Journal of Chromatography, 171 (1979) 448–452
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,591

Note

Use of high-performance liquid chromatography as a clean-up procedure in analysis of polycyclic aromatic hydrocarbons in alcoholic beverages

GUY TOUSSAINT and ERNEST ALBERT WALKER

International Agency for Research on Cancer, Unit of Environmental Carcinogens, 150 cours Albert Thomas, 69372 Lyon Cédex 2 (France)

(Received November 7th, 1978)

In Northern France, in the regions of Brittany and Normandy, the relatively high risk of cancer of the oesophagus has been associated with the intake of alcoholic drinks[1]. In support of these epidemiological studies, the possibility that alcoholic drinks might contain chemical carcinogens is being explored.

Since Horie *et al.*[2] have shown that benzo[*a*]pyrene administered orally to rats as a solution in ethanol produced cancer of the oesophagus, we wished to carry out an analysis for the possible presence of polycyclic aromatic hydrocarbons (PAHs). Several procedures suitable for the determination of PAHs in food have been described[3–5]. Initial studies using thin-layer chromatography (TLC) showed[6] benzo[*a*]-pyrene to be present in a number of samples of apple brandy, but the method had a restricted value since it was difficult to identify more than a small number of PAHs. TLC was also suspected of being more subject to interference than gas chromatography (GC). GC on packed columns[5,7] and high-performance liquid chromatography (HPLC)[8,9] both appear to be more reliable and more informative, but in both cases the preliminary sample clean-up is lengthy and therefore less suited than TLC to screening the large number of samples requiring analysis in an epidemiological study. The high separation factors which may be obtained for GC using capillary columns[10] may be utilized to minimize the clean-up procedure, but the sensitivity was found to be insufficient for the determination of levels in the μg/l range. Although Novotny *et al.*[11] and Doran and McTaggart[12] have successfully used HPLC as a clean-up technique on columns packed with relatively large particle-size material, clean-up was only partial and offered little advantage in the present problem.

In previous work on the analysis of aflatoxins, it was found[13] that, by using a 10-μm particle-size silica, a single HPLC stage could be employed effectively in the clean-up technique. It was possible to elute, in a small volume, aflatoxins B_1, B_2, G_1 and G_2 as a group separated from the bulk of other interfering constituents. This relatively clean solution was then suitable for subsequent analysis by HPLC or TLC. By adopting this approach using 5-μm size particulates, a method has been developed which employs HPLC for the essential clean-up of extracts obtained from alcoholic drinks by continuous liquid–liquid extraction followed by GC on a packed column

for the determination step. The efficiency of the HPLC clean-up also permits the direct use of fluorimetric measurement, with an HPLC separation on a reversed-phase column for confirmation of the benzo[*a*]pyrene.

MATERIALS

The solvents employed were cyclohexane (RDH, Hannover, G.F.R.; analytical grade), redistilled, and *n*-pentane (Merck, Darmstadt, G.F.R.; analytical grade) which was first extracted with concentrated sulphuric acid to remove unsaturated compounds, then washed with water, dried over anhydrous sodium sulphate, redistilled and finally stored over sodium.

The purity of each solvent was checked by reducing a 100-ml volume to between 1 and 2 ml in a rotary evaporator, transferring to a mini-vial where the volume was further reduced to *ca.* 25 μl in a current of nitrogen and finally injecting 10 μl into the gas chromatograph using the normal analytical conditions. The solvent was accepted as satisfactory if no peak appeared after the solvent peak.

The HPLC reversed-phase eluent, distilled water–analytical grade redistilled methanol (10:90), was degassed by sparging with nitrogen before use[14].

HPLC clean-up of the prepared extract was performed using an ACS 750-01 constant-pressure pump and a column (25 cm $\times$ $\frac{3}{8}$ in. O.D.) packed with LiChrospher Si 100 (5 μm, Merck) fitted with a Rheodyne injection valve. The eluent flow-rate was 3 ml/min at 1000 p.s.i., and an ACS variable-wavelength ultraviolet (UV) detector set to read at 290 nm was used for detection. The selected HPLC fraction containing the PAHs was analyzed using a Packard Model 417 gas chromatograph fitted with a glass column (4 m $\times$ $\frac{1}{4}$ in. O.D.) packed with 5% OV-101 on Chromosorb W (80–100 mesh) and a flame ionization detector (FID). The nitrogen flow-rate was 60 ml/min and the oven temperature 260° (isothermal). For HPLC–fluorimetry confirmation of benzo[*a*]pyrene, the system consisted of an Altex Model 110 constant-flow pump with a reversed-phase column (25 cm $\times$ $\frac{1}{4}$ in. O.D.) packed with Merck RP-18 (5 μl) (Reeve Angel ODS II is equally suitable). A Jobin Yvon JY 3D spectrofluorimeter fitted with a micro cell was used for detection.

EXPERIMENTAL

Extraction

A 100-ml sample of an alcoholic drink was extracted continuously with 150 ml of cyclohexane for 20 h in a liquid–liquid extractor. The extract was transferred to a separator, washed with 50 ml distilled water, dried over anhydrous sodium sulphate and the volume reduced to 1 ml in a rotary evaporator at 40° under reduced pressure using a water-pump. The extract was then quantitatively transferred to a reactivial by means of a Pasteur pipette and the volume further reduced almost to dryness in a water-bath at 40° in a current of nitrogen. The volume was finally adjusted to 100 μl with cyclohexane.

Purification of extract by HPLC

Selection of a large pore (100 Å), small specific surface area silica (250 m^2/g) for the column packing provides a compromise in separation characteristics between

size exclusion and adsorption. Under these circumstances, all the family of PAHs from anthracene to coronene may be eluted together in a small volume when using pentane as the eluting solvent. Provided the pentane is scrupulously dried, the column has sufficient resolving power to separate PAHs from other hydrocarbons and non-polar constituents, retaining the polar constituents in the extract by adsorption on the column.

The separation is carried out using a 50-μl aliquot of the 100-μl residue in cyclohexane. About ten sample injections can be made before there is a risk that material from preceding sample injections adsorbed on the column will begin to elute and contaminate the PAH fraction. Therefore, after 10 injections, the column is purged with 20–30 ml of methanol and then re-equilibrated using 200 ml of the dry pentane.

GC determination

The HPLC fraction containing PAHs is collected, evaporated to small bulk in a stream of nitrogen then transferred to a graduated reactivial and finally evaporated almost to dryness. The volume is finally adjusted with pentane to 25 μl and a 10-μl aliquot injected into the gas chromatograph. Under the GC conditions employed, a standard mixture of 15 PAHs elute in 40 min in the following order: anthracene, phenanthrene, fluoranthene–pyrene, chrysene–benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene–benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*e*]pyrene, perylene, benzo[*ghi*]perylene, benzo[*b*]chrysene, 3-methylcholanthrene. The method is not restricted to these PAHs; for example, coronene, if present, is collected in the same HPLC fraction. It was not included in the standard mixture since its retention time is too long under the GC conditions employed and the sensitivity therefore too low. For a more extensive PAH profile, it would be necessary to take a larger sample and to adjust the GC conditions accordingly. Under these conditions, the time for analysis would be increased correspondingly. The selection of standards was therefore made to give a compromise between the data per sample and the rate at which samples could be analyzed.

Confirmatory test

In principle, HPLC with UV detection, which is more selective than an FID, can be employed, but since the levels of PAH in the samples examined were in fact very low the sensitivity is inadequate. Fluorimetric detection using an excitation wavelength of 380 nm and measurement at 407 and 430 nm for determination of benzo[*a*]pyrene is considerably more sensitive. The residual extract is therefore analyzed using the reversed-phase column with a micro flow-through fluorescence detector. Alternatively, since the HPLC clean-up provides a fraction of adequate purity, benzo[*a*]pyrene may be determined in the residual extract without further HPLC separation, by diluting to 2.5 ml in methanol and recording the emission spectrum of the solution between 390 and 450 nm using excitation at 380 nm, with excitation slits at 10 nm and fluorescence slits at 2 nm in a 1-cm cell.

RESULTS

Fig. 1 shows the GC chromatogram of the 15 PAHs recovered from a sample

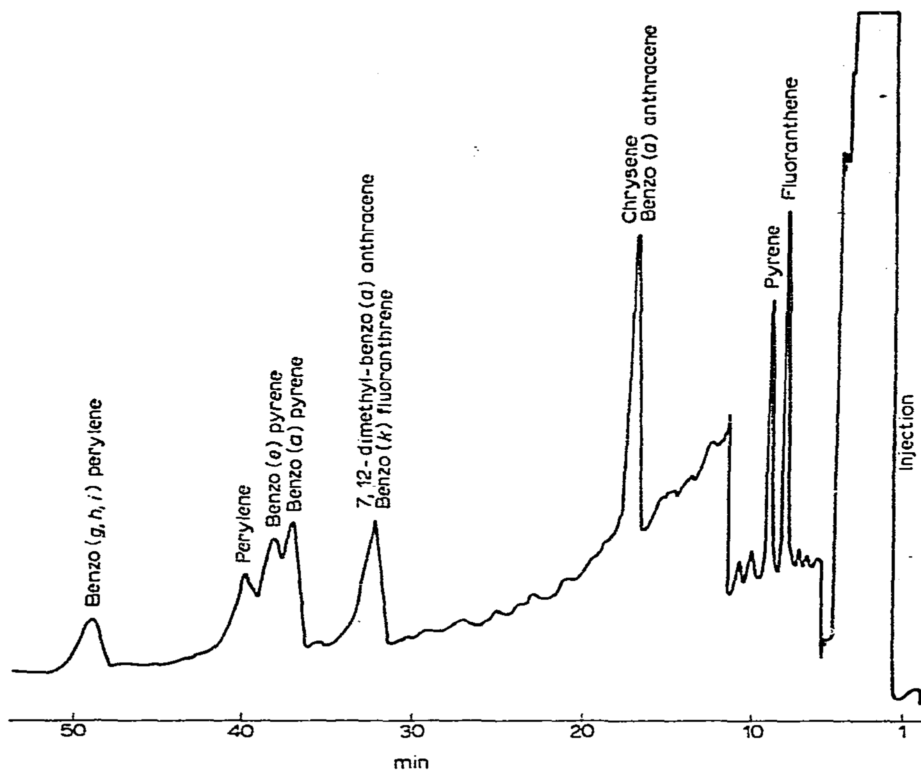

Fig. 1. Gas chromatogram of sample of apple brandy spiked at the 10 μg/l level.

of apple brandy spiked at the 10 μg/l level. The limit of detection was 1 μg/l using HPLC with a micro flow-through cell. Studies on samples spiked at the 10 μg/l level showed 60 ± 5% recovery for the HPLC purification and an overall recovery of 50 ± 5%. Although the losses are significant, probably due to irreversible adsorption, the reproducibility is good for this low level and superior to that which could be achieved using classical methods of separation on open silica or alumina columns or by TLC[15].

The method has been tested on a range of alcoholic drinks, all of which were found to contain very low levels of the non-carcinogenic PAHs anthracene, phenanthrene, fluoranthene and pyrene at levels of 1–10 μg/l which is consistent with a general background of environmental contamination by PAHs. Carcinogenic PAHs such as benzo[*a*]pyrene were mostly below the limit of detection. However, three out of seven samples of apple brandy, one sample of cognac, one sample of whisky and one sample of cider, taken at random, were found to contain benzo[*a*]pyrene at a level near the limit of detection. This compound was not found in samples of armagnac, rum, beer or wine.

Since PAHs are ubiquitous in the environment[16–18], the levels found would be consistent with a general environmental background level. It should be noted that, for successful application of the method, care must be taken during the concentration stages. If evaporation is allowed to proceed to dryness, severe loss of the 3- and 4-ring PAHs occurs. Care should also be taken to rinse all plastic and rubber pieces of

equipment used with cyclohexane (*e.g.*, funnels, plastic tubing from nitrogen supplies, etc.) since they are frequently a source of contamination[7]. PAHs, particularly at low concentrations, can be degraded by exposure to light and therefore all samples should be stored in the dark.

REFERENCES

1 A. J. Tuyns, G. Péquignot and O. M. Jensen, *Bull. Cancer*, 64 (1977) 45.
2 A. Horie, S. Kohchi and M. Kuratsune, *Gann*, 56 (1965) 429.
3 J. W. Howard, T. Fazio, R. H. White and B. A. Klimeck, *J. Ass. Offic. Anal. Chem.*, 51 (1968) 122.
4 *IUPAC Information Bulletin Technical Reports, No. 4*, International Union of Pure and Applied Chemistry, 1972.
5 G. Grimmer and H. Böhnke, *J. Ass. Offic. Anal. Chem.*, 58 (1975) 725.
6 G. Chatot, M. Castegnaro, J. L. Roche and R. Fontanges, *Chromatographia*, 11 (1970) 507.
7 E. D. John and G. Nickless, *J. Chromatogr.*, 138 (1977) 399.
8 H. Guerrero, E. R. Biehl and C. T. Kenner, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 989.
9 D. C. Hunt, P. J. Wild and N. T. Crosby, *Rapp. P.-V. Réun. Conc. Int. Explor. Mer*, 171 (1977) 41.
10 R. C. Lao, R. S. Thomas and J. L. Monkman, *J. Chromatogr.*, 112 (1975) 681.
11 M. Novotny, M. L. Lee and K. D. Bartle, *J. Chromatogr. Sci.*, 12 (1974) 672.
12 T. Doran and N. G. McTaggart, *J. Chromatogr. Sci.*, 12 (1974) 715.
13 G. Toussaint, F. Lafaverges and E. A. Walker, *Arch. de l'Institut Pasteur de Tunis*, 3-4 (1977) 325.
14 M. A. Fox and S. W. Staley, *Anal. Chem.*, 48 (1976) 992.
15 F. de Wiest, D. Rondia and H. della Fiorentina, *J. Chromatogr.*, 104 (1975) 399.
16 M. Suess, *Sci. Total Environ.*, 6 (1976) 239.
17 J. W. Howard and T. Fazio, *J. Agr. Food Chem.*, 17 (1969) 527.
18 *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, No. 3*, International Agency for Research on Cancer, 1973.