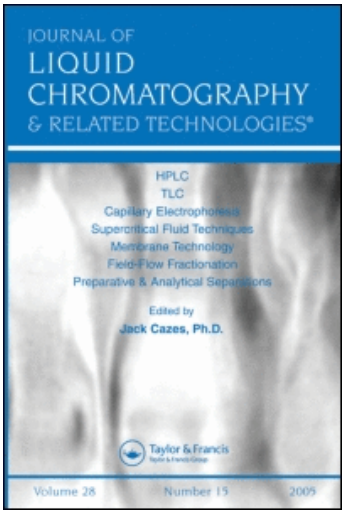

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DETERMINATION OF SELECTED POLYCYCLIC AROMATIC HYDROCARBONS IN TOASTED BREAD BY SUPERCRITICAL FLUID EXTRACTION AND HPLC WITH FLUORIMETRIC DETECTION

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DETERMINATION OF SELECTED POLYCYCLIC AROMATIC HYDROCARBONS IN TOASTED BREAD BY SUPERCRITICAL FLUID EXTRACTION AND HPLC WITH FLUORIMETRIC DETECTION

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ABSTRACT

A new method for PAHs determination in toasted bread samples is proposed. The method is based on supercritical-fluid extraction, using CO_2 and acetonitrile as modifier. The extracted PAHs were collected in 1 mL acetonitrile. Quantitation was carried out by HPLC with fluorimetric detection. A Hypersil Green PAH column, acetonitrile-water gradient mobile phase, and a program of 11 excitation and emission wavelength pairs for fluorimetric detection were used. Recoveries at concentration levels in the range 0.15-3.56 μg/kg bread were close to 100% for all PAHs except fluoranthene, chrysene, and benzo[*ghi*]perylene. Some PAHs were detected in these samples within the range 0.323-9.40 μg/kg toasted bread; the relative standard deviations were in the range 2 - 12% (n=4).

INTRODUCTION

As it is well known, polycyclic aromatic hydrocarbons (PAHs) are included in the European Community (EC) and in the Environmental Protection Agency (EPA) priority pollutant lists, due to their mutagenic and carcinogenic properties. There are quite a range of sources of PAHs contamination in foods, contaminated soils and water, polluted air, food processing such as roasting, smoking, and frying, contribute to PAHs concentration levels in different foods.[1-3] There is much investigation on PAHs determination in environmental samples but not so much in food samples.[4-6] PAHs determination in food samples is based generally on a previous treatment with alkali in methanol-water, followed by liquid-liquid extraction (LLE); when necessary, a clean-up step is carried out on alumina, florisil, or silica columns. Solid-phase extraction (SPE) on florisil or C-18 cartridges has been also used.[7-10] Supercritical-fluid extraction (SFE) has found an extensive use in PAHs determination in environmental samples, but in food samples only a study on PAHs determination in smoked and broiled fish has been found;[11] regarding bread samples, only benzo[*a*]pyrene has been determined.

The procedures used are based on extraction in c-hexane or n-hexane, followed by a clean-up step on silica gel or alumina columns; determinations were carried out by GC-MS or fluorimetry.[12, 13]

In this paper, we propose a rapid method for PAHs determination in toasted bread samples, based on supercritical-fluid extraction (SFE) followed by RP-HPLC with fluorimetric detection.

EXPERIMENTAL

Chemicals

PAHs analytical standards: naphthalene, acenaphthene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo[*e*]pyrene, benzo[*a*]pyrene, dibenzo [*a,h*]anthracene, and benzo[*ghi*]perylene from Sigma (St. Louis, MO). Stock standard solutions containing 200-795 mg/L were prepared by dissolving the solid product in methanol. The PAHs working standard mixture was prepared by dilution of the stock standard solutions with methanol according to their fluorimetric properties.

Solvents: HPLC grade acetonitrile and methanol from Scharlau (Barcelona, Spain) were used. Other solvents and chemical reagents were also of HPLC purity. Water used was obtained by means of a Milli-Q apparatus from Millipore (Milford, MA). CO_2 supercritical fluid used was from Carburos Metálicos (Madrid, Spain).

Apparatus and Material

The HPLC system consisted of the following components: a Milton-Roy CM 4000 high-pressure-gradient pump (Rivera Beach, FL); a Rheodyne 7125 with a 20 µL loop injector (Cotati, CA); a Perkin Elmer LS 30 luminescence spectrometer (Norwalk, CT), and a Milton Roy CI 4100 integrator were used. For PAHs separation a Hypersil Green PAH (100 x 4.6 mm) 5 µm particulate size column by Shandon (England) was used; to maintain the column temperature at 23°C a P-Selecta Precisterm bath (Barcelona, Spain) was used.

A Star SFE Varian (Sunnyvale, CA) supercritical-fluid extraction system for PAHs extraction from toasted bread samples and a Cisa sieve, (Spain) were also used. A P-Selecta ultrasonic bath was also used for the preparation of PAHs solutions.

The solvents used to prepare the mobile phase and sample extracts were filtered through nylon membrane filters with a 0.45 µm pore size (Ann Arbor, MI) and PTFE membrane filters with a 0.5 µm pore size MFS-13 (MFS, Dublin, CA), respectively. The mobile phase was degassed with helium.

Bread Samples

Four toasted bread samples, all of them available at the supermarket, were analyzed.

Procedure

Supercritical-Fluid Extraction

The toasted bread sample was triturated and screened through a sieve with a 0.1-1 mm pore size. Then, 2 g of the sample modified with 0.5 mL of acetonitrile were placed in the SFE vessel, extracted with CO_2 firstly statically at 60°C and 300 atm for 2 min, and then dynamically for 15 min; the PAHs were collected in 1 mL of acetonitrile. This extract was filtered through a PTFE membrane filter and then analyzed by RP-HPLC by injecting 20 µL. The HPLC method described below was applied for the PAHs quantitation.

For recovery studies, the sample toasted bread (2 g) was spiked with a PAHs mixture in the range 0.150-28.5 µg/kg.

HPLC Method

The acetonitrile-water mobile phase gradient specified in Table 1, with a flow-rate of 0.8 mL/min at 23°C, and the program of eleven excitation and

Table 1

Gradient of the Mobile Phase

Time (Min)	Acetonitrile (%)	Water (%)
0	55	45
2	55	45
20	90	10
28	100	--
36	100	--
36.1	55	45

emission wavelength pairs specified in Table 2, were used An injection volume of 20 μL was used, and PAHs quantitation was attained from data on the peak areas. Calibration graphs at five concentration levels were prepared from standard solutions containing PAHs in the range 0.300 - 114 μg/L.

RESULTS AND DISCUSSION

Chromatographic Analysis

Eleven PAHs were selected for this study: naphthalene, acenaphthene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo[*e*]pyrene, benzo[*a*]pyrene, dibenzo[*a,h*]anthracene, and benzo[*ghi*]perylene. Based on previous studies,[4-6,9,10] an acetonitrile/water mobile phase was selected.

Table 1 shows the gradient optimized at a flow-rate of 0.8 mL/min at 23°C using the Hypersil Green PAH column specified in the experimental section. In order to attain maximum sensitivity, a program of eleven selected excitation and emission wavelength pairs, was optimized; Table 2 shows the program. The chromatogram obtained in these conditions is shown in Figure 1.

Analytical Characteristics of the Chromatographic Method for Standards

Table 3 summarizes the analytical characteristics of the proposed chromatographic method for standards. Five PAH concentration levels were used to prepare calibration graphs in the range 0.3-114 μg/L. Linearity was found in all cases, with regression coefficients close to 0.999. The relative standard deviation percentages (RSD, %) were determined from four replicates, at concentration levels in the range 1.20-28.5 μg/L, obtaining values between 0.43 - 7.7%.

Table 2

Program of Excitation and Emission Wavelength Pairs

Detected Compound*	Time (Min)	λ_{ex} (nm)	λ_{em} (nm)
Naphthalene	0	270	335
Acenaphthene	8.7	285	330
Phenanthrene	11	250	365
Anthracene	11.9	254	402
Fluoranthene	13.5	285	465
Pyrene	14.5	270	390
Chrysene	17.2	270	384
B[*e*]P	19	290	390
B[*a*]P	21	295	405
Db[*a,h*]a	22.5	290	395
B[*ghi*]P	23.8	300	420

* B[*e*]P, Benzo[*e*]pyrene; B[*a*]P, Benzo[*a*]pyrene; Db[*a,h*]a, Dibenzo[*a,h*]anthracene; B[*ghi*]P, Benzo[*ghi*]perylene.

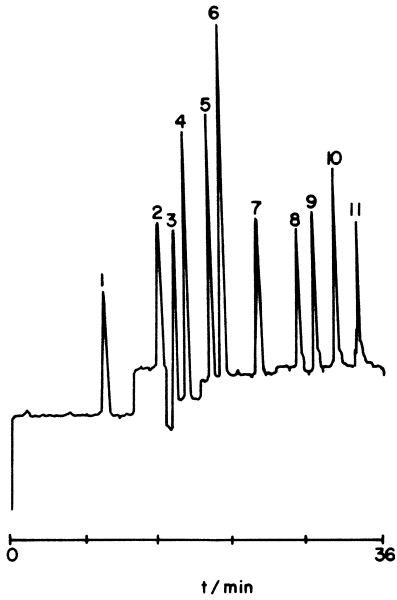

Figure 1. Chromatogram of a standard mixture of eleven PAHs. Conditions: Hypersil Green PAH (100 x 4.6 mm) column; Temperature, 23°C; Mobile phase, gradient of acetonitrile/water, see Table 1; Flow-rate, 0.8 mL/min; Fluorimetric detection, see Table 2; Injection volume, 20 µL. Peaks: 1, Naphthalene; 2, acenaphthene; 3, phenanthrene; 4, anthracene; 5, fluoranthene; 6, pyrene; 7, chrysene; 8, B[*e*]p; 9, B[*a*]p; 10, Db[*a,h*]a; 11, B[*ghi*]p.

Table 3

Analytical Characteristics of Chromatographic Method

Peak	PAH	Linearity Range µg/L[a]	RSD[b] (%)	DL[c] ng/L	t_R/min	RSD[d] (%)
1	Naphthalene	7.12 -114	0.95	11	6.49	1.5
2	Acenaphthene	2.42 - 38.7	7.6	2.9	10.45	1.5
3	Phenanthrene	2.00 - 32.0	0.43	3.1	10.58	1.5
4	Anthracene	0.75 - 12.1	1.4	0.34	12.40	1.0
5	Fluoranthene	0.51 - 8.16	2.6	0.40	14.12	1.0
6	Pyrenen	1.00 - 16.0	7.4	0.40	15.10	0.5
7	Chrysene	0.54 - 8.64	1.5	0.47	17.51	1.4
8	B[*e*]P	0.55 - 8.88	1.3	0.24	20.38	1.5
9	B[*a*]P	0.30 - 4.80	0.86	0.20	21.56	1.6
10	Db[*a,h*]a	1.25 - 20.1	0.75	4.0	23.10	1.6
11	B[*ghi*]P	0.70 - 11.3	7.7	1.5	24.60	1.0

[a] Studied range. [b] Relative standard deviation at a concentration level in the middle of the range studied (n=4). [c] Detection limit, DL = 3S/N. [d] Relative standard deviation of retention time (n=4).

Detection limits (DL), evaluated as three times the signal to noise ratio (3 S/N), ranged from 0.2 ng/L for benzo[*a*]pyrene to 11 ng/L for naphthalene. The retention times and their relative standard deviation are also specified in Table 3.

Optimization of Variables in Supercritical-Fluid Extraction

Variables such as time, pressure, and temperature of extraction were optimized. The nature and volume of the solvent used to collect the PAHs from the samples, and the organic modifier effect to improve extraction were also optimized.

In all experiments, 2 g of the toasted bread sample, number 1, spiked with PAHs in the range 0.6-14.2 µg/Kg, were placed in a SFE vessel. The PAHs were collected in a vial containing the solvent, and the registered chromatograms were compared with a reference chromatogram of the PAHs standard mixture.

The following extraction time values were tested: 2 and 10 minutes in static mode and, 15 and 45 minutes in dynamic mode, at 300 atm and 60°C. The

best results were obtained when static extraction and dynamic extraction were carried out for 2 and 15 minutes, respectively. The chromatographic peak total areas decreased 20% for higher times in the dynamic mode; the losses observed of the analytes may be due to volatilization during the CO_2 bubbling through acetonitrile.

Regarding the extraction pressure, pressures of 250, 300, and 400 atm were applied. According to the obtained results, a pressure of 300 atm was selected. At this pressure, the selectivity most favorable was achieved, and extraction of bread endogenous compounds was minimum. At 250 atm the chromatographic peak total areas were lower, and, although at 400 atm the total areas of higher molecular weight PAHs increased, the extraction of interference compounds was also favored, decreasing thus, the selectivity of the developed method.

On the other hand, temperatures of 50, 60, and 100°C were tested, 60°C being selected, given that at this temperature the total areas of the chromatographic peaks were higher, increasing the sensitivity of the developed method. Higher temperatures were not adequate due to the nature of the analyzed samples.

Regarding the nature and volume of the solvent used to collect the PAHs, acetonitrile, methanol (1 and 2 mL), and methylene chloride (2 and 5 mL) were tested. Given that an acetonitrile-water mobile phase was used, acetonitrile was selected for PAHs collection from the sample, also taking into account the better recoveries obtained, as well as a lower volatilization during CO_2 bubbling than for methanol and methylene chloride, which gave rise to a higher reproducibility.

As reported in the literature,[11,14] the use of organic modifiers improves the efficiency of the SFE for PAHs, in particular for those PAHs containing more than three rings, because their solubility increases by adding the modifiers directly into the sample, also increasing the extraction selectivity. Modifiers such as methanol, acetonitrile, methylene chloride, ethanol, and a methylene chloride/acetonitrile mixture were tested using volumes in the range 0.1-1 mL.

The best results for most of the PAHs were obtained with 0.5 mL of acetonitrile or of a methylene chloride/acetonitrile mixture (v/v = 1/1), with a 22 and a 25% increase, respectively, in the chromatographic peak total areas. Acetonitrile was selected because an increase in the extraction selectivity was also observed with this solvent.

The studied variables and the obtained results are summarized in Table 4.

Table 4

Organization of Variables in Supercritical Fluid Extraction

Variable	Studied Values	Recommended
Program		
Pressure, atm	250, 300, and 400	300
Temperature, °C	50, 60, and 100	60
Static time, min	2 and 10	2
Dynamic time, min	15 and 45	15
Collection solvent, mL		
Acetonitrile	1 and 2	1
Methanol	1 and 2	
Methylene chloride	2 and 5	
Modifier, mL		
Methanol	0.5 and 1.0	
Acetonitrile	0.1, 0.2, 0.5 and 1.0	0.5
Methylene chloride	0.5 and 1.0	
Ethanol	0.5 and 1.0	
Methylene chloride/ acetonitrile (v/v = 1/1)	0.5 and 1.0	

Recovery Studies of PAHs from Toasted Bread Samples

Recovery studies were used to evaluate the validity of these results. The proposed optimized extraction method was applied to 2 g of toasted bread, sample number 1, spiked with PAHs at four concentration levels in the range 0.15-28.5 μg/kg. The attained results are shown in Table 5. These results were obtained considering the PAHs amounts in samples 1; the highest recoveries (close to 100%) were achieved for the lower concentrations tested; the relative standard deviations were in the range 1-12% (n=4).

As can be seen in Table 5, high molecular weight PAHs present lower recoveries in all cases, due to the fact that a higher pressure should be applied to solubilize them.[15] A typical chromatogram is shown in Figure 2.

Table 5

PAH Recoveries from Toasted Bread Samples*

PAHs	C_1	R_1 (%)	C_2	R_2 (%)	C_3	R_3 (%)	C_4	R_4 (%)
Naphthalene	3.56	100	7.12	100	14.2	100	28.5	88
Acenaphthene	1.21	100	2.42	72	4.84	92	9.70	70
Phenanthrene	1.00	100	2.00	100	4.00	100	8.00	100
Anthracene	0.38	92	0.76	72	1.51	72	3.02	74
Fluoranthene	0.25	80	0.51	65	1.02	66	2.04	70
Pyrene	0.50	100	1.00	64	2.00	78	4.00	74
Chrysene	0.27	84	0.54	56	1.08	50	2.16	84
B[*e*]P	0.28	100	0.55	95	1.11	94	2.22	70
B[*a*]P	0.15	100	0.30	50	0.60	67	1.20	64
Db[*a,h*]a	0.63	100	1.26	69	2.52	46	5.03	64
B[*ghi*]P	0.35	54	0.70	41	1.41	38	2.83	60

C_1, C_2, C_3, and C_4 (μg/kg): PAHs concentrations in spiked bread sample. * R_1 mean percentage recovery of four determinations. Relative standard deviations were in the range 1 - 12%. Toasted bread sample (number 1): 2 g.

Determination of PAHs in Toasted Bread Samples

The proposed method was applied to determine these PAHs in four types of toasted bread samples, available at the supermarket. The obtained results are summarized in Table 6. In all samples, naphthalene, acenaphthene, phenan-

Table 6

Determination of PAHs in Toasted Bread Samples (μg/kg)*

PAH	Samples 1	2	3	4
Naphthalene	6.63	5.37	6.95	9.40
Acenaphthene	0.516	1.20	1.23	2.08
Phenanthrene	2.73	2.32	1.65	4.05
Fluoranthene	---	---	---	0.175
B[*e*]P	---	---	---	1.25
Db[*a,h*]a	0.323	0.441	0.361	1.41

* n = 4; relative standard deviations were in the range 2 - 12%.

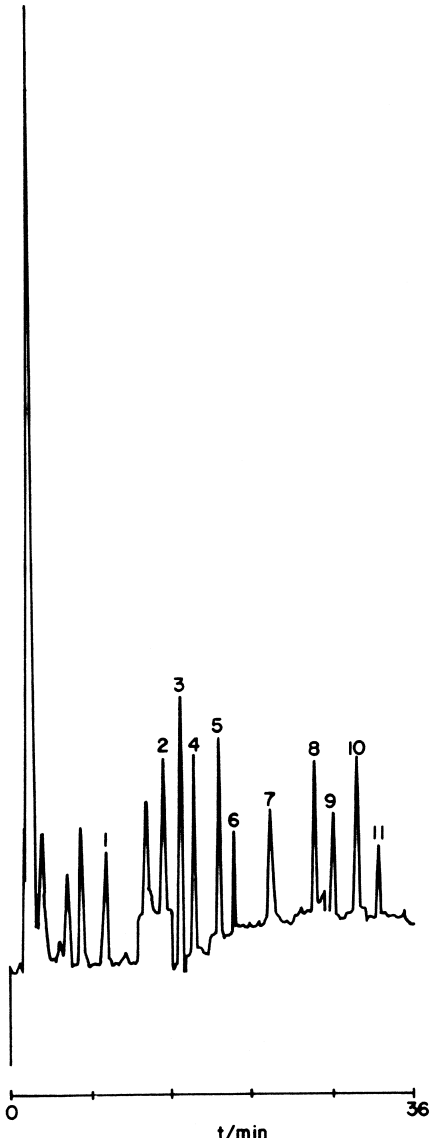

Figure 2. Chromatogram of a spiked toasted bread sample with PAHs standard. Conditions: Hypersil Green PAH (100 x 4.6 mm) column; Temperature, 23°C; Mobile phase, gradient of acetonitrile/water, see Table 1; Flow-rate, 0.8 mL/min; Fluorimetric detection, see Table 2; Injection volume, 20 µL. Peaks: 1, Naphthalene; 2, acenaphthene; 3, phenanthrene; 4, anthracene; 5, fluoranthene; 6, pyrene; 7, chrysene; 8, B[*e*]p; 9, B[*a*]p; 10, Db[*a,h*]a; 11, B[*ghi*]p.

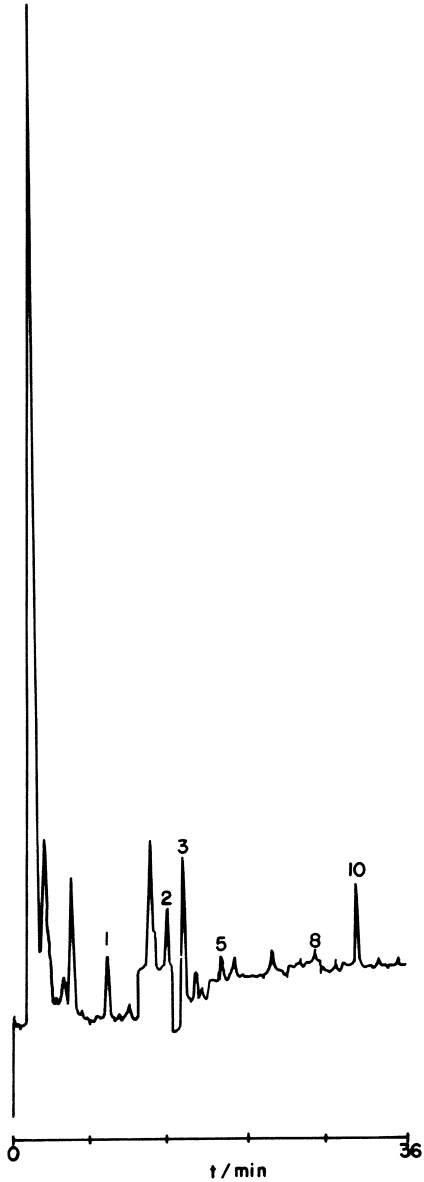

Figure 3. Chromatogram of a toasted bread sample. (Sample 4). Conditions: Hypersil Green PAH (100 x 4.6 mm) column; Temperature, 23°C; Mobile phase, gradient of acetonitrile/water, see Table 1; Flow-rate, 0.8 mL/min; Fluorimetric detection, see Table 2; Injection volume, 20 µL. Peaks: 1, Naphthalene; 2, acenaphthene; 3, phenanthrene 5, fluoranthene; 8, B[*e*]p; 10, Db[*a,h*]a.

threne, and dibenzo[*a,h*]anthracene were detected. Figure 3 shows the chromatogram obtained for toasted bread sample number 4. Fluoranthene and benzo[*a*]pyrene were only detected in this sample. The presence of these PAHs was confirmed by GC-MS. They were found at concentration levels of μg/kg bread with relative standard deviations within the range 2 - 12% (n=4).

CONCLUSIONS

The proposed method is rapid, selective, and sensitive enough for the determination of PAHs in toasted bread samples, where some PAHs were detected. The presence of acetonitrile as modifier, improved both solvent strength and selectivity in the PAHs extraction.

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