P.

Peak 12 at 22.2 min was identical with the reference B[a]P-4,5-dione (Fig. 5a) in its MS spectrum, UV spectrum and retention time. This is the first time that a fourth B[a]P-dione was identified along with the three main degradation products B[a]P-1,6-dione, B[a]P-3,6-dione and B[a]P-6,12-dione. The mutagenicity and toxicity of this newly found B[a]P-4,5-dione was found to be similar to those of the other three B[a]P-diones [12].

A further B[a]P metabolite structure could be verified whose occurrence was only suspected previously [18]. Substances 13 and 14 have been identified as two lactones. Peak 14 shows the same RT and the same fragmentation pattern as the lactone reference substance (Fig. 5b), whereas peak 13 seems to be an isomer with the same fragmentation pattern, but with a different retention time. These lactones are described as highly mutagenic compounds comparable to the mutagenic and carcinogenic potential of B[a]P [27].

After elution of the unreacted B[a]P (no.1), which remained after the clean-up step, the B[a]P-6,12-dione (no.2) eluted at 25 min. Although occurring at only very low concentrations, substances 15 and 16

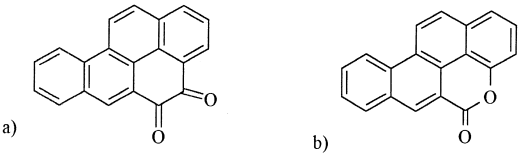

Fig. 5. Structures of the B[a]P degradation products identified with reference materials: (a) B[a]P-4,5-dione (no. 12), $C_{20}H_{10}O_2$, M=282 g/mol, RDB=16; (b) 4-oxa-benzo[*d,e,f*]chrysene-5-one (no. 14), $C_{19}H_{10}O_2$, M=270 g/mol, RDB=15.

were present in all samples and produced characteristic, but not readily interpretable, mass spectra.

3.3.5. Identification of compounds in the MeOH fraction

The above-described clean-up procedure lead to fractionation into a group of less polar B[a]P degradation products, which are found in the DCM fraction, and more polar metabolites, which are eluted by MeOH from the clean-up column.

Fig. 3 shows a chromatogram of the MeOH fraction recorded with DAD–UV detection at 254 nm. Many of the substances are UV absorbers and can thus be detected by DAD. However, without suitable reference substances, it is virtually impossible to identify the separated compounds by DAD–UV, whereas the use of APCI–MS enables the tentative identification of the investigated compounds. The chromatogram shown in Fig. 6 is a total ion chromatogram (TIC) and demonstrates the higher sensitivity for some of the compounds compared to the results obtained using DAD (Fig. 3). In addition, MS detection proves to be more useful than DAD, since it provides characteristic spectra even in this low concentration range. However, the first part of the chromatogram of the MeOH fraction (Fig. 6) shows a number of smaller peaks and, although mass spectrometric detection was applied, most of detected substances do not yield a characteristic mass spectrum that enables their tentative identification. These peaks as well as components that were also found in blank samples are not numbered and are not listed in Table 4.

Peak no. 17, with a RT of ca. 5 min, exhibits characteristic ions at m/z 213 (most likely the quasi-molecular peak $[M+H]^+$) and a protonated fragment at m/z 171 (corresponding to $[M+H-42]^+$, which probably is the loss of a $CH_2C=O$ fragment).

The fragmentation of peaks 18, 19 and 20 gave similar results, as described elsewhere [18]. Koeber

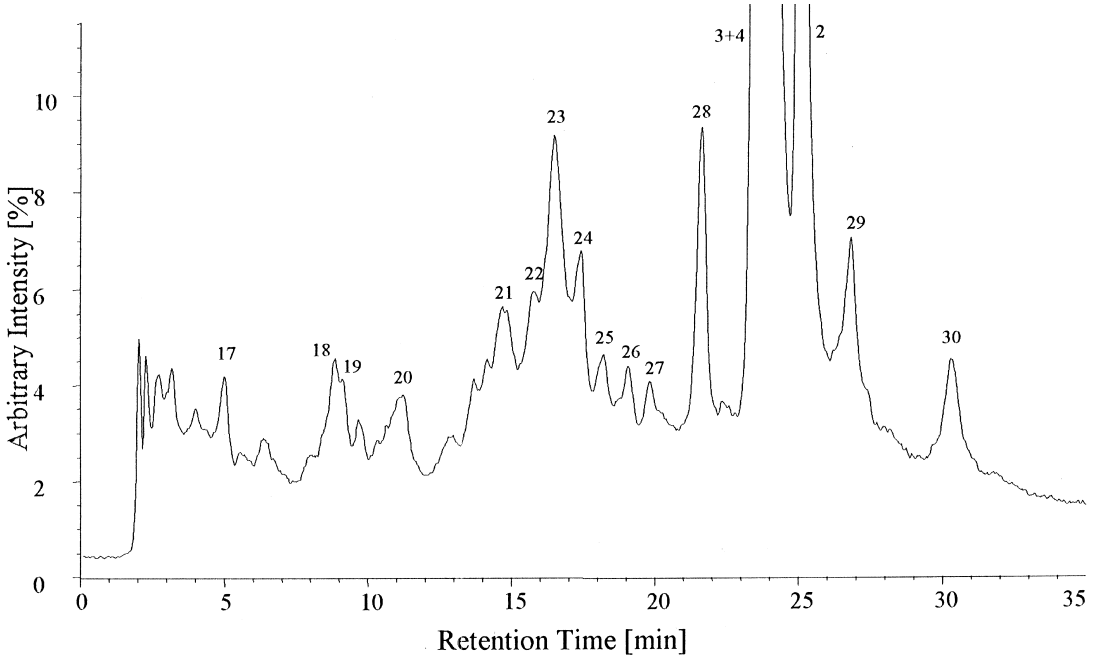

Fig. 6. TIC MS-chromatogram of the separation of the MeOH fraction using the improved clean-up procedure. Chromatographic conditions: Nucleosil 120-7C6H5 column, 250×4 mm, 7 μm; gradient separation with 1 ml/min MeOH–H_2O (50:50, v/v) for 2 min, linear to MeOH–H_2O (65:35, v/v) in 10 min, linear to MeOH–H_2O (80:20, v/v) in 11 min, hold for 12 min, then back to the initial conditions and equilibration for 5 min. Detection conditions: TIC (m/z 100–450) of APCI–MS with a fragmentor voltage of 120 V, positive-ion mode. MS-detectable peaks are assigned in Table 4.

et al. [18] described these substances as carboxylic acids. A loss of water (m/z 18) is confirmed, but the fragmentation pattern obtained with LC–APCI–MS in this study showed no more features of the typical fragmentation pattern observed for organic acids.

The next four peaks (nos. 21–24) are tentatively identified as B[a]P-diols from the characteristic mass spectral peaks, but, in contrast to the B[a]P-dihydrodiols that were available as reference substances, they have larger retention times, between 14.7 and 17.4 min. This supports the hypothesis discussed above. The parent ion m/z 285 undergoes a loss of H_2 to give m/z 283 $[M+H-2]^+$ (the same mass as B[a]P-diones), further loss of CO to give m/z 255 $[M+H-30]^+$and a second loss of CO to give m/z 227$[M+H-58]^+$. Two further protonated fragments are observed at m/z 241 $[M+H-44]^+$ and m/z 239 $[M+H-46]^+$, probably derived from the protonated molecule and the molecule after preceding loss of a hydrogen molecule (H_2).

Peak 25 could not be identified, but had two characteristic ions at m/z 285 and 275. Since there is no reasonable fragmentation reaction that can account for a loss of ten mass units from the parent ion, both peaks might be fragments of a higher-molecular-mass parent ion (e.g. m/z 303) or might be due to the coelution of two different substances.

Peak 26 shows a loss of CO (m/z 28) from the suspected quasi-molecular ion m/z 313 to the protonated fragment m/z 285, but the main ion fragments at m/z 275 and 258 could not be accounted for.

Peak 28 has a similar RT and fragmentation pattern to those of peak 11; a possible assignment has been described above.

The major peaks 4, 3 and 2 are B[a]P-1,6-dione, B[a]P-3,6-dione and B[a]P-6,12-dione; the latter is sometimes also observed in the MeOH fraction.

Peaks 29 and 30 could be detected in every sample, but could not be identified.

4. Conclusion

In this study, the clean-up and analysis of filter samples containing B[a]P and its oxidation products was optimized. The normal-phase column chromatographic clean-up procedure yielded two solvent fractions containing reaction products of different polarities. For the subsequent HPLC separation, different RP materials were tested whereby the phenyl stationary phase material showed the best resolution, which is probably due to π-electron interaction between the stationary phase material and the analytes. An optimized gradient was developed for the separation of the degradation products on this column.

For the identification of unknown degradation products, different approaches were used. When available, reference substances were used for a direct comparison of HPLC retention times and mass and UV spectra. However, LC–APCI–MS detection proved to be a valuable tool for elucidation of the structure of unknown compounds, even when no reference substances were available. The combination and interpretation of mass spectral data and chromatographic behavior enabled the elucidation of structural elements or even the tentative identification of the analytes.

For the first time, B[a]P-4,5-dione and a B[def]C-lactone were identified as products of B[a]P degradation with ozone. Three further previously unknown products could be tentatively identified. The B[def]-C-lactone is known in the literature as a molecule with a mutagenic potential similar to that of B[a]P, and B[a]P-4,5-dione is said to have the same mutagenic potential as the three main degradation products B[a]P-1,6-dione, -3,6-dione and -6,12-dione.

We therefore suggest that these two B[a]P degradation products should be considered in further environmental measurements and that they should be included in detailed mutagenic potential tests.

Acknowledgements

We thank Robert Koeber for technical assistance, Ulrich Pöschl for helpful discussions and two anonymous reviewers for valuable suggestions. We also thank Chromatographie Service GmbH for providing test columns and gratefully acknowledge the financial support by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie.

The authors would appreciate receiving any other available PAH metabolite for further investigations.

References

[1] S.M. Rappaport, Y.Y. Wang, E.T. Wei, R. Sawyer, B.E. Watkins, H. Rapoport, Environ. Sci. Technol. 14 (1980) 1505.
[2] T. Vo-Dinh (Ed.), Chemical Analysis, Vol. 101, Wiley, New York, 1989, p. 1.
[3] M.L. Lee, M. Novotny, K.D. Bartle, Anal. Chem. 48 (1976) 405.
[4] A. Bjorseth, B.S. Olufsen, in: A. Bjorseth (Ed.), Handbook of Polycyclic Aromatic Hydrocarbons, Marcel Dekker, New York and Basel, 1983, p. 507.
[5] J. Heyder, J. Gebhart, F. Rudolph, C.F. Schiller, W. Stahlhofen, J. Aerosol Sci. 17 (1986) 814.
[6] U. Heinrich, R. Fuhst, S. Rittinghausen, O. Creutzenberg, B. Bellmann, W. Koch, C. Levsen, Inhalation Toxicol. 7 (1995) 533.
[7] B.N. Ames, J. McCann, E. Yamasaki, Mutation Res. 31 (1975) 347.
[8] D.M. Maron, B.N. Ames, Mutation Res. 113 (1983) 173.
[9] I.A. Clark, A.C. Allison, Nature 252 (1974) 326.
[10] S.W. Tjioe, R.J. Hurtubise, Talanta 42 (1995) 59.
[11] J.P. Barry, C. Norwood, P. Vouros, Anal. Chem. 68 (1996) 1432.
[12] J.L. Durant, J.W.F. Busby, A.L. Lafleur, B.W. Penman, C.L. Crespi, Mutation Res. 371 (1996) 123.
[13] P.M. Kim, U. DeBoni, P.G. Wells, Free Radic. Biol. Med. 23 (1997) 579.
[14] J.N. Pitts Jr., K.A.V. Cauwenberghe, D. Grosjean, J.P. Schmid, D.R. Fitz, W.L. Belser, G.B. Knudson, P.M. Hynds, Science 202 (1978) 515.
[15] F. Valerio, M. Pala, C. Borgogni, Int. J. Environ. Anal. Chem. 38 (1990) 343.
[16] J.F. Thomas, M. Mukai, B.D. Tebbens, Environ. Sci. Technol. 2 (1968) 33.
[17] J.M. Bayona, M. Casellas, P. Fernandez, A.M. Solanas, J. Albaiges, Chemosphere 29 (1994) 441.
[18] R. Koeber, J.M. Bayona, R. Niessner, Int. J. Environ. Anal. Chem. 66 (1997) 313.
[19] J. König, E. Balfanz, W. Funcke, T. Romanowski, Anal. Chem. 55 (1983) 599.
[20] R. Koeber, R. Niessner, J.M. Bayona, Fresenius' J. Anal. Chem. 359 (1997) 267.
[21] J.G. Dorsey, W.T. Cooper, B.A. Siles, J.P. Foley, H.G. Barth, Anal. Chem. 70 (1998) 591R.
[22] B. Eliasson, U. Kogelschatz, Ozone Sci. Eng. 13 (1991) 365.
[23] E. Rosenberg, R. Krska, R. Wissiack, V. Kmetov, R. Josephs, E. Razzazi, M. Grasserbauer, J. Chromatogr. A 819 (1998) 277.
[24] C.v. Sonntag, H.-P. Schuchmann, Angew. Chem. Int. Ed. Engl. 30 (1991) 1229.
[25] R.H. Bieri, J. Greaves, Biomed. Environ. Mass Spectrosc. 14 (1987) 555.
[26] E.A. Elnenaey, W.P. Schoor, Anal. Biochem. 111 (1981) 393.
[27] S. Amin, S.S. Hecht, E. LaVoie, D. Hoffmann, J. Med. Chem. 22 (11) (1979) 1336.