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Review

Chromatographic methods for the determination of toxicants in faeces

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ABSTRACT

Modern chromatographic techniques and their application in the determination of toxic compounds in faeces are reviewed. Faecal analysis may be of importance in toxicokinetic studies of xenobiotics in order to determine factors such as metabolism, body burden and major routes of elimination. Compounds of interest include various food constituents, drugs and occupational or environmental factors. Further, various mutagenic or carcinogenic compounds which are excreted by faeces have been indicated to represent risk factors for colorectal cancer. In this context, the chromatographic determination of the endogenously generated fecapentaenes and bile acids, both postulated etiological factors in colorectal carcinogenesis, is reviewed. For fecapentaene determination, several high-performance liquid chromatographic (HPLC) methods are available; however, the applicability of some of these methods is limited owing to insufficient separation of various isomeric forms or discrimination between fecapentaenes and their precursors. For the determination of bile acids in faeces, many chromatographic procedures have been reported, and the characteristics of the most relevant methods are compared and discussed. It is concluded that separation by gas chromatography (GC) in combination with mass spectrometry provides the highest selectivity and sensitivity. A relatively rapid alternative analysis for the determination of total and aqueous

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faecal bile acids is proposed. Further, methods for the determination of polycyclic aromatic hydrocarbons (PAHs) are reviewed. Although the use of radiolabelled PAHs in animal studies has many advantages, it cannot be applied for human biological monitoring and HPLC and GC provide sensitive alternatives. An HPLC method for the determination of non-metabolized PAHs in faeces is described.

CONTENTS

LIST OF ABBREVIATIONS

AU	Absorbance units
B[a]P	Benzo[*a*]pyrene
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
cpm	Counts per minute
CRC	Colorectal cancer
DCA	Deoxycholic acid
DCM	Dichloromethane
DEAP	Diethylaminohydroxypropyl
FBA	Faecal bile acids
FID	Flame ionization detection
FP-12	Fecapentaene-12
GC	Gas chromatography
HPLC	High-performance liquid chromatography
LCA	Lithocholic acid
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PAHs	Polycyclic aromatic hydrocarbons
PHP	Piperidinohydroxypropyl
PUFA	Polyunsaturated fatty acids
RIA	Radioimmunoassay
TEA	Triethylamine
TLC	Thin-layer chromatography
TMS	Trimethylsilyl

1. INTRODUCTION

1.1. Faecal matrix, a complex source of toxicants

The composition of faeces is tremendously complex and actually changes constantly during passage from the proximal to the distal end of the large bowel. Of the total faecal mass, 50% or more consists of bacteria giving viable counts between $2 \cdot 10^{11}$ and $4 \cdot 10^{11}$ organisms per gram dry mass. Over 95% of these bacteria are obligatory anaerobes [1]. Of the remaining solid material, 10–20% is formed by inorganic salts (such as calcium and phosphates), and another 10–20% consists of fatty acids, neutral fats, phospholipids and steroids [2]. Further, undigested food residues (referred to as fibre), mucus and intestinal secretions form the main constituents of faeces

[3,4]. In normal human stool, the total water content, including intracellular water in bacteria, amounts to 70–85%.

In toxicological research, the usual points of interest concern the route of exposure to a specific compound, toxicokinetics in the organism, mode of action at a specific site and elimination of the (biotransformed) agent from the body. Within this context, faecal composition can be studied from different perspectives. First, faeces may contain ingested toxic compounds that to a certain extent are absorbed in the large bowel, thus presenting an exposure route. On the other hand, xenobiotics may be eliminated by faecal excretion. Bilary excretion contributes considerably to this route of elimination. In general, most xenobiotics are excreted via bile after phase I and/or phase II biotransformation, resulting in more hydrophilic products that are less readily (re)absorbed than the parent compound [5]. Third, microbial modification by the faecal (an)-aerobic flora may both activate and detoxify chemicals, or alter their chemical nature in such a way (*e.g.*, hydrolysis of glucuronides and of glutathione conjugates) that the resorption characteristics are considerably changed. Therefore, depending on whether the objective is to study exposure, distribution, metabolism or excretion, quantitative analysis of faecal toxicants can be approached with different goals.

Because of the complex nature of faeces, modern analytical and chromatographic techniques are indispensable in order to determine faecal composition or to identify one specific faecal compound. Appropriate extraction and purification procedures are required, usually followed by chromatographic separation by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) or gas chromatography (GC) in combination with various detection systems. In this review, general analytical and chromatographic procedures applicable to faeces are presented. Further, the analysis of three different groups of compounds which have been suggested to represent etiological factors in colorectal carcinogenesis is discussed.

1.2. General methods for the determination of faecal toxicants and applications

The first important step in the determination of chemicals excreted in faeces is the proper collection of stool samples. Factors such as stability and volatility of the compound of interest and faecal microbial activity may seriously interfere with both the quality and amounts of the extracted compound [6]. Depending on the physicochemical characteristics of the compound of interest, faeces are prepared for extraction by drying as is usually applied for lipophilic chemicals, or by direct mixing with aqueous solutions when water-soluble compounds are being studied. Drying of faeces can be performed in a vacuum desiccator, by air/nitrogen or addition of Na_2SO_4 (anhydrous) or by lyophilization. Again, factors such as stability, reactivity and volatility have to be considered in making a correct choice. Dried faeces are subsequently extracted in a Soxhlet extractor or by shaking and homogenizing in various solvents. In some instances, faeces may by directly applied to a suitably prepared Florisil, silica gel or other column. These semipurified extracts containing compounds with equal polarity and/or molecular mass may be further purified by TLC, column chromatography or repeated extractions. The compounds of interest are usually identified and determined by comparison of their chromatographic (*e.g.*, TLC, GC, HPLC) and spectroscopic properties (ultraviolet or infrared absorption, mass spectrometry (MS), nuclear magnetic resonance (NMR)) with purified or synthetic standards. For reliable quantification, the recovery of each isolation and purification step has to be determined and optimized. This can be performed by mixing known amounts of the pure compound of interest with faeces, and subsequently applying identical procedures as for the experimental material.

HPLC is widely applied in faecal analysis. Applications have been described for all kinds of endogenous factors and also food contaminants or toxicants originating from environmental or occupational exposure. HPLC analyses have been developed for quantification of, *e.g.*, alfla-

toxins [7], 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ) [8–10] and the food additives butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) [11,12], all dietary compounds with (possibly) carcinogenic effects. HPLC analyses have also been applied in quantitative studies on the metabolism and disposition of drugs or antibiotics [13–15]. Further, analyses for pesticides and polycyclic aromatic hydrocarbons (PAHs) may provide data on environmental and occupational exposure to these agents. The application of HPLC in the determination of PAHs and fecapentaenes, a class of faecal mutagens produced by the intestinal flora, is discussed below. For the determination of glucuronides, sulphates, etc., in toxicokinetic studies, HPLC is often preferable to GC as phase II biotransformation products are generally less stable at high temperatures. On the other hand, GC or GC–MS is well suited for faecal analysis of various types of volatile toxicants, fatty acids, steroids and dioxins [16–18]. The main benefit of this technique is the high resolution of GC compared with HPLC. The disadvantage that volatility of the compounds is required can be partially overcome by derivatization. GC–MS provides an ideal combination for the characterization and identification of numerous factors and has been found to be useful in many applications. GC methods for faecal analysis of bile acids are discussed below.

Measurements of the faecal content of metals can be performed by atomic absorption spectrometry, which permits identification and quantification in one step. The determination of various heavy metals in faeces may be of relevance in relation to environmental and occupational exposure [19]. Further, enriched stable isotopes are increasingly used for the measurement of trace elements in humans. For instance, the determination of unabsorbed zinc (^{67}Zn) in faeces using various detection methods has been suggested to be useful in human nutrition studies [20,21]. Finally, methods using labelled compounds are frequently applied in animal metabolism studies. After single administration, distribution can be determined by measurement of radioactivity either directly or after separation using liquid or gas chromatographic methods. Body burden can be calculated as the retained dose after determination of the amount of radioactivity excreted in urine and faeces [22]. Examples have been described for metals [23], antibiotics and other drugs [13–15] and food and environmental contaminants [24–26].

2. CHROMATOGRAPHIC METHODS APPLIED TO THE DETERMINATION OF ETIOLOGICAL FACTORS IN COLON CARCINOGENESIS OCCURRING IN HUMAN FAECES

The origin of colorectal cancer has been suggested to be related to the presence of carcinogenic initiators and promoters in the colonic environment. Apart from epidemiological approaches trying to correlate environmental factors with the incidence of this disease, there is major interest in the identification of the specific factors that cause (pre)carcinogenic lesions in the bowel. Therefore, much attention is being paid to the determination of faecal genotoxic compounds. The importance of chromatographic methods in the isolation, identification and quantification of a selected group of faecal compounds which have been suggested to play a major role in colorectal cancer is discussed.

2.1. Fecapentaenes, faecal mutagens from bacterial production

2.1.1. Isolation and identification of fecapentaene isomers

In attempts to identify major mutagenic compounds in human faeces, a mutagenic fraction in diethyl ether faecal extracts has been purified and characterized. It was shown that the isolated mutagen has a UV spectrum with maxima at 320, 340 and 365 nm [27]. Further, it showed a green fluorescence with absorption at 340 nm and emission at 490 nm. Both the UV spectrum and fluorescence characteristics appeared indicative of polyenes containing five or six conjugated double bonds. It was also shown that the mutagenic fraction is very labile and that this instability is in-

creased by exposure to daylight, oxygen and acidity. In order to obtain relatively large amounts of the purified compound, 2 kg of faeces were dried by mixing with anhydrous Na_2SO_4, Na_2CO_3 and cellulose powder and extractive chromatography was performed on a Florisil column [28]. Mutagenic fractions were revealed on TLC plates as green fluorescent spots using long-wavelength UV lamps. Purification was performed on silica gel and fractions containing mutagens as shown by TLC were subsequently separated on Sephadex LH-20. Combined fractions were subjected to preparative HPLC on silica and reversed-phase columns. The structure was essentially deduced by taking ^1H NMR spectra of the isolated compounds. This analysis suggested the presence of a glycerol moiety, 1-substituted with an unsaturated ether. The length of the aliphatic chain was determined by HPLC interfaced with a mass spectrometer (chemical ionization), showing for one compound the presence of a C_{12} unsaturated chain (M + 1 ion at m/z 251) and for another a C_{14} chain (M + 1 ion at m/z 279). These two forms are referred to as FP-12 and FP-14, respectively (Fig. 1) [28–32]. As two other compounds in this faecal extract with chromatographic properties comparable to FP-12 also showed a mass spectrum with a molecular ion at m/z 251, it was concluded that these compounds are *cis trans* isomers of FP-12. In addition, we described an HPLC analysis with gradient elution on a Sperisorb ODS-2 column which separates eight analogues [33]. Using photodiode-array detection, all these analogues showed the typical pentaene chromophore UV absorption spectrum. As no mass spectra were available it was decided to distinguish these compounds on the basis of their retention times. A cluster of three compounds eluting at 13.2, 13.5 and 14.0 min was suggested to represent the same *cis–trans* isomers as mentioned above, as synthetic FP-12 co-eluted with FP (14.0 min), and FP (13.2 min) and FP (13.5 min) originated from FP (14.0 min) after exposure to UV light. This was confirmed by LC–MS (unpublished results). After UV exposure of FP (18.2 min), isomerization products FP (17.7 min) and FP (17.4 min) were observed, presumably analogues of FP-14. Further, additional fecapentaene analogues, FP (10.9 min), FP (12.2 min) and FP (19.5 min), have been described; identification of their structures by LC–MS is currently under investigation. Incidently, peaks were found with an identical pentaene UV spectrum, but eluting with a retention time of 15 or more than 20 min (unpublished results). In view of their less frequent occurrence (< 5%) and relatively low levels (< 250 μg/kg wet faeces), they appear not to contribute substantially to total fecapentaene excretion levels.

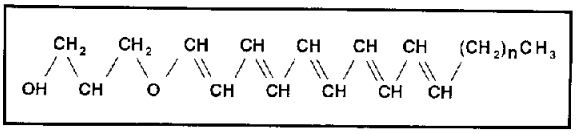

Fig. 1. Structure of fecapentaene-12 (n = 1) and fecapentaene-14 (n = 3).

In addition to these typical pentaene structures, compounds showing a UV spectrum with two absorption maxima shifted upscale (360–380 nm) or downscale (310–330 nm) as compared with the pentaenes are also found in these human faecal dichloromethane (DCM) extracts (Fig. 2). Peters *et al.* [34] first reported the presence of these substances and suggested their structure to be hexaenes (spectrum shifted upscale) or tetraenes (spectrum shifted downscale). Analysis of the mutagenic potential of synthetic fecaenes revealed that FP-12 has the strongest mutagenic potential, and that fecahexaene-14 is more mutagenic than fecatetraene-10 [35].

2.1.2. Fecapentaene determination in human populations

The occurrence of fecapentaenes in human populations has been investigated in order to determine (1) the level of colonic fecapentaene exposure, (2) whether groups at different risk of colorectal cancer excrete different concentrations of fecapentaenes and (3) possible effects of the diet on fecapentaene excretion. In order to do so, an appropriate and well defined extraction procedure is needed. Depending on whether determination of total fecapentaene concentrations is

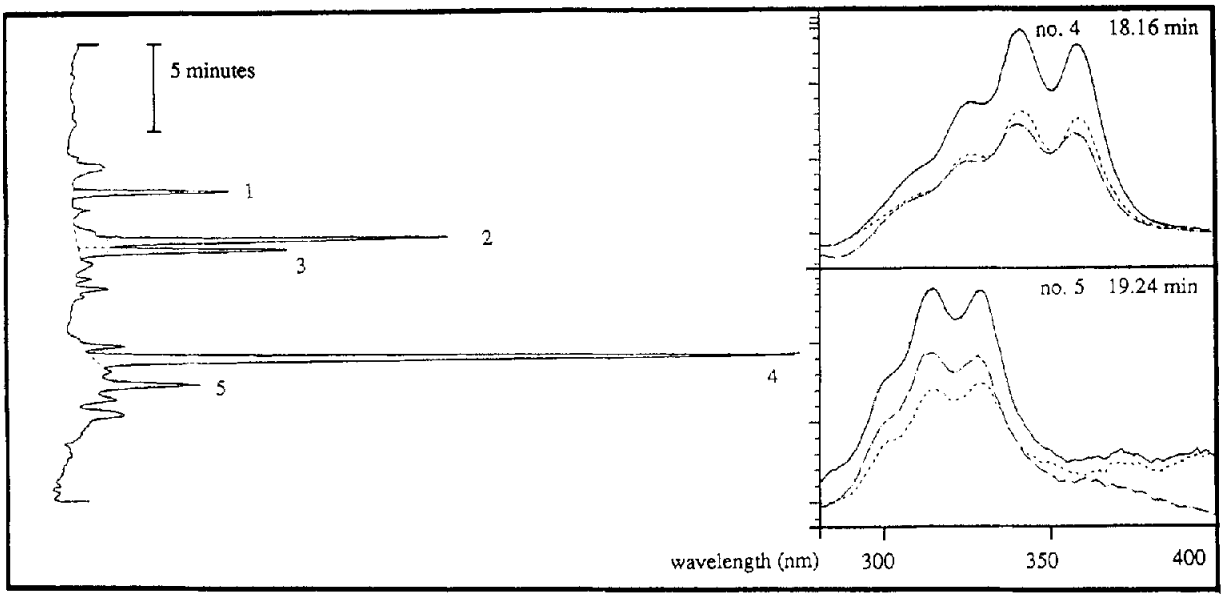

Fig. 2. HPLC of a faecal dichloromethane extract containing four fecapentaene analogues, all characterized by a typical pentaene absorption spectrum (spectra of compounds 1–4 are identical) and one hypothesized fecatetraene showing absorption maxima at 310–330 nm (compound 5).

sufficient or FP-12, FP-14 and their isomers have to be quantified separately, simple or relatively more complicated gradient chromatography is required. For fecapentaene extraction, several procedures have been described, using diethyl ether [36–39], acetone [40–44] or DCM [33,45,46] as extraction solvents and several antioxidants as stabilizing factors. We showed that the addition of triethylamine (TEA) is to be preferred to BHT for the stabilization of FP-12 [47]. Further, acetone and DCM extraction appeared more efficient than the use of diethyl ether [33]. These recoveries could be improved by homogenizing faeces with the extraction solvent using a Potter homogenization instrument. The overall recovery using DCM–TEA (9:1) as extraction solvent and subsequent purification on silica columns is 78% [33]. Unfortunately, the literature reports only one fecapentaene extraction recovery of 20% after diethyl ether extraction [34]. This hampers inter-assay validation. An overview of HPLC analyses is given in Table 1.

Apart from optimization of fecapentaene extraction recovery and reproducibility, several other methods may provide reliable fecapentaene quantifications. Isotopic dilution techniques have been employed using ^{3}H-labelled FP-12 and FP-14. After spiking with both [^{3}H]FP-12 and [^{3}H]FP-14, diethyl ether extraction and silica clean-up can be performed without regard to quantitative recovery followed by HPLC analysis. Endogenous fecapentaene concentrations may be calculated from ratios between radioactivity (cpm) per absorption unit (AU) of spiked fecapentaene and cpm per AU of the recovered fecapentaene [34]. Considerable loss of fecapentaenes during extraction and purification procedures may also be avoided by chemical conversion of fecapentaenes and their precursors to more stable methoxytetraenols [48]. A clear restriction in this approach is that actual fecapentaene exposure levels cannot be determined, as both fecapentaenes and their phospholipid precursors are converted without discrimination. Finally, synthetic fecapentaenes not naturally occurring in human stool may be added as internal standards, allowing for correction for fecapentaene loss. Synthetic FP-13 has been shown to be suitable as such an internal standard [48].

Although fecapentaenes may be separated by

TABLE 1

OVERVIEW OF ANALYTICAL AND CHROMATOGRAPHIC PROCEDURES IN FECAPENTAENE ANALYSIS

Ref.	Extraction/stabilization	Purification	HPLC	Characteristics
Baptista *et al.* [45]	Wet faeces is mixed with Na_2SO_4, Na_2CO_3 and cellulose powder; subsequent elution with benzene, dichloromethane and dichloromethane–20% acetone and acetone (total volume 5.5 l!) on a Florisil column; 0.01% BHT as antioxidant	μPorasil Sep-Pak, eluted with isopropanol	Zorbax ODS (25 cm × 4.6 mm I.D.); acetonitrile–water–methanol–tetrahydrofuran (36.2:32:25.4:6.4)	Separation of FP-12 and FP-14 areas
Schiffman *et al.* [40]	Freeze-dried faeces is extracted with BHT-stabilized acetone, with or without anaerobic incubation	Filtration through an Acrodisc LC-13 filter (0.5 μm)	Silica cartridge; chloroform–isopropanol (95:5)	No separation of FP-12 and FP-14
Peters *et al.* [34]	Freeze-dried faeces is extracted with hexane (which is discarded); remaining sample is extracted 5 times with diethyl ether; 0.01% BHT and 1% TEA as antioxidants	Silica gel eluted with 5% methanol in diethyl ether	Chemcopak 5-ODS-H; stepwise gradient elution with 0.01 *M* Na_2PO_4 acetonitrile–methanol	Extraction recovery of 20%, corrected by isotopic dilution techniques. Detection of fecatetraenes and -hexaenes. Occurrence of pre-FP-12 and pre-FP-14 after incubation.
Kivits *et al.* [48]	Wet faeces is mixed with aqueous solution of Na_2CO_3 and methanol. Extraction with diethyl ether; 0.02% BHT as antioxidant	Silica gel; elution of methoxytetraenols with diethyl ether	LiChrospher 100 RP-18 (5 μm) (250 × 4.6 mm I.D.); tetrahydrofuran–acetonitrile–water methanol (6:30:24:40)	Quantification after conversion of FP to methoxytetraenols with 70% recovery. No differentiation between FPs and precursors. FP-13 is added as internal standard
De Kok *et al.* [33]	Freeze-dried faeces is extracted with dichloromethane; rigorous homogenization; 5% TEA as antioxidant	Silica gel, eluted with diethyl ether–TEA–methanol (7:1:2)	Spherisorb ODS-2; stepwise gradient elution with acetonitrile–methanol water–tetrahydrofuran	Separation of 8 different FP analogues and fecatetraenes and fecahexaenes. Extraction recovery 78%

GC [49], in quantitative analyses fecapentaenes are separated without exception by HPLC. As the stationary phase, various reversed-phase columns such as Spherisorb ODS-2 [33], Chemcopak 5-ODS [48], LiChrosorb 5-RP-18 [47] and silica cartridges [40] have been used. In reversed-phase HPLC, quaternary eluents composed of methanol, acetonitrile, tetrahydrofuran and water have usually been used, whereas silica is eluted with chloroform–isopropanol [40]. Isocratic systems may be used for the determination of total fecapentaenes or FP-12 and FP-14 separately. Gradient elution variants [33,34] permit further separations of various geometrical isomers and chemically related compounds such as tetraenes and hexaenes. Application of the described method for fecapentaenes to faeces from human populations has shown inter-individual variations in excreted concentrations, varying from undetectable levels (< 5 μg/kg wet faeces) to several mg total fecapentaenes/kg wet faeces. In contrast to quantitative data on excretion of fecapentaene isomers, little is known about the occurrence of other fecaenes. Preliminary results of measure-

TABLE 2

OCCURRENCE OF HYPOTHESIZED FECATETRAENES AND FECAHEXAENES IN DICHLOROMETHANE EXTRACTS OF FAECES FROM 54 INDIVIDUALS, QUANTIFIED IN mAU AT 310 nm

Compounds	Occurrence (%)	Median value in mAU[a]	n
Fecatetraenes	31	7.2 (3.2;22.3)[b]	19
Fecahexaenes	5	12.1	3

[a] Compared with 10 mAU for 347.2 μg FP-12/kg wet faeces (at 335 nm).

[b] (10th; 90th percentiles).

ments using our analytical system [33] showed excretions of detectable levels of suggested tetraenes at retention times of 15.4 and 9.2 min in 31% of a group of 54 individuals (Table 2). Fecahexanes were found in only 5% of these samples. As the molar absorptivities of fecatetraenes and fecahexaenes are unknown, it is impossible to give absolute concentrations.

Quantitative analyses have clearly shown dietary influences on excreted fecapentaene concentrations [41,50,51]. Remarkably, vegetarians at relatively low risk of colorectal cancer and also colorectal cancer patients have been found to excrete significant lower fecapentaene concentrations in comparison with omnivores or control patients [40,50]. The relatively high genotoxic capacity of synthetic FP-12 [52–55] suggests an involvement in colorectal cancer initiation [56–58], but carcinogenicity studies in rodents have given predominantly negative results [57,59,60]. These contradictory results in epidemiological studies and studies on genotoxic effects indicate that further research is required to establish the role of fecapentaenes in colorectal carcinogenesis. Therefore, the availability of accurate and well described methods for fecapentaene determination is of the utmost importance. As indicated in Table 1, various methods have been developed for the determination of total fecapentaenes [40], total fecapentaenes and precursors without anaerobic incubation [48] and various fecapentaene analogues [33,34]. The method described by Baptista *et al.* [32], which also separates FP-12 and FP-14, appears too laborious for application in large populations and therefore cannot be recommended.

2.2. Bile acids, animal neutral steroids and long-chain fatty acids

2.2.1. Biological activity

The hypothesis that faecal bile acids are involved in colorectal carcinogenesis was first postulated several decades ago [61–63], and still receives much attention [64–68]. Indeed, epidemiological data demonstrate correlations between excretion of bile acids and both the incidence of colorectal cancer and the consumption of high-risk diets [65,69,70]. Further, it has been shown that secondary bile acids generated by metabolism of intestinal bacteria are co-mutagenic in microbial test systems [71,72] and co-carcinogenic in various animal models [73–77]. However, controversial results have been reported in case-control studies comparing faecal bile acid concentrations in cases of colorectal cancer or adenomatous polyps with controls [70,78–82].

Apart from bile acids, faecal neutral steroids have also been shown to be related to colorectal malignancy. Studies comparing the animal neutral steroids and long-chain fatty acids in the faeces of populations at different risk of colorectal cancer indicated that a higher concentration of oleic acid was excreted by high-risk populations, and that neutral steroids were more extensively metabolized by the colonic flora [63,83]. Further, patients with colorectal cancer were found to excrete higher concentrations of faecal neutral steroids [84], and high-risk diets were found to increase faecal concentrations of cholesterol and coprostanol [85–87]. Like neutral steroids, long-chain fatty acids have also been shown to be toxic to the human colon [88], and were found to be excreted in higher concentrations in both high-risk groups and populations on high-risk diets [89,90]. A low-risk vegetarian diet was shown to reduce fatty acid concentrations in faecal water [91].

2.2.2. Extraction and purification procedures

Quantitative bile acid excretion, determined from single daily stool collection, has been shown to exhibit wide day-to-day variations in faecal levels, and it has been indicated that a minimum of 4-day collections is necessary to determine reliably average faecal excretion [92]. After homogenization, faeces may be extracted directly or after lyophilization. Both samples can be stored at −20°C. Procedures for extraction and purification can be divided into seven basic methods, with many modifications reported. These are reviewed below and summarized in Table 3. Bile acids can be extracted from human faeces by refluxing with chloroform–methanol [93,94], glacial acetic acid and toluene [95], alkaline methanol [96], ethanol and methanol–chloroform [97–99] or ethylene chloride–methanol [100]. Extraction recoveries from samples containing endogenous radiolabelled bile acids vary from 85 to 97%. For the determination of bile acids in the aqueous phase, faecal water can be prepared by centrifugation at 25 000 *g* for 2 h [101]. As the colonic epithelium is relatively more exposed to bile acids in the aqueous phase, it has been suggested that the determination of bile acids in faecal water may be of greater importance than total bile acids [91,101].

In faeces, bile acids occur as primary compounds, such as cholic and chenodeoxycholic

TABLE 3

BASIC METHODS FOR EXTRACTION AND PURIFICATION IN THE DETERMINATION OF FAECAL BILE ACIDS

Method	Pretreatment	Extraction	Purification
Grundy *et al.* [93]	Homogenization, mild and rigorous saponification	Chloroform–methanol (2:1, v/v)	(1) Removal of neutral steroids with light petroleum. (2) Florisil and/or TLC.
Ali *et al.* [100]	Homogenization, lyophilization	Ethylene chloride methanol (3:1, v/v)	Lipid extraction with light petroleum.
Evrard and Janssen [95]	Homogenization, lyophilization	Acetic acid; addition of toluene after extraction	(1) Saponification. (2) Light petroleum extraction of neutral steroids. (3) Diethyl ether extraction of bile acids. (4) Chromate oxidation.
Eneroth *et al.* [94]	Homogenization	Hot chloroform–methanol (1:1, v/v)	(1) Saponification in KOH–dioxane. (2) Diethyl ether extraction. (3) Silicic acid chromatography.
Setchell *et al.* [99]	Homogenization	Reflux with (1) 90% ethanol, (2) 80% ethanol, (3) chloroform–methanol (1:1, v/v)	(1) Lipidex 1000 and Bond-Elut. (2) Amberlyst A-15. (3) DEAP-LH-20. (4) Enzymatic or alkaline hydrolysis of conjugates.
Van Faassen *et al.* [96]	Homogenization, lyophilization	(1) Enzymatic deconjugation. (2) Reflux with 0.1 *M* NaOH in 70% methanol	Light petroleum extraction of neutral steroids.
Korpela *et al.* [98]	Homogenization	Reflux with (1) 90% ethanol, (2) 80% ethanol, (3) chloroform–methanol (1:1, v/v)	(1) DEAE-Sephadex A-25. (2) Enzymatic and alkaline hydrolysis of conjugated fractions. (3) Radiolabelled taurocholic acid as internal standard.

acid, or secondary products, such as deoxycholic and lithocholic acid. Bile acids may also be conjugated with glycine or taurine, sulphated or glucuronidated. Separation of the various bile acid groups and neutral steroids can be achieved by TLC on silica gel [102–105] or by column chromatography with materials such as Sephadex LH-20, diethylaminohydroxypropyl (DEAP)-Sephadex LH-20 or piperidinohydroxypropyl (PHP)-Sephadex LH-20 [78,106,107]. The use of the anion exchangers DEAP- and DHP-Sephadex LH-20 requires the removal of cations before separation, which can be performed on Amberlyst A-15 [78], Sep-Pak C_{18} cartridges [106] or XAD-2 [107]. After these purification and separation steps, bile acid esters can be determined following mild saponification [96,98,99]. Glycine and taurine conjugates may be hydrolysed under alkaline conditions [108,109] or by the enzyme cholylglycine hydrolase [96,98,99,110]. During alkaline hydrolysis, loss of some bile acids may occur, whereas application of enzymatic hydrolysis has been shown to induce only minimal destruction and artifacts [110–112]. Sulphates and glucuronidates may be hydrolysed by various methods, all under acidic conditions [107,112–116]. However, in healthy subjects without intestinal pathology, only a minor amount (< 5%) of the faecal bile acids occur as glycine or taurine conjugates or as sulphates [78,83,92,117]. Bile acid esters, however, may account for 25% of total faecal bile acids [98]. Whether total bile acids or also individual bile acids and the degree of conjugation have to be determined depends on the scientific issues to be studied, and this will also indicate the analytical procedures to be followed.

2.2.3. Quantitative chromatographic analysis

An extensive set of separation and quantification techniques have been developed for the determination of bile acids, including paper chromatography, counter-current distribution, ion-exchange chromatography, TLC, HPLC, GC, enzymatic methods, spectrophotometric and fluorimetric methods, radioimmunoassay (RIA), MS and NMR. Detection limits and applicability have been extensively compared elsewhere [118]. Owing to non-specificity or relatively low sensitivity, methods such as paper chromatography, counter-current distribution and spectrophotometry have been replaced with more modern techniques, whereas enzymatic methods and RIA are generally used for the determination of bile acids in serum or urine. As the concentration of bile acids in faecal water is considerably lower than the concentration in total faeces, measurement in the aqueous phase generally requires more sensitive techniques such as GC. An overview of chromatographic methods applied in analyses for bile acids is given in Table 4 and their characteristics and applicability are discussed below.

Although TLC is still in use for the identification of determination of bile acids [119], it is mainly applied as a purification step or for the determination of extraction recoveries [120]. The main groups that can be separated on silica TLC plates are free bile acids and their tauro and glyco conjugates. This can be achieved by development with three successive solvent systems [102], and solvent systems have been described which separate the glycine and taurine conjugates of lithocholic, chenodeoxycholic, deoxycholic and cholic acids on silica gel G [121]. Also, a unidirectional single-solvent development system for the separation of chenodeoxycholic and deoxycholic acid or their methyl esters has been described [122]. Other separation methods for various conjugated bile acids on silica gel 60 [123] and $KC_{18}F$ reversed-phase TLC plates have been developed [124]. After chromatography, bile acids may be revealed by spraying with a variety of reagents *e.g.*, sulphuric acid [125,126]. Non-destructive methods for detecting bile acids on TLC plates such as exposure to UV radiation after spraying with 0.05% pyrene in light petroleum or with water permits the removal of the bile acids from the plates for further analysis. However, these methods have been reported to be insensitive [126].

Application of HPLC to bile acid determination results in an improved resolution as compared with TLC, although the complete separation of some specific bile acids may not be achieved on various types of columns [102,127–

TABLE 4

CHROMATOGRAPHIC METHODS AND THEIR APPLICATION IN THE DETERMINATION OF FAECAL BILE ACIDS

Method	Ref.	Bile acid measurement	Detection limit	Application
TLC:				
On silica gel plates	102,119	Total bile acid	0.5–3 nmol	Owing to the relatively low sensitivity and selectivity, TLC is used for purification rather than determination of faecal bile acids
On $KC_{18}F$ reversed-phase plates	120,125,126	Group separation of free and conjugated bile acids and neutral steroids		
HPLC:				
UV absorbance	130–132	Free bile acids (as *p*-bromophencayl, phenacyl, nitrobenzyl and chlorobenzyl esters	2–265 pmol	Despite the improved sensitivity and selectivity as compared with TLC, determination of faecal bile acids by HPLC has been performed only infrequently
Differential refractometer	102,127	Free and glycine- or taurine-conjugated bile acids; in combination with TLC	1 nmol	
Electrochemical detection	133	Free bile acids on LiChrosorb RP-18	1.25 nmol	
Immobilized enzyme	129,134,135	Free and conjugated bile acids	20–30 pmol	
Enzymatic/spectrophotometry	136,137,155–157	Total bile acids	10 nmol	Applied in human/animal studies on the relationship between diet and bile acid excretion
GC:				
Packed column; FID	78,93	Trifluoroacetate methyl esters	0.2 nmol	GC is widely applied for the determination of bile acids in animal and human faeces. Effects of dietary habits, dietary interventions and levels of faecal bile acids in colorectal cancer patients and controls have been compared using GC
	96,147	Trimethylsilyl ether methyl esters		
	95	Methyl esters		
Wall-coated capillary column; FID	87	Trifluoroacetate methyl esters	1 pmol	
	99,106	Trimethylsilyl ether methyl esters		
	17	Butyl ester acetates		
Mass spectrometry	65,92,97,158	Trimethylsilyl ether methyl esters	1 pmol	

129]. Further, other faecal constituents and derivatization compounds may interfere during analysis [130,131]. A combination of group separation by TLC and subsequent HPLC may increase the specificity of the analysis of complex samples. Such a method has been described by Shimada *et al.* [102] for the measurement of free and conjugated bile acids on a μBondapak C_{18} column. This analysis is performed in two stages using different mobile phases, which permits the determination of conjugates directly in biological samples without prior hydrolysis. Conjugated

bile acids may be detected by UV spectrophotometry or differential refractometry [102], whereas unconjugated bile acids generally require derivatization as they show only weak UV absorption. Derivatives such as *p*-nitrophenyl esters [129], *p*-nitrobenzyl esters, *p*-chlorobenzoyl esters [132] and phenacyl esters [130] have been described. The feasibility of electrochemical detection of bile acids has also been demonstrated [133], but has never been evaluated with biological samples. Enzymatic analysis can be performed after fractionation of the eluate, or by using a postcolumn conversion of NAD^+ to NADH in the reaction with bile acids and immobilized 3α-hydroxysteroid dehydrogenase [134,135]. Compared with the other HPLC detection systems, this last method is the most sensitive. Despite the fact that bile acids occur in faeces at relatively high concentrations, which makes HPLC a suitable technique, this method has not been widely applied in faecal bile analysis.

GC is most frequently used for the quantification of faecal bile acids. Derivatization is performed after deconjugation and solvolysis. Methylation of the carboxyl group can be achieved by using reagents such as acetyl chloride–methanol [138], HCl–2,2′-dimethoxypropane–methanol [96,139] or diazomethane [99,140,141]. The last method is frequently used although the formation of byproducts has been reported and diazomethane is known to be highly toxic [141]. Resolution can be improved by using propyl or butyl esters rather than esterification with methanol or ethanol. This may be advantageous in the simultaneous determination of faecal neutral steroids and bile acids, as several late-running steroids overlap with the bile acid region as methyl esters. In that event, the application of TLC purification to purify the methyl esters of the bile acids [93,113] may be omitted [17]. The hydroxyl groups may be converted into ketones [95] or derivatized as acetates [17], formates [142], trifluoroacetates (TFA) [78,96] or silyl derivatives such as di- or trimethylsilyl ethers (TMS) [106,143]. As the stationary phase either packed [78,96,126] or capillary columns [99,106] can be applied. Concentrations of bile acids are determined by comparison of peak areas with known standards. Several compounds have been used as internal standards, including radiolabelled compounds, 7α,12α-dihydroxy-5β-cholanoic acid [106], 5β-cholanic acid [144], heptadecanoic acid, nordeoxycholic acid [17] and hyodeoxycholic acid [87]. In comparison with flame ionization detection (FID), the specificity may be improved by MS (GC–MS). This method has been applied to the determination of bile acids in all kinds of biological samples, including serum, urine, bile and faeces. Electron impact MS is more generally used than chemical ionization MS, and mass spectra of various types of derivatives and conjugates have been well documented [139,141,145,146].

Animal neutral steroids can be determined by GC after extraction with light petroleum [147,148]. The oxosteroids (coprostanone, etc.) have been separated by normal-phase chromatography on a Lipidex 5000 column before GC analysis as TMS ethers on an SE-30 column [149]. Immediate separation of the oxosteroids from the other neutral steroids can be obtained by GC on a CP-Sil 5 column as trifluoracetates [87], on a CP-Sil 19 CB column as TMS ethers [150] or as acetates on a DB-1701 capillary column [17].

GC is also suitable for the determination of faecal long-chain fatty acids in hexane extracts of the acidified alkaline ethanolic extract of faeces, derivatized as methyl esters [89]. More recently, these compounds have been determined as butyl esters, but this method does not separate linoleic and oleic acid [17]. Sufficient separation of the faecal long-chain fatty acids has been described for methyl esters on methylsilicone-coated fused silica [151].

In conclusion, several methods are available for the determination of faecal bile acids, which are especially useful when the amount of saponifiable conjugated and/or sulphated bile acids has to be measured [97–99]. However, these methods are time consuming. Further, a disadvantage of the method described by Grundy *et al.* [93] is the introduction of artefacts due to the strongly alkaline hydrolysis [109], whereas the original method of Evrard and Janssen [95] cannot distinguish be-

tween primary and secondary bile acids. This requires an additional dehydrogenation step [152]. A disadvantage of the procedure of Korpela *et al.* [98] is the requirement for radiolabelled internal standards. For the complete resolution of different groups of bile acids (*e.g.*, sulphates, conjugates) and neutral steroids, the method of Setchell *et al.* [99] can be recommended. The method that we described previously [87] determines rapidly the amount of unconjugated bile acids, including sulphates, and the amount of neutral steroids. The major steps applied for extraction and purification are shown in Fig. 3. During the purification procedure, the light petroleum extract is retained for the subsequent measurement of neutral steroids. In order to determine also the isoform of deoxycholic acid, we changed to the measurement of TMS ethers on a CP-Sil 19 CB column. A gas chromatogram of the analysis is shown in Fig. 4.

Recently, we applied this method to faecal samples of habitual vegetarians and omnivores,

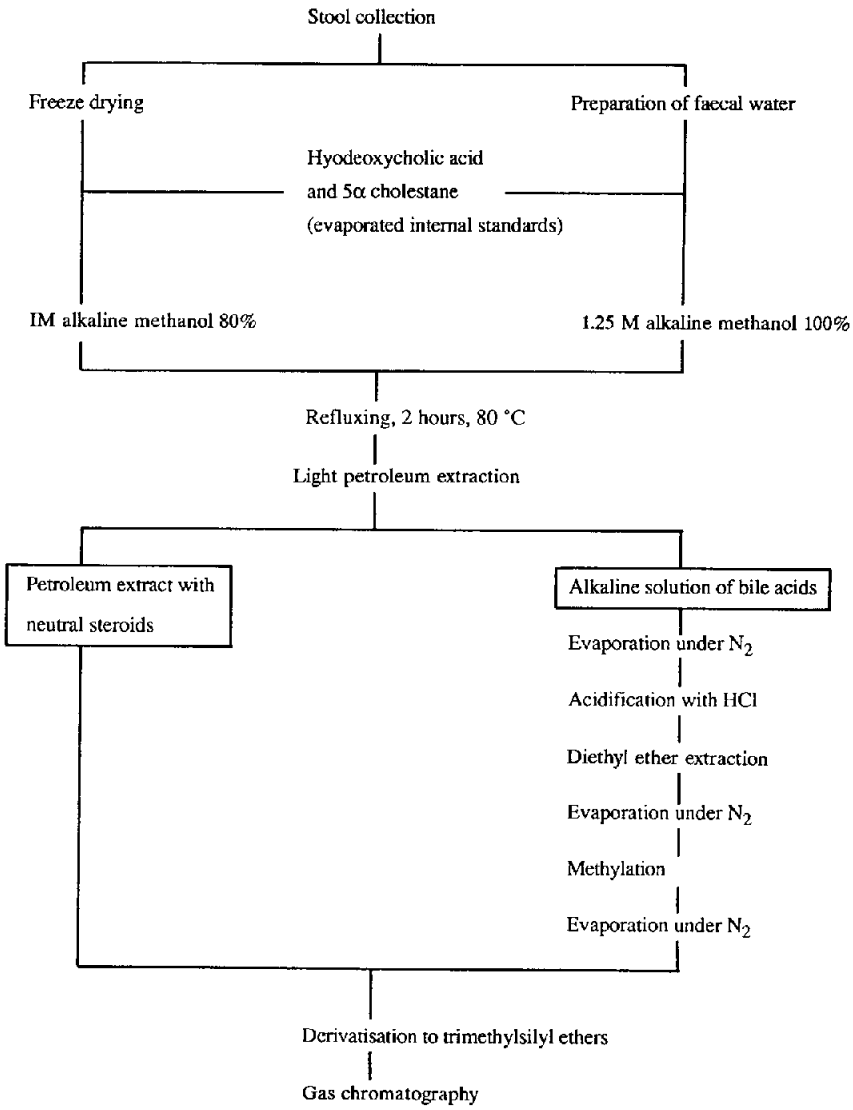

Fig. 3. Major preparation steps in the analysis of faecal bile acids and neutral steroids.

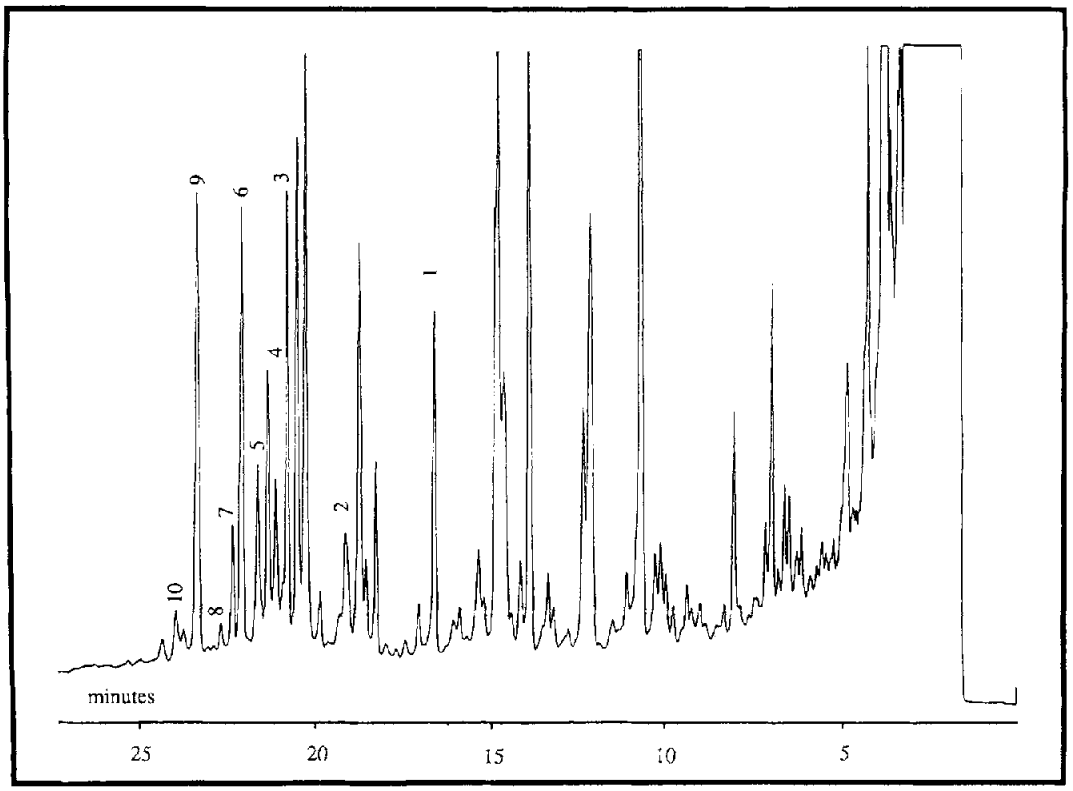

Fig. 4. Gas chromatogram of a faecal extract from an omnivorous donor. Peaks: 1 = coprastonol; 2 = cholesterol; 3 = isolithocholic acid; 4 = lithocholic acid; 5 = isodeoxycholic acid; 6 = deoxycholic acid; 7 = cholic acid; 8 = chenodeoxycholic acid; 9 = hyodeoxycholic acid (internal standard); 10 = ursodeoxycholic acid.

TABLE 5

BILE ACID CONCENTRATIONS IN FAECAL WATER AND TOTAL FAECES FROM HABITUAL OMNIVORES (n = 20) AND VEGETARIANS (n = 19)

Sample	Bile acid	Concentration (μmol/l water)[a]	
		Omnivores	Vegetarians
Faecal water	Isolithocholic acid (ILC)	10 (2–28)	8 (0–23)
	Lithocholic acid (LC)	23 (7–97)	21 (6–94)
	Isodeoxycholic acid (IDC)	27 (10–77)	15 (3–38)[b]
	Deoxycholic acid (DC)	87 (23–247)	34 (4–77)[b]
	Chenodeoxycholic acid (CDC)	6 (0–19)	2 (0–10)[b]
	Cholic acid (C)	19 (0–83)	5 (0–22)[b]
	Ursodeoxycholic acid (UDC)	11 (2–23)	13 (2–48)
	Sum (ILC + LC + IDC + DC + CDC + C + UDC)	187 (59–442)	98 (34–213)[b]
Total faeces	Sum (ILC + LC + IDC + DC + CDC + C + UDC)	21 (8–38)[c]	19 (12–30)[c]

[a] Mean values with minimum and maximum values in parentheses; log transformed in t-test.

[b] Significantly different from omnivores ($p < 0.05$).

[c] Concentration in μmol/g dry weight.

groups at different risk of colorectal cancer [153]. Some results for faecal bile acids in the aqueous phase and bile acids in total faeces are given in Table 5. The concentrations of bile acids in dried total faeces did not differ between the omnivorous and vegeterian groups. In faecal water, the concentrations of isodeoxycholic, deoxycholic, chenodeoxycholic and cholic acid and the sum of all the bile acids were significantly lower in faeces from vegetarians than from omnivores. The ratio of deoxycholic acid in the aqueous phase to that in total faeces was significantly lower in vegetarians than in omnivores: 0.018 ± 0.016 (mean ± S.D.) *versus* 0.044 ± 0.024, indicating that deoxycholic acid in faecal water represents only a small percentage of total faecal deoxycholic acid. As conjugated and sulphated bile acids have been shown not to cause adverse effects on the colonic mucosa and only a minor part of faecal bile acids is present in conjugated or sulphated form [154], this method can be recommended for the determination of toxic faecal steroids.

2.3. Polycyclic aromatic hydrocarbons (PAHs)

2.3.1. Intake, kinetics and carcinogenicity of PAHs

The group of polycyclic aromatic hydrocarbons (PAHs) includes several hundred organic compounds [159] which, in their simplest form, are constituted of two or more benzene rings. Many PAH sources have been identified. Generally, PAHs originate from *e.g.*, incomplete combustion (pyrolysis) of fossile fuels during industrial processes (such as aluminium and coke production, oil refining and wood conservation) or from motor exhaust fumes [159,160]. PAHs are deposited on the soil and surface water via the air. Via this route, drinking water and vegetables may be contaminated. Also, PAHs can be formed by food processing, *e.g.*, high levels of PAHs can be detected in cooked, charcoal broiled or smoked meats. Further, cigarette smoking contributes to PAH intake. Active smoking of 25 cigarettes per day can lead to a PAH intake of up to 160 μg/day [161,162]. The total PAH intake may vary from 25 to 300 μg/day, depending on life style [162]. Environmental intake of PAH in non-polluted areas is only minor in comparison with indirect PAH intake from the consumption of food. Only 3% of the body burden derives from the air, 0.04% is taken in by drinking water and about 97% of the body burden of PAHs derives from food. However, at sites of former gas works or coke washeries and coal tar factories, the soil concentration of PAHs can rise to harmful levels. If dwellings have been built on these sites human health may be at risk, as can be derived from several environmental risk assessment models that have been developed by the National Institute of Public Health and Environmental Protection in The Netherlands [163,164]. Occupational exposure of PAHs is at another order of magnitude, and may be considerable for coal tar and pitch workers [165,166]. In these highly exposed populations, increased incidence of lung, skin, bladder and gastrointestinal cancers have been found [167,168].

PAHs are readily absorbed by the intestine, with absorption being facilitated by the presence of bile. Gastrointestinal uptake is at least 30% [169,170]. The major route of excretion of PAHs and metabolites involves accumulation in bile after conjugation with, *e.g.*, glucuronic acid, and subsequent faecal excretion [171–174]. Also nitrated PAHs (nitro-PAHs) are mainly eliminated via this route [175]. Biliary metabolites are partly reabsorbed, which means that an entero-hepatic circulation of these substances exists [171].

PAHs such as benzo[*a*]pyrene (B[a]P) and dimethylbenz[*a*]anthracene reveal high carcinogenic activity in mice after subcutaneous injection or by topical application to the skin [176–178], whereas other PAHs show only moderate carcinogenic effects (*e.g.*, dibenz[*a*,*h*]anthracene, methylbenz[*a*]anthracene) or are completely inactive (anthracene, phenanthrene, perylene and benzo[*a*]pyrene) [178,179]. As a substantial part of PAHs is excreted in faeces, the colon and rectum present a putative target for PAH-induced carcinogenicity. Many of the food pyrolysis products, including B[a]P, have been shown to induce significant elevations in the frequency of nuclear aberrations in the colon epithelium [180–

182], thus representing presumptive colon carcinogens. Further, colorectal sarcomas and carcinomas have been induced by 20-methylcholanthrene and dimethylbenzanthracene [183,184], whereas B[a]P has been shown to be metabolised by cultured human colon resulting in DNA adduct formation [185]. However, the relevance of PAH in colorectal cancer etiology has not yet been established and is still under discussion [186,187].

2.3.2. General methods in the determination of PAHs

As PAHs are widely distributed through the environmental compartments, analytical methods have been developed to detect PAHs in various matrices. Most of these methods involve an extraction step, in which the PAHs are recovered from the original matrix into an organic solvent, followed by a purification or fractionation step. PAHs may be determined by HPLC with UV or fluorescence detection and by GC with flame ionization or MS detection. Table 6 gives an overview of methods for PAH determination in the different environmental compartments.

Further, many methods have been developed for the determination of PAHs in animal or human tissues and excreta (liver, blood, bile or urine; faeces are discussed below). The great differences in the characteristics of these media resulted in special analytical approaches to PAH analysis. Here also several steps can be discerned: isolation/extraction (sometimes in combination with a deconjugation step), purification and separation/detection. Table 7 presents examples of the main methods.

2.3.3. Faecal PAH determination

The determination of PAHs in faeces has been performed in a limited number of investigations, which are compared in Table 8. The determination of PAHs from faeces mostly involves saponification in an aqueous solution of 0.1 *M* KOH, followed by extraction into an organic solvent, *e.g.*, toluene, hexane, cyclohexane, DCM, methanol, ethanol, benzene–methanol, ethyl acetate or butanol [172,173,200,201,206]. For the extraction of PAH metabolites with organic solvents a deconjugation step is necessary, in order to cleave off the glucuronide or sulphate groups. This can be done by acid hydrolysis with 10 *M* HCl [172,206], alkalinization with KOH [201,203] or enzymatic digestion with glucuronase–sulphatase mixtures [201]. After cleavage, the free PAHs are extracted by organic solvents. The extracts are then mostly purified by filtration on silica gel using the above-mentioned organic solvents as eluent or Lipidex 1000 with methanol–chloro-

TABLE 6

EXAMPLES OF METHODS FOR THE DETERMINATION OF PAH COMPOUNDS IN SOIL, WATER AND AIR

Sample	Extraction	Fractionation or purification	Detection	Detection range or limit	Ref.
Soil	Soxhlet: benzene–hexane	Al_2O_3–silica gel	MS	ng/g	188
	Soxhlet: ethanol–toluene	XAD-2	GC–FID	ng/g	189
	Saponification, dichloromethane	Al_2O_3	GC–MS	0.2 ng/g	190
	Acetone–hexane	0.5-μm PTFE filter	HPLC–fluorescence	ng/g	191
Water	Dichloromethane	Silica gel	HPLC–fluorescence or UV	0.01–2 μg/l	192
		Sep-Pak	HPLC–fluorescence	10 ng/l	193
			GC–MS	–	194
Air	Soxhlet: dichloromethane	HPLC	MS	ng/m^3	195
	Soxhlet: dichloromethane	Silica gel	GC–MS	ng/m^3	196
	Soxhlet: cyclohexane	Liquid–liquid, silica gel/Sephadex	GC–MS	0.05 ng/m^3	197

TABLE 7

EXAMPLES OF METHODS FOR THE DETERMINATION OF PAH COMPOUNDS IN TISSUES AND EXCRETA

Matrix	Extraction	Fractionation or purification	Detection	Detection range or limit	Ref.
Lung, spleen, testis, lymph, bile, blood	–	Oxidation by combustion	Liquid scintillation	ng/ml	198
Lung, liver, blood	KOH–DMSO–hexane	Neutralization/bleaching	Liquid scintillation	0.1 nmol/ml	199
Urine, blood, mucus	Alkali and acid hydrolysis or ethyl acetate	Neutralization/bleaching	Liquid scintillation and RP-HPLC	–	200
Urine	Acid hydrolysis, cyclohexane	Silica gel, Sephadex LH-20	Capillary GC	< 1 ng	172
Urine	Methanol–chloroform, methanol–water	Sep-Pak C_{18}, SP-Sephadex C_{25}, TEAP-LH-20	RP-HPLC, UV, silica gel TLC, UV. Also: liquid scintillation	–	201
Urine	Soxhlet: HCl–toluene, methanol, cyclohexane	Sephadex LH-20, GC–MS	Capillary GC	< 1 ng	202
Blood, liver, spleen	Protein precipitation ethyl acetate	TLC	MS	–	203
Urine	Enzymatic digest, ethyl acetate	TLC	MS		203

TABLE 8

METHODS FOR THE DETERMINATION OF PAH COMPOUNDS IN FAECES

Extraction	Fractionation or purification	Detection	Detection range or limit	Ref.
Ethyl acetate–butanol	Sephadex LH-20	RP-HPLC, liquid scintillation	–	173
Alkali and acid hydrolysis or ethyl acetate	Neutralization/bleaching	RP-HPLC, liquid scintillation	–	200
NH_3 solution methanol and ethyl acetate	–	RP-HPLC, UV or fluorescence; also liquid scintillation	pmol	204
Soxhlet: benzene–methanol cyclohexane, DMSO or nitromethane	Silicar CC-7	RP-HPLC, UV and fluorescence; also liquid scintillation	1 10 ng/g	205
Saponification with KOH, acidification, cyclohexane	Silica gel, Sephadex LH-20	Capillary GC	< 1 ng	172
Ethanol–water, hexane–propanol	Lipidex-1000, Sep-Pak C_{18}, Sephadex LH-20 TEAP-LH-20, silica gel, Lipidex 5000. Also: enzymatic hydrolysis	RP-HPLC, UV, silica gel TLC, UV. Also: liquid scintillation	–	201
Saponification with KOH, Soxhlet: HCl–toluene, methanol, cyclohexane	Sephadex LH-20	Capillary GC, GC–MS	< 1 ng	206
Ethyl acetate, chloroform, toluene	Enzymatic digestion, ethyl acetate, TLC	MS		203
KOH digestion, chloroform–methanol	–	Liquid scintillation	–	207
Homogenization, lyophilization	–	Liquid scintillation	–	208
Water methanol	HPLC	Liquid scintillation	–	209

form mixtures as eluent [172,173,205,206]. The purified and eluted PAHs are then separated by LC on, *e.g.*, Sephadex LH-20 [172,201] or several kinds of reversed-phase HPLC columns [204], and detected by fluorescence or UV (diode-array) spectrophotometry [205]. Both gradient and isocratic HPLC analyses have been described using various C_{18} columns [200,201,205].

Another kind of technique involves the measurement of radioactivity in a liquid scintillation counter in experiments using radioactively labelled PAHs. After extraction in an organic solvent, the components may be purified or separated by ion-exchange chromatography or HPLC. Further, capillary GC, GC–MS and TLC–MS have been applied in faecal PAH determinations [172,202,203].

Reviewing the different methods for the determination of PAHs in faeces, several conclusions may be drawn. First, the use of radioactively labelled PAHs in combination with liquid scintillation counting is a sensitive method for detecting the fate of the administered dose, and is therefore applicable in kinetic studies. Another advantage is that the sample pretreatment can be very simple. However, in its simplest form this method cannot discriminate the original PAH from its metabolites. For this a purification and separation step, preferably by (reversed-phase) HPLC, is necessary. Also, liquid scintillation counting can be used only after administration of radioactive PAHs, which makes this method unsuitable for biological monitoring after *e.g.*, environmental exposure to PAHs. For this application, reversed-phase HPLC combined with fluorescence detection (after extraction and purification procedures) or capillary GC or GC–MS can be recommended because of their sensitivity (with a detection limit in the ppb–ppt range). Whereas in GC or GC–MS the various components are lost, HPLC has the advantage of the possibility of recovering the different PAHs or metabolites, especially if preparative HPLC columns are used. On the other hand, unknown components cannot be positively identified by HPLC with fluorescence detection. Even diode-array UV detection has it limitations. For this purpose MS is the preferred method.

It can be concluded that the various methods for the determination of faecal PAHs have advantages and limitations and that the method selected will depend on the aim of the investigation.

In the context of a study to assess the bioavailability of soil-bound PAHs after oral intake, we have recently adapted a method for determining non-metabolized PAHs in faeces. With this method we performed a pilot experiment to determine the amount of non-metabolized PAHs that is excreted via faeces after oral intake. Rat faeces (0.3–0.4 g) were spiked with 2–24 ng of anthracene, then stored at room temperature for several days and subsequently suspended in water (18 ml/g faeces) by sonication for 5 min and shaking for 1 h. An equal volume of hexane was added, after which the mixture was shaken for 1 h. Half of the hexane phase was collected, evaporated to dryness under nitrogen and the residue dissolved in a small volume of hexane (0.5 ml/g faeces). From this solution, aliquots of 150–250 μl were purified by chromatography on a silica gel 60 column (4.5 × 0.5 mm I.D.; gel volume 3.5 ml). After elution with hexane, six fractions of 2 ml each were obtained. HPLC analysis was performed by isocratic elution with acetonitrile–water (60:40) on a Chrompack 5 ODS Hypersil column, using fluorescence detection (λ_{ex} = 253 nm; λ_{em} = 370 nm). With this HPLC method the detection limit for (pure) anthracene is about 1–2 pg in 20 μl and the retention time of anthracene is between 11.7 and 12.0 min (Fig. 5A).

Of the initially spiked dose, 83.4 ± 6.3% (n = 5) was recovered. Apart from the anthracene peak, on the HPLC trace a second peak was observed having a retention time of 10.8 min (Fig. 5B). This component is slightly more polar than anthracene itself and probably represents a contaminant present in the extraction solvent, as the same component was found in reference measurements only using hexane. From these consistent results, we conclude that this method is well suited to the measurement of non-metabolized PAHs in faeces.

Therefore, we used this method for the determination of bioavailability of oral doses of PAHs. In a pilot experiment, 40 μg of anthracene

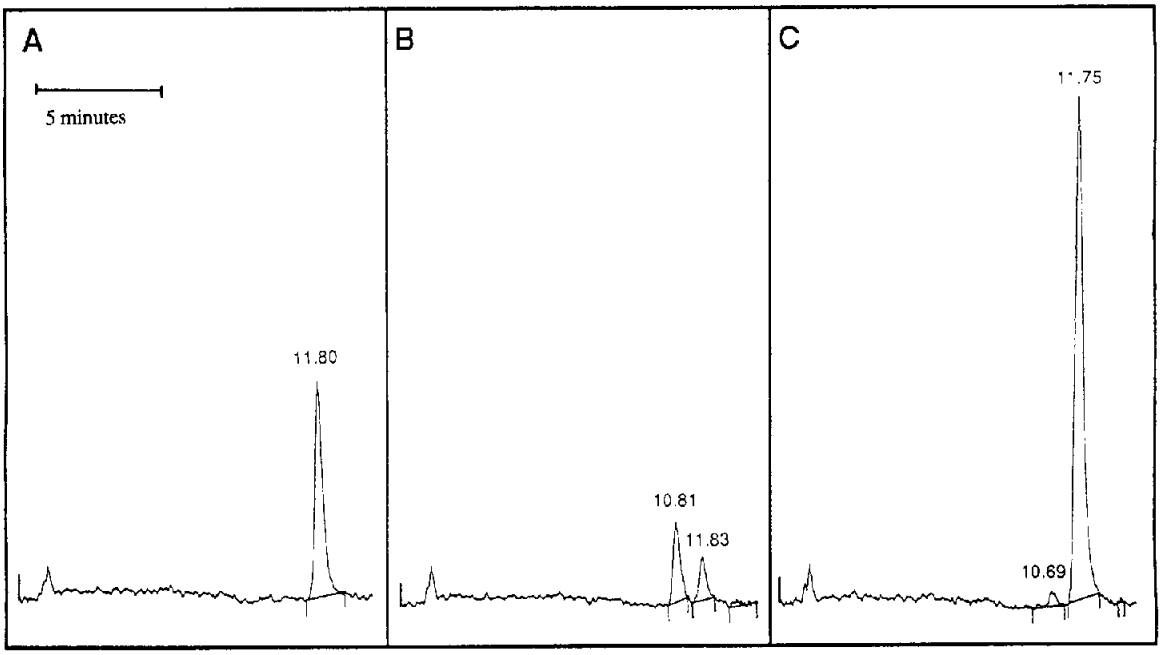

Fig. 5. HPLC of (A) 40 μg of anthracene (retention time = 11.8 min), (B) silica elution fraction 1 of faecal extract (t = 0) and (C) silica elution fraction 3 of rat faecal extract after oral administration of anthracene. Both B and C show an additional peak (retention time = 10.81 or 10.69 min) identified as a contaminant in the extraction solvent.

(Sigma) were dissolved in 0.5 ml of sunflower oil and administered orally to rats by gavage (n = 2). Control rats (n = 2) were dosed with 0.5 ml of sunflower oil only. The rats were housed in stainless-steel metabolic cages and were allowed to eat standardized lab chow and drink water *ad libitum*. Faeces were collected during 24 h prior to administration (0-h sample) and at 24 and 48 h after the oral dose. All samples were treated as described above (modified method).

Preliminary results indicate that the 0-h samples contain several components, one of which yields a peak with the same retention time as anthracene (Fig. 5A and B). Quantification of these peaks as anthracene rendered an excretion of 10.4 ± 3.8 ng per 24 h. In control rats, administration of 0.5 ml of sunflower oil did not significantly change the anthracene excretion over a 48-h period (9.1 ± 0.9 ng per 24 h). Based on measurements on reference extractions (only using hexane) we conclude that the detected peaks in the 0-h samples and the samples from the control rats are caused by a contaminant in the extraction solvent.

The oral administration of 40 μg of anthracene in sunflower oil resulted in a faecal excretion of 1646–2245 ng of non-metabolized anthracene within a period of 48 h after administration (Table 9; Fig. 5C), which is 3.6–5.2% of the original dose (these values have been corrected for background peaks). Taking into account that via this

TABLE 9

DETERMINATION OF NON-METABOLIZED ANTHRACENE IN RAT FAECES COLLECTED DURING 24 h BEFORE AND AFTER AN ORAL DOSE OF 40 μg OF ANTHRACENE

Amounts in ng; measured in duplicate.

Animal	t = 0 h	t = 24 h	t = 24 h
1	98.7 ± 35.0	1529.2 ± 221.0	116.6 ± 7.1
2	77.3 ± 0.8	2112.8 ± 452.4	131.7 ± 5.8

extraction method about 83% of the PAHs in faeces are recovered, the corrected dose recoveries are 4.3–6.3%.

The pilot experiment indicates that only a very small part of the orally administered dose of anthracene is excreted in faeces in unchanged form. This would imply that a large amount (*ca.* 95%) of the oral dose is absorbed and/or metabolized, indicating that the bioavailability of pure anthracene after oral intake is high. In a study by Hecht *et al.* [205], it was found that in rat faeces 74–79% of an oral dose of B[a]P was excreted via faeces, of which 5.6–13% consisted of unchanged B[a]P. Further experiments are planned in order to extend our knowledge about the bioavailability of soil-bound PAHs after oral intake.

3. CONCLUSION

It is clear that the determination of faecal toxicants requires properly evaluated sample preparation and chromatographic methods. This has been illustrated by three examples of hypothesized etiological factors in colorectal carcinogenesis. In fecapentaene determination, HPLC is preferred as the main chromatographic method. Various elution systems are available for the determination of total fecapentaenes or for the separation of several fecapentaene analogues and chemically related fecaenes. Analytical problems related to the chemical instability of this type of compound may be avoided by addition of stabilizing agents and applications of standardized high-recovery extractions. Loss of fecapentaenes can also be diminished by conversion into more stable products. Further, the use of internal standards or isotopic dilution has been applied in order to correct for fecapentaene decay during sample preparation. Future research applying these methods to populations at different risk of colorectal cancer and in dietary studies may elucidate the role of fecapentaenes in the initiation of cancer of the large bowel.

For determination of faecal bile acids, many chromatographic methods are available. Sample preparation for bile acid analysis has been shown to be laborious, involving several consecutive column chromatographic or other purification steps. Although TLC, HPLC and enzymatic method are still in use for the determination of faecal bile acids, a tendency in favour of the application of GC has been observed. Combination of GC and MS appears to be the most sensitive and specific method. Further, a relatively rapid method for the determination of unconjugated bile acids and neutral steroids has been described and recommended. Application of these methods in epidemiological studies suggests a role for faecal bile acids in colorectal cancer, although the results are not always unequivocal.

Finally, the determination of PAHs in faeces has been discussed. Faecal PAHs mainly originate from the diet or occupational exposure and can be determined by both HPLC and GC methods. In previous studies, excretion of PAHs in faeces has generally been quantified by measurement of the recovered radioactivity after oral intake or dermal application. However, it is suggested that the intake of PAHs from the environment may be considerable in certain cases of soil contamination. In order to study the bioavailability of PAHs after oral intake of contaminated soil, an HPLC method for the determination of non-metabolized PAHs in faeces has been described. This method results in a total recovery of about 80% after extraction and purification on silica gel. Application of this method in the determination of non-metabolized anthracene in rats showed that over a 48-h period between 4 and 6% of the orally applied dose of pure anthracene was excreted unchanged in faeces. It is concluded that the described method is applicable to the determination of the bioavailability of oral doses of PAHs.

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