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Determination of bile acids in human faecal samples using supercritical fluid extraction and high-performance liquid chromatography

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Abstract

A supercritical fluid extraction (SFE) method for the extraction of bile acids from faeces is described. HPLC with pulsed amperometric detection was used to examine and confirm the recovery of bile acids. The analytes were extracted within a period of 75 min using supercritical carbon dioxide at a pressure of 34.5 MPa and a temperature of 90°C. In developing this method the following parameters were investigated: temperature, pressure, and extraction time. Two alternative methods of sample preparation were also investigated with a view to reducing the overall analysis time. The method was validated for the major primary and secondary bile acids found in faeces. It was found that the overall mean±SD recoveries were 102.1±7.92%, 111.6±9.91%, 112.1±9.92% and 113.7±9.92% for dry samples and 108.5±15.77%, 110.0±7.22%, 115.9±11.11% and 106.6±9.16% for wet samples with respect to cholic, deoxycholic, chenodeoxycholic and lithocholic acid. The SFE is an alternative to the traditional methods available. The extraction is relatively easy to conduct and does not utilise as much glassware, solvents or time.

Keywords: Bile acids

1. Introduction

Bile acids are steroid acids of bile, produced during the metabolism of cholesterol in the liver. Common bile acids possess a characteristic structure consisting of a 24 carbon atom skeleton whose terminal three carbon atoms of the cholesterol side chain are cleaved during its synthesis from cholesterol. Fig. 1 illustrates the characteristic structure of the major bile acids found in human faeces.

Studies have interlinked faecal bile acids with cholesterol lowering effects and colorectal cancer. The latter is one of the most common malignancies reported in developed countries being second only to lung cancer in men and breast cancer in women. In the United Kingdom there are approximately 17 000 deaths from large bowel cancer annually, nearly half of which are due to colorectal cancer [1]. Bingham [2] stated that countries with the highest risk include Australia, New Zealand, USA and parts of Northern Europe, those with the lowest risk include Africa, China and India.

In order to establish the effect of certain dietary factors upon bile acid metabolism, faecal bile acids are normally analysed. A plethora of methods concerning bile acid extraction have been proposed by a

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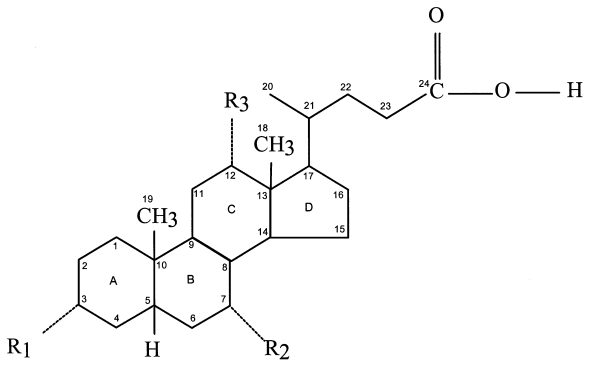

	Cholic	Chenodeoxycholic	Deoxycholic	Lithocholic
R_1	OH	OH	OH	OH
R_2	OH	OH	H	H
R_3	OH	H	OH	H

Fig. 1. Characteristic structure of the major bile acids found in human faeces.

number of researchers over the last three decades [3–8]. The method proposed by Grundy et al. [3] is one of the most frequently cited for faecal bile acid measurements. Unfortunately, the methods mentioned above are not simple or rapid but instead are both time consuming and costly in terms of the quantities of solvents used during the extraction stages.

Supercritical fluid extraction (SFE) is a relatively new technique, which possesses unique physicochemical properties of both liquids and gases that make them attractive as solvents for extraction. Due to its simplicity it has found a growing interest in recent years, evolving to replace many of the standard extraction procedures [9]. Their solvent strength can approach those of liquids and can be easily varied by changing the extraction pressure or temperature.

The major advantage of SFE is that it is a rapid method of extraction that has the capability for on-line analysis. The use of carbon dioxide as a supercritical fluid eliminates the concern of environmental pollution with organic solvents. The potential for loss of analytes and sample contamination is reduced as sample handling and the numbers of steps are minimised.

To date the only research that has been conducted upon SFE faecal extraction is using hamster faeces, and this has been done by two groups of researchers in America. The first group Pinkston et al. [10] researched components of hamster faeces ranging from low molecular mass fatty acids through the expected range of triglycerides using off-line SFE. The extraction chamber was heated to 60°C or 100°C and pressurised to 39.7 MPa. Whilst the second group Merkt et al. [11] produced an abstract on the analysis of faecal bile acids and neutral sterols using SFE and GC–MS. There was no paper or documentation produced on the exact SFE method employed.

A third group has analysed free bile acid standards by supercritical fluid chromatography and evaporative light scattering detection [12]. They found that this chromatographic method was more sensitive than UV detection and had the potential to be applied subsequently to biological samples such as stools. Scalia and Games have used supercritical fluid chromatography for the analysis of conjugated bile acids in bile samples [13,14] and have developed a new procedure for the assay of chenodeoxycholic and ursodeoxycholic acid in capsule and tablet formulations [15].

The aim of this paper was to present a rapid method for the extraction of human faecal bile acids using a supercritical fluid as an alternative to the long solvent extraction methods available. This method is a three-step process, consisting of extraction by SFE, purification with Sep-Pak tC_{18} cartridges followed by quantification with HPLC. This study was initiated with a view to reducing the time required for the isolation of the bile acids, thereby reducing the analysis time required for the entire procedure. Two different methods of sample preparation were investigated with a view to reducing the overall analysis time further.

2. Experimental

2.1. Chemicals

Bile acids: cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid were all purchased from Sigma (Poole, Dorset, UK). Ricinoleic acid was used as an internal standard purchased from Fluka.

Packing materials: octylsilane and octyldecylsilane

were purchased from Jones (Lincoln, NE, USA) Florisil activated magnesium silicate (particle size 74–149 μm) purchased from Sigma and Florisil deactivated (particle size 149–250 μm) and alumina oxide were purchased from BDH Merck (Poole, Dorset, UK).

Hydromatrix was purchased from Varian (Walton-on-Thames, UK).

Anhydrous sodium sulphate (analytical grade), sodium acetate and all other solvents used and were of HPLC-ECD (electrochemical detection) grade and purchased from Fisher (Loughborough, UK).

2.2. Apparatus

Extraction work was carried out using an Isco SFX 2-10 extractor (0°C–150°C) and an Isco single pump system (Model 260D) with manual refill valves (Lincoln, NE, USA). The flow of carbon dioxide was controlled by the use of an adjustable variable restrictor and was heated to 100°C by an Isco restrictor heater.

HPLC study was performed on a Dionex DX-300 HPLC system fitted with a PAD-2 pulsed amperometric detector with a gold working electrode. Bile acids were eluted using a 250×4 mm I.D Dionex Carbopac PA-100 column (bead diameter 8.5 μm) protected by 50×4 mm I.D Dionex Carbopac PA-100 guard column maintained at 60°C.

2.3. Standards

Working bile acid standard solutions 0.05, 0.1, 0.2, and 0.4 mg/ml, containing mixtures of cholic, chenodeoxycholic, deoxycholic and lithocholic acid made up to volume with methanol were used to spike human faecal samples.

2.4. Samples

All faecal samples collected were homogenised with an equal amount of 2 m*M* HCl using a stomacher until no longer particulate. Using a wide tipped pipette, a 1 or 1.5 g aliquot was taken and placed into a preweighed beaker. Excess samples were stored at −31°C until analysis. These samples were defrosted at room temperature using tepid water and then shaken until homogenous, prior to extraction.

2.5. SFE

2.5.1. Method (A) dry faecal samples

A 1.5 g of faecal sample was freeze-dried overnight. Then using a medium sized spatula the sample was ground into fine particles. Samples were prepared in Isco extraction cartridges, which are both temperature and pressure resistant. A 1.5 g mass of octylsilane packing material was placed into an extraction cartridge via a plastic funnel, followed by the dry powdered faecal sample. This was then mixed using a small spatula until uniform in constitution. Three grams of anhydrous sodium sulphate was placed on top and then mixed with the rest of the cartridges content, until the faecal sample was fully dispersed among the contents of the extraction cartridge. The extraction cartridge was then packed with shredded tissue to remove any excess void volume.

The SFE chamber was heated to 90°C and allowed to equilibrate for 20 min prior to the first extraction of the day and set to a pressure of 34.5 MPa. Five ml of 12.5% (v/v) dimethyl sulphoxide (DMSO) in methanol was added to the top of the extraction cartridge as a modifier. A static extraction of 15 min followed by a 15 min dynamic extraction was carried out. The bile acids were collected into a 25 ml volumetric flask containing 5 ml of methanol and 0.2 ml of 1 mg/ml ricinoleic acid. The volumetric flask was placed in a beaker of tepid water to reduce rapid condensation and build up of ice around it. The extraction was repeated under the same conditions with a further 5 ml of modifier and then once again with a static extraction of 5 min and a dynamic extraction of 10 min. In each of these extractions the flow of carbon dioxide was maintained between 1–2 ml/min whilst the restrictor was heated to 100°C.

2.5.2. Method (B) wet faecal samples

A 1 g faecal sample was mixed with approximately 1 g of hydromatrix (derivative of diatomaceous earth) until the sample was no longer wet and grained particles were separate. The hydromatrix support helps to disperse the sample evenly, allowing the supercritical fluid to solvate the analytes of

interest efficiently and without interference of moisture. The amount needed varies with the moisture content of the sample matrix, but for best results the sample–support mixture should be free-flowing and appear dry. Care was taken to ensure all the contents of the beaker were transferred to an Isco cartridge containing 1.5 g of octylsilane packing material. The contents of the tubes were not homogenised, hence the packing material would act as a filter bed. Shredded tissue was also added to the cartridge to allow for the addition of modifier. Five ml of 12.5% (v/v) DMSO in methanol was added to the beaker that had contained the wet faecal sample and swirled. This was to ensure that any bile acids that may have adsorbed onto the glassware would be transferred to the extraction cartridge. Extraction times and conditions used were exactly the same as for the dry sample.

2.6. Solvent extraction

Samples were extracted according to the validated method by Davies et al. [16]. A 2.0±0.3 g mass of human faeces with 0.2 ml of 1 mg/ml ricinoleic acid were lyophilised overnight. Once samples appeared light in colour they were extracted with 24 ml methanol with the aid of a glass rod and refluxed (EMEA Electromantle Range) for 110 min (heat setting 3, stirrer setting medium). Six ml of water was added and refluxed for another 10 min. The contents were then centrifuged at 925 *g* for 10 min, the supernatant was retained and the pellet was suspended in 30 ml chloroform: methanol (50:50, v/v) and refluxed for 120 min using the conditions stated above. The contents were then centrifuged at 925 *g* for 10 min. Both organic supernatants were pooled and then evaporated to dryness using a 500 ml centrifugal evaporator (Savant sc 110 speed vac) overnight on medium heat setting. The residue obtained was then dissolved in 4 ml methanol and the procedure in Section 2.7 was followed.

2.7. Sample purification and concentration

For both methods (A) and (B) the contents were transferred to 10 ml graduated test tubes and placed into a water bath heated to 60°C and evaporated to 4 ml under a flow of nitrogen. Six ml of 0.1 *M* sodium phosphate pH 7.5 buffer was added to the remaining contents of the tubes. The stoppered tube was heated briefly at 60°C and then centrifuged at 925 *g* for 20 min. Samples were extracted using Sep-Pak tC_{18} cartridges. Cartridges were preconditioned twice with 5 ml methanol followed by 5 ml water. The clarified solution containing bile acids was then passed through the cartridge. The cartridge was then washed with 5 ml of methanol–0.1 *M* phosphate buffer pH 4.5 (40:60, v/v), followed by 2 ml water. Finally the cartridge was eluted twice with 2 ml of methanol and collected. These were then evaporated and reconstituted with a mixture of 0.4 ml methanol, 0.2 ml sodium hydroxide (46–48%, w/v) and 0.6 ml water, whereupon they were injected onto the HPLC.

2.8. HPLC

Bile acids were eluted under gradient conditions using 4.5% acetonitrile in 0.7 *M* sodium acetate, 0.1 *M* sodium hydroxide (mobile phase A) and 20% acetonitrile in 0.7 *M* sodium acetate, 0.1 *M* sodium hydroxide (mobile phase B). The PAD conditions chosen were $V_1=0.0$ V, $t_1=720$ ms, $V_2=+0.6$ V, $t_2=120$ ms, $V_3=-0.6$ V, $t_3=60$ ms. The gradient profile used was as follows: 100% A from 0.0–3.0 min, 100% B from 26.5–37.0 min, 100% A from 37.1–42.0 min. Flow-rate was set to 1 ml/min and 25 μl of sample was injected. The columns were preequilibrated for 30 min using mobile phase B at 0.5 ml/min at the beginning of each day. A 5-min equilibration time was allowed between each injection.

3. Results and discussion

A number of investigations, varying the temperature, pressure, extraction time, modifier and sample preparation technique, were undertaken in order to determine the optimal conditions required for the extraction of bile acids from faecal samples.

Preliminary investigations identified octylsilane as the appropriate packing material to be used for the extraction of bile acids. Table 1 illustrates the percentage recoveries of bile acids from different packing materials. With any extraction method a possible limiting factor is how strongly the analyte is

Table 1
Percentage mean recovery and standard deviation of bile acids with different packing materials at a pressure of 41.4 MPa and a temperature of 70°C for a 10-min static extraction and a 20-min dynamic extraction period

Packing material	n[a]	Cholic	Deoxycholic	Chenodeoxycholic	Lithocholic
Activated Florisil	4	<1%	<1%	<1%	<1%
Deactivated Florisil	4	0.0	0.0	0.0	0.0
Alumina oxide	4	0.0	0.0	0.0	0.0
Octylsilane	4	79.0±2.47	76.7±3.18	84.5±1.58	82.6±2.41
Octyldecylsilane	4	78.5±2.03	72.2±2.74	79.3±1.41	76.8±2.45

[a] n=no of samples extracted.

absorbed onto the matrix. This was limited in this investigation by the use of octylsilane, which gave the maximum recovery of the bile acids in comparison to the others. The slight difference in the recovery of bile acids between octylsilane and octyldecylsilane can be explained by the fact that the former sorbent is less retentive than the latter when retention is based solely on nonpolar interactions.

Pressure and temperature conditions between the range of 20.7–48.3 MPa and 50–80°C respectively were studied further (n=45 extractions) using a 0.2 mg/ml bile acid solution. This was to ascertain the optimal extraction conditions. It was found that ricinoleic acid, the internal standard was not extracted efficiently, when added directly to the extraction cartridge. It was decided to add the internal standard directly to the volumetric flask in which the analyte was collected. Ricinoleic acid may have a strong affinity for the octylsilane packing material hence not extracted efficiently. It was necessary to include an internal standard in this particular protocol, to account for any bile acid losses through purification and transfer procedures and for the quantification during HPLC. A pressure of 34.5 MPa and a temperature of 70°C gave the highest recovery.

A number of modifiers were used to increase the polarity of the carbon dioxide, hence enhance the recovery of bile acids (Table 2). This experiment was based upon single determinations to provide data for the selection of the appropriate modifier. Methanol was the ideal modifier as it gave the best recovery and required no preparation. Bile acids, in particular lithocholic acid are hydrophobic compounds and do not dissolve significantly in aqueous media. This was shown by the use of modifiers such as 90% absolute ethanol and 80% absolute ethanol. As the aqueous content increased, the extraction recovery decreased, especially for lithocholic acid, from 97.6 to 82.4% for the modifiers mentioned above respectively.

A 0.4 mg/ml bile acid standard was extracted for 110 min at 34.5 MPa and 75°C, the per cent recoveries were 89, 95.5, 90.5 and 89 for cholic, deoxycholic, chenodeoxycholic and lithocholic acid respectively. These per cent values were found to be lower when a faecal sample (1.5 g) spiked with 0.4 mg/ml bile acid standard was extracted; 82.5, 93.1, 95.2 and 81.8. Optimal conditions using only bile acid standards may be different from those for actual sample matrices. The bile acids may be strongly bound to the nonsoluble components of faeces (e.g. fibre).

Table 2
Modifiers used for the modification of carbon dioxide[a]

Entrainer	Cholic	Deoxycholic	Chenodeoxycholic	Lithocholic
Methanol	114.2	111.8	104.0	97.6
100% abs. ethanol	110.4	108.7	116.8	86.1
90% abs. ethanol	120.7	113.5	104.4	97.6
80% abs. ethanol	99.6	101.2	91.3	82.4
Chloro:meth (1:1, v/v)	79.4	75.1	66.8	66.3

[a] An extraction time of 60 min was employed (a 10-min static and 20-min dynamic extraction carried out twice) at a pressure of 34.5 MPa at 70°C.

A higher temperature of 100°C improved extraction recoveries and reduced the extraction time to 90 min, owing to increased solute diffusion coefficients at higher temperatures. Under these new conditions the overall mean bile acid per cent recoveries and standard deviations from faecal samples spiked with bile acid standards were as follows: the dry ($n=8$) and wet ($n=10$; a 1.5 g wet faecal sample) samples were found to be 97.2 ± 7.57, 100.5 ± 20.02 110.3 ± 20.02, 114.6 ± 12.16 and 89.3 ± 11.15, 131.1 ± 23.26, 106.0 ± 16.59, 110.8 ± 17.25 for cholic, deoxycholic, chenodeoxycholic and lithocholic acid respectively. There appeared to be a possibility of an interfering compound being extracted with the bile acids at this higher temperature. Since percentage recoveries for some of the faecal samples spiked with bile acid standards were relatively high, it was thought that some fatty acid or other lipid was present.

This led to the partial modification of the protocol to eliminate the interference problem and to investigate if the extraction time could be reduced further. Different modifiers were investigated within the range of 90–100°C with a 0.4 mg/ml bile acid solution and were extracted for 90 min to establish a difference in extraction recoveries (Table 3). A temperature of 90°C at 34.5 MPa with a modifier 12.5% DMSO in methanol were found to be just as efficient for the extraction of bile acids. Using these new extraction conditions, the method was validated by spiking faecal samples with a range of bile acid standards (0.05–0.40 mg/ml). Table 4 illustrates the per cent recoveries for the wet and dry methods. A lower temperature reduced the interference problem with a reduction in wet weight, hence extraction time.

Calibration curves for bile acids were established in the range of 0.05–0.48 mg/ml for both dry and wet SFE methods. The limit of quantification for the bile acids was approximately 0.04 mg/ml. The curves for the regression line and correlation coefficient for the dry and wet SFE methods were as follows: $y=4.20x+0.05$, $r=0.995$; $y=3.53x-0.005$, $r=0.995$; $y=2.31x-0.02$, $r=0.998$; $y=1.00x-0.02$, $r=0.974$ and $y=5.29x+0.17$, $r=0.982$; $y=4.10x+0.01$, $r=0.997$; $y=3.73x-0.01$, $r=0.997$; $y=1.83x-0.01$, $r=0.998$ for cholic, deoxycholic, chenodeoxycholic and lithocholic acid respectively.

With respect to the final extraction methods developed it was found that it was necessary to either lyophilise the wet faecal sample or to mix it directly with hydromatrix. Sodium sulphate alone was not efficient at absorbing the moisture completely and led to restrictor blockages. The size of the extraction cartridge (9 ml) dictated that the wet faecal sample weight be reduced from 1.5 g to 1 g, for it to be dried efficiently by the hydromatrix and for the total transfer of contents into the extraction cartridge quantitatively.

SFE samples appeared to be relatively clean in comparison to the solvent extracted ones after the extraction stage. The Sep-Pak purification step was still necessary. Blank extractions upon shredded tissue paper revealed that an orange dye was extracted, it is recommended that any material used to remove excess void volume should be preextracted by the SFE method used.

Statistical analysis indicated there was a significant difference between the overall dry and wet SFE methods percent mean values (Table 4) for the bile acids cholic ($10\%>P>5\%$) and lithocholic ($5\%>P>2\%$), but none for deoxycholic ($P>10\%$) and

Table 3
Investigation of different modifiers from 90–100°C at a pressure of 34.5 MPa

Entrainer and conditions	Cholic	Deoxycholic	Chenodeoxycholic	Lithocholic
Methanol at 90°C	90.4	86.8	87.1	109.4
Methanol at 95°C	80.8	79.4	81.8	94.7
Methanol at 100°C	89.9	89.7	84.0	118.9
10% DMSO in methanol at 90°C	96.9	102.2	96.2	95.8
10% DMSO in methanol at 95°C	97.0	91.9	93.7	107.6
12.5% DMSO in methanol at 90°C	99.0	104.8	108.0	109.9
12.5% DMSO in methanol at 95°C	67.0	72.0	69.7	67.0

Table 4
Percentage recoveries of faecal bile acid samples by SFE (34.5 MPa for 90°C) and solvent extraction

Spike (mg/ml)	Parameter	Cholic	Deoxycholic	Chenodeoxycholic	Lithocholic
Extraction at 34.5 MPa and 90°C[a]					
0.05	Dry mean±SD[b]	103.9±14.01	103.9±14.01	103.9±14.01	103.9±14.01
(n=3)[c]	%C.V.	13.5	10.8	11.4	3.9
0.1	Dry mean±S.D	103.5±14.48	108.5±16.79	108.9±14.76	107.5±13.07
(n=4)	%C.V.	3.68	15.47	13.55	12.16
0.2	Dry mean±S.D	97.0±3.75	111.2±8.83	112.0±10.40	112.3±14.22
(n=4)	%C.V.	3.86	7.95	9.29	12.67
0.4	Dry mean±S.D	103.5±8.86	111.8±4.38	114.7±6.19	117.9±5.23
(n=6)	%C.V.	8.56	3.91	5.40	4.45
Overall	Dry mean±S.D	102.1±7.92	111.6±9.91	112.1±9.92	113.7±9.92
(n=17)	%C.V.	7.76	8.88	8.85	8.72
Extraction at 34.5 MPa and 90°C[d]					
0.05	Wet mean±S.D	119.3±12.23	116.9±3.32	122.2±0.21	113.1±0.85
(n=2)	%C.V.	10.26	2.84	0.17	0.75
0.1	Wet mean±S.D	121.2±13.15	110.2±0.41	121.9±13.15	108.3±12.81
(n=3)	%C.V.	10.85	9.45	10.79	11.83
0.2	Wet mean±S.D	111.1±13.17	109.1±5.68	117.0±11.70	104.4±9.37
(n=5)	%C.V.	11.85	5.20	10.00	8.97
0.4	Wet mean±S.D	96.3±13.42	108.4±7.90	110.0±10.47	105.5±9.52
(n=6)	%C.V.	13.93	7.29	9.53	9.03
Overall	Wet mean±S.D	108.5±15.77	110.0±7.22	115.9±11.11	106.6±9.16
(n=16)	%C.V.	14.55	6.56	9.59	8.59
Solvent extraction					
Overall	Mean±S.D	103.7±14.61	104.5±27.84	115.7±21.62	125.7±22.05
(n=15)	%C.V.	14.08	26.63	18.69	17.55

[a] Extraction time of 75 min was employed using 1.5 g of faecal sample.
[b] SD=standard deviation.
[c] n=no. of samples analysed.
[d] Extraction time of 75 min was employed using 1 g of wet faecal sample.

chenodeoxycholic acid ($P>10\%$). A possible explanation for such a variability occurring is the water content in both methods and the homogeneity of the aliquot of faecal sample. In the dry method most of the moisture is removed by freeze-drying, all percentage recovery calculations are based upon dry weight. In the wet method one cannot account for the exact contents of the faecal aliquot, how much faecal water or biomass is actually present. Therefore in the latter case there appears to be a chance of in-built error. The dry method appears to be best and the overall standard deviation of the bile acids is less in comparison to the wet and solvent extraction methods (Table 4).

A plausible reason for the significant difference in the variances between the solvent extraction and SFE (dry and wet) methods is that with the solvent extraction method there are more steps which involve the use of different glassware items and thus, the potential for loss and experimental error to occur anywhere in the protocol.

There was no significant difference between the percentage mean values of the solvent extraction method and SFE (dry and wet) methods for all bile acids, apart from lithocholic extracted by the wet SFE method ($0.1\%>P>0.2\%$). This finding is important as it reveals that there are potentially no losses on freeze-drying apart from lithocholic acid.

An important aspect of the comparitive study between the three methods has been in the similarities of the faecal bile acid profiles exhibited by all three methods (Fig. 2). It is highly recommended

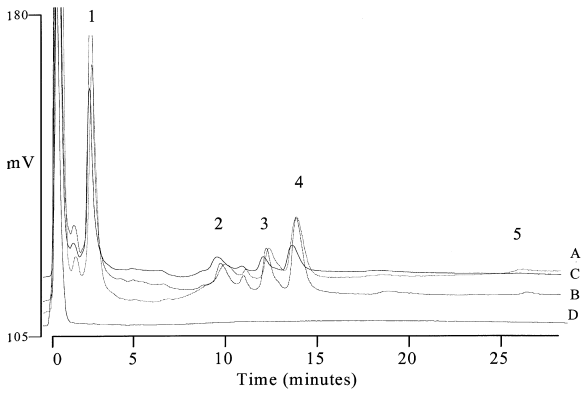

Fig. 2. Bile acid profiles of a subject generated by the SFE and solvent extraction methods. A=SFE dry method; B=SFE wet method; C=solvent extraction method and D=blank extraction. 1=Cholic acid; 2=deoxycholic acid; 3=chenodeoxy-cholic acid; 4=ricinoleic acid and 5=lithocholic acid.

when working with faecal samples and HPLC, that the column should be cleaned on a regular basis to prevent the build up of organic/inorganic contaminants on the column, hence allowing data to be analysed at its optimum.

4. Conclusion

This method enables very fast determination of faecal bile acids in less than 3 h including extraction time, purification step and HPLC analysis time in comparison to traditional techniques. The supercritical extraction stage has the potential to be automated by the addition of a second pump to mix pure organic modifiers and an autosampler to analyse numerous samples. Supercritical carbon dioxide has been used for the extraction of bile acids from faeces using octylsilane packing material. Mean±SD bile acid recoveries were 102.1±7.92%, 111.6±9.91%, 112.1±9.92% and 113.7±9.92% for dry samples and 108.5±15.77%, 110.0±7.22%, 115.9±11.11% and 106.6±9.16% for wet samples with respect to cholic, deoxycholic, chenodeoxycholic and lithocholic acid. The results obtained from this study demonstrate the use of SFE-HPLC as an alternative method for faecal bile acid extraction.

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