RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2004; **18**: 2593–2600
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/rcm.1662

RCM

A streamlined approach to the analysis of volatile fatty acids and its application to the measurement of whole-body flux

Douglas J. Morrison[1,2]*, Karen Cooper[2], Susan Waldron[2], Christine Slater[1], Lawrence T. Weaver[1] and Tom Preston[2]

[1]Division of Developmental Medicine, University of Glasgow, Royal Hospital for Sick Children, Yorkhill, Glasgow G3 8SJ, UK
[2]Scottish Universities Environmental Research Centre, Rankine Avenue, East Kilbride G75 0QF, UK

Received 5 July 2004; Revised 1 September 2004; Accepted 1 September 2004

Volatile fatty acids (VFAs) are produced in the human colon by the bacterial breakdown of carbohydrates that escape digestion and absorption in the small intestine. They have important local and systemic effects on gastrointestinal and nutritional functions. Measuring their production is difficult because of inaccessibility of sampling sites and low circulating concentrations. Stable isotope tracer techniques are a way to measure VFA production but require measurement of isotope dilution in blood and other biological fluids. We have developed a streamlined and robust method to measure the concentration and enrichment of [^{2}H]-labelled VFAs by gas chromatography/mass spectrometry (GC/MS) and [^{13}C]-labelled VFAs by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). Both types of analysis were carried out on the same samples allowing multiple tracer studies to be conducted. Good accuracy and repeatability were found for GC/MS analysis of [^{2}H]-labelled VFAs. Careful handling of the background contribution, especially acetate, allowed quantitation of concentration and enrichment within the analysis. GC/C/IRMS analysis of [^{13}C] VFAs was also achieved with good accuracy and repeatability. This methodology was used to determine whole-body acetate production in two subjects using multiple tracers ([$^2H_3$]- and [1-^{13}C]acetate) and blood and urine sampling. Whole-body acetate flux was similar when measured either with [$^2H_3$]- or [1-^{13}C]acetate, and when flux was determined from plasma or urine tracer enrichment. This new method will permit rapid and accurate measurement of VFA flux using [^{2}H]- and/or [^{13}C]-labelled VFAs as tracers. Measurements of the contribution of colonic VFA production to whole-body VFA flux are now possible. Copyright © 2004 John Wiley & Sons, Ltd.

Volatile fatty acids (VFAs) are important biological products in the human colon and in the rumen of farm animals. They refer to linear fatty acids of up to 4–6 carbons in chain length. Short-chain fatty acids (SCFAs) refer to broadly similar fatty acids in human nutrition, up to 6 carbons in length. Both definitions refer primarily to the acids, acetic (ethanoic), propionic (propanoic) and butyric (butanoic), because they are the most abundant substrates of physiological significance, although lactic acid (2-hydroxypropionic acid) can be more abundant in the colon of infants.[1] In addition to dietary sources, exogenous VFAs are produced by the bacterial degradation of carbohydrates and proteins that escape digestion and absorption in the small intestine.[2] This fermentation process is carried out by the predominantly anaerobic bacterial flora in the colon. The main substrates for fermentation are dietary fibre, resistant starches, non-digestible oligosaccharides, plant cell and cell wall remnants—collectively referred to as non-digestible carbohydrates (NDC).

Recent evidence suggests that VFAs produced in the colon have several local and systemic effects. Butyric acid and, to a lesser extent, propionic and acetic acids, are oxidised by the colonocytes and account for the majority of their energy needs.[3] Butyric acid is also a key mediator in the inflammatory process in the large intestine.[4] VFAs produced from fermentation stimulate water absorption and mineral uptake and therefore may prevent mineral deficiency[5] and hasten recovery from diarrhoea.[6] Acetic and propionic acids are mostly absorbed across the colonic epithelium and transported to the liver. A substantial fraction of exogenous acetate production escapes hepatic metabolism but little propionate appears in the circulation.[7] Acetate can be used as an energy source and induces an insulin response,[8] while propionate may reduce hepatic cholesterol synthesis.[9] The optimum VFA production to maintain health has not been determined,

**Correspondence to*: D. J. Morrison, Division of Developmental Medicine, Child Health Section, University of Glasgow, Royal Hospital for Sick Children, Yorkhill, Glasgow G3 8SJ, UK.
E-mail: douglas.morrison@clinmed.gla.ac.uk
Contract/grant sponsor: Mars Nutrition Research Council.
Contract/grant sponsor: Commission of the European Communities; contract/grant number: QLK 1-2001-00431.

mainly owing to the inaccessibility of the human colon to direct sampling. Almost every documented investigation of exogenous VFA production has relied on indirect measurements. For example, faecal VFA concentrations are often used to make inferences about colonic production but they can only realistically reflect the residual pool in the distal colon and cannot be used as a proxy for VFA production because no index of absorption or metabolism is determined. Changes in faecal VFA concentration may better reflect changes in absorption and not changes in production.[10] A few notable studies have measured exogenous VFA production either by measuring the portal appearance of VFAs[11] or by isotope dilution techniques.[12–15] Many studies have been conducted in animals using stable and radioisotope techniques to measure VFA production and these have been largely successful because of invasive sampling techniques and the relatively high concentration of VFAs in the large intestine/rumen.[16] However, it is not appropriate to extrapolate animal data to humans because of nutritional, anatomical, physiological, and microbiological differences. With limited availability of isotope-labelled NDC, isotope dilution is one technique with which to measure exogenous VFA production.

VFAs in biological fluids have been measured by a number of techniques, including enzymatic methods,[17–19] gas chromatography,[20,21] and liquid chromatography.[22,23] Before analysing VFAs from biological fluids, sample clean-up and deproteinisation are necessary.[14] Moreover, because of the low abundance of VFAs, especially of propionate and butyrate, in plasma and urine, concentration of samples by liquid/liquid extraction is often required. Solid-phase microextraction has been employed to clean up and concentrate samples before analysis by gas chromatography/quadrupole mass spectrometry (GC/MS).[24–26] GC/MS is the analytical method of choice for many isotope-dilution tracer studies but requires relatively high tracer enrichment in the biological pool being sampled. Derivatisation is often used to improve sample handling by reducing volatility, and to improve quantitation of fragment ions. The *tert*-butyldimethylsilyl (*t*BDMS) derivatives of VFAs have been successfully used in a number of isotope dilution studies because they produce characteristic high-mass ions for each VFA that can be used for quantitation.[13,20,21,27] The ubiquitous nature of VFAs, especially acetate, has often hampered their analysis and correcting for this background is a problem for the analyst when measuring isotope ratios.[28]

Gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) is a technique whereby complex mixtures are separated by GC prior to combustion of all eluting molecules to CO_2 (H_2O is also formed). GC/C/IRMS has greater precision than GC/MS for measuring isotope ratios at low enrichment because the mass spectrometer is designed to measure isotope ratios in simple gases.[29] IRMS can measure ~100 times lower isotopic enrichments than GC/MS when using [^{13}C]-labelled tracers with one labelled carbon. This has obvious cost savings for tracer administration but also allows the labelled substrate to be considered a true metabolic tracer, analogous to a radioisotope tracer, which is unlikely to alter tracee flux.[30] The current method describes both GC/MS and GC/C/IRMS analysis of VFAs from the same plasma or urine sample and has been used to measure whole-body acetate flux.

EXPERIMENTAL

Chemicals and standards

All reagents and solvents were analytical grade or better. VFAs were purchased from Sigma-Aldrich (Poole, UK) as sodium salts or free acids. Solutions of the sodium salts of VFAs in 0.15 M NaOH were prepared at a concentration of 100 mM for sodium acetate and 20 mM for propionic, butyric, isobutyric (2-methylpropionic), 2-methylbutyric, valeric (pentanoic), isovaleric (3-methylbutanoic) and caprylic (octanoic) acids to serve as external standards. A 20 mM solution of 3-methylvaleric (3-methylpentanoic) acid (3MV) in 0.15 M NaOH was also prepared to serve as an internal standard. A second internal standard, caproic (hexanoic) acid, was prepared in dry acetonitrile (1 mM) to permit calculation of a derivatisation blank.

Two sets of external standards were prepared by accurate gravimetric methods, i.e. all solids and solutions used to make up standards were accurately weighed using an appropriate balance. The first set of standards was used to determine the accuracy of the VFA concentration assay using 3MV as an internal standard. The ratio of VFAs in these standards was kept constant (100:1:1 for acetate/propionate/butyrate) but the concentration ranged from 10–1600 μM acetate in the equivalent of 2.5 mL plasma or urine. The second set of isotopically enriched standards contained the equivalent of 400 μM acetate in 2.5 mL plasma or urine and they were spiked with either [$^2H_3$]acetate for GC/MS (0–10 mole percent excess (MPE)) or [1-^{13}C]acetate for either GC/MS (0–10 MPE) or GC/C/IRMS (0–1 MPE). [1-^{13}C]acetate enriched standards were prepared from the same stock (by dilution with acetate at natural abundance) to allow intercalibration of the two instruments.

Human whole-body acetate flux study

Two male subjects (mean age 39.5 years; mean body mass index 29.1 kg/m^2) were recruited into a study to measure whole-body acetate production. A combined [$^2H_3$]- and [1-^{13}C]acetate solution was taken in repeated oral doses (every 20 min) to achieve a constant entry rate of tracer into the body of 1.5 μmol · kg^{-1} · min^{-1} [$^2H_3$]acetate and 0.2 μmol · kg^{-1} · min^{-1} [1-^{13}C]acetate. The subjects consumed a diet free of fibre, alcohol and low ^{13}C abundance[31] during the period before the test (~40 h) and reported to the patient investigation suite at Glasgow Royal Infirmary at 8:30 am after an overnight fast. No food was consumed through the protocol but water was allowed *ad libitum*. 10 mL whole blood were collected into EDTA-coated vials every 20 min through an indwelling venous cannula placed in the forearm.[12] Each urine sample passed in the 24-h period from the beginning of the study was collected and measured for total volume and a 20-mL aliquot stored. The times of blood-sampling and micturition were noted. Whole blood was centrifuged to remove cells. Plasma and urine samples were both stored at −20°C until processed. Whole-body acetate production (WBAP) was calculated from the plateau acetate enrichment using the formula:

$$\text{WBAP}\ (\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}) = \frac{\text{Tracer infusion}\ (\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}) \times \text{Tracer enrichment (MPE)}}{\text{Acetate plateau enrichment (MPE)}} - \text{Tracer infusion}\ (\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1})$$

An estimate of the error of WBAP was calculated from the standard deviations (SDs) of samples at plateau enrichment, which were used to calculate WBAP.

Ethical approval for this study was granted by the ethical committee for investigations in human volunteers, University of Glasgow.

Preparation of plasma and urine samples for MS analysis

All reusable apparatus was acid washed before use in 0.2 M HCl to help minimise acetate blanks. Proteins and other high molecular weight components were removed from plasma and urine by ultrafiltration using Amicon Centriplus YM-30 centrifugal filter devices (30 kDa cut-off, Millipore Ltd., Watford, UK). The ultrafiltration units were stored in 0.1 M NaOH and rinsed before use with 0.2 M HCl by centrifuging at 3000 *g* for 10 min.

Urine samples were stored frozen at −20°C and thawed prior to analysis. Aliquots (2.5 mL) were pipetted into disposable 25-mL plastic universal bottles. Salt (2.5 mL, 4 M NaCl) was added to bring the NaCl concentration to 2 M. Internal standard (400 nmol 3MV) was added and mixed. The pH was adjusted to 7–8 using 0.15 M NaOH, if necessary. A 'process blank' was prepared containing freshly deionised water, salt and internal standard 3MV. Samples were transferred to ultrafiltration units and centrifuged at 3000 *g* for 2 h. The capacity of the centrifuge dictated that each batch comprised seven samples and a process blank. The resulting 5 mL of ultrafiltrate was acidified to pH 2–2.5 by adding 6 M HCl (~15 μL at a time) and loaded immediately onto a conditioned solid phase extraction (SPE) column as described below.

Venous blood was sampled into EDTA tubes that were held on ice until cell removal by centrifugation. Plasma was aliquoted into labelled vials and frozen at −20°C. Plasma samples were thawed prior to analysis. From each sample a 3-mL aliquot was taken and, after the salt concentration had been adjusted by adding 3 mL 4 M NaCl, 400 nmol 3MV was added as internal standard and the pH was adjusted to 7. A 'process blank' was prepared as described above. Samples were centrifuged at 2000 *g* for 20 min to exclude any clots, decanted into an ultrafiltration unit and centrifuged at 3000 *g* for 3 h. Blanks were processed with each batch of samples. The resulting ultrafiltrate (4–5 mL) was acidified to pH 2 by adding 6 M HCl prior to SPE.

VFA concentration and purification by SPE

VFAs were purified and concentrated[32] from the ultrafiltrate using disposable SPE cartridges (Bakerbond SDB-2 200 mg, 3 mL capacity; Mallinckrodt Baker UK, Milton Keynes, UK). SPE cartridges were mounted on a 10-place SPE manifold (International Sorbent Technology, Hengoed, UK). SPE cartridges were conditioned with 2 × 2 mL methanol, followed by 1 × 2 mL distilled/deionised water and 1 × 2 mL 0.1 M HCl before loading the samples. The SPE manifold was operated without applying vacuum. The acidified ultrafiltrate was loaded onto the conditioned SPE column. VFAs were eluted with 3 × 500 μL methanol without either a wash or drying step between loading and elution, as both had been proven to cause VFA loss. VFAs were eluted directly into 100 μL 1M NaOH held in a 2-mL crimp-cap vial (2-CV; Chromacol, Welwyn Garden City, UK). The vial contents were mixed and the VFA sodium salts taken to dryness in an oven at 55°C overnight. Sample vials were capped for storage at room temperature.

GC/MS analysis

Each sample batch contained an enriched (5 MPE [$^2H_3$]acetate) and unenriched external standard as described before, a derivatisation blank, a process blank and seven samples. Internal standard (400 nmol 3MV) was added to external standards but not to the derivatisation blanks.

After addition of 50 μL 4 M HCl, VFAs were extracted into 400 μL diethyl ether with vortex mixing. External standards and derivatisation blanks were extracted into 1.2 mL diethyl ether. Aliquots of 50 μL of the ether phase were transferred to a 1-mL tapered vial (1.1-CV; Chromacol) containing 100 μL dry acetonitrile (and hexanoic acid) and 20 μL *tert*-butyldimethylsilylimadazole (*t*BDMSIM; Fluka Chemical Co., Poole, UK). The remainder of the ether phase became available for GC/C/IRMS analysis (described later). The stock acetonitrile was held over anhydrous sodium sulphate and contained a second internal standard (hexanoic acid: 100 nmol per 100 μL reagent). All samples, standards and blanks had this addition of hexanoic acid. The *t*BDMSIM reagent was stored in a desiccator and exposed to ambient air for a minimum period. VFA *t*BDMS esters were formed by heating at 60°C for 60 min. Concentration and [^{13}C]/[^{2}H] VFA enrichment were measured by GC/MS (Hewlett Packard 5890 II GC; Optic II temperature programmable injector; Fisons Instruments A200S autosampler; VG Trio-1000 quadrupole MS with electron ionisation). The GC system was operated in splitless mode with helium (CP grade) as carrier gas. The injector temperature was ramped from 60 to 300°C at 16°C s^{-1}, the transfer line between the gas chromatograph and the mass spectrometer was operated at 300°C and the EI source was held at 200°C. The analytical column was an Rtx-5MS (Thames Restek, Saunderton UK), length 30 m, i.d. 0.25 mm, film thickness 0.5 μm. The temperature programme started at 50°C and was held for 0.2 min before ramping to 200°C at 10°C min^{-1}, then from 200 to 300°C at 30°C min^{-1}, followed by 2 min at 300°C. The solvent delay was 5 min and the injection volume was 0.4 μL.

The Trio-1000 quadrupole mass spectrometer was operated in the EI mode with a trap current of 150 μA and electron energy of 70 eV. The detector photomultiplier was operated at 350 V, approximately 1/5 of its maximum useable gain. Instrument control and selected ion monitoring (SIM) data

acquisition were performed using the MASPEC II software package (Mass Spectrometry Services Limited, Manchester, UK). Under electron ionisation, the VFA *t*BDMS esters have a prominent ion at [M−57], i.e. $[M-(C(CH_3)_3)]^+$. The ions used for quantitation of acetate were thus *m/z* 117, 118, 119, and 120. The dwell time on each mass was 0.04 s, with a mass window span of ±0.2 u.

When $[^2H_3]$acetate was used in studies as a biological tracer, acetate concentration was determined only by reference of the (unlabelled) acetate base peak (*m/z* 117) to the internal standard, 3MV (*m/z* 173). When $[^2H_3]$acetate (*m/z* 120) was not present in samples (i.e. when only VFA concentration was determined), isotope dilution (*m/z* 120:117 ratio) was used to calculate acetate concentration by adding a known amount of $[^2H_3]$acetate to the sample. $[^2H_3]$acetate is preferable as an internal standard to avoid differential derivatisation yields because all isotopomers of acetate should be derivatised to the same extent. Similarly, $[^2H_5]$propionate and $[^2H_7]$butyrate were exploited. Formate (*m/z* 103 and 104) was monitored in a specified SIM time window, as were propionate (*m/z* 131 and 136), butyrate and isobutyrate (*m/z* 145 and 152), valerate, 2-methylbutyrate and isovalerate (*m/z* 159 and 160), 3-methylvalerate, isocaproate and hexanoate (*m/z* 173 and 174), and octanoate (*m/z* 201 and 202). The resulting peak areas were exported into a spreadsheet (Microsoft Excel 2000). Each block of unknowns (seven samples plus process blank) was bracketed by external standards and derivatisation blanks. The average response of preceding and following standards was used in all calculations. The blank contribution from the derivatisation reagent (principally acetate) was subtracted, using the ratio of the ions for the VFA isotopomers to the *m/z* 173 ion of hexanoic acid. The response factor of each VFA was then calculated from the ratio of its base peak to 3MV in the unenriched external standard. Any remaining acetate (or other VFA) apparent in the process blank was subtracted from the unknowns using the ratio of its base peak to the *m/z* 173 ion of 3MV. The response of each VFA to 3MV, after blank subtraction, was converted into concentration units using the external standard response factors. Finally, the isotopic enrichment was calculated in units of MPE using the ratio of each isotopomer to its base peak. For example, $[^2H_3]$acetate enrichment was calculated, after subtraction of the analytical blank and natural abundance of the isotope, using the ratio (120/3MV)/(117/3MV). The natural abundance subtracted ratio, R (120/117), was transformed to MPE using the formula:[33]

$$\mathrm{MPE} = \frac{\mathrm{R}}{\mathrm{R}+1} \times 100$$

Calculated MPE values were drift-corrected using the values obtained from the gravimetrically prepared five MPE $[^2H_3]$acetate standards, which bracketed each group of samples.

To test the accuracy of the internal standard method of calculating acetate concentration, a spike of $[^2H_3]$acetate was added to a group of urine samples (n = 64) that had been processed solely for the determination of VFA concentration. These samples had no labelled VFAs added as metabolic tracers and therefore allowed the addition of $[^2H_3]$acetate to measure concentration by isotope dilution.

GC/C/IRMS analysis

Description of equipment

The ether phase remaining after removal of an aliquot for GC/MS, containing free (underivatised) VFAs, was analysed by GC/C/IRMS. The analysis was carried out using a gas chromatograph coupled to an in-house designed oxidation system, which was interfaced to an isotope ratio mass spectrometer (PDZ Europa 20-20), controlled using proprietary software. The principal feature of this design is twin 0.53 mm GC columns with matching helium flow and pressure. At any one time, the effluent from one column is directed to a flame ionisation detector (FID) while the other column is directed to the mass spectrometer via a capillary combustion interface. A high temperature, zero dead volume, automated 4-port valve, housed in a separate isothermal oven, controls this function. The use of 0.53 mm columns permits high sample loading at flows sufficient to avoid the use of make up gas elsewhere in the interface.

A schematic diagram of the GC/C/IRMS system employed for the separation of VFAs and the determination of their concentration and [^{13}C] enrichment is shown in Fig. 1. The gas chromatograph used was an Ai model 93 (ATAS, Cambridge, UK). The column used for VFA separation was a polar ZB-FFAP column, 30 m × 0.53 mm × 1 μm phase (Zebron, Phenomenex, Torrance, CA, USA). The helium carrier flowed at a constant rate of 7.5 mL min^{-1} through both columns, controlled by analogue flow controllers. Both injectors were of the packed column type without split or septum purge. These were held at 250°C, as was the FID. Aliquots (0.5–5 μL, typically 3 μL) of the ether phase from samples and standards, prepared as above, were injected manually or using an A200S autosampler (Finnigan MAT, Bremen, Germany). When using the autosampler, typically batches of only 12 samples were used to avoid ether evaporation. VFAs in diethyl ether were separated on the FFAP column using the following temperature programme: hold at 80°C for 3 min, ramp to 140°C at 4°C min^{-1}, ramp to 240°C at 16°C min^{-1}; hold for 1 min. By activating the Valco 4-port valve (4C4WT, VICI, Switzerland) at 8 min and again at 25 min, the mid portion of the helium carrier stream was directed towards the combustion furnace. The early and late parts of the carrier stream, including the solvent, were directed to the FID. The combustion furnace, which was held at 820°C, consisted of a 2 mm i.d., 30 cm long, silica tube filled with a spiral of 16 Cu and 4 Ni wires of 0.25 mm diameter and 20 cm length. A second 4-port valve allowed oxygen to pass through the combustion system to oxidise the wires while the combustion furnace was held at 550°C. This was operated after a new combustion tube had been installed and occasionally to regenerate the oxidation catalyst. No reduction stage was included, as nitrogenous compounds were not being analysed. After combustion, the carrier stream (now He, CO_2 and H_2O) was dried using a Nafion membrane-based water vapour trap (GV Instruments, Manchester, UK). A countercurrent flow of 60 mL min^{-1} dry helium was provided by an elemental analyser also interfaced to the isotope ratio mass spectrometer (valves 3 and 4, Fig. 1).

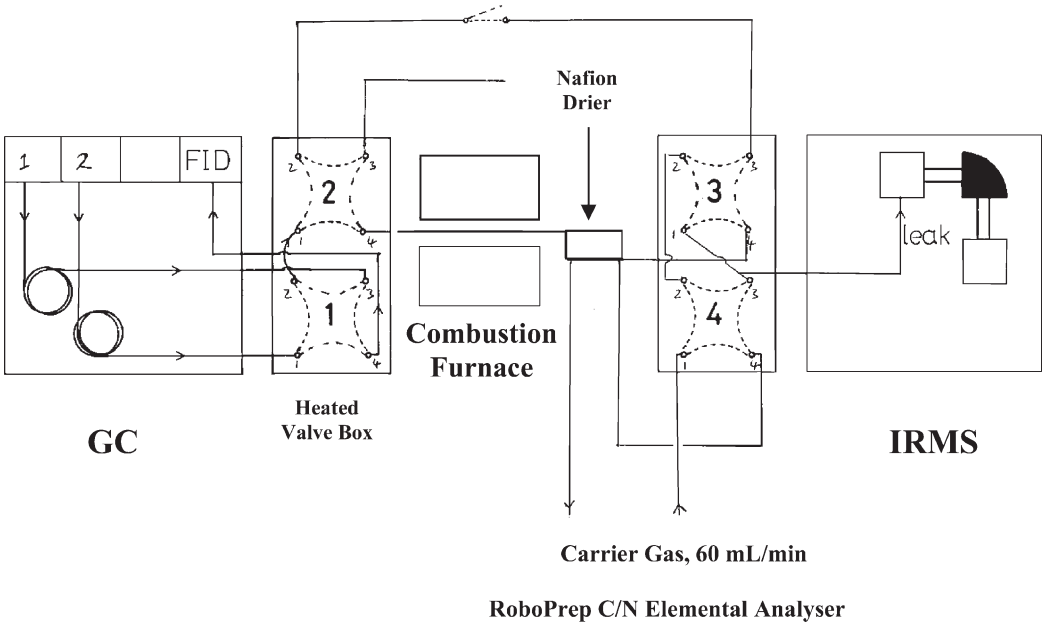

Figure 1. Schematic of the GC/C/isotope ratio mass spectrometer (see text for details).

The carrier entered the isotope ratio mass spectrometer ion source through an open split made of 95 cm long × 0.075 mm i.d. deactivated silica tubing and a zero dead volume T piece (SGE, UK). The length of the tubing was trimmed to obtain an ion source pressure of 3×10^{-6} mbar, measured by cold cathode gauge, unadjusted for response to helium. The remaining carrier vented to waste, resulting in some 3% of the sample injected into the gas chromatograph entering the isotope ratio mass spectrometer ion source. In the case of the 3MV internal standard, this equated to 0.2–1 nmol CO_2 entering the ion source. The isotope ratio mass spectrometer ion source was tuned for high sensitivity by optimising the ion repeller, beam focus, electron energy and accelerating voltage and operating the ion source with an electron trap current of 600 μA. The isotope ratio mass spectrometer was operated without CO_2 reference gas. 3MV was used as both a chemical and isotopic standard for this analysis. Raw results of the second-by-second signal intensities of CO_2 at m/z 44, 45 and 46 were exported to a spreadsheet where peak areas and peak are ratios were quantified.[34] This method models the minor beams as a function of the major. It has been validated against the proprietary software (PDZ Europa) and an in-house spreadsheet routine that calculated the response ratio regression in a similar fashion.[35] The three methods yield essentially the same isotope ratios but the chosen method produces more reliable peak areas for concentration analysis than the proprietary software and is faster than the in-house method. Further processing converts the ion beam ratios into ^{13}C abundance through elemental delta units, including a correction for ^{17}O,[36] and then to atom % ^{13}C,[33] with reference to 3MV. This internal standard had been calibrated in a way traceable to the international standard, VPDB.[37] The calculation included a correction for linearity (in IRMS, the term linearity describes the variation of measured isotope ratio with peak area) because measured isotope ratio is a function of peak area on this mass spectrometer and analyte peak areas can vary over an order of magnitude. This correction was facilitated by relating the offset ($\delta^{13}C$ of an acetate standard measured at a particular peak area ratio of the analyte to the internal standard, minus that measured at a peak area ratio of unity) to the peak area ratio. The offset was zero when analyte and internal standard peak areas were equal. The gradient of the plot of offset against $\log_{10}$ peak area ratio was calculated (Fig. 2) and used to predict any offset during routine analysis. This relationship was robust over an 80-fold range of major beam area ($R^2 = 0.96$). The gradient of the 'offset plot' was checked after maintenance such as a filament change. Peak area ratios with respect to the internal standard were also used to derive VFA concentration. In both calculations, the process blank was subtracted from each sample. Circulating acetate is at considerably greater concentration than propionate or butyrate (typical values in plasma: 150, 7 and 1 μM, respectively). Analysis of the latter required the sample to be more concentrated, which was accomplished by concentrating the ether phase ten-fold in a stream of nitrogen. Samples

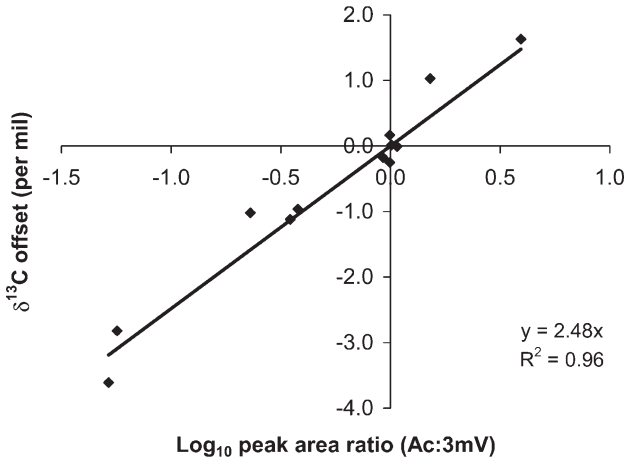

Figure 2. Analysis of the linearity of $\delta^{13}C$ measurements of acetate at varying concentrations (with respect to internal standard, 3MV) by GC/C/IRMS.

were processed individually, taking great care to leave 20–50 μL ether. This procedure takes less than 1 min, while samples remain at sub-ambient temperature.

RESULTS

Optimisation of extraction and derivatisation procedure

Ultrafiltration prior to SPE and increased salt strength prior to ultrafiltration were necessary to maximise VFA recovery. SPE was found to be robust for concentrating VFAs from as little as 2.5 mL plasma or urine. Samples could be processed as far as elution from SPE columns and stored for subsequent analysis as the dry sodium VFA salts. Derivatisation of VFAs directly in the ether extract was found to produce good yields for the higher VFAs (propionate—3MV) but acetate yields were low and variable. Increasing the polarity of the derivatisation mixture, by addition of acetonitrile, was found to normalise yields for all VFAs. As only 50 μL of the ether extract containing VFAs as their free acids was used for GC/MS analysis, the remainder was available for GC/C/IRMS analysis and re-analysis by either technique, if necessary.

Quantitation of VFA concentration and enrichment by GC/MS

The contribution of background or blank VFAs to the analysis is a real and problematic issue and is greatest for acetate analysis. Two internal standards were used for quantitation. Hexanoate was used to account for the blank contribution from the derivatisation reagents and 3MV was used to account for process blanks and to determine VFA concentrations. A comparison of calculated acetate concentrations using 3MV (internal standard method) versus [$^2H_3$]acetate (isotope dilution method) resulted in a linear model of $y = 1.07x + 21.83$ ($R^2 = 0.60$) and illustrates the good correlation between the two methods. Therefore, VFA concentrations were determined with respect to a standard addition of the internal standard, 3MV. The accuracy of VFA concentration analysis using 3MV as internal standard is demonstrated by the linear models for acetate of $y = 0.95x + 13.54$ ($R^2 = 0.996$), propionate $y = 1.02x - 1.00$ ($R^2 = 0.996$) and butyrate of $y = 0.86x - 2.20$ ($R^2 = 0.993$). The accuracy of determining the isotopic enrichments of [1-^{13}C]- and [$^2H_3$]acetate by GC/MS is demonstrated by the linear models for [1-^{13}C]acetate of $y = 0.96x + 0.05$ ($R^2 = 0.9991$) and for [$^2H_3$]acetate of $y = 1.04x + 0.01$ ($R^2 = 0.9997$). Both analyses showed good accuracy at the concentration found in biological fluids. The blank contributions and precision of the analysis are shown in Table 1.

Quantitation of VFA concentration and enrichment by GC/C/IRMS

The GC/C/IRMS instrumental set-up resulted in good chromatographic separation of VFAs, as illustrated in Fig. 3. Baseline resolution of all VFAs was achieved. This is necessary as considerable isotope fractionation can occur by chromatographic resolution of heavy and light molecules,[38] resulting in errors in calculated isotope enrichment of unresolved peaks. Separation was adequate but, at this gain, some tailing was evident. This was caused by the use of up to 5 mL injection volumes and choice of a packed column injector without septum purge. Constant flow and lack of an injector vent remove the possibility of discrimination in the injector of these volatile, low molecular weight analytes. The amount of CO_2 entering the ion source is a major factor governing analytical precision. Use of wide bore capillary columns, as opposed to higher resolution columns, increases sensitivity as it permits greater sample loading relative to carrier gas. For a fixed flow through the open split, more CO_2 enters the ion source. Figure 4 shows that the response ratio of acetate to 3MV was linear over an 80-fold concentration range allowing accurate determination of acetate concentration in blood and urine. The blank acetate contribution for the analysis was determined from the process blanks that had been processed with sample batches. This was calculated to be 49.5 (24.5) nmol (SD). The ether phase that remained after GC/MS analysis (~250 μL) was concentrated to ~50 μL in a tapered vial which permits propionate and butyrate analysis by GC/C/IRMS. Experiments with standard solutions at ambient concentration levels suggest that it is possible to measure acetate ^{13}C natural abundance with a precision of 0.29‰ (1 SD) over a concentration range of 10–800 μM, propionate to 2.7‰ ^{13}C (1 SD) over a concentration range of 2.3–17.9 μM, and butyrate to 3.3‰ ^{13}C (1 SD) over a concentration range of 1.8–7.2 μM. The accuracy of measuring [^{13}C]acetate enrichment is illustrated by the linear model, $y = 0.99x$ ($R^2 = 0.9996$).

Whole-body acetate flux

The results shown in Fig. 4 highlight the good comparison between the concentration determination of acetate by GC/MS and GC/C/IRMS in plasma and urine. Whole-body acetate flux measured by [$^2H_3$]- and [1-^{13}C]acetate dilution in plasma and urine is shown in Table 2.

Table 1. Parameters from the analysis of VFAs in plasma (n = 54 analyses)

	Acetate	Propionate	Butyrate
GC/MS			
Mean derivatisation blank (SD), nmol	310.3 (130.1)	1.2 (2.1)	3.2 (4.0)
Mean process blank (SD), nmol	36.3 (18.5)	14.0 (16.9)	1.4 (0.6)
Within analysis RSD (%)	9.4	5.4	4.8
Between analyses RSD (%)	15.5	10.6	10.5
GC/C/IRMS			
Mean process blank (SD), nmol	49.5 (24.5)	n.d.	n.d.

n.d. not determined.

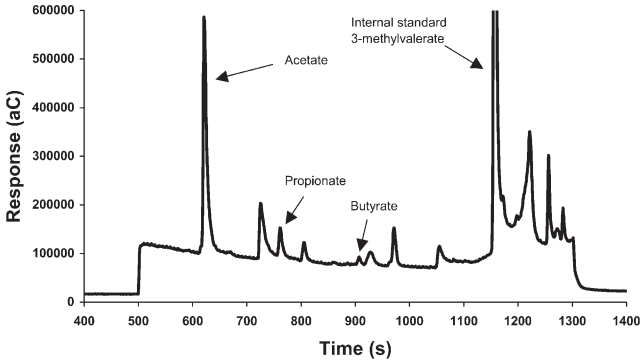

Figure 3. GC/C/IRMS chromatogram illustrating VFA separation in a sample processed from 2.5 mL urine.

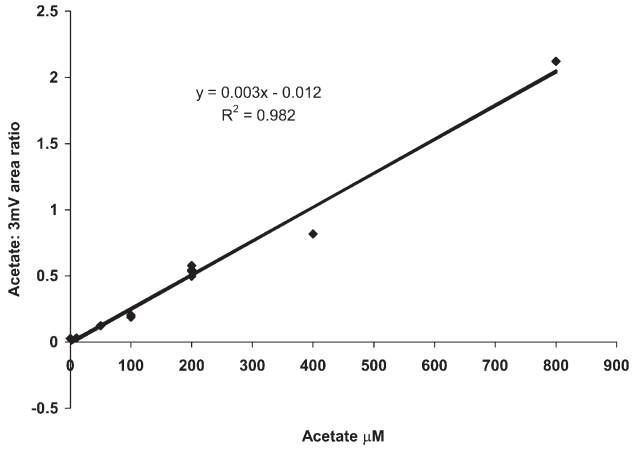

Figure 4. Acetate concentration determination by GC/C/IRMS. The plotted acetate/3MV (internal standard) ratio is linear over a large concentration range (0–800 μM) and within the concentrations found in plasma and urine.

DISCUSSION

A robust and sensitive method has been developed for the simultaneous determination of VFA concentration and enrichment from blood or urine samples by GC/MS and/or GC/C/IRMS. Because of the choice of sample preparation, each sample can be analysed by both techniques allowing studies to be conducted which employ multiple isotope tracers, e.g. [^{2}H] and [^{13}C] VFAs.

The design of any tracer study must recognise the limitations of the analytical methodology. The few published studies of VFA production have often used [1-^{13}C] VFAs. The natural abundance of ^{13}C in all biological carbon sources of ~1.1% (i.e. ~98.9% ^{12}C) results in a constant background at 1 mass unit greater [M+1] than the mass of any fragment ion [M^+] containing carbon. With respect to VFA *t*BDMS esters, the abundance of [M+1] ions is further added to by the contribution of silicon isotopes (^{29}Si natural abundance ~4.6%) when monitoring ions containing the *t*BDMS group. The practical consequences of using this tracer are that, with GC/MS analysis of VFA *t*BDMS esters, enough tracer must be added to the biological system to ensure that enrichments are above ~1 MPE.[39] The use of multiple-atom VFA tracers is justified to improve signal-to-noise in the analysis and reduce tracer requirements. One major issue encountered in this analysis (and highlighted by others[28]) is the ubiquitous nature of acetic acid which results in a considerable background problem both during sample processing and from the derivatisation reagent. The sources of background acetate are numerous and probably include reagents, the laboratory environment and contamination by laboratory staff. This problem was overcome by including two internal standards in the analysis. One internal standard (hexanoate) was used to account solely for VFAs from the derivatisation blank. This source of VFAs is most likely contaminants in the derivatisation reagent and was found to be the greatest source of acetate blank (by almost an order of magnitude). The other internal standard (3MV) was used to quantify the blank introduced in the processing of samples. This source of background VFAs was considerably lower when compared with the derivatisation blank (Table 1). The acetate process blank was similar when measured by GC/MS or GC/C/IRMS (36.3 vs. 49.5 nmol acetate, respectively). There was minimal blank contribution of other VFAs either at derivatisation or processing suggesting significant levels of acetate in the derivatisation reagent. Considerable variation was observed in the derivatisation blank and hence this method of quantifying the blank was incorporated into our analytical procedure.

The results from the *in vivo* tracer study to measure whole-body acetate flux in two subjects illustrate the potential of these two techniques to measure VFA turnover using different isotopic tracers. The results of acetate flux are in good agreement when the two different tracers are compared ([$^2H_3$]- vs. [1-^{13}C]acetate) highlighting the compatibility of the two techniques for measuring VFA concentration and enrichment in plasma and urine. Good agreement was also found when acetate flux was determined in urine vs. plasma, indicating that urine VFA concentration and enrichment measurements may be a suitable non-invasive way to measure whole-body VFA flux.

Table 2. Whole-body acetate production (WBAP) measured in plasma and urine using [$^2H_3$]- and [1-^{13}C]acetate

	Plasma		Urine	
	[1-^{13}C]acetate ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)	[$^2H_3$]acetate ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)	[1-^{13}C]acetate ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)	[$^2H_3$]acetate ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)
Subject 1	16.2 (3.5)*	12.4 (1.2)	15.4 (2.3)	13.2 (1.2)
Subject 2	28.7 (26.8)	23.4 (20.8)	26.3 (4.1)	24.2 (7.2)

*Error estimates for WBAP (SD) were determined from the standard deviation of the plateau enrichment measurements that were used to calculate flux.

There is little need to improve the sensitivity of the urine assay, as sample size is not a great issue. However, a reduction in blood volume sampled would be beneficial. This method currently uses 3 mL plasma (~6 mL whole blood). If only VFA concentration assay was required with analysis by GC/MS, this process could readily be scaled accordingly and an increase in detector gain would permit analysis of VFAs in 1 mL whole blood. For GC/C/IRMS analysis there was an estimated 0.2–1 nmol CO_2 entering the mass spectrometer ion source. This amount of CO_2 is close to the lower limit of sensitivity for GC/C/IRMS at natural abundance levels thus requiring sample concentration to remain above the sensitivity threshold. After taking a 50-μL aliquot for GC/MS, ~250 μL of the ether phase remained available for GC/C/IRMS and, as only between 0.5 and 5 μL (typically 3 μL) of the ether phase was injected, sensitivity could be increased by concentrating the ether phase. This was necessary to measure propionate and butyrate in plasma and urine.

A reduction in [^{2}H] tracer dose could be achieved by using an alternative analytical technique. GC/pyrolysis/IRMS is a technique similar in many ways to GC/C/IRMS that yields [^{2}H] isotopic enrichment.[40,41] The function of pyrolysis is to convert all hydrogen atoms in an analyte into hydrogen gas (H_2). The isotope ratio mass spectrometer analyses the [^{2}H]/[^{1}H] ratio of the hydrogen produced. This technique has high precision for isotope ratio analysis and therefore would permit significant reduction of [^{2}H] VFA doses for biological studies. This technique might be advantageous for studying the production of [^{13}C]-labelled VFAs, from [^{13}C]-labelled NDC, using [^{2}H] VFAs as biological yield tracers. However, the isotopomer information yielded by GC/MS analysis can be invaluable as GC/pyrolysis/IRMS of H_2 is less sensitive than GC/C/IRMS of CO_2.

The method described herein permits [^{13}C] and/or [^{2}H] isotopically labelled VFAs to be used as biological tracers to measure exogenous or colonic VFA production. Moreover, this methodology allows the simultaneous use of [^{13}C] and [^{2}H] VFA tracers in a single experiment and the processing of blood or urine samples generated allows both tracer enrichments to be measured from the same sample. Tracer studies to measure acetate, propionate and butyrate turnover are now possible using [^{13}C]-labelled substrates and GC/C/IRMS analysis.

Acknowledgements

The authors thank Sandra Small at SUERC for technical assistance, Dr Donny McMillan and Lynn Prentice at Glasgow Royal Infirmary for their assistance in conducting blood-sampling protocols, and Dr Les Bluck at MRC-HNR, Cambridge, for a copy of his Excel spreadsheet routine for calculating isotope ratios from GC/C/IRMS data. This work was carried out with the financial support of the Mars Nutrition Research Council through a post-doctoral fellowship. This work was also financially supported by the Commission of the European Communities, specific RTD programme 'Quality of Life and Management of Living Resources', QLK 1-2001-00431, 'Stable isotope applications to monitor starch digestion and fermentation for the development of functional foods' (www.eurostarch.org). It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

REFERENCES

1. Parrett AM, Edwards CA. *Arch. Dis. Child.* 1997; **76**: 249.
2. Cummings JH, Englyst HN. *Am. J. Clin. Nutr.* 1987; **45**: 1243.
3. Roediger WE. *Gastroenterology* 1982; **83**: 424.
4. Luhrs H, Gerke T, Boxberger F, Backhaus K, Melcher R, Scheppach W, Menzel T. *Dig. Dis. Sci.* 2001; **46**: 1968.
5. Younes H, Coudray C, Bellanger J, Demigne C, Rayssiguier Y, Remesy C. *Br. J. Nutr.* 2001; **86**: 479.
6. Topping DL, Fukushima M, Bird AR. *Proc. Nutr. Soc.* 2003; **62**: 171.
7. Dankert J, Zijlstra JB, Wolthers BG. *Clin. Chim. Acta* 1981; **110**: 301.
8. Piloquet H, Ferchaud-Roucher V, Duengler F, Zair Y, Maugere P, Krempf M. *Am. J. Physiol. Endocrinol. Metab.* 2003; **285**: E561.
9. Delzenne NM, Kok N. *Am. J. Clin. Nutr.* 2001; **73**: 456S.
10. Vogt JA, Wolever TM. *J. Nutr.* 2003; **133**: 3145.
11. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. *Gut* 1987; **28**: 1221.
12. Pouteau E, Piloquet H, Maugeais P, Champ MMJ, Dumon H, Nguyen P, Krempf M. *Am. J. Physiol. Endocrinol. Metab.* 1996; **271**: E58.
13. Pouteau E, Dumon H, Biourge V, Krempf M, Nguyen P. *J. Nutr.* 1998; 2663S.
14. Pouteau E, Vahedi K, Messing B, Flourie B, Nguyen P, Darmaun D, Krempf M. *Am. J. Clin. Nutr.* 1998; **68**: 1276.
15. Simoneau C, Pouteau E, Maugeais P, Marks L, Ranganathan S, Champ MMJ, Krempf M. *Biomed. Environ. Mass Spectrom.* 1994; **23**: 430.
16. Breves G, Schulze E, Sallmann HP, Gadeken D. *Z. Gastroenterol.* 1993; **31**: 179.
17. Seufert CD, Mewes W, Soeling HD. *Eur. J. Clin. Invest.* 1984; **14**: 163.
18. Ballard FJ. *Am. J. Clin. Nutr.* 1972; **25**: 773.
19. Buckley BM, Williamson DH. *Biochem. J.* 1977; **166**: 539.
20. Schooley DL, Kubiak FM, Evans JV. *J. Chromatogr. Sci.* 1985; **23**: 385.
21. Richardson A, Calder A, Stewart C, Smith A. *Lett. Appl. Microbiol.* 1989; **9**: 5.
22. Guerrant GO, Lambert MA, Moss CW. *J. Clin. Microbiol.* 1982; **16**: 355.
23. Mentasti E, Gennaro MC, Sarzanini C, Baiocchi C, Savigliano M. *J. Chromatogr.* 1985; **322**: 177.
24. Kim H, Nochetto C, McConnell LL. *Anal. Chem.* 2002; **74**: 1054.
25. Pinho O, Ferreira IM, Ferreira MA. *Anal. Chem.* 2002; **74**: 5199.
26. Mills GA, Walker V, Mughal H. *J. Chromatogr. B, Biomed. Sci. Appl.* 1999; **730**: 113.
27. Moreau NM, Goupry SM, Antignac JP, Monteau FJ, Le Bizec BJ, Champ MM, Martin LJ, Dumon HJ. *J. Chromatogr. B, Biomed. Sci. Appl.* 2003; **784**: 395.
28. Powers L, Osborn M, Yang D, Kien C, Murray R, Beylot M, Brunengraber H. *J. Mass Spectrom.* 1995; **30**: 747.
29. Preston T. *Plant Cell Environ.* 1992; **15**: 1091.
30. Wolfe RR. In *Radioactive and Stable Isotopes in Biomedicine: Principles and Practice of Kinetic Analysis*, Wolfe RR (ed). Wiley-Liss: New York, 1992; 1–17.
31. Morrison DJ, Dodson B, Slater C, Preston T. *Rapid Commun. Mass Spectrom.* 2000; **14**: 1321.
32. Waldron S, Preston T. *Abstr. Pap. Am. Chem. Soc.* 2001; **221**: 92-GEOC, Part 1.
33. Slater C, Preston T, Weaver LT. *Rapid Commun. Mass Spectrom.* 2001; **15**: 1270.
34. Bluck LJC, Coward WA. *Meas. Sci. Technol.* 2004; **15**: N21.
35. Thorne GC, Gaskell SJ. *Biomed. Mass Spectrom.* 1985; **12**: 19.
36. Craig H. *Geochim. Cosmochim. Acta* 1957; **12**: 133.
37. Coplen TB. *Geochim. Cosmochim. Acta* 1996; **60**: 3359.
38. Matthews DE, Hayes JM. *Anal. Chem.* 1978; **50**: 1465.
39. Preston T, Slater C. *Proc. Nutr. Soc.* 1994; **53**: 363.
40. Hilkert AW, Douthitt CB, Schluter HJ, Brand WA. *Rapid Commun. Mass Spectrom.* 1999; **13**: 1226.
41. Scrimgeour CM, Begley I, Thomason ML. *Rapid Commun. Mass Spectrom.* 1999; **13**: 271.