In situ derivatisation and extraction of volatile fatty acids entrapped on anion-exchange resin from aqueous solutions and urine as a test matrix using pentafluorobenzyl bromide in supercritical carbon dioxide

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Abstract

The simultaneous extraction and derivatisation of anion-exchange resin-trapped volatile fatty acids (C_2–C_5) as their pentafluorobenzyl esters has successfully been performed under CO_2 supercritical fluid extraction conditions. Volatile fatty acid standards of acetic, propionic and n-butyric acids (at 20 and 100 ppm) as their ester derivatives were recovered at 78.0–101.5% (C.V. 3.5–7.5%, $n=6$ and 7). Likewise, acrylic acid recoveries were 57.0–61.0% (C.V. 5.5–5.6%, $n=6$ and 7). This methodology was applied to the quantitation of acetic, propionic, n-butyric and n-valeric acids in spiked urine as a test matrix. Initial clean-up of phosphate and sulfate in the urine was required prior to anion-exchange application and this was achieved by barium salt precipitation. Recoveries ranged from 36 to 66.5% (C.V. 5.9–14.4%, $n=9$ and 6).

Keywords: Carbon dioxide; Fatty acids; Pentafluorobenzyl bromide; Acetic acid; Propionic acid; n-Butyric acid; n-Valeric acid

1. Introduction

Although supercritical fluid extraction (SFE) is still a relatively new extraction technique to the testing laboratory, increasingly more laboratories possess equipment and seek wider applications for its use. As an extraction medium, supercritical CO_2 has a solvent power similar to that of non-polar hydrocarbon solvents such as hexane. This characteristic is often the major factor that limits extraction efficiency in CO_2 supercritical fluid extraction (SFE) and thereby restricts the extraction range of CO_2 to relatively non-polar substances. Modified supercritical CO_2 containing a small percentage of an organic solvent such as methanol increases the polarity of the supercritical solvent, which results in an increase in the range of substances that can be extracted satisfactorily. However, the amount of undesirable co-extractives also increases. An alternative strategy, first highlighted by Hawthorne et al. [1], minimises the extraction of matrix constituents while enhancing the isolation of polar compounds by the simultaneous derivatisation of polar compounds during the supercritical extraction process. An advantage of this

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technique is that it allows the isolation of analytes as stable, relatively non-volatile, derivatives, which may be analysed directly by gas chromatography (GC). These derivatives may also provide enhanced detection with selective detectors.

Simultaneous derivatisation and extraction using various reagents and supercritical CO_2 has been used successfully to isolate derivatives of a number of acidic and phenolic compounds by various workers. These include the extraction as methyl esters of 2,4-dichlorophenoxyacetic acid from soils and sediments, phenols from C_{18} disks using BF_3–methanol in the presence of phenyltrimethylammonium hydroxide [1], chlorophenoxyacetic acids from aqueous solutions containing tetrahexylammonium hydrogen sulfate and methyl iodide [2], and fatty acids and resin acids from sediments as pentafluorobenzyl esters using α-bromo-2,3,4,5,6-pentafluorotoluene in the presence of triethylamine [3]. Recently, phenols were extracted as acetate derivatives following isolation on strong anion-exchange disks [4], and chlorophenoxyacetic acids, pentachlorophenol, sulfonamides, benzimidazoles and quinoxaline-2-carboxylic acid were methylated using methyl iodide from anion-exchange resin following entrapment and preconcentration on the resin [5].

Problems associated with the isolation of volatile fatty acids (VFAs) from complex aqueous matrices have been a major hindrance to their facile routine analysis at low levels. A number of methods have been employed for the determination of VFAs in biological fluids, such as solvent extraction and steam distillation and these have been comprehensively reviewed [6]. These methods, however, are hindered by a range of analytical complications. For example, organic solvent extraction is difficult due to unfavourable partition coefficients for aqueous volatile organic acids, steam distillation suffers from low recoveries, and concentration steps that are often required suffer losses due to volatilisation. Vacuum distillation has been efficiently employed for the isolation of VFAs from faeces [7] and serum [8], with subsequent determination by GC with flame ionisation detection (FID). Ion pair extraction has also been used to transfer VFAs from an aqueous to an organic phase prior to alkylation with pentafluorobenzyl bromide [9] and, likewise, tri-n-octylphos-

phine oxide has been employed as a phase transfer reagent prior to similar alkylation using 18-crown-6 as the catalyst [10]. The latter work was successful in the determination of acrylic acid from pore and sediment waters at nanomolar concentrations using GC with electron capture detection (ECD). A number of VFAs were also co-extracted and derivatised, but were not quantified.

The work undertaken in this study was part of a program to extend SFE's prospective applications. It was aimed at exploring the potential of in situ derivatisation and extraction from anion-exchange resins [5] using pentafluorobenzyl bromide in supercritical CO_2 for the determination of highly polar VFAs. Urine was chosen as the test matrix to demonstrate this technique given that, as a matrix, it was analytically challenging. Also, the presence of various VFAs in urine are related to several metabolic disorders, such as propionic acidaemia and isovaleric acidaemia, and are clinically relevant to some medical conditions [7,8], including renal failure, impaired hepatic function and some bacterial infections. However, while urine matrix was investigated as part of this study, the development of a clinical method was not the aim.

This paper describes a technique for the determination of resin-trapped VFAs using simultaneous esterification by a suitable reagent, pentafluorobenzyl bromide, and extraction by CO_2 SFE, with subsequent analysis by GC.

2. Experimental

2.1. Reagents

Acetonitrile (Hypersolv) was obtained from BDH (Poole, UK) and was used as supplied. n-Butanoic acid (99%), propanoic acid (99%) and n-pentanoic acids were obtained from Sigma (St. Louis, MO, USA), acrylic acid and glacial acetic acid AR was obtained from BDH. Pentafluorobenzyl bromide (bromo-2,3,4,5,6-pentafluorotoluene) was obtained from Aldrich (Milwaukee, WI, USA). Industrial grade carbon dioxide (99.5%) was used as supplied by Commonwealth Industrial Gases (Australia).

2.2. Resins and support columns

2.2.1. Resins

Analytical grade anion-exchange resin AG MP-1, 63-150 µm (chloride form) was supplied by Bio-Rad (Sydney, Australia) and Dowex 50X8 10-20 mesh (sodium form) was supplied by BDH (Kilsyth, Australia).

2.2.2. Resin preparation

The AG MP-1 resin was converted to either the fluoride or hydroxide form by treatment with at least 20 bed volumes of aqueous 1 M sodium fluoride or 1 M sodium hydroxide, followed by thorough rinsing with distilled water and then methanol before being dried by vacuum aspiration.

2.2.3. Resin storage

AG MP-1 resins were stored in the presence of dry silica gel under vacuum.

2.2.4. Resin columns

Resin columns were prepared by placing 100 mg of AG MP-1 resin in a disposable glass Pasteur pipette containing a glass wool plug to which sample/standard solutions were applied directly. Where larger amounts of resin (200–300 mg) were employed, empty (discarded) solid phase cartridges that still contained the bottom frit were used as column supports. In these cases, the resin was packed by adding sufficient distilled water to the resin in the column, shaking whilst being stoppered with a 2-ml syringe plunger and then allowing it to drain.

2.3. Standard solutions

Three stock solutions of VFAs were used, each containing a combination of acids, each at approximately 1000 mg/ml by weight as follows:

Stock standard (1): this contained acetic, propanoic and 1-butanoic acids in 0.1 M aqueous NaOH, from which working standards were prepared at 100 mg/ml (1a) and 20 mg/ml (2b) in 0.1 M NaOH.

Stock standard (2): this contained acetic, acrylic, propanoic and 1-butanoic acids in 0.1 M aqueous NaOH, from which working standards were prepared at 100 mg/ml (2a) and 20 mg/ml (2b) in 0.1 M NaOH.

Stock standard (3): this contained acetic, propanoic, 1-butanoic and 1-pentanoic acids in distilled water. This was used to spike directly into aqueous solutions.

Internal standard: this was prepared in acetonitrile using dimethyl phthalate (~1000 mg/ml) for addition to SFE extracts as a marker.

External standard: pentafluorobenzyl acetate was prepared by acetylation of pentafluorobenzyl alcohol (Aldrich). This was synthesised and purified by the R&D Section of the Australian Government Analytical Laboratories, Pymble, Australia. Standard solutions of pentafluorobenzyl acetate containing external standard were prepared from 5 to 500 mg/ml in acetonitrile.

2.4. Urine samples

2.4.1. Collection

Urine specimens were collected from an apparently healthy adult male.

2.4.2. Storage of urine

Samples of urine (2 ml) were immediately frozen in sealed disposable centrifuge tubes, without preservative, and were stored in a freezer at −18°C until required. For analysis, each sample was thawed by heating the centrifuge tube in warm water until the sample became translucent.

2.4.3. Pretreatment of spiked urine for addition to anion-exchange resin

Samples of urine (2 ml) were individually spiked with 200 and 40 µl of stock standard (3). Interfering anions, such as phosphate and sulfate [11,12], were removed as their barium salts by the addition of 0.1 M barium hydroxide (1 ml) and vortex-mixing (1 min). An additional clean-up for chloride was carried out using Dowex 50X8 converted to the silver (Ag$^+$) form, which was added (~100 mg) to the Ba(OH)$_2$-treated urine before vortex-mixing. This step was not employed as part of the final protocol. The precipitate was separated from the urine by centrifugation

(5 min, 500 g) and the supernatant was added to 200 mg of AG MP-1 resin.

2.5. Addition of aqueous solutions and pretreated urine to the anion-exchange resin

All solutions were applied to AG MP-1 resin columns dropwise using a Pasteur pipette, with care being taken to prevent disturbance of the resin bed. After elution of the added liquid was complete, the column was air aspirated to remove excess alkali. The column was then rinsed with distilled water (~1 ml) followed by two column volumes of methanol. The resin was thoroughly dried by air aspiration, with intermittent mixing of the resin by gentle tapping of the column.

2.6. In situ derivatisation and extraction of the resin-entrapped acids

The dried AG MP-1 resin containing trapped acids was placed either in a 0.5-ml ISCO extraction cartridge (100 mg resin) or in a 2.5-ml ISCO extraction cartridge (200–300 mg resin), to which pentafluorobenzyl bromide (typically 50 μl) was added directly to the top of the resin in the cartridge. The 2-ml cartridge was then filled with acid-washed sand to reduce the dead volume. The cartridge was placed in the SFE unit and extracted using the conditions described in Section 2.7.

2.7. Supercritical fluid extraction

2.7.1. SFE equipment
An ISCO (Lincoln, NE, USA) SFX 2-10 extraction unit fitted with an ISCO restrictor heater and a 50 μm I.D. fused-silica capillary restrictor was used. Pressurised liquid CO_2 was supplied to the extraction unit by an ISCO 260D syringe pump.

2.7.2. SFE conditions
Optimal extraction parameters, determined experimentally, consisted of a constant temperature of 110°C and a pressure of 30 MPa, with a static derivatisation–extraction period of 20 min, followed by dynamic extraction using a total CO_2 volume of 30 ml. An extraction flow-rate of approximately 1.5 ml/min was regulated by the 50 μm restrictor I.D.

The restrictor heater was maintained at 80°C. Extract collection was into 5 ml of acetonitrile.

2.8. Gas chromatography and data acquisition

2.8.1. Extract preparation
All extracts were spiked with the internal standard (100 μl; marker) and made up to 20 ml with acetonitrile.

2.8.2. Equipment
Analyses were carried using a Hewlett-Packard 5890 series II gas chromatograph that was fitted with a Hewlett-Packard 5971A mass selective detector (MSD) and a DB5 MS (15 m×0.32 mm I.D.×0.25 μm film thickness) column and a split/splitless injector.

2.8.3. GC MSD operation
Operating conditions for the GC MSD were: A 1-ml injection volume, the injector maintained at 260°C in splitless mode with the purge valve closed initially for 0.75 min; helium carrier gas with a column head pressure of 17.2 kPa; an initial oven temperature of 60°C for 2 min then ramped to 120°C at 12°C/min then to 220°C at 35°C/min, where it was held for 2 min; a MSD temperature of 280°C; MSD solvent delay was 3 min; MSD ionisation was by electron impact; data acquisition was from 40 to 510 m/z in scan mode using negative DC polarity; standard autotune calibrations of the MSD were routinely carried out.

2.9. Quantitation and MSD confirmation

2.9.1. Data analysis
MSD data processing and analysis was carried out using the Hewlett-Packard Chemstation data analysis software. The spectra for each derivative were confirmed for all acid derivatives by matching with spectra from the Wiley spectrum library database (HP G1035A) and by m/z comparison. Chromatogram peak areas were integrated using either the Chemstation integrator or the RTE integrator in the HP Chemstation software.

2.9.2. Quantitation

The chromatographic responses for each acid derivative were expressed in terms of the ratio of their respective peak areas versus the internal standard, dimethyl phthalate. The recovery of each acid derivative was determined by comparison with a similarly prepared external standard calibration curve of pentafluorobenzyl acetate.

2.10. Analysis of VFAs in spiked aqueous solutions and pretreated spiked urine using in situ derivatisation–extraction with pentafluorobenzyl bromide in supercritical carbon dioxide

2.10.1. Working standard (1) and (2) acids

Volumes (2 ml) of 1a and 1b standards were applied directly to 100 mg of the hydroxide form of AG MP-1 resin and, likewise, 2a and 2b standards to the fluoride form (Section 2.5) for SFE (Section 2.6) followed by GC MSD analysis.

2.10.2. Spiked urine

Analysis was also carried out on the supernatant from pre-treated spiked urine samples [20 and 100 ppm; stock standard (3)] trapped on AG MP-1 in the fluoride form (Section 2.5) for SFE (Section 2.6) followed by GC MSD analysis.

2.10.3. Stock standard (3)

Distilled water samples (2 ml) were individually spiked with 200 and 40 μl of stock standard (3) VFAs and then were basified with 0.1 M $Ba(OH)_2$ (1 ml) and were applied to AG MP-1 in the fluoride form (Section 2.5) for SFE (Section 2.6) followed by GC MSD analysis.

3. Results and discussion

3.1. Reagent selection

A number of factors influenced the selection of the derivatisation reagent. Apart from being suitable for resin-mediated esterification, the resulting VFA derivatives were expected to be both significantly less polar and less volatile than the parent compounds, thereby facilitating efficient CO_2 SFE and better chromatography through improved peak shape and detection by GC. Preliminary investigations were carried out using a number of reagents including iodobutane, iodopentane, 2-iodoethylbenzene and pentafluorobenzyl bromide, to produce VFA derivatives using this methodology. Since the chemical reactivity of pentafluorobenzyl bromide is similar to that of iodomethane, it was envisaged that the efficiency of derivatisation under resin-mediated SFE conditions would be similar. This proved to be the case. Pentafluorobenzyl VFA derivatives were substantially less volatile than the products of most iodo reagents tested, and they separated well chromatographically from the solvent front and excess reagent. These derivatives were also particularly suitable for selective detection, therefore, pentafluorobenzyl bromide was selected as the preferred reagent.

3.2. Quantitation

The MSD response for each pentafluorobenzyl ester was considered to be equimolar to that of the acetate. This was considered valid for the group of compounds used in this work because of the structural similarity between the acid derivatives studied and the predominant contribution of the pentafluorobenzyl 181 m/z ion to the total ion current. The similarity in the total ion current response of the MSD to each acid derivative was confirmed by analysis of a single derivatised SFE extract following serial dilution over a range from 4.0 to 0.4 times that used for quantitative analysis. The plot of peak area versus relative concentration was found to be linear for each acid derivative, and the similarity in slope between the derivatives was confirmed by the coefficient of variation between the slopes (4.6%). Accuracy of the technique could not be thoroughly tested due to the lack of certified standards.

3.3. In situ derivatisation and extraction of the resin-entrapped acid standards using supercritical carbon dioxide

3.3.1. Optimised extraction–derivatisation conditions

The most suitable supercritical fluid extraction–derivatisation conditions found were similar to those obtained by Chatfield et al. [5]. At least 50 μl of

pentafluorobenzyl bromide were required to ensure optimal derivatisation efficiency. Increasing the amount of pentafluorobenzyl bromide above 50 μl had a negligible effect. Reduced recoveries occurred at temperatures below 90°C, with best recoveries in the 100–120°C range. Increasing the extraction pressure from 15 to 35 MPa marginally improved recoveries. A temperature of 110°C and pressure of 30 MPa was used for routine work. At least 20 min static reaction time was required to ensure maximum derivatisation. A dynamic extraction CO_2 volume of at least 25 ml was needed for optimum recoveries under these conditions, however, 30 ml was used to ensure that complete extraction occurred.

3.3.2. Recoveries from aqueous standard SFE extracts

Recoveries from preliminary experiments using working standard (1a) on AG MP-1 resin in the hydroxide form ranged from 40.0–126.8% with a C.V. (%) ranging from 3.4–36.1% (Table 1). The recovery of the propionate derivative was compounded by co-extractive contamination in the chromatograms and recoveries in general were poor. The contamination occurred intermittently and, although not confirmed, was considered to be a result of alkaline hydrolysis of the resin, indicated by an ammonia-like odour, which appeared after short-term storage. However, this problem was overcome by using the AG MP-1 resin in the inert fluoride form and this significantly improved recoveries for the

Table 1
Recoveries of volatile fatty acid standards as pentafluorobenzyl esters from Ag MP-1 anion-exchange resin (OH⁻), derivatised in situ with pentafluorobenzyl bromide and extracted using supercritical carbon dioxide

Fatty acid[a]	Concentration (μg/ml)	n	Yield (%)	C.V. (%)
Acetate	20	5	40.0	14.2
	100	5	44.8	18.6
Propionate	20	5	126.8[b]	36.1
	100	5	63.1	11.6
n-butyrate	20	5	62.2	3.4
	100	5	68.2	18.6

[a] Combined solution of acids at two concentrations applied to 100 mg of AG MP-1 resin, hydroxide form.
[b] Value inflated by co-extractive contamination.

Table 2
Recoveries of volatile fatty acid standards as pentafluorobenzyl esters from Ag MP-1 anion-exchange resin (F⁻ form), derivatised in situ with pentafluorobenzyl bromide and extracted using supercritical carbon dioxide

Fatty acid[a]	Concentration (μg/ml)	n	Yield (%)	C.V. (%)
Acetate	20	7	88.0	6.1
	100	6	78.0	7.5
Propionate	20	7	98.0	6.0
	100	6	90.3	5.1
n-Butyrate	20	7	101.5	6.2
	100	6	93.7	3.5
Acrylate	20	7	61.0	5.5
	100	6	57.0	5.6

[a] Combined solution of acids at two concentrations applied to 100 mg of AG MP-1 resin, fluoride form.

same acids; (standard 1b) 78.0–101.5% with a C.V. (%) ranging from 3.5–7.5% (Table 2). As expected, a major contributing factor to decreasing recoveries with shorter fatty acid chain length is the relative loss of affinity of each acid for the anion-exchange resin.

3.3.3. Chromatography of aqueous standard SFE extracts

Typical chromatograms for the pentafluorobenzyl derivatives at two concentrations, 20 and 100 ppm, are provided in Fig. 1. Chromatograms of extracts of VFA derivatives extracted using other reagents, such as the iodo compounds, contained interfering reagent peaks. However, the relatively early elution of residual pentafluorobenzyl bromide allowed it to be vented from the MSD prior to acquisition.

3.4. In situ derivatisation–extraction of the resin-entrapped acids from spiked urine using supercritical carbon dioxide

3.4.1. Pretreatment of urine samples

First attempts at employing the above technique, as applied to aqueous standards, on spiked urine resulted in negligible spike recoveries. This was attributed to the high concentration of inorganic anionic material in the urine that was competing for the resin sites. Barium hydroxide has been used previously to precipitate sulfates and phosphates in
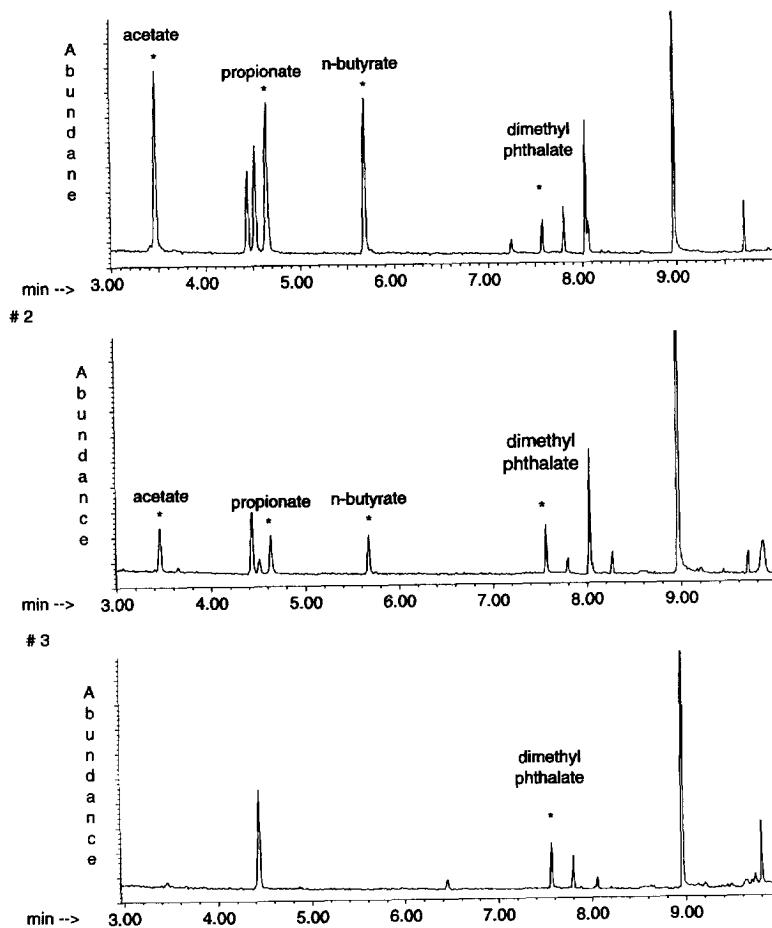
Fig. 1. GC–MSD chromatograms of volatile fatty acids, entrapped on AG MP-1 anion-exchange resin (F⁻), from standard solutions, derivatised in situ and extracted as pentafluorobenzyl esters using SFE. 1=100 µg/ml acids; 2=20 µg/ml acids; 3=blank.

urine prior to extraction of urinary acids using DEAE Sephadex anion-exchange [11,12]. A range of additions of Ba(OH)$_2$ (0.1 M, 0.5–8 ml) to spiked urine samples (2 ml) were investigated. Higher Ba(OH)$_2$ concentrations reduced recoveries significantly (Fig. 2). Addition of 1–2 ml of 0.1 M Ba(OH)$_2$ resulted in satisfactory yields of most spiked acid derivatives, therefore, 1 ml of Ba(OH)$_2$ was used routinely. This was sufficient to create alkaline conditions and complete fatty acid ionisation, therefore, Ba(OH)$_2$ was used in place of NaOH.

The effect of chloride as another possible interfering ion was also investigated. Chloride precipitation from urine samples as silver chloride was induced by the addition of Dowex 50X8 (Ag⁺) cation-exchange resin. However, silver chloride precipitation did not

improve the recoveries of spiked acid derivatives, demonstrating that chloride is not a major competitive ion for the resin.

3.4.2. Recoveries from urine SFE extracts

The results obtained for spiked urine at 100 and 20 µg/ml concentrations are presented in Table 3. Propanoic, butyric and 1-pentanoic acid recoveries were from 52.6 to 66.5%, with C.V.s ranging from 5.9 to 10.1%, while the recovery of acetic acid ranged from 36.0–40.8% (C.V., 12.8–14.4%). Determination of acetic acid spikes was complicated by the presence of endogenous acetic acid in the urine. Accurate determination of endogenous acids in urine was beyond the nature and scope of this work. The
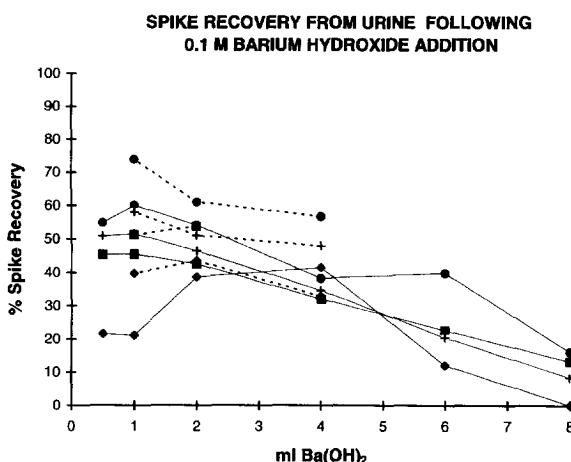
**SPIKE RECOVERY FROM URINE FOLLOWING
0.1 M BARIUM HYDROXIDE ADDITION**

Fig. 2. Effect of the addition of 0.1 M $Ba(OH)_2$ at different volumes on the recovery of volatile fatty acid derivatives from spiked (20 μg/ml) urine (2 ml). Single determinations from AG MP-1 (F⁻), 200 mg (- - - -), 100 mg (——). Pentafluorobenzyl acetate (♦); propionate (■); n-butyrate (+); n-valerate (●).

results for acetic acid can only be regarded as approximate.

Aqueous blanks were basified using $Ba(OH)_2$ for comparison with results using NaOH. Recoveries of stock standard (2) at 100 and 20 μg/ml were 70.3–100.1%. These were comparable with the results obtained using NaOH, indicating that some additional anionic competition for resin sites may be present in the urine. Improvement in the pre anion-exchange clean-up may resolve this. Increasing the amount of

Table 3
Recoveries of volatile fatty acids in spiked urine as pentafluorobenzyl esters from Ag MP-1 anion-exchange resin (F⁻ form), derivatised in situ with pentafluorobenzyl bromide and extracted using supercritical carbon dioxide

Fatty acid	Concentration (μg/ml)	n	Yield (%)	C.V. (%)
Acetate	20	9	36.0	14.4
	100	6	40.8	12.8
Propionate	20	6	52.6	10.1
	100	6	52.6	9.8
n-Butyrate	20	9	56.3	7.8
	100	6	57.7	8.4
n-Valerate	20	9	63.8	7.1
	100	6	66.5	5.9

ᵃ Combined solution of acids at two concentrations applied to 200 mg of AG MP-1 resin, fluoride form.

AG MP-1 resin from 100 to 200 mg marginally improved yields and this amount was used for subsequent urine analysis, however, increasing the amount of resin to 300 mg was of no observable benefit.

3.4.3. Chromatography of urine SFE extracts

The GC–MSD chromatograms of blank and spiked urine extracts, in Fig. 3, are of comparable quality to those in Fig. 1.

4. Conclusion

This investigation was designed to test the suitability of the resin-mediated in situ extraction and derivatisation technique, using supercritical fluid extraction, for highly polar and volatile fatty acid analysis. As expected, the most significant contributing factor to reduced recoveries was the relative affinity of the VFAs for the AG MP1 anion-exchange resin and this was reflected in the recoveries. However, adequate recoveries can be achieved from aqueous solutions with minimal matrix interference or where matrix effects can be suppressed. This approach may be particularly useful where conventional acid extraction and derivative preparation is difficult.

This report offers a simple and effective technique for the determination of VFAs by resin-mediated in situ extraction and derivatisation, as their pentafluorobenzyl esters, using supercritical CO_2 solvent extraction. It has been demonstrated that the methodology can be applied to a complex matrix such as urine. With the increasing availability of SFE in laboratories, this methodology may contribute to its wider application.
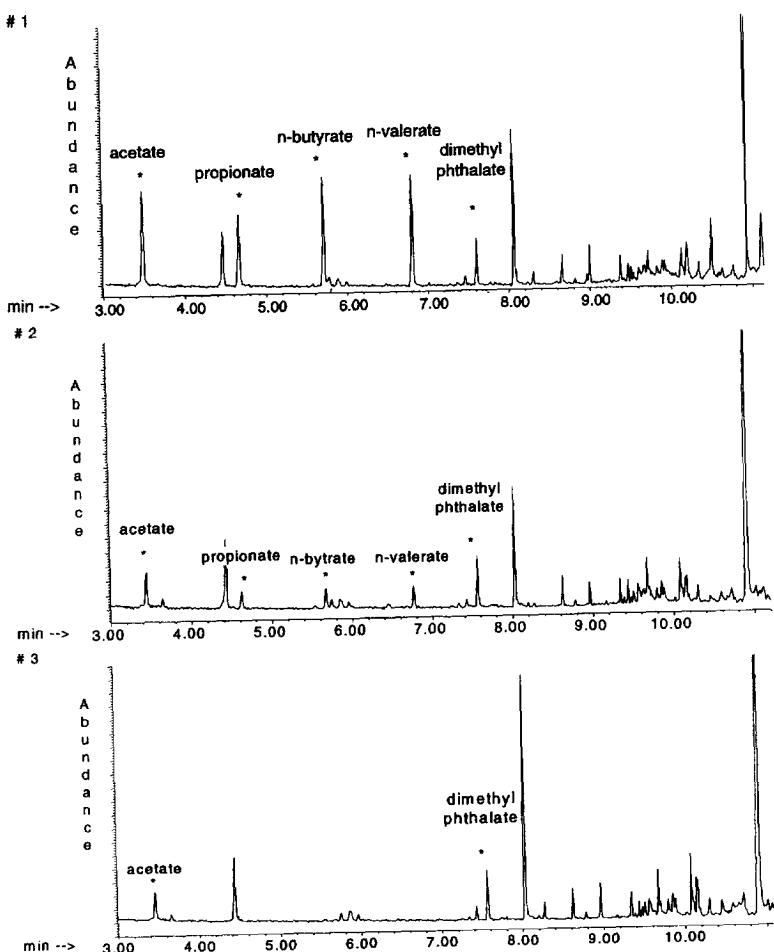
Fig. 3. GC–MSD chromatograms of volatile fatty acids, entrapped on AG MP-1 anion-exchange resin (F⁻) from spiked urine, derivatised in situ and extracted as pentafluorobenzyl esters using SFE. 1=100 µg/ml spike acids; 2=20 µg/ml spike acids; 3=urine blank; note; endogeneous acetic acid.

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