Pure & Appl. Chem., Vol. 63, No. 11, pp. 1659–1666, 1991.
Printed in Great Britain.

INTERNATIONAL UNION OF PURE
AND APPLIED CHEMISTRY

APPLIED CHEMISTRY DIVISION
COMMISSION ON OILS, FATS AND DERIVATIVES*

DETERMINATION OF BENZO[*a*]PYRENE IN OILS AND FATS BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Results of a collaborative study and the standardized method

(Technical Report)

Prepared for publication by

P. W. HENDRIKSE[1] and A. DIEFFENBACHER[2]

[1]Willem de Zwijgerlaan 29, 3136 AR Vlaardingen, The Netherlands
[2]Nestec Ltd, Central Quality Assurance Laboratory, Avenue Nestlé 55, CH-1800 Vevey, Switzerland

*Membership of the Commission during the period of the collaborative study (1987–89) was as follows:

Chairman: J. Beare-Rogers (Canada); *Vice-Chairman*: A. Dieffenbacher (Switzerland); *Secretary*: W. D. Pocklington (United Kingdom); *Titular Members*: P. W. Hendrikse (Netherlands); É. Kurucz (Hungary); G. Zwerenz (Austria); *Associate Members*: H. Brüschweiler (Switzerland); E. Fedeli (Italy); T. Hashimoto (Japan); G. Hølmer (Denmark); A. Karleskind (France); F. H. Koelman (Netherlands); Ö. Levin (Sweden); R. Manning (USA); R. Ohlson (Sweden); G. Ostermann (Germany); M. Pike (United Kingdom); A. Visapää (Finland); *National Representatives*: R. R. Brenner (Argentina); A. Johnson (Australia); P. Dysseler (Belgium); J. Pokorný (Czechoslovakia); J. V. Holm (Denmark); A. Gad (Egypt); R. Linko (Finland); J. P. Wolff (France); H. Wessels (Germany); D. Boscou (Greece); M. Jeránek (Hungary); A. Mathew (India); A. Sharkey (Ireland); A. Dolev (Israel); E. Tiscornia (Italy); S. Watanabe (Japan); S. Ong (Malaysia); A. Krab (Netherlands); L. Eyres (New Zealand); W. Zwierzykowski (Poland); R. Stoica (Romania); L. Du Plessis (South Africa); M. Gassiot Matas (Spain); T. Tuğlular (Turkey); A. E. Waltking (USA); V. L. Roubailo (USSR).

Determination of benzo(a)pyrene in oils and fats by reversed phase high performance liquid chromatography. Results of a collaborative study and the standardized method

Abstract - A description is given of the development by collaborative study of a standardized method for the determination of benzo[a]pyrene in crude and refined oils and fats by reversed phase high performance liquid chromatography and fluorimetric detection. The procedure is rapid and allows determination at levels of 1 - 10 µg/kg.

INTRODUCTION

In some cases crude edible oils have been contaminated with polycyclic aromatic hydrocarbons (PAHs). The light polycyclic aromatic hydrocarbons are removed during the usual refining steps, the heavy polycyclic aromatic hydrocarbons, however, have to be removed by a treatment with carbon.

In the oils and fats industry a reliable and rapid method to determine benzo[a]-pyrene is very important for process control purposes (range 1-5 µg/kg). A reversed phase high performance liquid chromatography (HPLC) method using fluorimetric detection has been developed and tested by means of a collaborative study.

The rapid determination of benzo[a]pyrene (BaP) can be used to estimate the level of the heavy polycyclic aromatic hydrocarbons in crude edible oils instead of a time consuming full polycyclic aromatic hydrocarbons analysis.

METHOD OF ANALYSIS

A suitable amount of oil dissolved in light petroleum or hexane is absorbed on an alumina column; subsequently any benzo[a]pyrene present is eluted with light petroleum. The final determination is carried out by reversed phase HPLC using fluorimetric detection. The method can be used to determine benzo[a]pyrene in crude and refined edible oils with a lowest limit of detection of about 0.1 µg/kg.

In order to establish the reliability of the method an international collaborative study has been organised.

COLLABORATIVE STUDY

In order to check the validity of the method as an international standard method for IUPAC and eventually for ISO[1], the method has been subjected to an international collaborative study by laboratories in 10 European countries.

Materials provided for the study were crude edible oils (fish oil and rapeseed oil) containing benzo[a]pyrene at three concentration levels (high, medium and low). Each concentration level was represented by two batches. Each sample was provided in duplicate (blind coded) so that participants received in total 24 samples. A statistical evaluation of the data was made for each level and for each type of sample separately in accordance to ISO 5725 - 1986 using a method published earlier (1).

RESULTS

Data screening From 18 laboratories data have been received for evaluation. Two laboratories submitted their results after the final closing date. These results were not included in the statistical evaluation. Eleven laboratories used exactly the prescribed method. Seven laboratories used different wavelengths or different HPLC conditions. It was decided to use only the statistical data obtained with the 11 laboratories which followed the prescribed method.
The data from these laboratories have been subjected to tests for outliers according to Cochran and Dixon.

Cochran tests As the 24 samples analyzed were in fact 12 pairs of corresponding samples, the differences between these blind (hidden) duplicates have been tested for stragglers and outliers according to Cochran's procedure.

Dixon tests After elimination of the significant outliers (i.e. significant at the 1% level) according to Cochran's test, the six batch averages per laboratory have been tested for stragglers and outliers according to Dixon's procedure.

Observation of the data resulted in the exclusion of the fish oil results of one laboratory. The outlying results have been excluded in the further statistical calculations.

[1] International Organisation for Standardization

Precision

In Tables 1 and 2 the summarized data for benzo[*a*]pyrene in Rapeseed oil and Fish oil respectively are given.

Table 1 Summarized data for BaP in Rapeseed oil (µg/kg)

Level	Batch	Actual value	Mean	Minimum	Maximum	Standard deviation
High	1	4.6	4.27	2.7	5.7	0.79
	2	5.1	4.52	2.7	5.6	0.76
Medium	1	2.8	2.50	1.3	4.1	0.65
	2	3.1	3.04	1.5	4.4	0.68
Low	1	0.8	0.79	0.0	1.7	0.40
	2	1.5	1.22	0.7	2.5	0.41

Table 2 Summarized data for BaP in Fish oil (µg/kg)

Level	Batch	Actual value	Mean	Minimum	Maximum	Standard deviation
High	1	5.2	4.63	2.9	6.6	0.95
	2	5.5	5.08	3.0	7.5	1.20
Medium	1	3.2	2.81	2.0	3.4	0.43
	2	3.7	3.34	2.2	4.3	0.66
Low	1	0.9	0.62	0.0	1.4	0.29
	2	1.4	1.13	0.6	1.5	0.28

In Tables 3 and 4 the batch averages at each concentration level in crude rapeseed oil and in crude fish oil respectively have been listed. Averages per concentration level have been calculated because the results of two batches at one concentration level are close enough to each other to be representative of the same concentration.

Table 3 Concentration levels, average recoveries (µg/kg) and precision parameters for Rapeseed oil [11 accepted laboratories]

Concentration level	Actual values	Averages		Repeatability			Reproducibility		
	Batch	Batch	Level	s_r	r	CV_r(%)	s_R	R	CV_R(%)
High	4.6	4.27	4.39	0.73	2.04	16.6	0.77	2.16	17.5
	5.1	4.52							
Medium	2.8	2.50	2.78	0.35	0.98	12.6	0.67	1.87	24.0
	3.1	3.04							
Low	0.8	0.79	0.99	0.27	0.76	27.4	0.41	1.14	41.0
	1.5	1.22							

Table 4 Concentration levels, average recoveries (µg/kg) and precision parameters for Fish oil [10 accepted laboratories]

Concentration level	Actual values	Averages		Repeatability			Reproducibility		
	Batch	Batch	Level	s_r	r	CV_r(%)	s_R	R	CV_R(%)
High	5.2	4.63	4.87	0.64	1.79	13.1	1.09	3.05	22.4
	5.5	5.08							
Medium	3.2	2.81	3.08	0.20	0.56	6.5	0.54	1.52	17.6
	3.7	3.34							
Low	0.9	0.82	0.98	0.24	0.67	24.6	0.29	0.80	29.4
	1.4	1.13							

S_r: repeatability standard deviation; r: repeatability limit; CV_r: repeatability coefficient of variation;
S_R: reproducibility standard deviation; R: reproducibility limit; CV_R: reproducibility coefficient of variation

DISCUSSION

The repeatability is better for the rapeseed oil than for the fish oil. The repeatability for the fish oil is comparable with the value obtained during the preliminary study within one industrial organisation (10 laboratories) reported separately to the Commission (2).

According to Horwitz (3) for an analytical method to be acceptable, the relative reproducibility (CV_R) should be about 45 % at the 1 µg/kg level, and 35 % at the 5 µg/kg level. The range of this criterion is fully met by the results in this study.

CONCLUSION

Reversed phase HPLC with fluorimetric detection is a rapid and sensitive method which allows reliable determination of the concentration of traces of benzo[*a*]pyrene in edible oils and fats.

After an extensive collaborative study it was concluded that the method meets the criterion for an analytical method to determine trace amounts of analyte as stated by Horwitz in 1982.

Based on the repeatability and reproducibility of the results obtained in the collaborative study the Commission has decided to adopt the method. The text of the standardized procedure is given on the following pages.

Acknowledgement

The authors wish to thank M.A.T. Kerkhoff, J.A.M. Arends and B. Folkersma of Unilever Research Laboratory Vlaardingen for their valuable practical assistance.

The Commission is indebted to the collaborators in Austria, Belgium, France, Federal Republic of Germany, Hungary, Italy, the Netherlands, Norway, Sweden and the United Kingdom for their participation and valuable cooperation.

REFERENCES

1. J. Zaalberg, J. Assoc. Off. Anal. Chem., 1989, **72** 34.

2. Applied Chemistry Division: Commission VI.3, Minutes of the Meeting in St Andrews, Scotland on 16th-18th August 1988, pp. A13 - A18.

3. W. Horwitz, Anal. Chem., 1982, **54** 67A.

2.608 DETERMINATION OF BENZO[a]PYRENE IN OILS AND FATS BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. SCOPE AND FIELD OF APPLICATION

This Standard describes a method for the determination of benzo[*a*]pyrene in crude or refined edible oils and fats by reversed phase high performance liquid chromatography (HPLC) with fluorimetric detection with a lowest limit of detection of 0.1 µg/kg.

2. PRINCIPLE

Absorption of a suitable amount of sample on an alumina column. Followed by elution of any benzo[*a*]pyrene present with light petroleum. Final analysis is carried out by reversed phase HPLC using a fluorimetric detector.

3. APPARATUS

3.1 Glass column for chromatography, length 30 cm, internal diameter 15 mm fitted with sintered glass discs and PTFE taps.
3.2 Waterbaths kept at 35 °C and 65 °C.
3.3 Flash evaporator (note 1).
3.4 High performance liquid chromatograph consisting of HPLC pump, injection valve with 10 and 20 µl sample loops, reversed phase column and electronic integrator with chart recorder.
3.5 Crimp top minivials of about 1 ml volume with teflon layered septa and aluminium caps.
3.6 Handcrimper for crimping the aluminium caps onto the minivials.
3.7 Disposable pipettes.
3.8 Columns for HPLC analysis:
Stainless steel pre-column, length 75 mm, internal diameter 4.6 mm, packed with Lichrosorb RP-18, 5 µm particle size.
Stainless steel reversed phase HPLC column, Vydac for PAHs, length 250 mm, internal diameter 4.6 mm.
3.9 Fluorimetric detector with excitation wavelength at 384 nm (slit 10 nm) and emission wavelength at 406 nm (slit 10 nm).

4. REAGENTS (NOTE 2)

4.1 Light petroleum (boiling point range 40 - 60 °C) or hexan, analytical grade, redistilled over potassium hydroxide pellets (4 g/l).
4.2 Acetonitrile, suitable for HPLC, e.g. Lichrosolv.
4.3 Tetrahydrofurane, suitable for HPLC, e.g. Lichrosolv.
4.4 Toluene, suitable for HPLC, e.g. Lichrosolv.
4.5 Sodium sulphate, analytical grade, granular, anhydrous.
4.6 Water, double distilled, filtered over a membrane filter of 0.45 µm pore size.
4.7 Alumina, activity grade 4, prepared as follows:
Neutral aluminium oxide, activity super I is deactivated by the addition of 10 ml distilled water to 90 g alumina. Be cautious because of the heat of reaction and the build-up of pressure. The vessel is shaken for about 15 min and equilibrated for 24 h before use. The alumina is stored in a closed vessel at ambient temperature.
4.8 Benzo[*a*]pyrene, purity 99.0 % (note 3).

CAUTION

Benzo[*a*]pyrene is a known carcinogen. All work using benzo[*a*]pyrene should be carried out in a fume hood and gloves should be worn to minimize exposure.

4.9 Preparation of Benzo[a]pyrene—standard solutions

4.9.1 Stock solution:
Weigh accurately about 12.5 mg benzo[*a*]pyrene in a graduated flask of 25 ml. Dissolve the benzo[*a*]pyrene in toluene and fill up to the mark. The benzo[*a*]pyrene concentration in the stock solution is about 0.5 mg/ml. When stored in the dark at 4 °C this standard stock solution is stable for at least 6 months.

4.9.2 Standard solutions:
Prepare benzo[*a*]pyrene standard solutions of about 0.2 µg/ml and 0.01 µg/ml by diluting aliquots of the standard stock solution (4.9.1) with acetonitrile.

5. PROCEDURE

5.1 Clean-up

5.1.1 Weigh to the nearest mg about 2 g of the oil sample into a graduated flask of 10 ml. Dissolve the oil in light petroleum and make up to the mark with light petroleum.

5.1.2 Fill the chromatography column (3.1) to half its height with light petroleum. Rapidly weigh 22 g of alumina, activity grade 4 (4.7), into a small beaker and transfer the alumina immediately to the column. Promote setting of the alumina by gently tapping the column.

5.1.3 Add anhydrous sodium sulphate on the top of the column in a layer of about 3 cm.

5.1.4 Open the tap and allow the light petroleum to become level with the top of the sodium sulphate layer.

5.1.5 Place a 20 ml graduated flask under the column.

5.1.6 Pipette 2.00 ml of the oil solution (5.1.1) onto the column. Rinse the column with minimal amounts of light petroleum allowing the solvent layer to run into the sodium sulphate layer between rinsings.

5.1.7 Elute the column with light petroleum with a flow of about 1 ml/min.

5.1.8 Discard the first 20 ml of eluate.

5.1.9 Collect a further 60 ml of eluate into a 100 ml round bottomed flask.

5.1.10 Concentrate the eluate in a waterbath of 65 °C to about 0.5 to 1.0 ml and transfer the concentrated solution into a pre-weighed (to the nearest 0.1 mg) crimp top minivial of about 1 ml (note 1).

5.1.11 Continue evaporation from the minivial in water of 35 °C under a gentle stream of nitrogen (about 25 ml/min) until nearly dry.

5.1.12 Rinse the round bottomed flask twice with about 1 ml of light petroleum and collect these rinsings quantitatively into the minivial.

5.1.13 Continue evaporation at 35 °C under a gentle stream of nitrogen till dryness.

5.1.14 Weigh the minivial to the nearest 0.1 mg and calculate the weight of the residue.

5.1.15 Stopper the minivial with the teflon layered septum and the aluminium cap and store at 4 °C.

5.2 High performance liquid chromatography

5.2.1 Elution solvent: Acetonitrile / water 88/12 (v/v)
Degass the eluant solvent to remove oxygen in order to avoid fluorescence quenching of the benzo[*a*]pyrene. Use helium purging.

5.2.2 Elute at a flow of about 1 ml/min.

5.2.3 Calibration curve (note 4):
Prepare a four point calibration curve by injecting 0.04 ng and 0.2 ng benzo[*a*]pyrene corresponding to 4 and 20 µl respectively of the standard solution (4.9.2) with the concentration of 0.01 µg/µl and by injecting 1 ng and 2 ng of benzo[*a*]pyrene by injecting 5 and 10 µl respectively of the standard solution (4.9.2) with the concentration of 0.2 ng/µl.
Use the peak area obtained by means of the integrator and chart recorder (4.4) to construct the calibration curve.

5.3 Sample analysis (note 5)

5.3.1 Inject 20 µl tetrahydrofuran into the minivial containing the cleaned residue (5.1.15).

5.3.2. Dissolve the residue in the vial in tetrahydrofuran by careful swirling, avoiding contact of the solvent with the septum.

5.3.3 Inject an accurately known volume of about 10 µl of the dissolved residue into the HPLC column and start the running of the chromatogram.

6. CALCULATION AND EXPRESSION OF RESULTS

6.1 Calculation

Calculate from the calibration curve (5.2.3) the amount of benzo[*a*]pyrene in the injected volume of the residue dissolved in tetrahydrofuran (5.3.2).
The benzo[*a*]pyrene content (BaP), in µg/kg, is given by the formula:

$$BaP = \frac{5 \times A \times (20 + 1.25\ G)}{V \times M}$$

where

A is the amount of benzo[*a*]pyrene, in ng, read from the calibration curve (5.2.3).
G is the mass of residue in the minivial (5.1.14) in mg with an assumed density of 0.8 and therefore of a volume of 1.25 *G* µl.
V is the volume of tetrahydrofuran injected into the chromatograph.
M is the mass of test portion in g weighed in (note 6).

6.2 Expression of results

Express the results as µg/kg (to two significant figures)

7. QUALITY ASSURANCE

7.1 For general principles of analytical quality control see the section on Quality Assurance in the introductory part of the Compendium of the Standard Methods.

7.2 For specific applications of analytical quality control see the Annexe to this standard method.

8. NOTES

1 A rotary evaporator with vacuum and a waterbath of 40 °C may be used. Care should be taken to prevent cross contamination. Clean the system thoroughly between subsequent determinations.

2 If other analytical grade solvents than the recommended ones are used a full blank analysis has to be carried out and the results of this blank analysis have to be reported.

3 Supplied by the Commission of the European Community Bureau of Reference (BCR), Rue de la Loi 200, B-1049 Brussels, Belgium.

4 If an autosampler is used the sample loop should be flushed with acetonitrile between two subsequent injections.

5 Tetrahydofuran proved to be the optimal solvent for residue analysis of oils and fats following the clean-up procedure described in 5.1. Injection of volumes in excess of the prescribed 10 µl will give rise to problems. Do not store the samples in tertrahydrofuran for a prolonged period because benzo[*a*]pyrene is not stable in this solvent.

6 With the calibration curve (5.2.3) benzo[*a*]pyrene levels of 0.1 to 10 µg/kg can be determined. For benzo[*a*]pyrene concentrations above 10 µg/kg the residue solution (5.3.2) should be diluted further with tetrahydrofuran or a smaller volume than 10 µl (5.3.3) should be injected.

ANNEXE

1. Repeatability limit

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (*r*) as given in the table with statistical and other data derived from the results of the interlaboratory test.

2. Reproducibility limit

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) as given in the table with statistical and other data derived from the results of the interlaboratory test.

3. Trueness (bias) the bias of the method was demonstrated in the collaborative study of the method (see table of statistical data below) to be negligible when used for the determination of concentration levels of benzo[*a*]pyrene in the range 1 - 5 µg/kg.

4. The sensitivity of the method is demonstrated by the low values for r and R at the low concentration levels studied (see table of statistical data below), the limit of detection is 0.1 µg/kg, the limit of determination is 1 µg/kg.

Interference by other poly aromatic hydrocarbons is not to be expected provided the measurements are carried out at the wavelengths specific for benzo[*a*]pyrene: excitation wavelength 384 nm and emission wavelength 406 nm.

5. Statistical and other data derived from the results of the interlaboratory test

The interlaboratory test carried out at the international level in 1989 by the IUPAC Commission on Oils, Fats and Derivatives, in which 23 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with ISO 5725-1986) summarized in the following table:

Sample	Fish oil			Rapeseed oil		
Batch	A	B	C	A	B	C
Number of laboratories retained after eliminating outliers	10	10	10	11	11	11
Number of outliers (laboratories)	2	2	2	1	1	1
Number of accepted results	240	240	240	264	264	264
Mean value (µg/kg sample)	4.87	3.08	0.98	4.39	2.78	0.99
True or accepted value (µg/kg)	5.35	3.45	1.15	4.85	2.95	1.15
Repeatability standard deviation (S_r in µg/kg)	0.64	0.20	0.24	0.73	0.35	0.27
Repeatability relative standard deviation (CV_r in %)	13.1	6.5	24.6	16.6	12.6	27.4
Repeatability limit (r) [2.8 x S_r]	1.79	0.56	0.67	2.04	0.98	0.76
Reproducibility standard deviation (S_R in µg/kg)	1.09	0.54	0.29	0.77	0.67	0.41
Reproducibility relative standard deviation (CV_R in %)	22.4	17.6	29.4	17.5	24.0	41.0
Reproducibility limit (R) [2.8 x S_R]	3.05	1.52	0.80	2.16	1.87	1.14

S_r: repeatability standard deviation; r: repeatability limit; CV_r: repeatability coefficient of variation; S_R: reproducibility standard deviation; R: reproducibility limit; CV_R: reproducibility coefficient of variation