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Development and Validation of Two Methods to Quantify Volatile Acids (C2-C6) by GC/FID: Headspace (Automatic and Manual) and Liquid-Liquid Extraction (LLE)

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Abstract

The concentration of the volatile fatty acids (VFA) is an important indicator of the status of anaerobic processes, but most of the existing methods require sample pretreatment and are labor-intensive. It was developed and validated a rapid Gas Chromatographic (GC) method to quantify seven VFA (acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic), acetone, methanol, ethanol and n-butanol by headspace (automatic and manual) and liquid-liquid extraction (LLE) with diethyl ether (only VFA). The determination was made in a Shimadzu Gas Chromatograph equipped with a Flame Ionization Detector (GC/FID), a headspace auto-sampler and an HP-INNOWAX column. Isobutanol and crotonic acid were utilized as internal standards (IS). The validation parameters evaluated were: precision (coefficient of variation—*C.V.*% for the retention times, from 0.02 to 0.87), linearity (R^2 = 0.9291 - 0.9997), limits of detection (from 3.97 to 36.45 $mg \cdot L^{-1}$) and instrumental precision (from 0.01 to 0.53), which provide evidence that the methods are adequate to determine these analytes in samples from anaerobic reactors and from the environment.

Keywords

Short Chain Organic Acids, Gas Chromatography, Ethyl Ether, Anaerobic Processes

1. Introduction

The factors that affect the performance of anaerobic reactors are related to the production of the so called vola-

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tile fatty acids (VFA): acetic, propionic, isobutyric, butyric, isovaleric and valeric acids, among others. The concentration of the VFA gives fast information of the status of the anaerobic processes and is also an important indicator for monitoring them compared to others such as alkalinity, chemical oxygen demand (COD) and pH [1].

Some of the most common sources of carbon used by anaerobic microorganisms are acetic acid, methanol and ethanol. Therefore, in the anaerobic processes of wastewater treatment it is important to monitor the levels of such substances as well as others by-products generated, acetone and n-butanol, in order to evaluate the status of the system.

Several studies have pointed the importance of the concentration of the individual VFA as an early warning indicator for process failure [2]-[5]. These include the concentration of isobutyric and isovaleric acids [2]-[4]. According to Ahring *et al.* [5] the accumulation of VFA suggests an imbalance in the process.

There are lots of methods described in the literature, like those proposed for offline individual VFA measurement, that involve sample filtration or centrifugation followed by direct injection into the gas chromatograph [6]; solvent extraction followed by GC for samples from leachate [7] or high performance liquid chromatography (HPLC) of acids from marine sediments after derivatization with 2-nitrophenylhydrazides [8] and solid phase micro extraction (SPME) directly from the aqueous phase, followed by GC/mass spectrometry [9]; determination by GC/FID of its methyl esters after derivatization [10], among others. Nevertheless, these methods are time consuming because most of them require sample preparation before the GC determination or can damage the chromatographic column by injecting directly very acidic solutions. Besides being a simple method, the headspace determination is a technique that preserves the life of the chromatographic column.

Liquid-liquid extraction (LLE) is a highly sensitive and selective technique that also contributes to increasing the useful life of the chromatographic column and is important as an alternative when it is necessary to determine only the organic acids.

This study aimed to develop and validate chromatographic methods to determine some VFA (acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic) by LLE using diethyl ether as the extracting solvent due to the compatibility of the polarities of the acids with the extractant; and the same acids simultaneously with acetone, methanol, ethanol and n-butanol by headspace (automatic and manual) in environmental samples as well as in samples from anaerobic reactors using the internal standard (IS) method for quantification. The proposed methods are based on GC/FID with an HP-INNOWAX capillary column, using an automatic headspace sampler or by manual headspace and, alternatively, by LLE with ethyl ether as the solvent.

It was added an inorganic salt to the samples to promote the salting out effect in both methods. It is a useful tool that has been widely used in determinations involving phase separations. In order to decrease the solubility of the substances in the aqueous phase and increase their transition to the vapor phase an inorganic salt as potassium carbonate (K_2CO_3) or sodium chloride (NaCl), for example, is added to the aqueous solution [11]. The behavior of the phase equilibrium changes significantly with the presence of a dissolved salt and it improves the efficiency of the process of extracting [12].

This paper describes an investigation of the following validation parameters for both the methods described: linearity, detection and quantification limits, precision of the method and instrumental precision, according to Ribani *et al.* [13], Miller & Miller [14], Duarte *et al.* [15] and Damasceno *et al.* [16] recommendations.

2. Materials and Method

2.1. Chemicals and Reagents

All the chemicals used during the experiments were of analytical reagent grade (>98%): acetone, methanol, ethanol, n-butanol, isobutanol and the VFA (acetic, propionic, isobutyric, butyric, isovaleric, valeric, crotonicand caproic—**Table 1**), diethyl ether, sodium chloride (NaCl), potassium carbonate (K_2CO_3), sulfuric acid (H_2SO_4) and sodium hydroxide (NaOH). It was used AccuBOND II SAX Cartridges, Agilent Technologies and cellulose microcrystalline Merck.

2.2. Equipment and Chromatographic Conditions

Chromatographic analyses were performed with an HP-INNOWAX (30 m × 0.25 mm × 0.25 μm) capillary column (Agilent Technologies), in a Shimadzu GC-2010 (Kyoto, Japan) gas chromatograph equipped with FID,

Table 1. Description of the substances studied—molecular formula and boiling point (B.P.)*.

Substance	Molecular formula	B.P. (˚C)
Acetic acid (1)	CH_2COOH	118.0
Propionic acid (2)	CH_2CH_2COOH	141.0
Isobutyric acid (3)	$(CH_3)_2CHCOOH$	155.0
Butyric acid (4)	$CH_3CH_2CH_2COOH$	163.5
Isovaleric acid (5)	$(CH_3)_2CHCH_2COOH$	177.0
Valeric acid (6)	$CH_3CH_2CH_2CH_2COOH$	96.0
Crotonic (IS)	$CH_3CH=CHCOOH$	93.0
Caproic acid (7)	$CH_3CH_2CH_2CH_2CH_2COOH$	205.0
Acetone (8)	CH_3COCH_3	56.5
Methanol (9)	CH_3OH	64.7
Ethanol (10)	CH_3CH_2OH	78.5
n-butanol (11)	$CH_3(CH_2)_2CH_2OH$	118.0
Isobutanol (IS)	$(CH_3)_2CHCH_2OH$	108.0

*The Merck Index [17].

a COMBI-PAL auto sampler using 10 mL headspace vials and a 2.5 mL HD type Hamilton gas-tight syringe. For the manual headspace method it was used a Fisatom hot plate and a SGE gas tight, with luer-lock—1000 µL syringe. For the LLE it was employed flasks type COD-10 mL (**Figure 1**), a Phoenix vortex stirrer, an Eppendorf centrifuge 5702 and the freezer of a common refrigerator; the syringe used for the injection was an Agilent 10 µL manual syringe for liquids. GC solution software was used for data treatment. After testing different possibilities, the chromatographic conditions to achieve better separation and peak resolution for the headspace method were: 35˚C (0 min) 2˚C/min 38˚C (0 min) 10˚C/min 75˚C (0 min) 35˚C/min 120˚C (1 min) 10˚C/min 170˚C (2 min), with total run time of 14.49 minutes (min). **OBS:** when there were many organic compounds in the sample it was necessary to increase the final temperature of the heating ramp up to 230˚C for 4 min (40˚C/min) to clean the column before the next injection. The column flow was 1.5 $mL \cdot min^{-1}$ with ultra-pure hydrogen (H_2) as the carrier gas. The detector used H_2 at 30 $mL \cdot min^{-1}$ with synthetic air at 300 $mL \cdot min^{-1}$ and nitrogen (N_2) at 30 $mL \cdot min^{-1}$ flows as flame and make-up gases, respectively. The temperatures of the detector and injector were held at 280˚C and 250˚C, respectively.

After testing different temperatures for heating time and temperature of the block for the COMBI-PAL auto sampler and different volumes for the injection of the sample, the best conditions achieved were: 13 min of heating of the block at 100˚C, injection of 400 µL of sample with the syringe at 100˚C and 3 minutes of washing the syringe with N_2 after each injection.

For the LLE method the better chromatographic conditions were:

Detector temperature: 300˚C; make-up: N_2 at 30 $mL \cdot min^{-1}$; delay: 2.5 min;

- Injected volume: 1.0 µL;
- Injector temperature: 250˚C; split: 10;
- Carrier gas (H_2): 3.2 $mL \cdot min^{-1}$;
- Oven temperature: 100˚C (1 min) 8˚C/min 150˚C (1 min) 35˚C/min 200˚C (1 min); 9 min of chromatographic run.

2.3. Sample Preparation

The preservation of the samples from anaerobic reactors was tested in three ways, after centrifugation: without adding any substance, with addition of 3% H_3PO_4 and with addition of 1 M NaOH, before to refrigerate or freeze the samples for 60 days prior to the analyses.

Because of the inability to analyze the samples from leachate directly due to saturation of the detector by the large amount of organic compounds present in the samples, the three methods tested for cleanup were: a) acidification with 2 M H_2SO_4 followed by filtration with membrane of 1.2 µm and then freezing and thawing; centrifugation again, filtration (0.45 µm) and freezing; b) cartridge SAX eluted with 2 M H_2SO_4; c) agitation of the sample in vortex with 2 M H_2SO_4 and microcrystalline cellulose, followed by centrifugation.

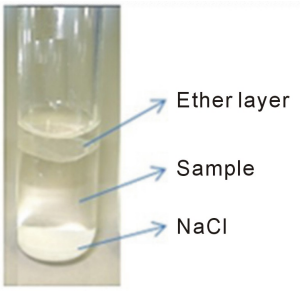

Figure 1. Flask with sample, NaCl and ethyl ether.

The influence of the salt added to the sample for the salting out effect was tested by adding 1 g of K_2CO_3 or NaCl.

2.3.1. Automatic Headspace

An aliquot of 2 mL of the sample (or standard solution) was placed in a 10 mL standard vial for automatic headspace analysis, fitted with Teflon tape and Teflon-faced blue septa, containing approximately 1 g of NaCl, as follows:

- 1 g NaCl;
- 2 mL sample;
- 70 µL isobutanol solution 1 $g \cdot L^{-1}$ (IS for acetone and alcohols);
- 100 µL crotonic acid solution 700 $mg \cdot L^{-1}$ (IS for the VFA);
- 200 µL 2 M H_2SO_4 solution.

2.3.2. Manual Headspace

It was the same preparation described in 2.3.1 and the flask with the sample was heated inside a Becker with boiling water on a hot plate for 13 min, before the injection of 400 µL of the headspace into the GC. The syringe must be kept at 90˚C before the injection to prevent condensation of the sample.

2.3.3. Liquid-Liquid Extraction (LLE)

An aliquot of 2 mL of the sample (or standard solution) was placed in a 10 mL COD type flask with Teflon tape, containing approximately 1 g of NaCl (**Figure 1**), as follows:

- 1 g NaCl;
- 2 mL sample;
- 100 µL crotonic acid solution 700 $mg \cdot L^{-1}$ (IS);
- 200 µL 2 M H_2SO_4 solution;
- 0.6 mL ethyl ether (previously stored in freezer);
- vortexing/1min, centrifuge at 2000 rpm/1min;
- freezer for at least 30 min before injection.

OBS: the syringe for the manual injection of the ether layer must stay in the freezer for, at least, 30 min before the injection because of the high volatility of the solvent.

2.4. Calibration Curves

The solutions for the calibration curves were prepared from stock solutions of each one of the substances at a concentration of 20 $g \cdot L^{-1}$ in ultra-purified water and in the synthetic medium [18] commonly used to feed the anaerobic reactors (**Table 2**). Due to the low solubility of the VFA in water, it was necessary to add sodium hydroxide to the stock solutions to achieve its complete dissolution. These solutions were used for both methods described. From the stock solutions it was prepared the so called "mother solution" (MS), containing all the analytes to be determined in a concentration of approximately 1.0 $g \cdot L^{-1}$. The concentrations of the calibration levels for all the substances to elaborate the calibration curves for the headspace method ranged from 1.00 to 800.00 $mg \cdot L^{-1}$ (11 levels) and, for the LLE, the concentrations ranged from 2.5 to 600.0 $mg \cdot L^{-1}$ (9 levels). The

Table 2. Composition of the synthetic substrate commonly used to feed anaerobic reactors [18].

Substance	Concentration ($g \cdot L^{-1}$)
Sucrose	4.000
Urea	0.080
Peptone	1.000
p-aminobenzoic acid	0.0004
Biotine	0.010
$NiSO_4 \cdot 6H_2O$	0.500
$FeSO_4 \cdot 7H_2O$	2.500
$FeCl_3 \cdot 6H_2O$	0.250
$CoCl_2 \cdot 2H_2O$	0.040
$CaCl_2 \cdot 6H_2O$	2.060
Se_2O	0.140
KH_2PO_4	0.0054
K_2HPO_4	0.0013
$Na_2HPO_4 \cdot 2H_2O$	0.0028

dilution was made with ultrapure water and also with a synthetic medium similar to the matrix of the samples from anaerobic reactors as the solvent using "Equation (1)" to obtain the desired concentration, in triplicate.

$$C_1 * V_1 = C_2 * V_2 \quad (1)$$

where C_1 is the concentration of the MS, V_1 is the volume of the MS, C_2 is the concentration of each level of the curves and V_2 is the final volume of each prepared solution.

2.5. Validation

The quantification of the analytes was made by the internal standard method, with the comparison of the response factor—*RF*, "Equation (2)" of each substance with the calibration curve under the same conditions.

$$RF = A_i / A_{IS} \quad (2)$$

where A_i is the peak area of each substance and A_{IS} is the peak area of the internal standard considered.

Linearity, precision of the method (*C.V.*% of the retention times) and limits of detection and quantification (*LD* and *LQ*, respectively) were assessed based on Ribani *et al.* [13], Duarte *et al.* [15] and Damasceno *et al.* [16] recommendations. Three replicates of each standard level were used to build the analytical curves, with the aid of the GC Solution software. The precision of the method was investigated through the *C.V.*% of the retention times (*RT*) of the replicates of the standard solution at the different concentrations, using "Equation (3)". The instrumental precision was evaluated by 10 successive injections of a unique standard solution with all the analytes at 50 $mg \cdot L^{-1}$. Finally, *LD* and *LQ* were determined from the calibration curves with the "Equations (4)" and "(5)", suggested by Ribani *et al.* [13].

$$C.V.\% = (SD/m) * 100 \quad (3)$$

where *C.V.*% is the coefficient of variation, *SD* is the standard deviation of *RT* and *m* is the average of RT of the analyte.

$$LD = 3.3 * (S/s) \quad (4)$$

$$LQ = 10 * (S/s) \quad (5)$$

where *S* is the standard deviation of the intercept and *s* is the slope of the calibration curve.

3. Results and Discussion

The smaller differences between the standard deviation (from 0.1 to 34 $mg \cdot L^{-1}$) of the responses were observed for the samples centrifuged and analyzed without addition of any preservative and frozen or refrigerated (for this

last option, in flasks without headspace), after 60 days.

The better cleanup for samples from landfill leachate was extraction from SAX cartridge with 2 M H_2SO_4 (*C.V.*% from 1.0 to 20.8 for the concentrations obtained).

Both of the salts tested for the salting out effect: K_2CO_3 and NaCl were appropriate to transfer the substances to the vapor phase. However, the former also promoted an increase of the pH, which is not suitable for this kind of determination, once the pH for the VFA determination must be ≤2.0. So, it was established the NaCl as the substance to promote the salting out effect.

3.1. Headspace

A typical chromatogram of the headspace analysis (that is similar for manual and automatic ways) is in **Figure 2**, **Table 3** (peaks between 6.0 and 7.0 min may be related with some substances present in previous injections, probably retained due to the high polarity of this column). In **Figure 3** (**Table 4**) there is a chromatogram of a standard solution of the acids (100 $mg \cdot L^{-1}$) by GC/FID after extraction with ethyl ether. The chromatograms exhibit good separation with well-resolved peaks for the analytes in a short time.

3.2. Calibration Curves

No considerable changes have been observed in the equations obtained from the standards diluted with water and with synthetic medium for both methods (headspace and ethyl ether extraction), which indicates that these methods were not affected by this kind of changes in the matrix of the sample.

3.3. Validation

The methods studied were linear at the considered range of concentrations (1.0 to 800.0 $mg \cdot L^{-1}$). The coefficient of correlation (R^2) for the headspace analysis of the standards diluted in synthetic medium ranged from 0.9291 (for caproic acid, which has the higher boiling point among the considered acids and, therefore, the lower volatility—**Table 1**) to 0.9997 (automatic, **Table 5**) and from 0.9614 to 0.9997 for the manual technique. It was observed the carry-over effect mainly for the automatic headspace, which is avoided with the introduction of "whites" every 3 samples (containing only water and sulfuric acid). The best results were obtained for the correlation coefficients by the method of extraction with ethylic ether (from 0.9951 to 0.9997, **Table 6**). These results show the higher extraction efficiency of this method; however, it does not allow the determination of acetone and alcohols, due to the fact that the retention times are identical to the ethylic ether, besides being more labor-intensive.

The *LD* and *LQ* for the headspace (from 5.8 to 28.6 $mg \cdot L^{-1}$) and for the LLE (from 3.97 to 36.45 $mg \cdot L^{-1}$) demonstrate the reliability of these methods.

The *C.V.*% of the retention times were very low (from 0.02 to 0.87) for the headspace and from 0.07 to 0.34 for the LLE methods, respectively; and from 0.01 to 0.53 for the 10 successive injections of the 50 $mg \cdot L^{-1}$ standard by all the techniques. These values reflect the good precision of the method and the instrumental precision, respectively.

The *LD* and *LQ* for the headspace (from 5.8 to 28.6 $mg \cdot L^{-1}$) and for the LLE (from 3.97 to 36.45 $mg \cdot L^{-1}$) demonstrate the reliability of these methods.

The tabulated values for the Ftest (ANOVA) are 3.0204 and 3.5004 for the headspace and the LLE methods, respectively and the calculated values are in **Table 7**. These values ($F_{calculated} > F_{tabulated}$) are further evidence of the linearity of the methods studied [19].

4. Conclusion

The gas chromatographic determination (GC/FID) by headspace (automatic and manual) described is a useful and rapid analytical method for monitoring eleven volatile substances: acetone, some alcohols and VFA in samples from anaerobic processes and from the environment. The method described for LLE with diethyl ether is an efficient analytical procedure for the determination of VFA. The validation parameters analyzed for the methods developed indicated good linearity, precision and both low detection and quantification limits, allowing its application to monitor the status of wastewater anaerobic treatment systems in a short time of analysis.

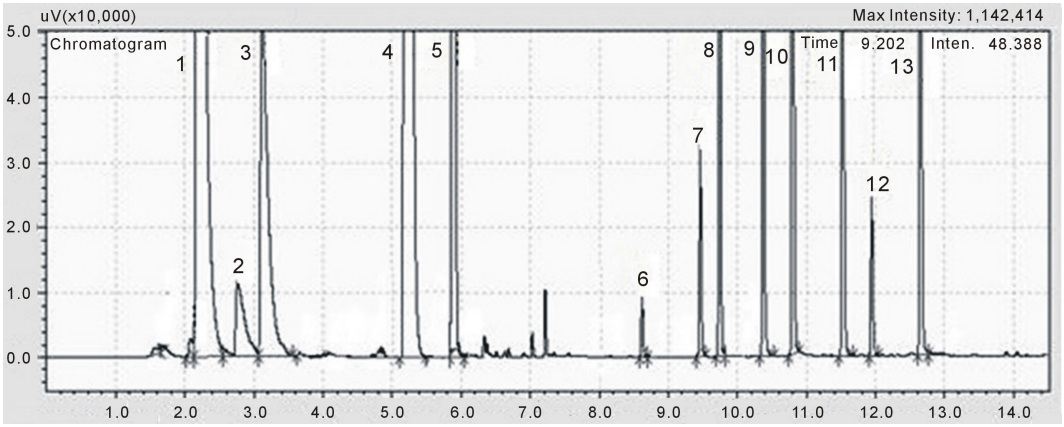

Figure 2. Chromatogram by automatic headspace analysis—GC/FID (100 mg·L^{-1}, **Table 3**).

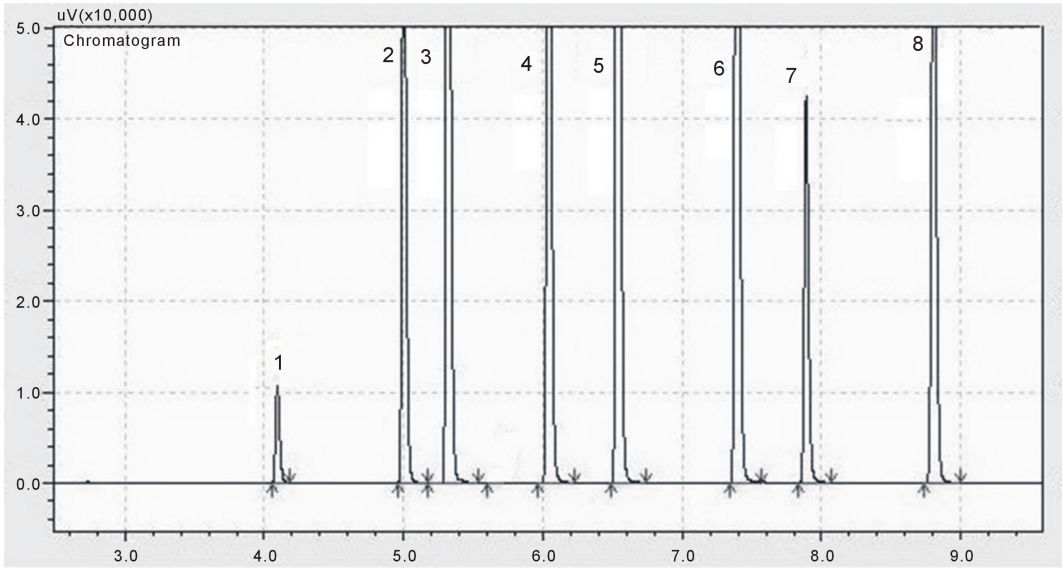

Figure 3. Chromatogram from GC/FID after extraction of the acids with ethyl ether (100 mg·L^{-1}, **Table 4**).

Table 3. Retention times of the substances by automatic headspace (GC/FID).

Number	Substance	Retention time (min)
1	Acetone	2.18
2	Methanol	2.76
3	Ethanol	3.12
4	Isobutanol	5.23
5	n-butanol	5.88
6	Acetic acid	8.62
7	Propionic acid	9.46
8	Isobutyric acid	9.74
9	Butyric acid	10.37
10	Isovaleric acid	10.80
11	Valeric acid	11.52
12	Crotonic acid	11.94
13	Caproic acid	12.65

Table 4. Retention times of the substances by LLE—ethyl ether (GC/FID).

Number	Substance	Retention time (min)
1	Acetic acid	4.09
2	Propionic acid	5.00
3	Isobutyric acid	5.32
4	Butyric acid	6.04
5	Isovaleric acid	6.54
6	Valeric acid	7.39
7	Crotonic acid	7.89
8	Caproic acid	8.81

Table 5. Linear regression equations from the analytical curves of the standards (diluted in synthetic medium/automatic headspace method).

Substance	Linear regression equations	R^2
Acetone	y = 0.0148x – 0.1280	0.9975
Methanol	y = 0.0004x – 0.0121	0.9832
Ethanol	y = 0.0038x – 0.0236	0.9897
n-butanol	y = 0.0188x + 0.0103	0.9953
Acetic acid	y = 0.0234x + 0.0659	0.9941
Propionic acid	y = 0.0231x + 0.4244	0.9984
Isobutyric acid	y = 0.0739x + 2.9494	0.9710
Butyric acid	y = 0.0545x + 1.9410	0.9902
Isovaleric acid	y = 0.1285x + 5.6741	0.9516
Valeric acid	y = 0.1085x + 3.9121	0.9681
Caproic acid	y = 0.0841x + 3.6989	0.9291

Table 6. Linear regression equations from the analytical curves of the standards (diluted in synthetic medium/LLE method).

Substance	Linear regression equations	R^2
Acetic acid	y = 0.0024x – 0.0118	0.9967
Propionic acid	y = 0.0154x – 0.0593	0.9977
Isobutyric acid	y = 0.0339x – 0.0757	0.9974
Butyric acid	y = 0.0330x – 0.0848	0.9983
Isovaleric acid	y = 0.0429x – 0.1167	0.9991
Valeric acid	y = 0.0430x – 0.1225	0.9997
Caproic acid	y = 0.0233x – 0.0874	0.9951

Table 7. Values of Fcalculated for automatic headspace and LLE methods.

Substance	F (headspace)	F (LLE)
Acetone	787.66	-
Methanol	584.41	-
Ethanol	964.38	-
n-butanol	2094.74	-
Acetic acid	1680.81	2418.93
Propionic acid	6397.21	3494.73
Isobutyric acid	336.00	3034.34
Butyric acid	1009.06	4814.36
Isovaleric acid	197.55	9137.69
Valeric acid	304.00	26924.05
Caproic acid	132.08	1630.68

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