ELSEVIER

Journal of Chromatography A, 753 (1996) 207–215

JOURNAL OF CHROMATOGRAPHY A

Enrichment of benzo[*a*]pyrene in smoked food products and determination by high-performance liquid chromatography–fluorescence detection

M.S. García Falcón, S. González Amigo, M.A. Lage Yusty*, M.J. López de Alda Villaizán and J. Simal Lozano

Department of Analytical Chemistry, Nutrition and Bromatology, Area of Nutrition and Bromatology, Faculty of Pharmacy, University of Santiago de Compostela, E-15706 Santiago de Compostela, Spain

Received 30 January 1996; revised 22 May 1996; accepted 28 May 1996

Abstract

We developed a procedure for trace enrichment of benzo[*a*]pyrene (BP) in extracts of smoked food products, and an HPLC–fluorescence detection (FL) method for determination of BP in the enriched extracts. The procedure consists in extraction/sonication of the lyophilized product in hexane, clean-up of the hexane extract by passage through a Sep-Pak Silica Plus cartridge and, subsequently, by partitioning between hexane and dimethyl sulphoxide, and concentration of the BP using a Sep-Pak C_{18} Plus cartridge. HPLC–FL and quantification limits were 0.049 μg/l in acetonitrile (<0.0067 μg/kg of smoked food) and 0.089 μg/l in acetonitrile (<0.012 μg/kg), respectively. Recovery (94.1%) and RSD (<8.65%) were satisfactory. When applied to 15 types of sausage, mean BP content was 0.022 μg/kg, and all but two samples (both treated with wood smoke) had BP contents below the 0.03 μg/kg limit imposed in EU legislation for smoking-flavour agents.

Keywords: Food analysis; Sample preparation; Polynuclear aromatic hydrocarbons; Benzo[*a*]pyrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of high lipophilic compounds that are generally formed by combustion, pyrolysis of organic matter. PAHs with a variety of chemical natures have been detected in tobacco smoke, air, soils, waters and sediments, aquatic organisms and foodstuffs [1]. Such contamination mainly occurs by atmospheric deposition and, in the case of processed foods, through contact with hydrocarbon-based materials and during severe (500–700°C) heat treatments or pseudo-curing with smoking-flavour agents or wood smoke, especially the latter [2].

Use of smoking-flavour agents (hydrophilic or lipophilic smoke-extracts) is becoming increasingly popular because it allows more uniform flavouring of foodstuffs and greater control of the levels of PAH contaminants [3]. Nevertheless, in Europe meat is still widely cured or flavoured using smoke generated by combustion of wood (several types of wood are used, but hardwoods are preferred to softwoods, which produce more soot and can impart a resinous flavour to the meat) [4]. But wood smoke contains appreciable amounts of carcinogenic PAHs, which

*Corresponding author.

0021-9673/96/$15.00
PII S0021-9673(96)00522-5

are the main cause of concern regarding its toxicity and, hence, its suitability for use in curing [5].

The potently carcinogenic PAH benzo[*a*]pyrene (BP) is often used as an indicator of the presence of PAHs in water and foodstuffs. Human exposure to BP and PAHs in general occurs almost exclusively (99%) through their ingestion with food [6]. Because of this, and the increasingly acknowledged link between diet and cancer, several legal measures aimed at limiting the levels of BP in foodstuffs have been adopted. For foods eaten without preparation, the EC (European Communities) [now the EU (European Union)] has set the maximum permissible level of BP derived from smoking-flavour agents at 0.03 μg/kg [7]. Currently, however, no such legislation exists regarding BP levels in other foods, though the EU intends to set a maximum of 1 μg/kg, and to prohibit severe treatments such as cooking food on an open flame [2]; and, for fish products, a health directive sets out the conditions under which fish should be smoked in order to limit PAH contamination [8].

PAHs have been detected and quantified in vegetable foods, in uncured, smoked and grilled animal foods, and also in smoking-flavour agents [9–24]. Of the methods recently proposed for PAH analysis [25–27], the best developed are HPLC–fluorescence detection (FL) methods HPLC offers advantages such as short analytical run-times and excellent selectivity that can be enhanced by detection methods exploiting molecular properties of PAHs such as their UV absorption and fluorescence, especially the latter [18,20–22].

The principal problems associated with determination of BP in smoked food products are the low analyte levels (μg/kg) and the diversity of potential interferents present. Because of the complexity of smoked food samples and their high lipid content, sample clean-up and PAH enrichment are problematic, usually necessitating the use of stepwise methods that are laborious and time-consuming. In this work, we developed a relatively straightforward procedure that is faster and uses less solvent than methods using saponification with potash [12,18,28–30], or the Soxhlet extraction method [31–33]. The procedure consists in extraction of the BP in the lipid fraction using hexane/sonication, clean-up on a Sep-Pak Silica Plus cartridge and by partitioning between hexane and dimethyl sulphoxide (DMSO), and concentration on a Sep-Pak C_{18} Plus cartridge. Final separation and quantification are by HPLC with fluorescence detection. Method precision and recovery are satisfactory, and the detection limit is well below the 0.03 μg/kg limit imposed for BP derived from smoke-flavourings added to foods that are eaten without preparation.

2. Experimental

2.1. Samples

All commercial smoked food products were purchased from supermarkets in Santiago de Compostela (N.W. Spain).

2.2. Reagents

Benzo[*a*]pyrene (BP) standard was purchased from Aldrich, residue analysis grade *n*-hexane and sodium chloride (NaCl) were from Merck, routine grade CH_3CN for HPLC was from Scharlau and DMSO for analysis was from Panreac. Sep-Pak Silica Plus and C_{18} Plus cartridges were purchased from Waters (Millipore), and nitrogen (SEO N-45) was from the Sociedad Española de Oxígeno.

2.3. Preparation of standards

Stock solutions containing 100 mg/l and 100 μg/l of BP were prepared in *n*-hexane, CH_3CN and DMSO, were stored at 4°C in volumetric flasks (with glass stoppers) wrapped in aluminium foil to avoid possible light degradation. BP standards (0.25, 0.5, 1, 2, 4, 8 and 16 μg/l) were prepared by appropriate dilutions of these stock solutions.

2.4. Apparatus

For lyophilization of smoked food products, a Liolabore 3 Telstar freeze-drier was used. For sonication during extraction, a Selecta Model 300513

ultrasonic bath (50 Hz) was used. Determination of BP during optimization of the clean-up procedure, was carried out on a Perkin–Elmer LS 50 luminescence spectrometer equipped with a xenon discharge lamp, Monk–Gillieson monochromators and 1 cm quartz cuvettes. Acquisition and processing of spectral data was by Fluorescence Data Manager software. For HPLC, a Spectra-Physics liquid chromatograph equipped with a P100 isocratic pump, Sugelabor reversed-phase Tracer Tr-C-160 C_{18} precolumn and Tracer PAH column (5 μm particle size; 15 cm×0.46 cm I.D.), an FL2000 fluorescence detector, and a Datajet integrator connected vía Labnet to a PC running Winner on Windows (WOW) data processing software were used. Water circulating through a thermostatted waterbath was used to keep the column temperature at 38±0.1°C.

3. Procedure

3.1. Extraction and clean-up

The stepwise procedure used is summarized in the flow-chart shown in Fig. 1. Samples of food products were weighed and diced before being lyophilized. Then, 5 g (dry mass) of the lyophilate was extracted with 25 ml (1 h), 15 ml (1 h) and then 10 ml (1 h) of *n*-hexane while being sonicated. The combined extracts (50 ml) were centrifuged at 2500 rpm for 10 min, and the supernatant was decanted and concentrated to 5 ml under a nitrogen stream. Then followed a series of clean-up steps, each of which was optimized. Firstly, the concentrated hexane extract was passed onto a Sep-Pak Silica Plus cartridge (particle size, 55–105 μm) and eluted with 10 ml *n*-hexane at flow-rate of 1 ml/min. Then, the hexane eluate (final volume, 15 ml) was extracted with 15 ml (5 min), 10 ml (5 min) and then 5 ml (5 min) of DMSO that had previously been equilibrated with *n*-hexane. The DMSO solution was diluted with 75 ml water and passed onto a Sep-Pak C_{18} Plus cartridge (particle size, 55–105 μm) that had previously been activated by passage of 5 ml of CH_3CN followed by 10 ml of distilled water. The eluates were discarded and the cartridge was eluted with 5 ml of *n*-hexane. Prior to HPLC, this hexane solution was concentrated to dryness under a nitrogen stream, and the residue was redissolved in 1 ml of CH_3CN, which was then filtered through 0.5-μm pore-size MFS-25 PTFE filters [Micro Filtration Systems (MFS)].

3.2. Spectrofluorimetry

For optimization of the purification procedure, BP was determined by constant-wavelength synchronous spectrofluorimetry [34].

3.3. Chromatographic method

An aliquot (20 μl) of the acetonitrile solution was injected into the HPLC system and eluted with acetonitrile–water (85:15, v/v) at a constant flow-rate of 0.5 ml/min. To quantify the BP, the detector was set at excitation wavelength 294 nm and emission wavelength 404 nm.

3.4. Confirmation of results

That the HPLC peak corresponded to BP was confirmed by two approaches.

(a) By HPLC under the conditions described but with emission wavelength of 424 nm (a slightly less intense emission band should be detected for BP).

(b) By setting the detector to sweep mode, recording emission and synchronous spectra of the eluting peak and comparing them with those of standards. For emission spectra, the excitation wavelength was 294 nm, starting emission wavelength 350 nm, scan length 100 nm, and step size 2 nm. For constant-wavelength synchronous spectra, scan type was delta (excitation emission wavelength difference=110 nm), starting excitation wavelength 250 nm, starting emission wavelength 360 nm, scan length 100 nm, and step size 2 nm.

Since these experiments require BP concentrations >1 μg/l (synchronous spectra) or >2 μg/l (emission spectra), for samples found to contain concentrations below these limits at the quantification stage, the remaining solution was concentrated before injection.

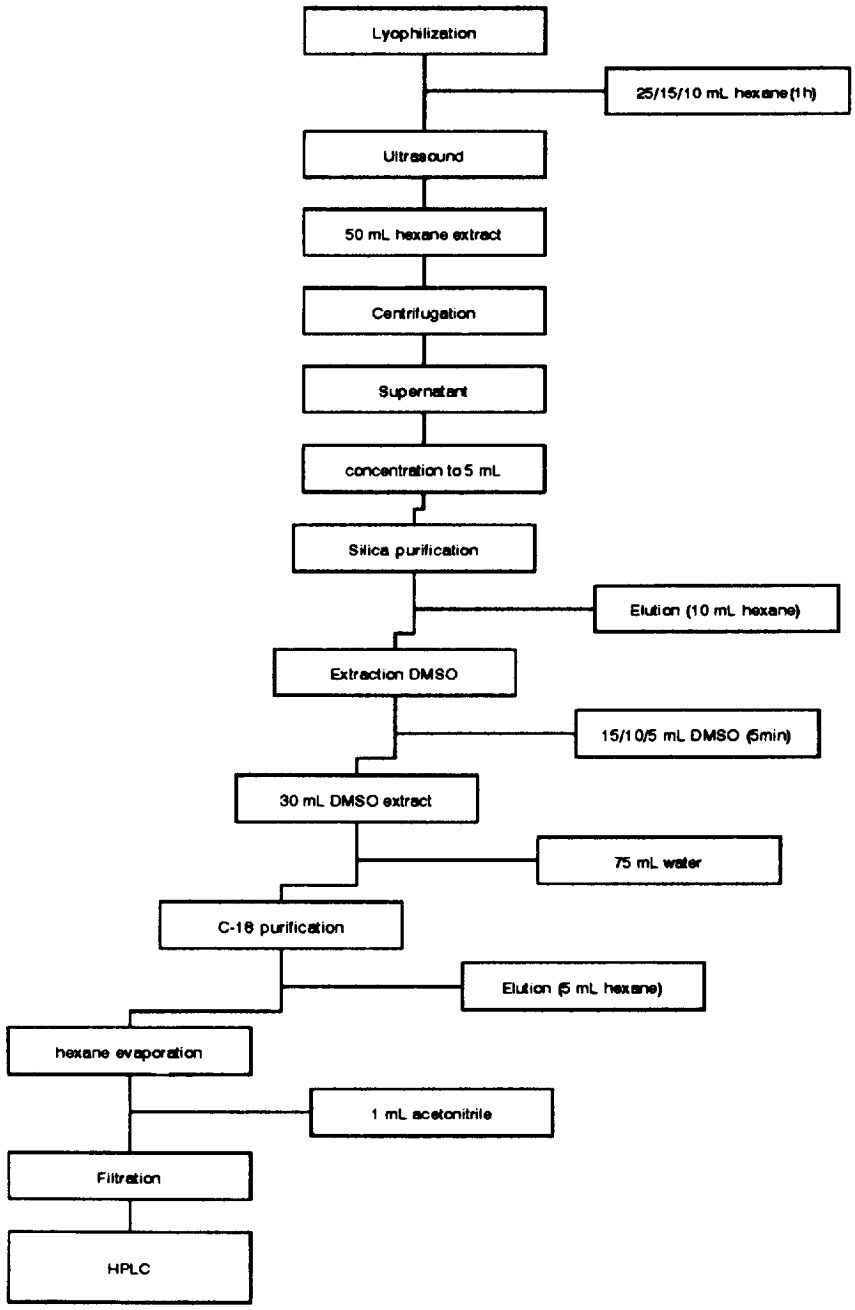

Fig. 1. Flow-chart summarizing treatment sample prior to HPLC analysis of BP.

4. Results and discussion

4.1. Extraction

Lyophilization of the smoked food products is necessary because otherwise water in the sample causes problems in the extraction step. In combination with ultrasound, extraction of the dried material with hexane allows easy isolation of the lipid fraction, including PAH components. Hexane was chosen as extractant because (a) it has been very widely used for extraction of organic compounds,

among them PAHs; and (b) it is an excellent solvent for fluorimetric determination of PAHs [35]. Ultrasound has previously been used for extraction of PAHs from plant tissues and soils [36,37], but we could find no references to its use for extraction of PAHs from foodstuffs. Note that hexane extraction following saponification with potash produced emulsions that were very difficult to break up.

4.2. Clean-up

In order to obtain a suitably pure and concentrated sample for determination of BP, several clean-up steps were required, each of which was optimized by determining BP in the cartridge eluates by synchronous spectrofluorimetry. This method was sufficiently precise (although it in fact has a RSD higher than the HPLC method) and, moreover, considerably speeded up the optimization process. In the first step, impurities apparently implicated in emulsification are removed by passage of the concentrated extract through a silica cartridge. The volume of *n*-hexane eluant was optimized for BP elution: aliquots (5 ml) of a solution of 8 μg/l BP in *n*-hexane were applied to the cartridge and then eluted with an increasing volume of *n*-hexane, and the BP in the eluant was determined by synchronous spectrofluorimetry. As little as 6 ml of hexane gave 100% recovery; however, to ensure complete recovery, 10 ml (total volume 15 ml) was used. Using this volume, assays were carried out (in sextuplicate) on of 5 and 20 ng standards; recoveries were 100 and 97.5%, and RSD values were 5.3 and 4.1%, respectively.

Next, the hexane eluate is extracted with DMSO. Partitioning of PAHs in food samples between hexane and DMSO (for which PAHs have greater affinity) has been described previously [14,15], and here allows elimination of lipid components that, in view of the fatty nature of the samples under study, are potentially a major source of interference. Emulsions obtained in this step are broken up by adding a few drops of 2% (w/v) NaCl solution. (It was noted that the tendency to emulsification increases with the time allowed to elapse between lyophilizing the sample and beginning the enrichment procedure.) BP recovery in this extraction step was spectrofluorimetrically determined for 1, 4 and 8 μg/l standard solutions extracted for 5 min each with 15, 10 and then 5 ml of DMSO that had previously been equilibrated with *n*-hexane. Mean ($n=6$) recoveries were 98.0, 101 and 99.0%, and RSD values were 5.4, 2,2 and 1.2%, respectively.

The final stage of clean-up involves dilution of the DMSO extract with water, and passage of this solution through an activated Sep-Pak C_{18} Plus cartridge. Careful balancing of the polarity of the solution allows simultaneous elimination of polar substances and concentration of the BP, which is retained on the cartridge for later elution with *n*-hexane. The results of experiments to maximize BP retention are shown in Fig. 2: 100% retention was obtained with a DMSO–water ratio of 1:2.4 (RSD 2.9%, $n=6$). Complete elution of the BP required 5 ml of *n*-hexane [24].

4.3. Confirmation of results

(a) Under the conditions described, a slightly less intense emission band than that used for BP quantification is detected, as expected (Fig. 3). The ratio of the areas of this peak and that obtained with emission wavelength 404 nm was constant and indepen-

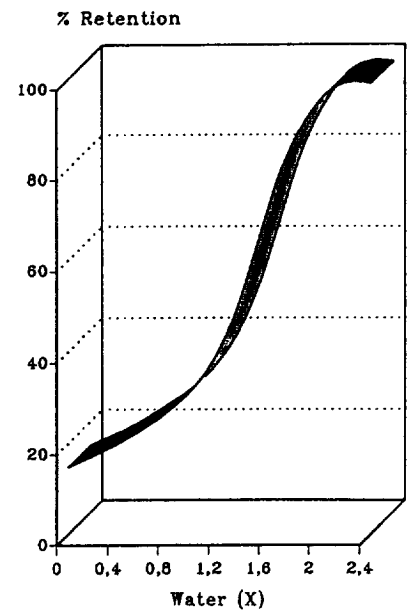

Fig. 2. Optimization of the polarity of the DMSO–water (1:*X*) mixture: cumulative retention (% retention) as a function of water fraction *X*. For DMSO–water (1:2.4), RSD = 2.9% ($n=6$).

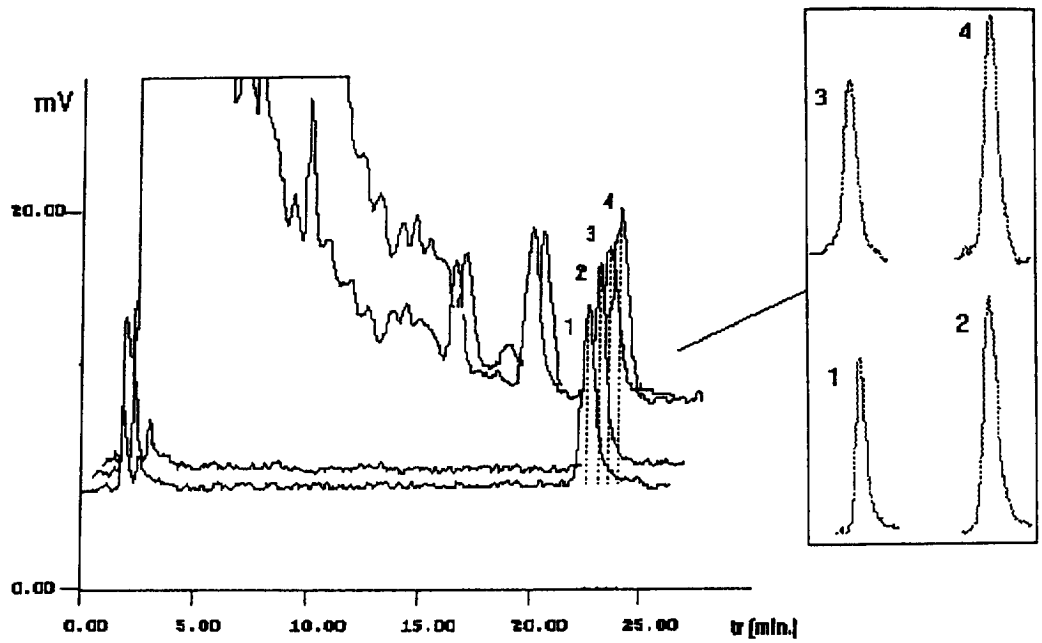

Fig. 3. HPLC–FL chromatograms of a 0.5 μg/l, benzo[*a*]pyrene (BP) standard obtained with: (1) $\lambda_{exc}=294$, $\lambda_{em}=424$, and (2) $\lambda_{exc}=294$, $\lambda_{em}=404$; and of sausage sample obtained under corresponding conditions (3) and (4), respectively. Mean peak-area ratios: 1/2=1.33; 3/4=1.30.

dent of BP concentration (mean peak-area ratio= 1.33, RSD 7.9%, $n=6$).

(b) For sample 6, emission and synchronous spectra of the eluting peak are compared with those of a BP standard in Fig. 4 Fig. 5, respectively.

4.4. Quantification and the linearity of the instrumental response

The signal due to BP was identified by comparison of sample chromatograms with the chromatogram of

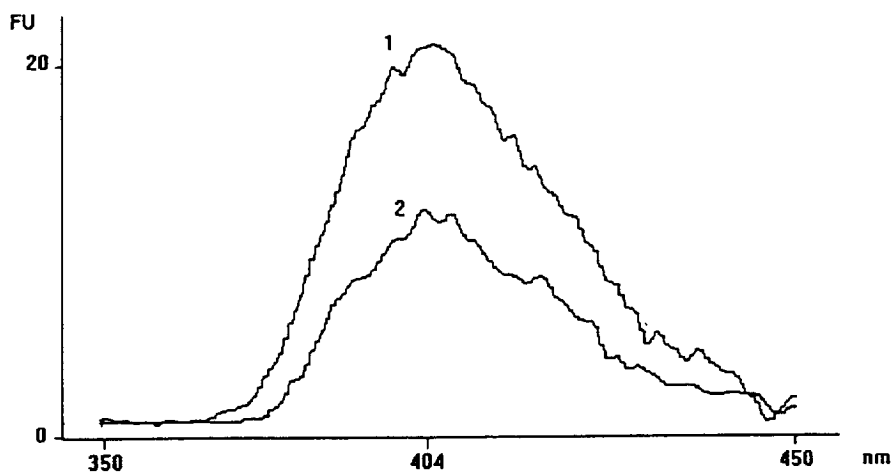

Fig. 4. Fluorescence emission spectra of (1) 3 μg/l benzo[*a*]pyrene in CH_3CN; and (2) sausage sample 6.

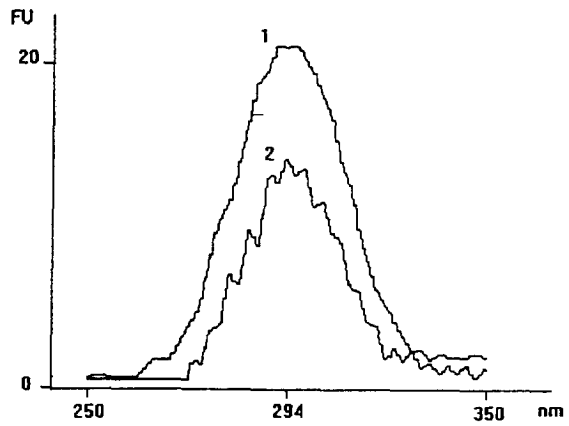

Fig. 5. Constant-wavelength synchronous fluorescence spectra of (1) 3 μg/l benzo[*a*]pyrene in CH_3CN; and (2) sausage sample 6.

the BP standard. Quantification was by the external standard method.

The calibration line was constructed by regressing mean ($n=3$) peak height on standard concentration (0.25–16 μg/l in CH_3CN). Response was highly linear ($r=0.9997$).

The calibration equation,

$$[BP]\ (\mu g/l) = 5.52 \cdot 10^{-5}\ \text{Height} - 0.0523$$

4.5. Detection and quantification limits

Detection and quantification limits for BP in the acetronitrile eluate are 0.049 and 0.089 μg/l, respectively (calculated, following ACS guidelines [38] as the concentration corresponding to the signals equal to the mean signal for 10 blanks (570±104) plus respectively, three and ten standard deviations). Conversion of these values to μg BP per kg of whole sample required division by the mass of sample analysed. Depending on the moisture content of the smoked food product, the detection limit varied between 0.0019 and 0.0067 μg/kg, and the quantification limit between 0.0036 and 0.012 μg/kg (lower and upper limits for 80 and 30%, w/w, water, respectively).

4.6. Precision

The precision of the method was determined by applying the full procedure to six replicate subsamples of one type of sausage (sample 6) that, according to the packaging label, had been smoke-cured by a traditional method. Mean ($n=6$) BP found was 0.051 μg/kg of smoked sausage (RSD 9.6%).

4.7. Recovery

Firstly, it was confirmed that the BP levels in a type of, according to the label, unsmoked sausage (63% water) were below the detection limit. Then, as a measure of the accuracy of the method, the mean recovery was calculated for six replicate subsamples of this unsmoked sausage spiked with 0.03 μg/kg of BP – the maximum BP level permitted in food or drink treated with a smoking-flavour agent and subjected to the full procedure. Mean ($n=6$) recoveries were 94.16% (RSD 8.65%).

The procedure developed was used to determine the BP in 15 types of sausage three of which (samples 1–3) had been treated with a smoking-flavour agent, while the other 12 (samples 4–15) had been treated with wood smoke. The results are given in Table 1, BP content varied from not detected (ND; sample 12) to 0.051 μg/kg (sample 6), the mean BP content being 0.022 μg/kg. For samples treated with smoking-flavour agents, all values were below the 0.03 μg/kg limit in EU legislation for BP derived from such agents; in all but two cases (samples 6 and 14), the BP content of smoke-cured samples was also below this limit.

5. Conclusions

We developed an extraction/clean-up procedure for trace enrichment of benzo[*a*]pyrene (BP) in smoked food products and an HPLC–FL method to determine the levels of BP in those enriched extracts. The detection limit is <0.0067 μg/kg and the quantification limit is <0.012 μg/kg (values for samples containing 30% water). Recovery is 94.1%, and RSD (precision) 8.65%. When the procedure was applied to 15 samples of smoked sausages, mean BP content was around 0.02 μg/kg, and all but two samples (both smoke-cured) had BP contents below the limit imposed in EU legislation for smoking-flavour agents.

Table 1
Nature, moisture content (%, w/w, water) and benzo[*a*]pyrene (BP) content of the 15 smoked sausage samples

Number sample	Sample	% Water	BP (μg/kg)
1	Cooked sausage	59.64	0.021
2	Cooked *frankfurter* style sausage	58.71	0.026
3	Cooked *frankfurter* style sausage	58.10	0.021
4	*Frankfurter* style sausage	54.97	dis.
5	*Frankfurter* style sausage	62.97	0.021
6	*Frankfurter* style sausage	66.90	0.051
7	*Frankfurter* style sausage	58.15	0.027
8	*Frankfurter* style sausage with bacon-cheese	62.16	0.016
9	Chorizo style *frankfurter*	46.66	0.027
10	*Vienna* style sausage	52.82	0.030
11	*Vienna* style sausage with bacon-cheese	63.97	0.008
12	*Cocktail* sausage	66.95	ND
13	*Cocktail* sausage	58.17	0.020
14	Sausage with Parmesan cheese	49.91	0.040
15	"*Snackis*" sausage	59.52	0.025

ND = not detected.
dis. = detection disturbed, quantification not possible.

Acknowledgments

The authors thank the Consellería de Educación y Ordenación Universitaria of the Xunta de Galicia (N.W. Spain), for the concession of bursaries to M.J. López de Alda Villaizán and M.S. García Falcón.

References

[1] S.K. Katiyar, R. Agarwal and H. Mukhtar, in H.S. Rathore and J. Sherma (Editors), CRC Handbook of Chromatography: Liquid Chromatography of Polycyclic Aromatic Hydrocarbons, CRC Press, Boca Raton, Florida, 1993, pp. 1–17.
[2] R. Derache, in Toxicology y Seguridad de los Alimentos, Ed. Omega, Barcelona, 1990, pp. 295–317.
[3] J.L. Multon, in Aditivos y Auxiliares de Fabricación en las Industrias Agroalimentarias. Ed. Acribia., Zaragoza, 1987, pp. 503–505.
[4] A. Beltrán García-Echaniz, Alimentación: equipos y tecnologia, (1989) 187–190.
[5] A. Beltrán García-Echaniz, Alimentaria, Junio (1989) 45–49.
[6] World Health Organization (WHO), Guidelines for drinking-water quality, 1, Geneva, (1985) 70–72.
[7] Council of the European Communities, Directive 88/388/EEC, Off. J. Eur. Communities, L 184 (1988) 61–66.
[8] Council of the European Communities, Directive 91/493/EEC, Off. J. Eur. Communities, L 268 (1991) 15–34.
[9] D.O. Alonge, J. Sci. Food Agric., 43 (1988) 167–172.
[10] B.K. Larsson, H. Pyysalo and M. Sauri, Z. Lebsensm. Unters. Forsch., 187 (1988) 546–551.
[11] K. Speer, E. Steeg, P. Horstmann, Th. Kühn and A. Montag, J. High Resolut. Chromatogr., 13 (1990) 104–111.
[12] K. Speer, P. Horstmann, E. Steeg, Th. Kühn and A. Montag, Z. Lebensm.-Unters.-Forsch., 191 (1990) 442–448.
[13] M.J. Dennis, M.I. Massey, G. Cripps, I. Venn, N. Howarth and G. Lee, Food Add. Contam., 8 (1991) 517–530.
[14] E. Meninchini, A. Bocca, F. Merli, D. Ianni and F. Monfredini, Food Add. Contam., 8 (1991) 363–369.
[15] E. Meninchini, A. Di Domenico and L. Bonanni, J. Chromatogr., 555 (1991) 211–220.
[16] W. Pfannhauser, Mitt. Gebiete Lebensm. Hyg., 82 (1991) 66–79.
[17] J.J. Vreuls, G.J. De Jong and U.A.Th. Brinkman, Chromatographia, 31 (1991) 113–118.
[18] G.A. Perfetti, J.P. Nyman, S. Fisher, F.L. Joe, Jr. and G.W. Diachenko, J. AOAC Int., 75 (1992) 872–877.
[19] B. Saint-Aubert, J.F. Cooper, C. Astre, J. Spiliotis and H. Joyeux, J. Food Compos. Anal., 5 (1992) 257–263.
[20] E.A. Gomaa, J.I. Gray, S. Rabie, C. López-Bote and A.D. Booren, Food Add. Contam., 10 (1993) 503–521.
[21] H. Yuco YabiKu, M. Satou Martins and M. Yanzasaki Takahashi, Food Add. Contam., 10 (1993) 399–405.
[22] V. Akpan, M. Lodovici and P. Dolara, Bull. Environ. Contam. Toxicol., 53 (1994) 246–253.
[23] H.A.M.G. Vaessen, C.G. Van De Kannp and A.A Jekel, Polycyclic Aromat. Compd. 3 (Suppl.) (1993) 65–75.
[24] M.S. García Falcól, S. González Amigo, M.A. Lage Yusty, M.J. López de Alda Villaizán and J. Simal Lozano, Talanta in press.
[25] M.A. Quilliam and P.G. Sim, J. Chromatogr. Sci., 26 (1988) 160–167.

[26] G.W. Kelly, K.D. Bartle, A.A. Clifford and O. Scammell, J. Chromatogr. Sci., 31 (1993) 73–76.
[27] M.J. López De Alda-Villaizán, S. García-Falcón, M.A. Lage-Yusty and J. Simal-Lozano, J. AOAC Int., 78 (1995) 402–406.
[28] K. Takatsuki, S. Suzuki, N. SATO and I. Ushizawa, J. Assoc. Off. Anal. Chem., 68 (1985) 945–949.
[29] J.A. Lebo, J.L. Zajicek, T.R. Schwartz, L.M. Smith and M.P. Beasley, J. Assoc. Off. Anal. Chem., 74 (1991) 538–544.
[30] H. Seto, T. Ohkubo, T. Kanoh, M. Koike, K. Nakamura and Y. Kawahara, Archiv. Environ. Contam. Toxicol., 24 (1993) 498–503.
[31] D.A. Birholz, R.T. Coutts and S.E. Hrudey, J. Chromatogr., 449 (1988) 251–260.
[32] J. Dujmov, P. Sucevic and M. Tonkovic, Toxicol. Environ. Chem., 46 (1994) 73–80.
[33] H.A. Bravo, S.L. Salazar, A.V. Botello and E.F. Mandelli, Bull. Environ. Contam. Toxicol., (1978) 171–176.
[34] J. Simal Lozano, M.A. Lage Yusty, L. Vázquez Odériz and M.J. López de Alda-Villaizán, J. Framçais d'Hydrologie, 23 (1) (1992) 37–55.
[35] M.J. López de Alda-Villaizán, E. Álvarez Piñeiro, S. García Falcón, M.A. Lage Yusty and J. Simal Lozano, Anulusis, 22 (1994) 495–498.
[36] J.T. Coates and A.W. Elzerman, J. Assoc. Off. Anal. Chem., 69 (1986) 110–114.
[37] U. Hechler, J. Fischer and S. Plagemann, Fresenius J. Anal. Chem., 351 (1995) 591–592.
[38] American Chemical Society (Subcommittee on Enviromental Analytical Chemistry), Anal. Chem., 52 (1980) 2241–2248.