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Development of a static headspace gas chromatographic procedure for the routine analysis of volatile fatty acids in wastewaters

J.A. Cruwys[a], R.M. Dinsdale[a,*], F.R. Hawkes[a], D.L. Hawkes[b]

[a]*School of Applied Sciences, University of Glamorgan, Pontypridd, RCT, CF37 1DL Wales, UK*

[b]*School of Technology, University of Glamorgan, Pontypridd, RCT CF37 1DL Wales, UK*

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Abstract

An optimised procedure has been developed for the routine analysis of volatile fatty acids in wastewater matrices, using static headspace gas chromatography with flame ionisation detection. Factors such as sample volume, sample pre-treatment and the time and temperature of sample equilibration have been included in an optimisation model designed to provide maximum detector response for acetic, propionic, iso- and *n*-butyric and iso- and *n*-valeric acids in the concentration range 0–1000 mg/l. Optimal headspace conditions were observed when equilibrating at 85 °C for 30 min, using a 2.0 ml sample volume with the addition of 1.0 ml of $NaHSO_4$ (62%, w/v) into standard 22.3 ml vials. 2-Ethylbutyric acid was used as an internal standard. The suitability of ordinary least squares regression and weighted least squares regression models for the purposes of calibration and quantification were investigated. A weighted least squares linear regression model applied to the heteroscedastic data provided lower detection limits, e.g. 3.7 and 3.3 mg/l for acetic and propionic acids.

Keywords: Headspace analysis; Wastewater analysis; Environmental analysis; Regression analysis; Weighted least-squares analysis; Volatile fatty acids; Volatile organic compounds

1. Introduction

Low-molecular-mass carboxylic acids (in this paper aliphatic short chain C_2–C_5) are important intermediates and metabolites in biological processes. Known as volatile fatty acids (VFAs) and short-chain fatty acids (SCFAs) these homologues and corresponding structural isomers include acetic, propionic, iso- and *n*-butyric and iso- and *n*-valeric acids. The presence of VFAs in a sample matrix is often indicative of bacterial activity. VFA analysis is of significance in studies of health and disease in the intestinal tract [1]. VFA measurements are required to monitor the operation of landfills and biological wastewater treatment plants carrying out anaerobic digestion, phosphorus removal or denitrification [2,3].

Many wastewater treatment and environmental applications require detection of VFA concentrations in the range 1–5000 ppm and involve a variety of matrices. These matrices often contain components that can result in the degradation of sensitive equip-

*Corresponding author. Tel.: +44-1443-48-2227; fax: +44-1443-48-2285.

E-mail address: rdinsdal@glam.ac.uk (R.M. Dinsdale).

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ment such as those used in gas chromatography (GC) and high-performance liquid chromatography (HPLC). Traditionally, the VFA content of wastewaters has been analysed by titrimetric methods and direct injection GC or GC preceded by solvent extraction. More recently, ion chromatography [4,5] and HPLC [6] have been applied to VFA analysis.

These procedures suffer from a number of disadvantages. Titrimetric procedures including the five-point method proposed by Lahav and Loewenthal [7], cannot determine particular species of VFA and results are commonly reported as total VFA content. HPLC and ion chromatography procedures often require extensive sample clean up and the use of derivatisation techniques or agents to suppress interference from coeluting ions, to reach acceptable limits of detection. The most commonly used method of VFA analysis, GC–flame ionisation detection (FID), has sufficient detection capability without derivatisation. VFAs have been analysed using direct aqueous injection [1]. However, such methods – though rapid – can lead to contamination of the GC injection port and column with sample matrix components that interfere with analysis and degrade chromatographic performance, and hence increase the incidence of maintenance [8]. Sample clean up by solvent extraction can prevent the introduction of sample matrix components and has been used extensively in the analysis of wastewaters. A number of disadvantages of this method are commonly quoted and include the extended sample preparation times and costly disposal of used solvent. Manni and Caron [9] highlighted potential problems associated with the quantitative transfer of acetic and propionic acid from the aqueous phase into diethyl ether, which could effect the reproducibility of extractions and the calibration of the procedure. Automated headspace gas chromatography (HS-GC) techniques avoid these problems and offer relatively rapid and solvent-free analyses. Headspace solid-phase microextraction (HS-SPME) techniques for VFA analysis, first described by Pan et al. [10] and extended by other authors including Ábalos and Bayona [11], offer similar advantages to HS-GC, including solvent-free sample preparation and sufficient detection capability for the determination of volatile acids in wastewater. HS-SPME calibration procedures can be complicated by the introduction of a third phase during the fiber absorption process [12], and by the use of derivatisation techniques.

Quantitative analysis of headspace samples is often carried out using gas chromatographic techniques. Kolb and Ettre [13] discuss the various calibration options available to chromatographers. The most suitable method for a given application is largely dependent on the time available for analysis and the nature of the sample matrix. For high sample throughput the methods of standard addition and calibration procedures associated with multiple headspace extraction (MHE) are not appropriate. In such cases a choice between traditional internal and external standardisation is required. However, accurate reproduction of the sample matrix should be performed for *both* calibration procedures, a process that is rarely possible in the analysis of unknown wastewater samples. Chen et al. [14] adopted an external standard (ES) approach for the analysis of malodorous compounds including VFAs in swine wastewaters, validating their results by direct aqueous GC injections. No indication of matrix matching and few details of the calibration procedure were given. Due to the highly complex and variable matrix found in human faecal samples, Stansbridge et al. [15] incorporated 2-ethylbutyric acid as an internal standard (IS) in a standard addition calibration method for the VFA analysis of faeces. Authors including Drozd and Vodáková [16] have drawn attention to problems associated with the composition of the sample matrix and the possible shortcomings of the I.S. calibration method, suggesting that unless careful consideration is given to the nature of the I.S., it cannot account for the effects of matrix components.

In analytical chemistry, calibration curves describing the relationship between factors and response are often determined by application of ordinary least squares (OLS) regression models. Replicate measurements taken at various predictor levels can sometimes show increasing variance (heteroscedasticity) with increasing concentration of standard. In such cases application of OLS can cause gross errors in the calibration model [17] and the use of a weighted least squares (WLS) regression model may be appropriate. Heteroscedasticity should also be accounted for when calculating detection and quantification limits [18].

Various authors have described the analysis of volatile fatty acids using static headspace techniques in faeces [15], for bacterial identification [19], and in wastewaters [14]. To the knowledge of the authors no single paper has described in detail an optimisation procedure for the direct HS-GC analysis of VFAs in wastewaters, and provided a discussion of the problems of calibration particular to HS-GC. This paper investigates techniques to minimise sample carry-over and procedures for calibration in routine high-throughput analysis of wastewaters.

2. Experimental

2.1. Chemicals and materials

Acetic (99.99%), propionic (99.5%), isobutyric (99%), *n*-butyric (99%), isovaleric (99%), *n*-valeric (99%) and 2-ethylbutyric (99.5%, internal standard) acids were purchased from Aldrich (Gillingham, UK). Formic acid (99.9%) suitably low in acetic acid (0.04%) was also obtained from Aldrich. All standards were used as received. The inorganic salts NaCl and $NaHSO_4$ used for the optimisation stage were acquired from Fisher Scientific (Loughborough, UK) as was analytical-grade concentrated HCl. All HS-GC analyses were performed using standard 22.3 ml glass vials, PTBE septa and patented closures each from Perkin–Elmer (Beaconsfield, UK).

2.2. Standard and sample preparation

Standards used for linearity trials and for the calibration procedures were prepared from a stock solution containing 2500 mg/l each of acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids. Appropriate dilution of the stock solution using certified glassware throughout produced VFA standards in the range 1000–1 mg/l.

2.3. Instrumentation

Headspace chromatographic analysis was performed using a HS 40XL automatic headspace sampler connected to an Autosystem XL GC system, both from Perkin–Elmer. All HS-GC analyses were performed using standard 22.3 ml glass vials, PTBE septa and patented closures each from Perkin–Elmer. The temperatures of the HS 40XL oven, needle and transfer line were set at 85, 105 and 135 °C, respectively. The HS 40XL unit also controlled the optimised vial thermostatting time of 30 min, vial pressurisation time of 3 min and sample injection period of 0.10 min. The HS 40XL sample shaker was used throughout the study. The GC injection port was operated in flow mode (split flow 5.0 ml/min) and maintained at 200 °C. The GC column used was a free fatty acid phase (FFAP) fused-silica capillary (30 m × 0.25 mm I.D., film thickness 0.25 μm) from Perkin–Elmer. The GC system was programmed to heat the column from 60 to 190 °C at 10 °C/min; the temperature was held at 190 °C for 1 min. Analyses were carried out using a flame ionisation detector set at 250 °C. Because of the overlapping function of the HS 40XL unit, the rate-limiting step in the analysis procedure was the GC cycle time, not the equilibration time.

2.4. Headspace optimisation strategy

VFA concentration was treated as a fixed variable in the entire optimisation procedure. The number of trials that could be conducted in one session was limited by the 40-vial carousel capacity of the HS 40XL headspace sampler and also by the inclusion of wash cycles to reduce the influence of analyte carry-over. The effects of sample volume and sample pre-treatment were evaluated at a fixed equilibration time of 30 min, chosen after initial range finding trials. It was not possible to change the temperature of equilibration during a 40-vial HS sequence; hence this factor was included as a blocking variable.

The optimisation trials were conducted at four volumes, at three temperatures and using four sample matrices: one prepared in ultrapure water and three matrices of comparable ionic strength prepared using NaCl and $NaHSO_4$. Four standards containing 250 mg/l each of acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids were prepared in four different matrices: (1) ultrapure water, (2) 9.7% (w/v) NaCl, (3) 9.7% (w/v) NaCl with sufficient HCl added to lower the pH to 1.0, and (4) 20% (w/v) $NaHSO_4$. Four sample volumes (13 μl, 0.5 ml 3.0 ml, 9.0 ml) were selected in order to assess the effect of changing the phase ratio, β, on FID signal

response. Each of the three VFA standards was analysed in duplicate using the four sample volumes. For the optimisation process the use of two 3.0 ml ultrapure water washes in between sample analysis was sufficient to reduce the effect of carry-over. The trials were conducted on consecutive days at vial equilibration temperatures of 45, 65, and 85 °C. A separate extended volume study was conducted using a total vaporisation volume (13 μl), a full evaporation volume (20 μl) and also using 0.1, 0.2, 0.5, 1.0, 3.0, 5.0, 7.0 and 9.0 ml volumes at the optimum equilibration temperature (85 °C) for all six VFAs. The extended volume study was completed using two matrices: an ultrapure-based matrix and a 20% (w/v) $NaHSO_4$-based matrix for comparison, both containing 250 mg/l each of acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids.

Following the work of Heitefuss et al. [20] the use of the acidic salt sodium hydrogensulphate was investigated for the salting out process. Addition of such a hygroscopic salt in pure form to individual vials proved to be a laborious task, not well suited to routine wastewater analysis. Five standard VFA 250 mg/l solutions with varying concentrations of $NaHSO_4$ (0, 5, 10, 20, 25%, w/v) were prepared for a $NaHSO_4$ salt concentration effect study. Four duplicate analyses were performed for each concentration using a sample volume of 3.0 ml. All samples were analysed using the optimised HS-GC procedure. The final concentration and amount of salt added to the sample was investigated using various sample/salt volume ratios.

Finally, in order to ensure that the optimised method represented a liquid–vapour equilibrium process, the effect of varying the sample equilibration time was investigated in the range 10, 20, 30, 40, 50, 60 and 120 min.

2.5. Carry-over study

A carry-over evaluation study was undertaken following the work of Van Eeaeme et al. [21]. A 3.0 ml volume of a 1000 mg/l standard solution of acetic, propionic and isobutyric acids made up in 12% (w/v) NaCl was introduced into a headspace vial and analysed under optimal HS conditions (85 °C, 30 min). This sample was followed by analysis of nine blank runs (washes) each consisting of 3.0 ml of ultrapure water in order to ascertain the percentage carry-over between runs. This process was repeated using washes containing 3.0 ml of 12% (w/v) NaCl and also for a 10% (w/v) solution of formic acid (low in low acetic acid content, 0.04%, w/v).

2.6. Calibration procedure

The VFA stock solution was diluted in a suitable manner to produce nine calibration standards (1000, 500, 250, 100, 50, 25, 10, 5 and 1 mg/l named levels 1–9, respectively). All standard solutions were equilibrated at 20 °C prior to preparation and use. The internal standard (99%, w/v, 2-ethylbutyric acid) was prepared at a concentration of 1800 mg/l. For the calibration procedure 2.0 ml of VFA standard was added to 1.0 ml of $NaHSO_4$ (62%, w/v) together with 100 μl of the I.S. solution into a glass vial, using the open vial technique. Ten replicates were analysed each separated by three wash vials containing 3.0 ml of ultrapure water, to reduce the effect of VFA carry over. A method blank solution was prepared by adding 1.0 ml of $NaHSO_4$ (62%, w/v) to 2.1 ml of ultrapure water. Ten replicate blank solutions were analysed. It was necessary to separate the calibration procedure into two distinct blocks, high calibration values (1000–100 mg/l) and low calibration values (50–1 mg/l) to avoid accentuating the carry-over problem by sequentially analysing low and high values. The analyses were carried out in two blocks under a Latin square design, in an attempt to reduce possible systematic errors.

Particular care was taken to follow the guidelines of MacTaggart and Farwell [22] and Baumann [23] with regards to the application of linear regression to the calibration data and validation of the proposed models. As a starting point ordinary least squares linear regression was applied to the data obtained for the six acids, for both external and internal standardisation. The analysis of variance (ANOVA) lack of fit test suggested by Draper and Smith [24] was used to check the adequacy of the linear models. Data were checked for departure from normality by application of the Shapiro–Wilk test and by inspection of normal probability plots. The homogeneity of the variance of the error terms at the various

concentration levels was investigated using the Levene test statistic, and also by inspection of a plot of the raw residuals against the response variable values predicted by the chosen model.

A separate study was conducted to evaluate performance characteristics of the method that provide an indication of its detection and quantification capability. The VFA stock solution was diluted in a suitable manner to produce seven limit of detection standards (10.0, 7.5, 5.0, 2.5, 1.0, 0.5, and 0.1 mg/l). The in-vial standard solutions were prepared as described in the previous paragraph. Ten replicate analyses were run for each standard using the optimised HS-GC method. Estimates of the detection capabilities of the calibration procedures, namely the critical value, L_C and the detection limit, L_D, which are defined by Curie [18] as a value used to distinguish a chemical signal from background noise and a measure of the inherent detection capability of a method, respectively, were obtained for the OLS and WLS models. The detection limit was also estimated using the classical $3\sigma_{blank}$ approach, i.e. calculation of L_D through analysis of the standard deviation of blank measurements. A procedural blank was obtained by mixing 2.0 ml of ultrapure water with 1.0 ml of 20% (w/v) $NaHSO_4$. A value of s_{blank} (the standard deviation of the estimated net signal of the procedural blank) was obtained by analysing 20 replicates of the signal obtained using the blank solution and multiplying the result by a factor, $k=3$.

2.7. Analysis of artificial and real samples

The chosen calibration model was subsequently applied to the analysis of artificial and real wastewater samples. An artificial wastewater matrix was prepared based on a mineral medium proposed by Cohen et al. [25] and commonly used as a feedstock in laboratory-scale anaerobic digesters. The effect of increasing the hydrogencarbonate alkalinity (BA), which is a measure of the buffering capacity of wastewater treatment systems, on the FID response for the six VFAs was investigated. The feedstock solution contained 8.4 g/l of $NaHCO_3$, which is the molar equivalent of a 5000 mg/l $CaCO_3$ solution on which the BA scale is traditionally measured. The feedstock was used in conjunction with the VFA stock solution (2500 mg/l) to produce six artificial wastewater standards across a concentration range representative of the BA range encountered in anaerobic digesters. Standards containing hydrogencarbonate alkalinities of 0, 1000, 2000, 3000, 4000 and 5000 mg/l $CaCO_3$ were analysed with the optimised HS-GC method for VFAs. Internal and external standard calibration procedures were applied to the data, using both OLS and WLS regression for the purpose of comparison.

A trial was conducted to test the repeatability of the HS-GC method using a sample taken from an expanded granular sludge bed (EGSB) anaerobic digester fed on a mineral medium containing 1% (w/v) glucose as the main carbon source, after Cohen et al. [25]. Approximately 25 ml of an EGSB reactor liquor sample was divided into ten 2.0 ml aliquots. To each vial 1.0 ml of a $NaHSO_4$ solution (62%, w/v) and 100 μl of a 1800 mg/l I.S. solution was added and the mixtures were analysed using the optimised HS-GC method.

The authors suggest that readers wishing to use this VFA method for wastewater treatment samples, check the time required for equilibration prior to routine real sample analysis, i.e. whether a 30 min equilibration time will produce the required level of repeatability.

3. Results

3.1. Selection of optimum headspace parameters

Both general method development and a strategy aimed at optimising VFA concentration in the headspace above aqueous samples followed the guidelines outlined by Kolb and Ettre [13]. Trials were conducted with the primary goal of maximising the headspace concentration of acetic, propionic and isobutyric acids since these acids are of particular importance in the monitoring of wastewater processes that include anaerobic digestion. Emphasis was also placed on high sample throughput as the procedure was to be designed for routine wastewater analysis.

Variables considered to be significant in maximising the response of the procedure (peak area counts of the VFAs) included sample volume and con-

centration, time and temperature of heating (the equilibration process) and the addition of salt and/or adjustment of pH. The results of the combined optimisation trials are summarised in Table 1.

3.1.1. Vial equilibration temperature

It can be seen from Table 1 that, as expected the temperature of equilibration had a dramatic effect on the FID response for all three acids. Peak area counts improved by a factor of between 1.7 and 4.0 when considering a 20 °C increase in temperature within a given standard series. Further increase in temperature and hence internal vial pressure was not attempted for safety reasons. A significant advantage with respect to acetic acid response was to be had by performing all subsequent HS extractions at 85 °C.

3.1.2. Volume of sample

Table 1 shows that for acetic, propionic, isobutyric and *n*-butyric acids, traditional headspace sample volumes in the series 0.5, 5.0 and 9.0 ml had a minimal effect on the FID response (measured as peak area count indexed against lowest value for each acid) in comparison to the equilibration temperature and pre-treatment of the sample. In general, response at the optimum equilibration temperature of 85 °C was at a maximum using a 3.0 ml volume for each of the acids and for all four matrices investigated with the exception of acetic acid. An extended volume study at the optimum operating temperature produced a negligible change in the peak area counts obtained for all six VFAs. A decrease in FID response was noted for propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids using total vaporisation volumes (13 μl) and full evaporation volumes (20 μl). Such a decrease is in accordance with the findings of Strassnig and Lankmayr [26], and suggests that the full evaporation technique

Table 1
Effect of pre-treatment, sample volume and equilibration temperature on the peak area counts for acetic, propionic, isobutyric and *n*-butyric acid

Pre-treatment		Acetic acid			Propionic acid			Isobutyric acid			*n*-Butyric acid		
		Equilibration temperature											
	Sample volume	45 °C	65 °C	85 °C	45 °C	65 °C	85 °C	45 °C	65 °C	85 °C	45 °C	65 °C	85 °C
None	13 μl	1.0	3.6	12.1	1.0	3.5	10.4	1.0	3.2	7.5	1.0	3.8	10.1
	0.5 ml	1.2	3.7	9.5	1.2	4.4	12.1	1.3	5.4	14.9	2.5	10.6	14.5
	3.0 ml	1.3	5.0	12.0	1.3	5.1	13.9	1.4	5.6	16.3	1.4	6.0	17.5
	9.0 ml	1.2	4.4	11.0	1.3	4.8	13.1	1.4	5.1	15.1	1.3	5.5	16.2
9.7% NaCl	13 μl	1.9	6.8	11.5	1.8	6.5	11.4	1.6	5.4	8.2	1.8	6.7	11.3
	0.5 ml	2.1	7.4	18.6	2.2	8.4	22.3	2.5	9.9	26.8	2.8	11.1	29.5
	3.0 ml	2.1	7.6	18.8	2.5	8.6	23.2	2.9	10.1	30.2	2.9	10.8	32.1
	9.0 ml	1.7	6.6	16.1	2.1	8.2	20.4	2.7	9.7	27.5	2.6	10.2	28.8
9.7% NaCl, pH 1	13 μl	2.2	7.8	19.2	2.1	7.1	15.6	2.0	5.7	9.7	2.1	7.3	14.4
	0.5 ml	2.9	8.1	18.6	2.6	9.0	22.5	2.7	10.3	26.6	3.8	13.1	31.8
	3.0 ml	2.2	8.8	18.9	2.4	9.1	23.2	2.9	10.4	30.3	2.9	11.1	32.1
	9.0 ml	2.1	7.5	18.0	2.3	8.9	22.0	2.8	10.3	28.6	2.7	11.1	30.3
20% $NaHSO_4$	13 μl	2.5	9.4	21.0	2.4	7.6	16.7	2.5	5.3	10.5	1.2	5.4	15.8
	0.5 ml	3.1	8.5	19.1	3.3	10.2	23.8	3.6	12.1	28.6	2.6	7.3	15.6
	3.0 ml	2.2	7.5	19.5	2.7	9.3	24.9	3.7	12.0	33.6	3.5	12.6	35.4
	9.0 ml	2.0	7.6	18.3	2.5	8.7	23.0	3.3	10.8	30.4	3.1	11.4	32.0

Data is indexed for each acid (lowest peak area count in bold set to 1.00). Percentage salt conc. is on a w/v basis. Analyses performed in duplicate.

pioneered by Markelov and Guzowski [27] is not suitable for the analysis of these VFAs if low concentrations are expected.

3.1.3. Effect of changing the activity coefficient of the analyte

The results of initial trials involving 9.7% (w/v) NaCl, 9.7% (w/v) NaCl with pH adjusted to 1.0 using concentrated HCl and 20% (w/v) $NaHSO_4$ which represent the same ionic strength of salt in solution, are displayed in Table 1. Use of NaCl in a salting out procedure increased the peak area count for acetic, propionic and isobutyric acids in all cases. For acetic acid the mean increases in response across the temperature due to adding NaCl (12%, w/v) were 1.5, 1.5 and 1.4 for the volume levels 3, 5 and 7 ml, respectively. The lowering of pH and addition of salt resulted in mean increases in response across the temperature range of 1.7, 1.5 and 1.5 for the same volume levels. Hence, the change of pH to 1.0 had a minimal effect for acetic acid. A similar pattern was evident for propionic and isobutyric acids.

Sample preparation time was significantly reduced with the use of the acidic salt $NaHSO_4$ when compared to that of acidified NaCl. The in-vial pH of the samples with $NaHSO_4$ (0, 5, 10, 20, 25%, w/v, and saturated $NaHSO_4$) was in the range 0.7–0.5. The results of these investigations are shown in Fig. 1 for each VFA. A linear increase in peak area counts for all acids with salt concentration was observed ($r^2 \geq 0.99$ for each acid). It was noted that the optimum sample-to-salt ratio involved use of

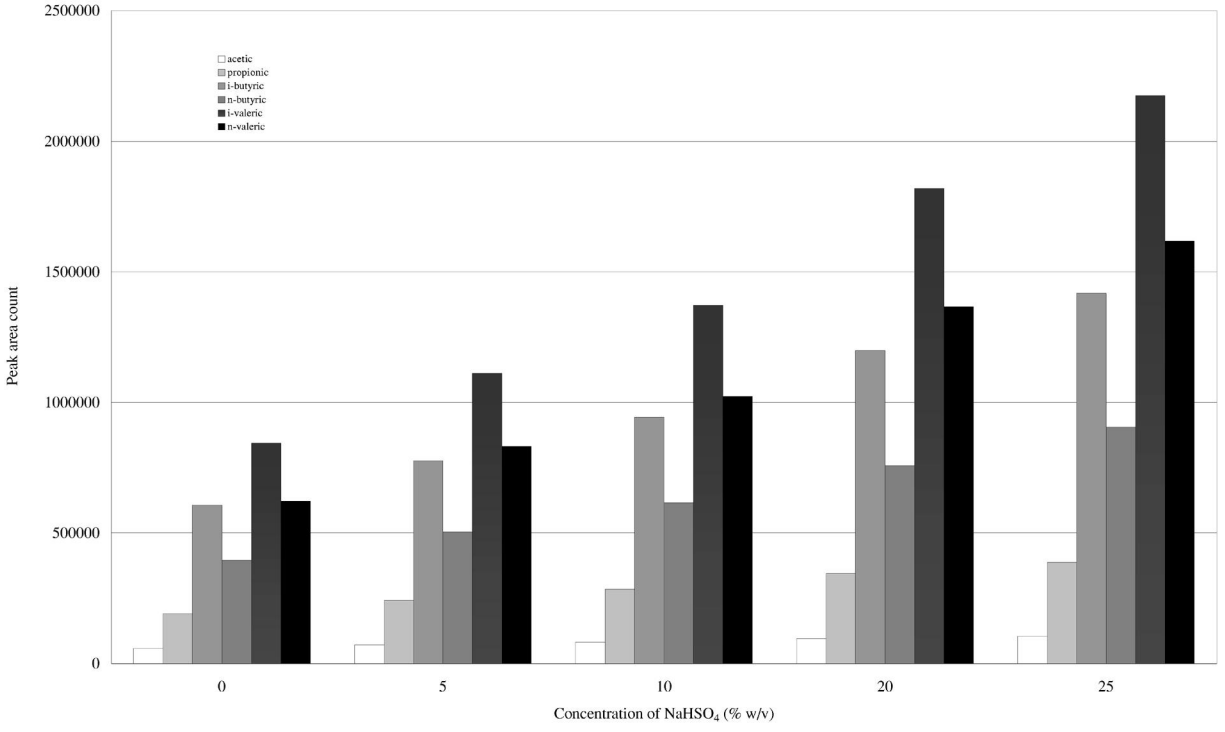

Fig. 1. Effect of $NaHSO_4$ concentration on the peak area counts of six VFAs. A solution containing 250 mg/l of acetic, propionic, isobutyric (''i-butyric''), *n*-butyric, isovaleric (''i-valeric'') and *n*-valeric acids was used for this trial. Details of sample preparation are given in Section 2.4. Data points are mean values of four replicate analyses in each case. Relative standard deviation $<4\%$ for each measurement level for all VFAs.

1.00 ml of the $NaHSO_4$ stock (60%, w/v) added to 2.00 ml of standard/sample.

3.1.4. Effect of equilibration time

Results of the equilibration trials demonstrated that liquid–vapour equilibrium at 85 °C with 1.00 ml of the $NaHSO_4$ stock (60%, w/v) added to 2.00 ml of standard/sample was achieved after 10 min. An equilibration time of 30 min was adopted. The overlapping mode of the HS 40XL unit prevented any loss in sample throughput for the method at this extended time. The effect of equilibration time on the peak area response for anaerobic digester samples should be assessed prior to analysis; the lowest appropriate equilibration time should be employed.

3.1.5. Addition of the internal standard

The addition of the I.S. to both standards and samples can be an important source of systematic error within the analytical process. The options investigated in this paper included direct addition of undiluted (99%) 2-ethylbutyric acid into the sample vial using the open or closed vial technique, addition of the I.S. to the $NaHSO_4$ stock solution (60%, w/v) at a suitable concentration to produce a sizeable peak upon GC analysis, and pipetting a known volume of an I.S. solution into the vial using the open vial technique. Each method was investigated for repeatability, and the standard deviations of measurement analysed. The I.S. was not suitably soluble in the $NaHSO_4$ (60%, w/v) stock solution to provide the degree of repeatability required. Addition of 5 μl of neat I.S. to 1.00 ml of the $NaHSO_4$ stock (60%, w/v) and 2.00 ml of standard/sample (1.54 g/l I.S.) did not produce a reasonably sized peak following HS-GC analysis. Addition of I.S. to the vials by pipetting 100 μl of a standard 1800 mg/l solution for calibration and for sample analysis proved to be the most convenient and repeatable method.

3.2. Carry-over of analytes

The aim of the carry-over study was to reduce the appearance of ghost peaks in wash cycles and to establish the minimum number of washes required in between standard/sample analyses to allow accurate calibration and sample quantitation. Fig. 2 summarises the results of ultrapure water and formic acid washes (10%, w/v) for acetic, propionic acid and isobutyric acids (1000 mg/l in 12%, w/v, NaCl solution) in which the carry-over peak areas are represented as the cumulative percentage of the initial analyte peak area. No significant difference was observed between the eluting capability of ultrapure water and 12% NaCl (w/v in ultrapure water). Consistent with the work of Van Eeaeme et al. [21] formic acid appeared to be the most efficient VFA eluter for acetic acid removing a cumulative percentage of 10.9% after nine identical washes as opposed to 6.5% when ultrapure water alone was used. However for propionic acid, ultrapure water and formic acid exhibited very similar eluting abilities removing a total of 5.7 and 5.3% of the initial peak area count for the acid. As the number of carbon atoms increased to four in the case of isobutyric acid, the eluting properties of ultrapure water and formic acid were reversed with a total 5.3 and 2.4% being removed, respectively. A very similar situation was also observed for *n*-butyric acid. The HS-GC chromatogram of the formic acid solution (10%, w/v) displayed significant impurities, which may prohibit its use as wash agent. After three washes with ultrapure water carry-over was reduced to below the repeatability level of the method for real samples, and hence three washes employed in future analyses.

3.3. Calibration procedures and method detection capabilities

Tests to check for model linearity, and the normality and homogeneity of the variances of the errors of the response variable, peak area count have been completed. An ANOVA table was constructed for each linear model proposed and extended to include the lack of fit test to validate the chosen model. The F-ratio $MS_{regression}/MS_{residual}$ obtained for acetic acid was significant at the 95% level in the absence of any lack of fit. Calculation of the F-ratio $MS_{lack\ of\ fit}/MS_{pure\ error} = 1.14$ which is less than the 95% point $F(9, 99, 0.95)$ indicates that the applied linear model is appropriate. Similar conclusions were drawn for the remaining acids using both external and internal standard calibration procedures.

The linear model error terms were then checked for normality by inspection of normal probability

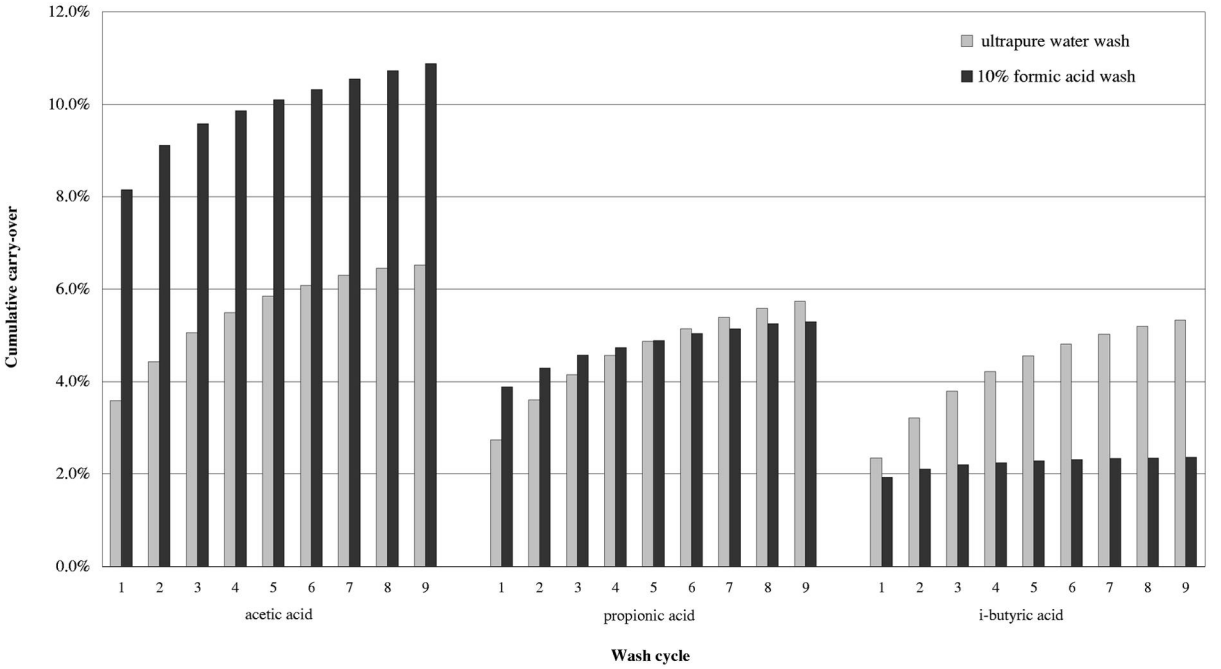

Fig. 2. Cumulative carry-over effect for acetic, propionic and isobutyric acids using two wash strategies. VFA standard containing 1000 mg/l acetic, propionic, isobutyric and *n*-butyric acid (not plotted) in 12% (w/v) NaCl using ultrapure water.

plots and by application of the Shapiro–Wilks test using SPSS 10.0. The Shapiro–Wilks statistic was not significant for each of the VFAs at all calibration levels. Probability plots of the calibration data sets displayed no major deviations from normality.

The assumption of constant variance across the calibration range for each acid was tested using the Levene statistic in SPSS 10.0. Significance values well below 0.05 for Levene tests based on means and medians for each VFA at all calibration levels suggest that the assumption of equal variances should be rejected. A plot of the residuals obtained by fitting a linear OLS calibration model against predicted response displayed a funnel shaped pattern for all six acids, using both ES and I.S. calibration. Such patterns are also reasonable indicators of increasing variability with increasing response (heteroscedasticity). In a situation of non-constant variance, an ordinary (unweighted) least squares model is inappropriate since all observations are treated equally and calibration levels with high variability can exert a strong influence on the least squares line-of-best-fit [28]. WLS regression models were subsequently fitted to the calibration data sets. As a first order approach to dealing with heteroscedastic data, weights were assigned as the inverse of the peak area count variance at each calibration level after Burdge et al. [29]. The calibration coefficients for both OLS and WLS regression models are displayed in Table 2. It is clear from Table 2, that the use of weighting in the estimation of the calibration curve has little effect on the gradient of the curve, but has a greater effect on the position of the intercept.

Currie [18] suggests use of the critical value for distinguishing a chemical signal from background noise, and use of the detection limit as a measure of the inherent detection capability of a method. The critical values and detection limits for the six VFAs based on externally calibrated data has been evaluated for OLS using confidence bands calculations presented by Burdge et al. [29]. For the WLS model approximations suggested by Oppenheimer et al. [28] have been employed so that the process of modelling of the variance function can be avoided, since the authors suggest this may indeed require

Table 2
Regression constant estimates and estimates of the detection capability parameters for ordinary and weighted least squares models

VFA	Regression	Estimated parameter						
		a	*b*	S_C	x_C	S_D	x_D	$x_D(3s_{bl})$
Acetic	OLS	−105.3	128.4	344.2	3.5	716.7	6.4	41.1
	WLS	21.9	120.6	102.2	0.7	427.6	3.7	39.2
Propionic	OLS	−73.8	456.6	1341.6	3.1	2711.3	6.1	37.7
	WLS	17.7	446.6	170.7	0.3	1474.5	3.3	36.0
Isobutyric	OLS	−174.4	1565.0	6085.6	4.0	12 189.1	7.9	36.1
	WLS	23.1	1540.0	348.1	0.2	1444.9	0.9	34.4
n-Butyric	OLS	−59.6	1007.9	3871.2	3.9	7802.0	7.8	38.6
	WLS	16.5	992.6	69.9	0.1	267.4	0.3	37.4
Isovaleric	OLS	−185.3	2374.3	9549.4	4.1	19 284.0	8.2	15.1
	WLS	13.9	2325.2	287.0	0.1	1585.7	0.7	14.5
n-Valeric	OLS	−90.3	1765.8	7326.1	4.2	14 742.4	8.4	35.5
	WLS	39.5	1736.7	74.9	0.0	470.3	0.3	33.4

OLS and WLS represent ordinary least squares and weighted least squares linear regression, respectively. Estimates of the regression coefficients are given by: *a* (the intercept estimate) and *b* (the gradient estimate). Estimates of the detection capabilities are given by: S_C the signal domain Critical Level, x_C the concentration domain Critical Level, S_D the signal domain Detection Limit, and x_D the concentration domain Detection Limit. The classical concentration domain Detection Limit is included for comparison $x_D(3s_{bl})$. Units in the concentration domain and signal domain are mg/l and peak area count, respectively.

more information than the calibration process itself. The estimated parameters are presented in Table 2 together with values obtained using the traditional method of three times the standard deviation of the blank measurement for the purpose of comparison. It is clear that the application of WLS regression procedures present lower values for the critical level and detection limit than those obtained using OLS for the heteroscedastic data. Weighted analysis uses a more appropriate estimate of the variability that reflects the situation at lower concentrations [28]. Table 2 also shows that for WLS treatment of the calibration data, the traditional $3-\sigma_{blank}$ values for the detection limits are considerably larger than those obtained using the regression approach for each VFA. Detection limits in the concentration domain using the WLS procedure were 3.7, 3.3, 0.9, 0.3, 0.7 and 0.3 mg/l for acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids, respectively. A typical chromatogram produced on analysis of the 250 mg/l VFA standard is displayed in Fig. 3a.

3.4. Analysis of artificial and real samples

The results of the analysis of artificial wastewater samples are displayed in Table 3 using both internal and external OLS and WLS calibration models for acetic acid. In both cases an attempt has been made to provide accurate 95% prediction intervals for the individual measurements using formulae suggested by Baumann [23]. The response variable values estimated by the individual regression models together with estimated 95% prediction intervals were plotted against concentration for each acid. Such plots can also be used to determine approximate 95% prediction intervals for estimated analyte concentrations [23]. The intervals produced for acetic acid using WLS are narrower than those predicted using OLS at the concentration analysed (250 mg/l) and provide a more realistic representation of the estimated quantity. Estimated concentrations for acetic acid using the internal standard calibration method appear to be higher and closer to the expected value

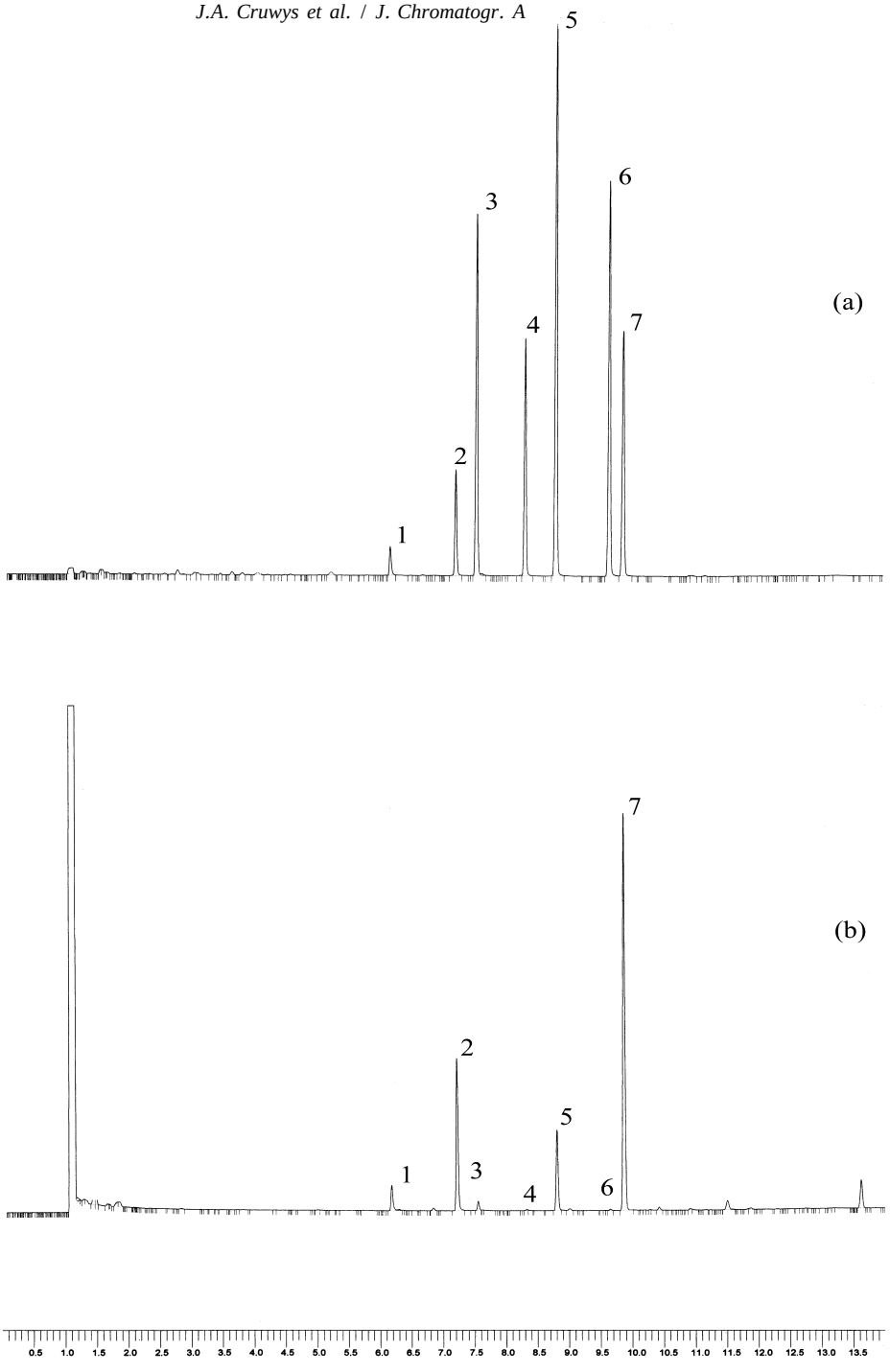

Fig. 3. HS-GC chromatogram of: (a) standard VFA 250 mg/l solution; (b) sample taken from an expanded granular sludge bed EGSB anaerobic reactor. Peak identification: (1) acetic acid; (2) propionic acid; (3) iso-butyric acid; (4) *n*-butyric acid; (5) isovaleric acid; (6) *n*-valeric acid and (7) 2-ethylbutyric acid (IS).

Table 3
Analysis of acetic acid in artificial wastewater matrices of varying BA using external and internal calibration methods with application of ordinary and weighted least squares linear regression models

WW matrix	Peak area y_0	External standard calibration method						Internal standard calibration method					
		OLS			WLS			OLS			WLS		
		x_0	x_U	x_L	x_0	x_U	x_L	x_0	x_U	x_L	x_0	x_U	x_L
0 mg/l BA	31 245.5	229.5	258.6	200.4	237.9	269.8	211.4	246.9	275.1	218.8	250.9	272.5	232.3
1000 mg/l BA	30 936.0	227.3	256.4	198.2	235.6	267.0	209.1	244.8	273.0	216.6	248.7	270.4	230.3
2000 mg/l BA	33 023.3	241.9	271.0	212.9	251.5	284.9	223.6	255.8	284.0	227.6	260.2	280.1	240.9
3000 mg/l BA	31 468.4	231.0	260.1	202.0	239.6	271.6	212.7	236.9	265.1	208.8	240.5	260.9	220.0
4000 mg/l BA	33 095.5	242.4	271.5	213.4	252.0	285.5	224.3	241.9	270.1	213.7	245.6	267.0	225.1
5000 mg/l BA	32 572.4	238.8	267.8	209.7	248.0	281.2	220.6	241.9	270.1	213.7	245.6	267.0	225.1

BA is the bicarbonate alkalinity (mg/l $CaCO_3$). OLS and WLS are the ordinary and weighted least squares models respectively. y_0 is the average of duplicate HS-GC analyses; x_o is the concentration predicted by the applied model; x_L and x_U are the lower and upper 95% prediction values for x_o. VFA standards (250 mg/l) were used throughout the study.

of 250 mg/l for each matrix investigated, than those provided by the external calibration procedure. Table 3, indicates that as anticipated, the effect of changing the sample matrix by increasing the amount of $NaHCO_3$ present was more pronounced when the external standard calibration method was applied to estimate VFA concentrations in comparison to the internal standard procedure. The internal standard calibration method was effective in accounting for a five-fold change in BA in the range commonly found in anaerobic digester samples.

The internal standard calibration method was applied to the analysis of ten replicate wastewater samples taken from an EGSB anaerobic reactor in a repeatability study. The residual standard deviation values for acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids were 5.26, 5.50, 3.91, 3.90, 5.48, and 6.47%, respectively. A typical chromatogram obtained during the analysis of EGSB reactor liquor is displayed in Fig. 3b.

4. Discussion

The analysis of VFAs in aqueous matrices is one of the oldest applications of gas chromatography, and still represents a considerable challenge for a number of reasons. Not least of these is the occurrence of sample carry-over that potentially is a significant source of measurement error both at the calibration stage and also during the quantitation of unknowns. Various sites within the chromatographic system can adsorb VFAs leading to potential difficulties with the repeatability of measurement results. In particular, adsorption at hot metallic surfaces (which include the GC injection port and in HS-GC the heated headspace sampling needle) can result in significant carry-over profiles being recorded during subsequent blank solvent runs. This phenomenon is not unique to HS-GC, and has also been reported in the analysis of VFAs using for example GC [30]. Solutions to the carry-over problem have been offered since the early days of VFA chromatographic analysis. Implementing a factorially designed experiment, Van Eeaeme et al. [21] investigated the carry-over effect and causal factors such as column packing, type of injector used, use of formic acid in the carrier gas and the type of compound used to indicate the effect. Acetic acid-free formic acid can however be difficult to purchase, and the acid has the potential to corrode stainless steel. Tangerman and Nagengast [2] have developed a direct injection method for faecal VFA analysis and report no signs of major column acid-damage after 10 years of use of a 10% formic acid solution. In the present paper, the efficacy of various wash cycle strategies used to reduce sample carry-over was investigated. On using a 1000 mg/l standard made up in 12% (w/v) NaCl, the cumulative percentage carry-over after three ultrapure washes and three 10% (w/v) formic acid washes were 5.1 and 9.6% for acetic acid; 4.2 and 4.6% for propionic acid; 3.8 and 2.2% for isobutyric acid and 4.4 and 3.9% for *n*-butyric acid, respectively. It is interesting that the efficiency of formic acid

as a wash agent rapidly decreases after acetic acid in the VFA series acetic acid, propionic acid and isobutyric acid, as shown in Fig. 2, whereas the efficiency of ultrapure water remains reasonably constant for each acid in comparison. A more comprehensive carry-over study, which is beyond the scope of this paper, would need to be conducted in order to validate any theories proposed with regard to differences in the behaviour of ultrapure water and formic acid wash strategies. The decline in elution efficacy together with the presence of impurity peaks in formic acid across the retention time window for VFAs, prevented its future use. Three ultrapure washes were included in the optimised method since the benefits of increasing to four proved to minimal with regard to cumulative elution.

Eq. (1) describes the relationship between the original analyte concentration (C_0) and the concentration of the analyte C_G in the headspace of a closed vial, for example, after liquid–vapour equilibrium has been achieved:

$$A \propto C_G = \frac{C_0}{K + \beta} \quad (1)$$

where K is the partition coefficient of the analyte and β is the phase ratio, i.e. the ratio of the volume of the analyte headspace vapour to the volume of the sample in the liquid phase at equilibrium. (Eq. (1) assumes that the volume of the sample phase at equilibrium equals the volume of the sample before the equilibration process is initiated). Ioffe [31] suggests that the largest K values may be the result of the formation of specific solvent–solute interactions, such as the formation of stable hydrogen bonds and establishment of acid–base equilibria. Both interactions can occur in the case of VFA–water solutions and have important implications in the analysis of VFAs particularly in the evaluation of K values as will be described in the following paragraph.

Kolb and Ettre [13] use Eq. (1) to describe the effects of temperature and sample volume (or β value) on the equilibrium headspace concentration C_G, and introduce two extreme cases in which either $K \gg \beta$ or $\beta \gg K$. In the case $K \gg \beta$, it follows that $(K + \beta) \rightarrow K$ and at constant initial analyte concentration, C_0, the headspace concentration is strongly influenced by $1/K$. Since K is temperature dependent, the temperature of equilibration has a large effect on the value of C_G which is proportional to the peak area count, A, of the analyte. This situation is clearly borne out for the six VFAs in this study on inspection of Table 1. If, on the other hand $\beta \gg K$ then $(K + \beta) \rightarrow \beta$, and C_G is influenced by $1/\beta$. The phase ratio, β, is not highly dependent on temperature and hence increasing equilibration temperatures have little effect on the analyte headspace concentration C_G and therefore its corresponding peak area count. This is not the case for VFAs. It can also be shown that at a constant temperature, changing the β value for compounds with a high partition coefficient value, K, has a minimal effect on C_G and hence on the peak area count. Indeed, as in the case of the extended volume trials, the difference in peak area counts for volumes in the range 0.1 to 5.0 ml will be difficult to distinguish from those obtained during repeatability trials of identical volumes. This situation may have important implications for VFA analysis if MHE is applied at the calibration and quantitation stages, or if MHE and also the phase ratio variation (PRV) method after Ettre et al. [32] are to be used for the determination of K values, since both MHE and the PRV method are rely on peak area differences obtained from analysis of samples with differing phase ratio values. Another procedure described by Kolb et al. [33] known as the vapour phase calibration (VPC) method, can be used for the determination of K values using HS-GC. The VPC method compares the response, A_C, obtained from analysis of a vapour calibration standard prepared using total vaporisation technique (TVT) volumes of pure analyte to the response, A_G, of a sample containing the same volume of analyte spiked into a known volume of blank sample matrix. For analytes with a high partition coefficient problems can also be encountered using the VPC method when determining K. In particular, it is possible that peak area values are hence at opposite extremes of measurement for the analyte, i.e. $A_G \ll A_C$ such that the value of A_G is outside the linear range of the method. From experiment, this appears to be the case for VFAs and therefore the K value were not obtainable using the VPC technique under optimum conditions.

As outlined in the previous paragraph, validation of the use of 2-ethylbutyric acid as an internal

standard by comparison of *accurate K* values for the I.S. and the VFAs in a given matrix, across the concentration range of the calibration procedure, can prove to be difficult using either VPC or PRV methods, particularly if K is large. If this is the case, as in this paper, alternative more empirical methods of validation should be sought. These methods can include variation of the ionic strength of the wastewater matrix, and subsequent application of I.S. calibration.

Calibration is defined by Danzer and Currie [34] as the operation that determines the functional relationship between measured values, and analytical quantities characterising types of analytes and their amount. In the case of linear regression and the method of least squares a number of conditions must hold if the model is to be applied correctly, and should be checked during method development. MacTaggart and Farwell [22] and Tranter [35] list the main assumptions. Chen et al. [14] employed an external standard calibration method for the analysis of the volatile components of swine wastewater using static HS-GC. This method of quantifying components such as VFAs requires accurate matrix matching of standards and samples particularly if severe matrix effects are expected. Such procedures are not always desirable or indeed feasible when complex matrices are to be analysed whose exact composition may not be known. Alternatively, the method of internal standardisation can be used. For headspace analysis in particular, the internal standard (IS) should be as chemically similar to the analyte(s) as possible. If components within the sample matrix are suspected or known to influence the headspace concentration of the analyte (the so-called matrix effect) then in theory both the analyte(s) and I.S. should encounter similar changes to their solvent–solute interactions within the sample. Drozd and Vodáková [16] derived an expression describing the use of an I.S. in HS-GC that differed from convention by the inclusion of a quotient shown enclosed in parenthesis in Eq. (2) below:

$$\frac{A_i}{A_s} = \frac{m_{io} f_s}{m_{so} f_i^m} \cdot \left(\frac{V_g + K_s V_l}{V_g + K_i V_l} \right) \quad (2)$$

where A_i and A_s are the peak area counts for the analyte and I.S. respectively; m_{io} and m_{so} are the masses of the analyte and I.S. originally in the sample; f_s and f_i^m are the mass response factors of the analyte and I.S.; K_s and K_i are the partition coefficients of the analyte and I.S.; V_g is the volume of the gaseous phase and V_l is the volume of the liquid phase. This added expression describes the effect of the matrix components on the concentration of analyte and I.S. in the headspace sample. In general the value of the quotient does not equal unity and hence should be multiplied by the gradient of the calibration curve obtained to allow for accurate quantitation. In the present paper the authors attempted to obtain values for the partition coefficients of the analytes, K_i and of the I.S., K_s using both the VPC and PRV methods. Since the other factors within the expression are constant for a given analysis, and the analytes and I.S. show a high degree of chemical similarity, it was envisaged that they will be affected in a similar manner by matrix components and hence share closely related K values and therefore the value of K_s/K_i would be constant across the concentration range investigated. The value of the quotient in Eq. (2) would then approach unity. Both the VPC and PRV methods rely on differences in peak area counts obtained from analysis of varying volumes of sample. As mentioned previously the β value and hence the volume of sample does not have a great influence on the headspace concentration, C_G, and hence on the peak area count, A. Values for the partition coefficient for VFAs using OLS linear regression as outlined by Kolb and Ettre [13] were difficult to obtain with the required degree of accuracy.

It is the intention of the authors to present a method that balances a vigorous calibration and quantification procedure with the ability to analyse a large quantity of wastewater samples, particularly from wastewater treatment systems, in a given period. If, following spiking experiments, severe sample matrix effects become apparent that cannot be accounted for by a method that uses an internal standard calibration, a standard addition procedure should be employed. Use of FET or TVT methods may be complicated by the difficulties in obtaining representative and repeatable microlitre aliquots from wastewater and wastewater treatment system samples, particularly when they have a high particulate content.

5. Conclusion

An optimised HS-GC method for the routine analysis of VFAs in wastewater matrices has been presented utilising 2-ethylbutyric acid as an internal standard. Addition of the acidic salt $NaHSO_4$ increased the analyte headspace concentration by reducing sample pH and through its salting out action. Optimal headspace conditions were observed when equilibrating at 85 °C for 30 min, using a 2.0 ml sample volume with the addition of 1.0 ml of $NaHSO_4$ (62%, w/v) into standard 22.3 ml vials. Internal standard and salt introduction methods have been optimised and the problem of sample carry-over has been accounted for, thus allowing a high degree of repeatability of measurement to be obtained. Analysis of real wastewater samples yielded residual standard deviation values of 5.26, 5.50, 3.91, 3.90, 5.48, and 6.47% for acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids, respectively. The procedure has been designed to provide a balance between high sample throughput and reliability of the quantitation process, by employing internal standard calibration with WLS regression, over a wide (0–1000 mg/l) and linear concentration range. The calibration method is able to account for changes in the sample matrix commonly encountered in wastewater treatment processes, and does not involve the complication of a third phase as in the case of SPME methods. Use of WLS on heteroscedastic calibration data provides lower VFA detection limits than those obtained using OLS, which are comparable to those reported in SPME–GC–FID methods, for example 3.7, 3.3, 0.9, 0.3, 0.7 and 0.3 mg/l for acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids, respectively.

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