MELiSSA

TECHNICAL NOTE

Departament d'Enginyeria Química
Escola Tècnica Superior d'Enginyeries
Universitat Autònoma de Barcelona

TECHNICAL NOTE 62.12

Instrumentation for on-line determination of VFA

Prepared by/Préparé par Núria Martínez, Julio Pérez, Joan Albiol and Francesc Gòdia
Reference/Réference ESTEC/CONTRACT13292/98/NL/MV
Issue/Edition 1
Revision/Révision 0
Date of issue/Date d'édition March 2007
Status/Statut Final

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

TECHNICAL NOTE

APPROVAL

Title *Titre*	**Instrumentation for on-line determination of VFA**	Issue *Edition*	1	Revision *Révision*	2

Author *Auteur*	Núria Martínez, Julio Pérez, Joan Albiol and Francesc Gòdia	Date *Date*	March 2007

Approved by *Approuvé par*	Brigitte Lamaze	Date *Date*	March 2007

CHANGE LOG

Issue/*Edition*	Revision/*Révision*	Status/*Statut*	Date/*Date*

Distribution List

Name/*Nom*	Company/*Société*	Quantity/*Quantité*

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

TABLE OF CONTENT

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

1. Introduction

The aim of compartment II of the MELISSA Pilot Plant is to consume volatile fatty acids generated in the first compartment. For a reliable implementation of compartment II and optimisation of the whole loop operation by the control software, it is necessary to have information on the type and concentration of volatile fatty acids (VFA) produced in the first compartment and its level of consumption by compartment II.

In the first part of this workpackage (Camargo *et al.*, 2005) different techniques have been considered for the analysis of VFA in compartment II. According to the requirements needed for this application, the GC technique coupled to a FID detector was chosen. A comparison between different equipment for GC was done and as a result and due to the harmonisation required with other MELISSA partners the Shimadzu gas chromatograph was the final proposal submitted to ESA for approval.

A Shimadzu GC 2010 (see next section "2 Description of the equipment purchased") was purchased and installed. A method for analysing VFA has been identified. Calibration with standards has been performed and liquid samples from compartment II have been analysed using the method described in this technical note.

Due to the final application of this method, which is the on-line monitoring of VFA in both liquid phases from compartments II and I, a first on-line test have been ran with standard samples.

It has been also considered the possibility to analyse VFA on the gas phase of compartments II and I simultaneously to the liquid phase, due to VFA are present in both liquid and gas phases. An offer to purchase the hardware needed for this possible future application is also presented in this technical note.

2. Description of the purchased equipment

The purchased equipment was a Shimadzu GC-2010 (Camargo *et al.*, 2005). The main characteristics of the equipment are the following:

GC Model: Shimadzu GC-2010AF
Detector: FID
Injector port: split/Splitless
Autoinjector: PALGC1, PALMR-S2010, PALCycComp
On-line valves system: P/AOC 5000
SW: GC solution (version 2)
Control SW AOC5000
Injection mode: Split/Splitless

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

A picture of GC equipment is in figure 1 and an example of the PC interface of the "GC Real Time Analysis" software which is included in GC-Solution software (v.2) is shown in figure 2.

Figure 1 General GC equipment installed at the MELISSA Pilot Plant, UAB.

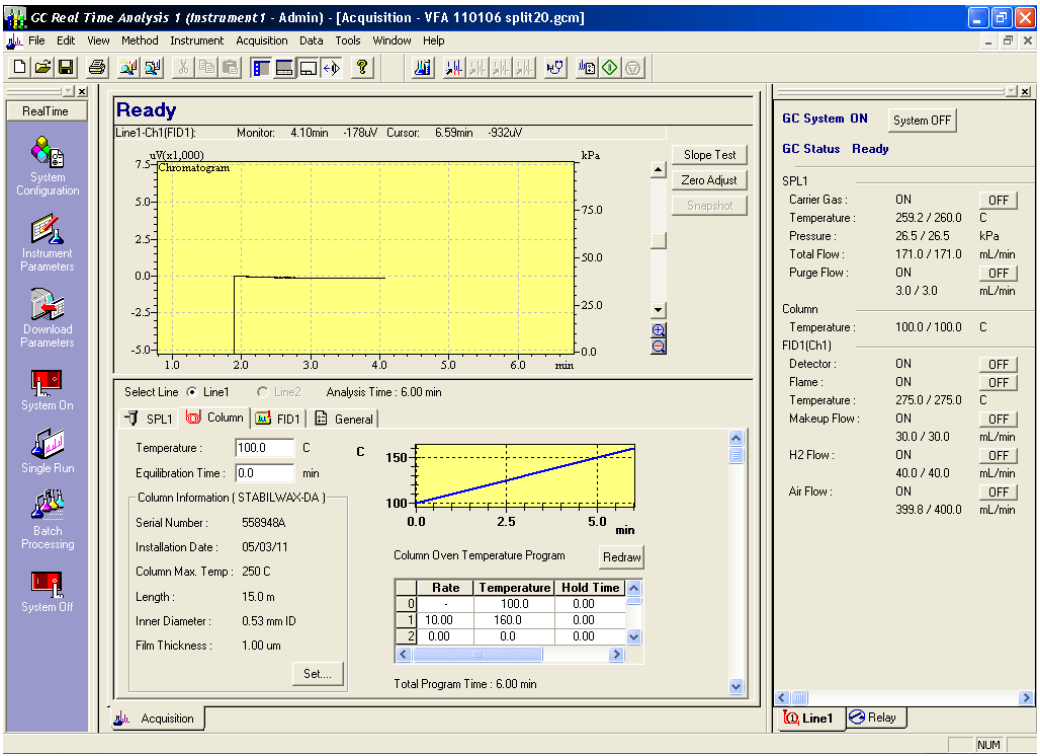

Figure 2 Example of the PC interface of the "GC Real Time Analysis" software. This software is used to introduce the method, start the analysis using batch sequences, if necessary, and follow the GC status and the chromatogram during analysis.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

3. Calibration phase

3.1. Materials and reagents

3.1.1.Gas chromatography column

The column used for these experiments was a fused silica STABILWAX-DA semi-capillary column from Restek. The dimensions of this column are 15m x 0.53mm x 1µm, corresponding to the length, inner diameter and thickness of the stationary phase film, respectively. The stationary phase is bonded PEG that has been specifically deactivated for acidic compounds.

3.1.2.Standards

We expect an amount around 5g/L total VFA, 80% of the total VFA being acetic acid. If we estimate the average molar molecular weight around 65g/Mol for an average formula with 2.3 Carbon atoms, then the corresponding Carbon concentration in Compartment II liquid input will be around 2gC/l.

The range of VFA concentration used for the calibration was from 0.01gC/L to 2gC/L, approximately, of each VFA. Two stock standard solutions of a mixture of VFA at concentrations of 0.5g/L and 10g/L of each compound were prepared in MilliQ water. Final solutions of the following concentrations: 0.01, 0.025, 0.075, 0.1, 0.2 and 0.4, 0.5, 1, 2, 3, 4g/L of each compound were also prepared with MilliQ water from stock solutions of 0.5g/L and 10g/L, respectively.

GC is capable of separating each VFA and analyse them one by one, regardless the number of different VFA that the sample contain or whether they are at the same concentration or not. Under this basic, standard samples are prepared with a mixture of VFA. Each standard sample could have been prepared with one VFA, but the number of components per sample does not affect the analysis.

All solutions were filtered with 0.22µm pore filters (Millipore).

Glacial acetic acid was obtained from Panreac Química. propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid were obtained from Merck analytical grade. In order to maintain clean the syringe methanol and acetone from Panreac Química analytical grade were used.

3.2. Method for analysing VFA and standardisation

3.2.1.Gas chromatography method

A chromatographic method was identified for the analysis of VFA. Helium was used as a carrier gas (6bar), and N_2 as make up gas was (2bar). H_2 (3bar) and Air (3bar) were used in order to get ignition from the Flame Ionisation Detector (FID). Two different split ratios, 10 and 40, were used depending on the concentration range of VFA. The parameters of this method are the following:

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Injector temperature:	220ºC
Column temperature program:	from 100ºC to 160ºC at a rate of 10ºC/min
Total flow:	171.0mL/min
Pressure:	26.5kPa
Column flow:	8.00mL/min
Injection size:	1µL
Injection mode:	split
Liner:	Deactivated with silica wool plug
Detector temperature:	275ºC

3.2.2.Calibration method

External standard method was chosen for calibration, using individual calibration curves done for each VFA. Calibration curves of standards and analysis of samples must be performed under identical conditions (Novák, 1988). No need to add an internal standard compound to samples is required for this method.

3.2.3.Autoinjection method

The autoinjection method was optimised for a better sampling performance and cleaning of syringe. When aqueous samples are injected a specific cleaning program has to be designed in order to maintain the syringe in good performing conditions. The final refined autoinjector method parameters are presented in table 1. Two wash stations are available; therefore two different solvents can be used. Mainly, post-injection clean is done 5 times with methanol followed by 5 times more with acetone. Pre-injection clean is solely done with the most volatile solvent: acetone.

The method had to be also optimised with the aim of avoiding bubbles in the syringe when sampling. For that reason the parameters “Filling speed”, “Filling Strokes”, “Pull-up Delay”, “Injection Speed”, “Pre-injection Delay” and “Post-injection Delay” were modified as required for optimisation.

Table 1 Autoinjector Method Macro Sequence

Autoinjector parameter	#
Air volume	1
Pre Clean with Solvent 1 (methanol)	0
Pre Clean with Solvent 2 (acetone)	2
Pre Clean with Sample	2
Filing Volume (µL)	3
Filling Speed (µL/s)	10
Filling Strokes	5
Pullup Delay (ms)	300
Injection Speed (µL/s)	100
Pre inject Delay (ms)	0
Post inject Delay (ms)	0
Post Clean with Solvent 1 (methanol)	5
Post Clean with Solvent 2 (acetone)	5

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

3.3. Results

3.3.1.Chromatography

Time of analysis is an important parameter for a control tool; hence retention time of VFA was decreased in order to achieve a shorter time of chromatographic analysis per sample.

The use of a short length column (15m) allowed to elute all VFA in few minutes and still good resolution of peaks was maintained. As it can be observed in Figure 2, all VFA are eluted separately in 6min.

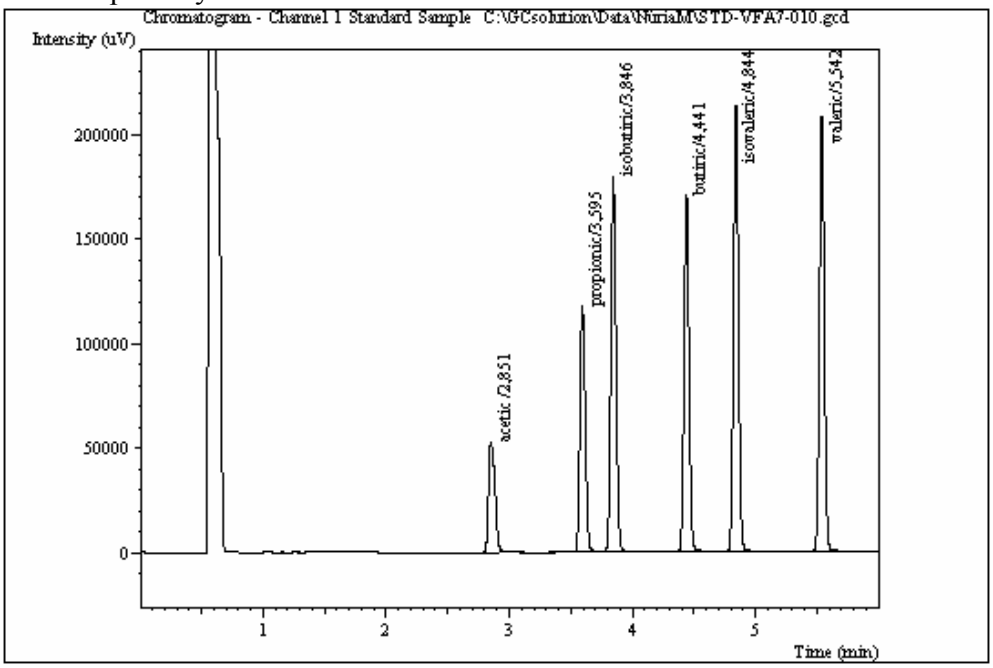

Figure 2 VFA Chromatogram

3.3.2.Linearity

Calibration curves were obtained by analysing all standard samples, which were analysed in triplicates. Due to the range of VFA to be analysed is quite broad, we can distinguish two different optimal conditions depending on the concentration range of VFA. For concentration ranges 0.025g/L-0.4g/L (25, 75, 100, 200 and 400 mg/L) and 0.1g/L-4g/L (0.1, 0.5, 1, 2 and 4g/L) split 10 and split 40 were used respectively. Correlation factors (R) were calculated for each compound at each split ratio (table 2). Good results of linearity ($R \geq 0.999$) for all VFA resulted from these calibration tests.

For the future application split ratio can be easily changed on the PC interface or remotely by the control software. However, an external standard calibration is required for each split ratio.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 2 Results of linearity and reproducibility

Compound	Split 10			Split 40		
	Conc. Range of compound (mg/L)	R	RSD* (%) n=3	Conc. Range of compound (mg/L)	R	RSD* (%) n=3
Acetic acid	25-400	0.9996	6.43	100-4000	0.9996	3.43
Propionic acid	25-400	0.9996	5.28	100-4000	0.9995	3.11
Isobutyric acid	25-400	0.9994	4.21	100-4000	0.9995	3.56
Butyric acid	25-400	0.9995	4.79	100-4000	0.9995	3.28
Isovaleric acid	25-400	0.9994	4.2	100-4000	0.9994	3.67
Valeric acid	25-400	0.9994	4.87	100-4000	0.9994	3.39

*Mean value of Relative Standard Deviations (RSD)

3.3.3.Precision and accuracy

Precision was studied by measuring the reproducibility of peak areas. Reproducibility was measured by calculating the relative standard deviation (RSD) of peak areas for three repeated analysis of each sample. Results of RSD of individual samples of all different concentrations range from 0.3 to 16%. The highest RSD values correspond to the lowest acetic and propionic acids concentrations analysed at a split ratio of 10. In addition, average values of RDS of each VFA were calculated from all the RSD values for a given VFA. These averages RSD are represented in table 2, and range from 3 to 6%.

To improve reproducibility with samples based on aqueous matrix the following parameters were implemented: (i) the amount of silica wool to the liner was increased to 12mg, (ii) injector temperature was decreased to 220ºC and (iii) autoinjector method was optimised as described above ("3.2.3 Autoinjection method").

Accuracy is measured by the relative error existing between the real concentration and the theoretical concentration obtained by the calibration curve of standard samples. Relative errors were calculated for all VFA for all concentrations. Values of relative error of each VFA are represented in table 3.

It is remarkable that at split ratio 40 the relative error calculated for the lowest concentration (100mg/L) is considerably high (see section "8 Appendix" tables 12 to 19). Therefore, the optimal range of concentration used in the analysis of VFA at split 40 must be from 200 to 4000mg/L of compound. Although the analysis of concentrations ranging from 200 to 400mg/L is more accurate at split ratio 10, concentrations from 200mg/L to 500mg/L can be also analysed at split 40, because the error is acceptable (see table 3 below).

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 3 Results of accuracy represented by the relative error of analysis of standard samples

Split ratio	Concentration of compound (mg/L)	Relative error (%)					
		Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid
10	25	18	6	4	0.8	6	3
	75	7	3	2	2	0.9	2
	100	3	4	4	4	4	4
	200	3	4	5	5	5	5
	400	0.3	0.6	0.9	0.8	1	1
40	200	6	8	6	7	4	5
	500	8	9	10	10	11	11
	1000	5	6	6	6	5	5
	2000	3	3	4	4	4	4
	4000	0.4	0.4	0.5	0.5	0.7	0.7

3.4. Conclusions

In this section of the TN a method for analysing VFA has been developed and described. Different aspects of the analysis method were selected taking into account the correspondent application. For example, time of analysis was decreased using a short length column due to this analysis is part of a control loop. Another aspect to be selected was the calibration method. Two different dilution rates: split 10 and split 40, were chosen, since the range of VFA concentrations to analyse is considerably broad. This fact allowed to achieve good linearity for both calibration curves, at high (split 10) and low (split 40) dilution rates corresponding to low and high concentrations of each compound, respectively.

According to our expectations and at initial time reproducibility had to be improved, because autoinjection method and some fungible material and reagents were not adapted to VFA analysis. After this optimisation process good results of reproducibility were achieved.

4. Analysis of liquid samples from compartment II

4.1. Materials and methods

4.1.1. Sample preparation

An anaerobic continuous culture of *Rhodospirillium rubrum* (ATCC 25903) was ran. Medium composition was based on the salts mixture described by Albiol (1994). 1g/L of acetic acid was used as a carbon source. Temperature of culture was maintained at 30ºC and pH at 6.9 by adding NaOH (1.5M) or HCl (1M) under a pH controller. Dilution factor was $0.0102h^{-1}$ which corresponds to a residence time of 4.1days. After two weeks of continuous culture, the outlet of the reactor was harvested and stored at –21ºC. When

required culture was centrifuged at 9000rpm during 15" and filtered by a 0.45µm pore filter (Millipore).

4.2. Results

Liquid samples from compartment II were analysed at split 10, because it was expected to find low concentrations of VFA at culture conditions used. Acetic acid and propionic acid were found in the samples. Results of this analysis are shown in table 4.

Table 4 Results of the analysis of liquid samples from compartment II

Split	Compound	Mean Conc. of compound (mg/L)	RSD (%)
10	Acetic acid	26.2	1.86
	Propionic acid	23.5	2.20

5. On-line determination of VFA in liquid phase

5.1. Materials and methods

With the aim of future sampling of liquid phase from compartment II and I two flow cells (figure 3) were installed in the GC. Liquid loop, made of 1/16" inner diameter Teflon® tubing, was connected to both flow cells and checked for viability by the distributor. VFA used for this test were acetic acid, propionic acid and butyric acid. 1L of standard solution was prepared in MilliQ water at a concentration of 0.1g/L of each compound.

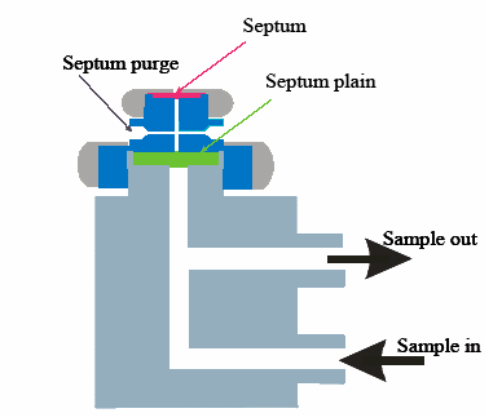

Figure 3 Flow cell

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

5.2. Results

5.2.1.Analysis of VFA

Standard solution of a mixture of three VFA at 0.1g/L each compound was analysed on-line using the flow cell 1. Analysis was repeated six times in order to check the reproducibility of peak areas using the flow cell. In table 5 are shown the results of reproducibility and accuracy represented by the Relative Standard Deviation (RSD) and relative error, respectively. Good results of accuracy were achieved in this analysis.

Table 5 Results of On-line analysis of a standard sample

Compound	Mean Conc. (mg/L)	Relative error (%)	RSD (%)
Acetic acid	100.1	0.2	5.15
Propionic acid	101.5	2	4.92
Butyric acid	103.1	4	4.00

5.2.2.Determination of the ratio Volume/Distance and dead time of the liquid loop

For the future implementation of the on-line analysis of VFA two parameters were determined corresponding to the distance D depicted in figure 4: (i) the ratio Volume/Distance of the liquid loop and (ii) dead time spent to reach the flow cell ($T_{d,FC}$). D is defined as the distance existing from the biomass separation unit to the flow cell (figure 4). Therefore, in these determinations it was not considered the time spent to separate the liquid samples from biomass.

To estimate the Volume/Distance ratio we calculate the loop volume as a function of distance D (see equation 1 below). Once the loop volume has been determined by equation 1, dead time of flow cell ($T_{d,FC}$) can be determined with the loop volume previously calculated and the volumetric flow used.

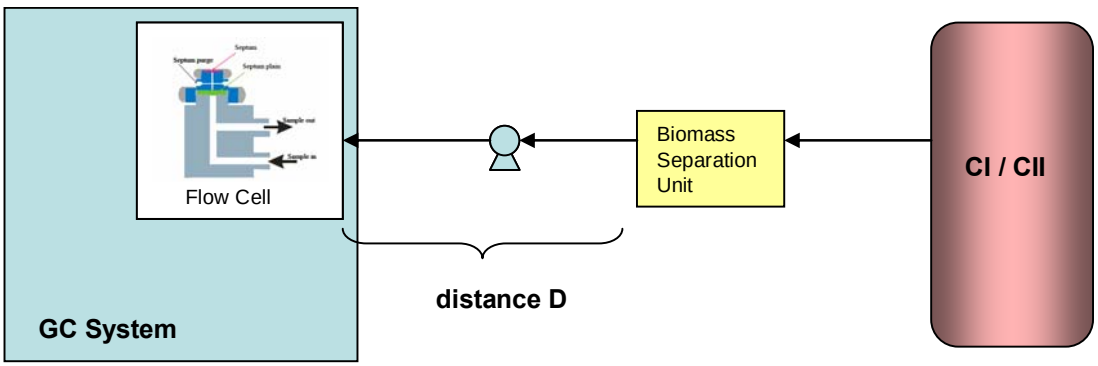

Figure 4 Scheme of liquid sampling from CII or CI. Dead time ($T_{d,FC}$) and ratio Volume/Distance are calculated for distance D.

Determination of the ratio volume/distance

Determination of the loop volume was done: (i) experimentally by weighting the MilliQ water that fits into the loop and (ii) a theoretical calculation was done using the tubing length and diameter. For the theoretical determination inner diameters and lengths of both pump and loop tubing were used. The volume of the flow cell was determined experimentally by weighting due to the impossibility to calculate it theoretically. The experimental volume of the flow cell was added to the theoretical determination of the loop volume as a constant value. Both experimental and theoretical results were compared (table 6) and a relative error of 2%[1] was estimated.

Table 6 Determination of the volume of the liquid loop

Type of determination	Volume of liquid loop (mL)	Relative error (%)
Experimental	**10.75** (±0.05)	
Theoretical	7.74 + 2.85(±0.05) ($F_{low\ cell}$) = **10.59**(±0.05)	1.5

Once it is demonstrated that experimental and theoretical values of loop volume are comparable the ratio volume/distance can be determined using the theoretical volume calculated and the length of the tubing used. Calculations are given in Appendix (see "8.2 Calculations for the determination of the ratio volume/distance and dead time"). The equation that relates the distance, in terms of tubing length (L), and the volume of the liquid loop can be expressed as follows:

$$V(mL) = 1.98567\left(\frac{mL}{m}\right)\cdot L(m) + V_{FC}(mL) + V_{PT}(mL) \qquad \text{eq 1}$$

where V is the loop volume in "mL", L is the length in "m" of the loop excepting the pump tubing length used, V_{FC} is the experimental volume in "mL" of the flow cell and V_{PT} is the volume in "mL" corresponding to the pump tubing. The constant value V_{FC} is the following:

$$V_{FC} = 2.85(\pm 0.05)mL$$

V_{PT} depends on the inner diameter and length of the pump tubing used. In this case V_{PT} it is the following:

$$V_{PT} = 1.37mL$$

[1] The value shown in table 6, 1.5%, is round up to 2% because it is an error value.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Determination of dead time

Dead time spent to reach the flow cell ($T_{d,FC}$) can be calculated as follows:

$$T_{d,FC} = \frac{V}{Q} \qquad \text{eq 2}$$

Where Q is the volumetric flow in "mL/min" and V is the loop volume in "mL". As an example, if L value is fixed to 2m of Teflon® tubing, the volume V it is also fixed and can be calculated by equation 1. Therefore, dead time can be expressed as a function of the volumetric flow:

$$L = 2m \Rightarrow V = 8.2mL$$

$$T_{d,FC}(\min) = \frac{8.2mL}{Q\left(\frac{mL}{\min}\right)}$$

Q value used for the test was 9mL/min. If we take an interval of possible values of Q from 5 to 10mL/min, the interval of dead time will be the following:

$$T_{d,FC} = 1.2 \pm 0.4 \min$$

In order to estimate the total time spent for the on-line analysis, these values of dead time must be added to dead time spent for biomass separation and to chromatographic analysis time (6min).

6. On-line analysis of the gas phase

It has been also considered the possibility to analyse on-line the gas phase of compartments II and I with the same equipment purchased. To this purpose it has been requested to the distributor an application for the on-line analysis of gas samples. The main requirement for the installation of the new hardware was that analysis of gas samples had to be compatible with analysis of liquid samples.

The system that the Spanish dealer IZASA offered us is based on the installation of a 6-way 2-position valve into the gas carrier line that goes to the injector port of the GC. This valve is also connected to the gas sample line. As it is depicted in figure 4, in valve position A gas sample is flown to a gas loop of 1mL of volume which will be connected to the carrier gas line and injected to the injector port when valve will be in position B.

When liquid sampling is required valve remains in position A in which gas carrier (He) is flown to the injector port without injecting any gas sample.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

The budget for the installation of this hardware is summarised in table 7. The original budget is in Appendix "8.3 Original offer for the on-line analysis of gas phase application".

Table 7 Summary of budget from IZASA for the on-line analysis of gas application

IZASA,S.A.	
Date	2006 February 13th
Budget Nº	1000012109 MA

Works to do

Installation of a 6-way 2-position valve from Valco and 1/16" tubing with the aim to inject gas samples at room temperature in GC 2010 system, adapting pneumatic connections, electrical control and software methods, in order to incorporate it into an on-line system.

Material	**Quantity**	**Price/unit**	**Total**	**%VAT**
6 PORT 2POS VALVE, STD ELECTRI	1	4,645.80	4,645.80	16.00
8PIN RELAY CABLE	1	264.64	264.64	16.00
Connection tubing 2mm to 1/16"	2	37.41	74.82	16.00
1/16" STAINLESS STEEL LOOP V=1 mL	1	146.75	146.75	16.00
TUBING SUS 316 1.6X0.8 MM, 2	1	52.63	52.63	16.00

Labour and journey	**Quantity**	**Price/unit**	**Total**	**%VAT**
Time spent	2	174.41	348.82	16.00
Time of journey	1.5	146.39	219.59	16.00

	Tax base	**%VAT**	**VAT**
	4,959.53	16.00	793.52
	TOTAL	**EUR**	5,753.05

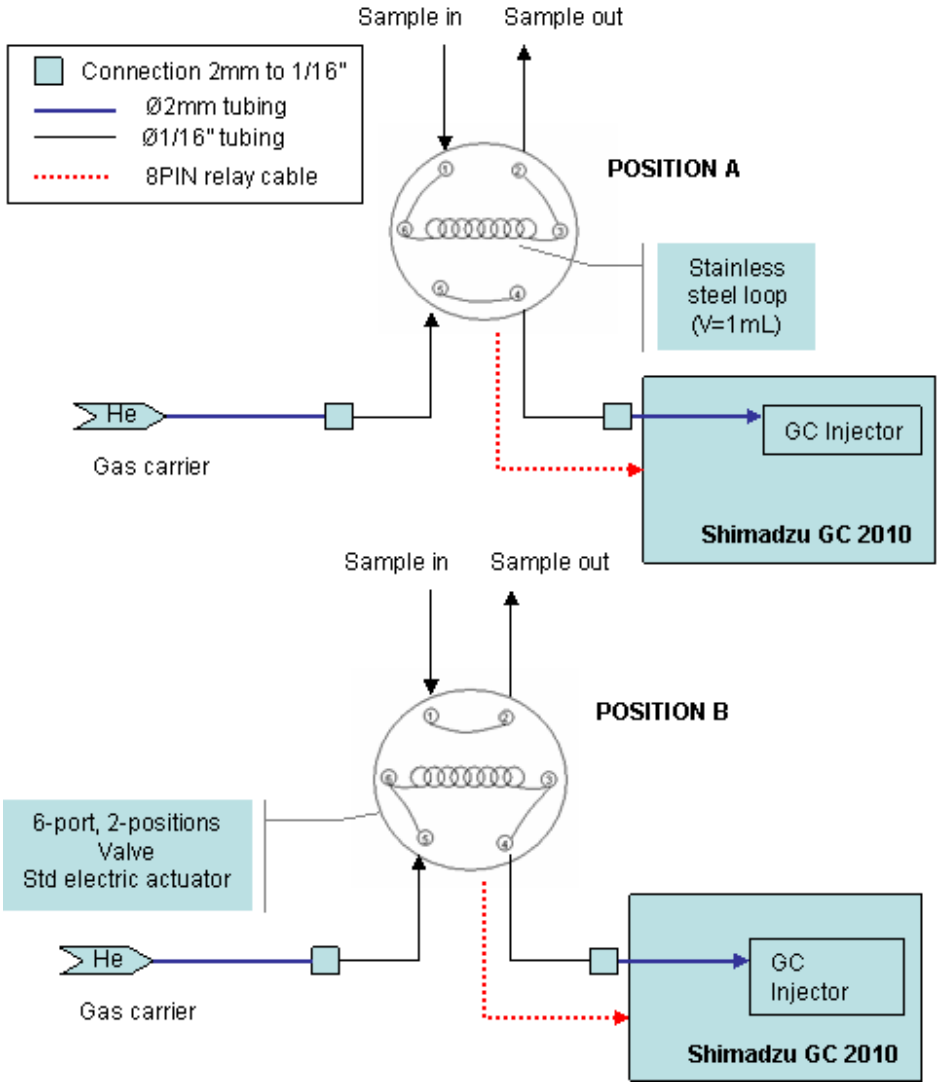

Figure 5 Hardware for on-line analysis of gas samples. The system consists on the installation of one 6-way 2-position valve connected to the gas carrier line and the gas sample line. In Position A, gas sample is flown to the 1mL of volume stainless steel loop and gas carrier gas is flown to the gas chromatograph injector port without injecting any gas sample. In Position B, the loop is connected to the gas carrier line and gas sample is injected to the GC injector port.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

7. References

Camargo, J.; Pérez, J. and Gòdia, F. (2005) Instrumentation for on-line determination of VFA. Technical Note 62.11. ESTEC/CONTRACT13292/98/NL/MV.

Creus, N.; Albiol, J. and Gòdia, F. (2002) Tests with 3 linked bench compartments using 3 different carbon sources. Technical Note 52.6. ESTEC/CONTRACT13292/98/NL/MV.

Albiol, J. (1994) Study of the MELISSA photoheterotrophic compartment. Kinetics and effects of C limitation. ESA/YCL/2148.JAS ESTEC Working Paper. ESA-EWP-1808.

Novák, J. (1988) Quantitative analysis by gas chromatography 2nd ed. (chromatographic science; v. 41) Ed. Marcel Dekker, INC. New York.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

8. Appendix

8.1. Data

8.1.1.Data of Calibration tests

Acetic acid (SPLIT 10)

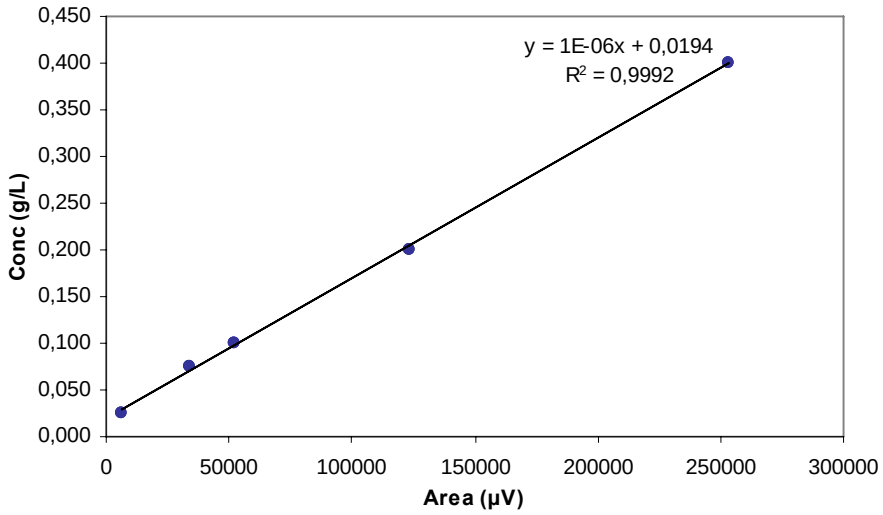

Figure 6 Acetic acid calibration curve at split 10

Conc (g/L)	SD	Average	%RSD	Area 1	Area 2	Area 3	Calculated Conc (g/L)	Rel error (%)
0,025	1061,51	6652,00	15,96	5593	6647	7716	0,029	17,35
0,075	3023,62	33797,67	8,95	30464	34566	36363	0,070	6,65
0,100	315,70	51998,00	0,61	52041	52290	51663	0,097	2,71
0,200	2427,34	123470,67	1,97	120731	125353	124328	0,204	2,19
0,400	11885,11	253323,33	4,69	241159	253903	264908	0,399	0,26
		MEAN	6,43					

Table 8 Data of peak areas of acetic acid calibration curve at split 10

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Propionic acid (SPLIT 10)

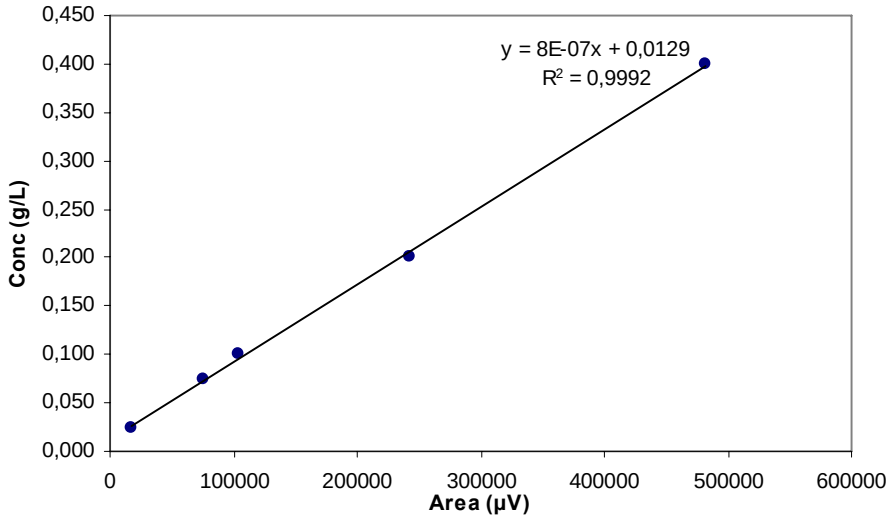

Figure 7 Propionic acid calibration curve at split 10

Conc (g/L)	SD	Average	%RSD	Area 1	Area 2	Area 3	Calculated Conc (g/L)	Rel error (%)
0,025	1762,33	16686,67	10,56	14979	16582	18499	0,026	5,10
0,075	2203,47	74857,33	2,94	72313	76137	76122	0,073	3,01
0,100	3693,05	104620,00	3,53	108302	104642	100916	0,097	3,48
0,200	3847,89	242767,67	1,59	241327	247128	239848	0,207	3,43
0,400	23621,90	481555,00	4,91	457705	482018	504942	0,398	0,60
		MEAN	4,71					

Table 9 Data of peak areas of propionic acid calibration curve at split 10

Isobutyric acid (SPLIT 10)

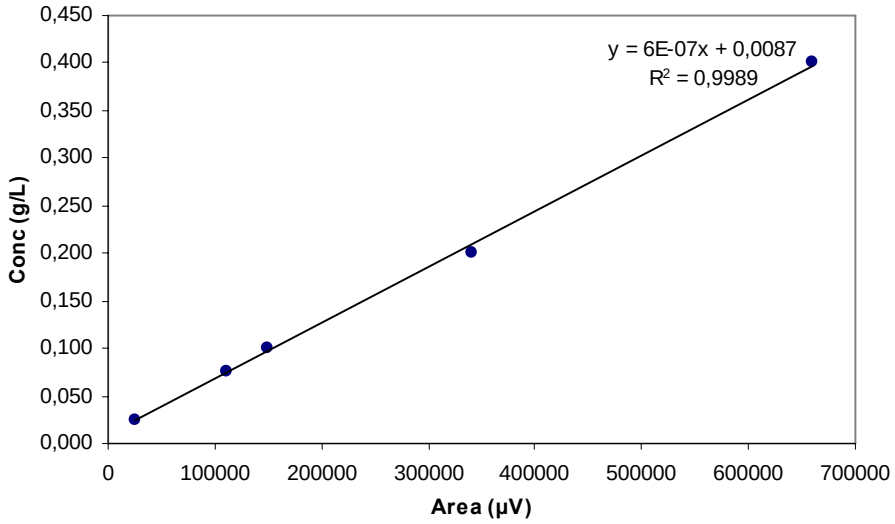

Figure 8 Isobutyric acid calibration curve at split 10

Conc (g/L)	SD	Average	%RSD	Area 1	Area 2	Area 3	Calculated Conc (g/L)	Rel error (%)
0,025	1340,88	26354,67	5,09	25371	25811	27882	0,024	3,42
0,075	1730,15	111418,67	1,55	110814	113370	110072	0,074	1,16
0,100	7357,35	149681,00	4,92	157670	148189	143184	0,097	3,39
0,200	6752,25	340287,33	1,98	340354	347006	333502	0,209	4,30
0,400	39621,71	660078,00	6,00	617981	665610	696643	0,397	0,87
			MEAN 3,91					

Table 10 Data of peak areas of isobutyric acid calibration curve at split 10

Butyric acid (SPLIT 10)

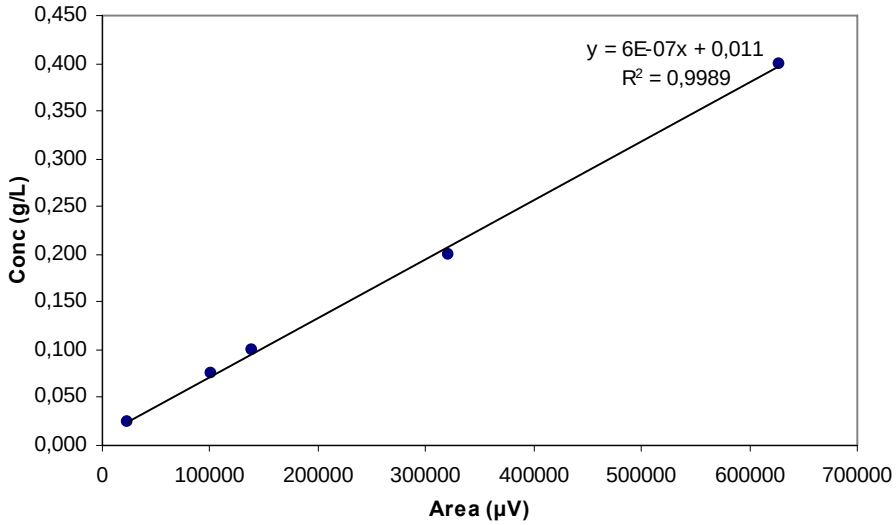

Figure 9 Butyric acid calibration curve at split 10

Conc (g/L)	SD	Average	%RSD	Area 1	Area 2	Area 3	Calculated Conc (g/L)	Rel error (%)
0,025	1861,31	23079,67	8,06	21397	22763	25079	0,025	0,80
0,075	1658,40	101714,33	1,63	100395	103576	101172	0,074	1,92
0,100	6308,59	138455,67	4,56	145087	137751	132529	0,096	3,85
0,200	6346,78	320691,33	1,98	319325	327610	315139	0,208	4,11
0,400	36165,68	627484,67	5,76	589932	630440	662082	0,397	0,78
		MEAN	4,40					

Table 11 Data of peak areas of butyric acid calibration curve at split 10

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Isovaleric acid (SPLIT 10)

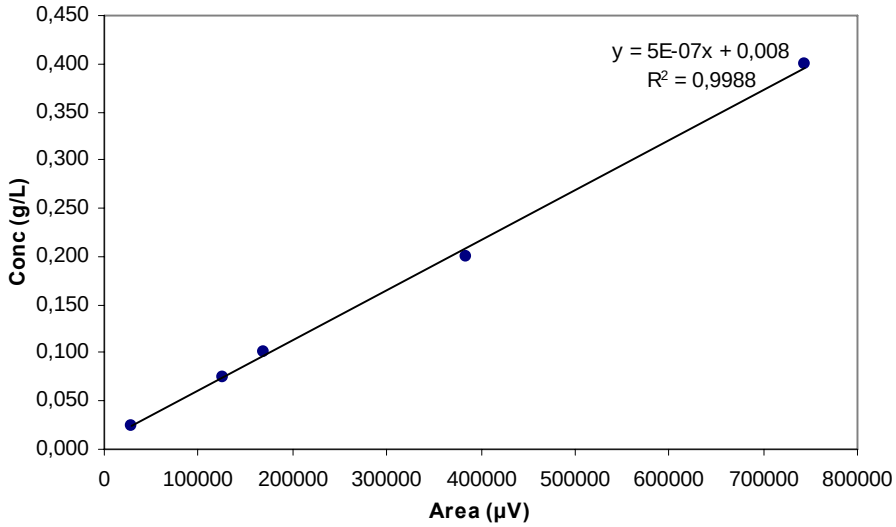

Figure 10 Isovaleric acid calibration curve at split 10

Conc (g/L)	SD	Average	%RSD		Area 1	Area 2	Area 3	Calculated Conc (g/L)	Rel error (%)
0,025	1571,98	29958,67		5,25	28824	29299	31753	0,024	5,30
0,075	2461,12	127093,00		1,94	126598	129764	124917	0,074	0,81
0,100	8693,95	169881,33		5,12	179223	168394	162027	0,097	3,27
0,200	7636,50	384700,67		1,99	384703	392336	377063	0,209	4,45
0,400	47605,51	743596,67		6,40	692755	750917	787118	0,396	0,93
			MEAN	4,14					

Table 12 Data of peak areas of isovaleric acid calibration curve at split 10

Valeric acid (SPLIT 10)

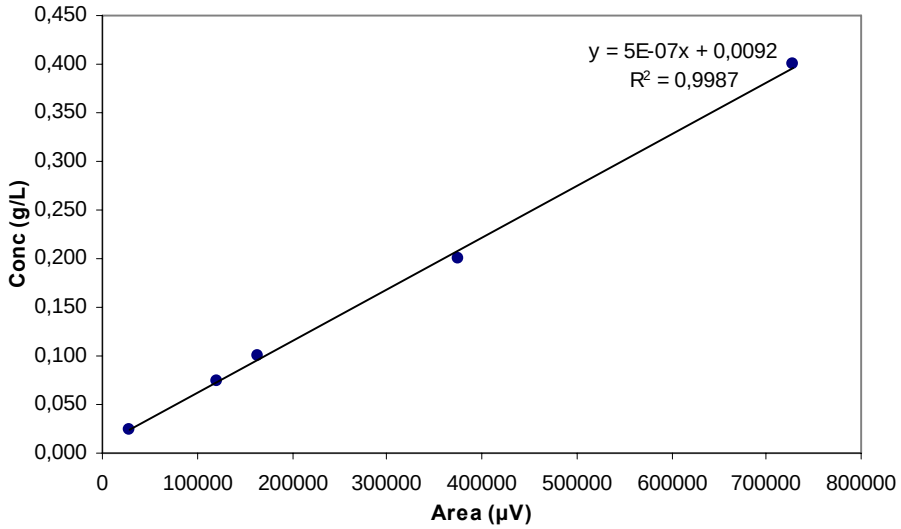

Figure 11 Valeric acid calibration curve at split 10

Conc (g/L)	SD	Average	%RSD	Area 1	Area 2	Area 3	Calculated Conc (g/L)	Rel error (%)
0,025	1850,50	28449,00	6,50	26752	28173	30422	0,024	2,70
0,075	2756,99	121823,33	2,26	120595	124981	119894	0,074	1,33
0,100	8667,79	163656,33	5,30	172827	162543	155599	0,096	3,74
0,200	7755,74	375665,00	2,06	374006	384116	368873	0,209	4,53
0,400	49546,76	727743,67	6,81	675020	734869	773342	0,396	0,91
		MEAN	4,59					

Table 13 Data of peak areas of valeric acid calibration curve at split 10

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Acetic acid (SPLIT 40)

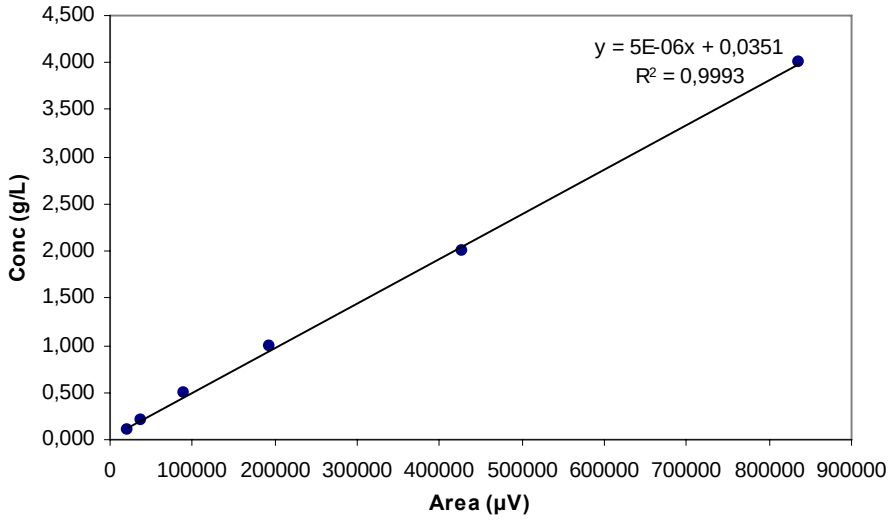

Figure 12 Acetic acid calibration curve at split 40

Conc (g/L)	SD	Average	%RSD	Area 1	Area 2	Area 3	Calculated Conc (g/L)	Rel error (%)
0,100	794,39	20229,3	3,93	20968	20331	19389	0,131	30,75
0,200	841,88	37116,7	2,27	36899	38046	36405	0,211	5,30
0,500	2523,03	90569,7	2,79	89119	93483	89107	0,463	7,33
1,000	7173,17	194261,0	3,69	197242	199463	186078	0,954	4,63
2,000	22164,33	427673,7	5,18	433298	446484	403239	2,057	2,87
4,000	21886,13	835091,7	2,62	815283	831405	858587	3,984	0,40
			MEAN 3,41					

Table 14 Data of peak areas of acetic acid calibration curve at split 40

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Propionic acid (SPLIT 40)

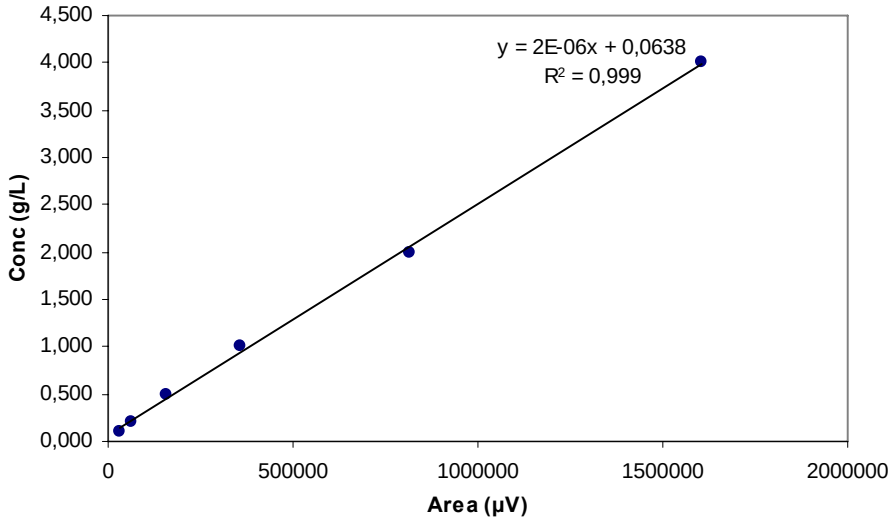

Figure 13 Propionic acid calibration curve at split 40

Conc (g/L)	SD	Average	%RSD	Area 1	Area 2	Area 3	Calculated Conc (g/L)	Rel error (%)
0,100	717,50	32519,7	2,21	33016	32846	31697	0,143	43,40
0,200	1613,53	62007,0	2,60	60645	63789	61587	0,216	7,79
0,500	4759,10	159970,0	2,97	156237	165329	158344	0,455	8,92
1,000	11470,34	358823,7	3,20	363049	367582	345840	0,942	5,78
2,000	46052,73	813740,0	5,66	830644	848952	761624	2,056	2,79
4,000	32396,38	1602817,7	2,02	1581710	1586625	1640118	3,988	0,31
		MEAN	3,11					

Table 15 Data of peak areas of propionic acid calibration curve at split 40

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Isobutyric acid (SPLIT 40)

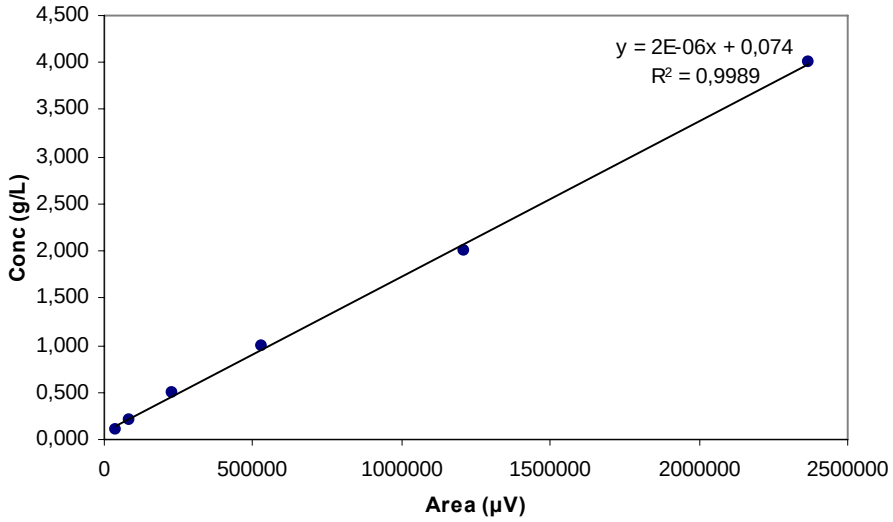

Figure 14 Isobutyric acid calibration curve at split 40

Conc (g/L)	SD	Average	%RSD	Area 1	Area 2	Area 3	Calculated Conc (g/L)	Rel error (%)
0,100	768,90	41881,0	1,84	41776	42697	41170	0,143	43,08
0,200	3168,24	83553,7	3,79	80478	86807	83376	0,212	5,89
0,500	8210,08	228295,3	3,60	220902	237131	226853	0,450	9,92
1,000	19295,76	529581,7	3,64	538652	542671	507422	0,947	5,29
2,000	76025,28	1207966,7	6,29	1238176	1264244	1121480	2,066	3,28
4,000	52025,83	2370318,7	2,19	2351705	2330160	2429091	3,982	0,45
			MEAN 3,56					

Table 16 Data of peak areas of isobutyric acid calibration curve at split 40

Butyric acid (SPLIT 40)

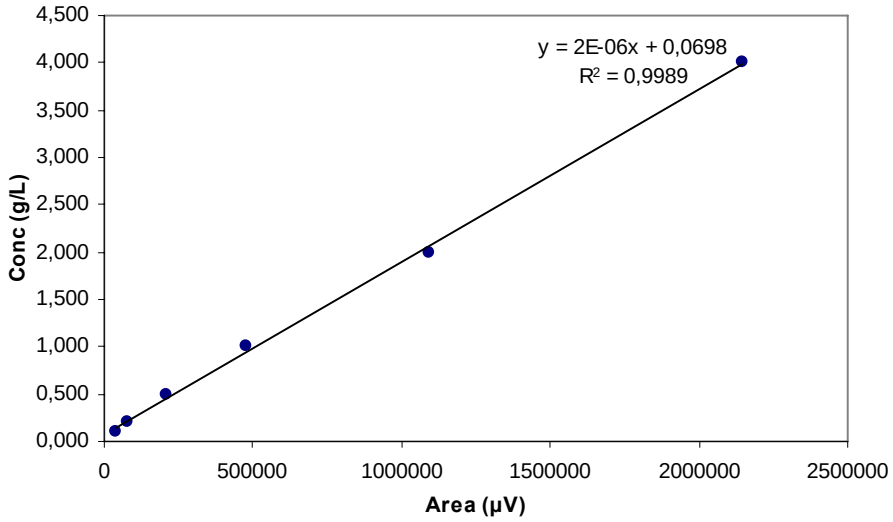

Figure 15 Butyric acid calibration curve at split 40

Conc (g/L)				Area			Calculated	Rel error
	SD	Average	%RSD	1	2	3	Conc (g/L)	(%)
0,100	906,09	40394,3	2,24	40525	41228	39430	0,144	43,51
0,200	2373,51	78359,7	3,03	75989	80736	78354	0,213	6,38
0,500	6760,73	208713,0	3,24	203107	216221	206811	0,451	9,89
1,000	15099,84	479786,3	3,15	486752	490146	462461	0,945	5,50
2,000	65912,22	1094249,3	6,02	1123086	1140829	1018833	2,066	3,30
4,000	42684,57	2144700,0	1,99	2120824	2119296	2193980	3,982	0,45
			MEAN 3,28					

Table 17 Data of peak areas of butyric acid calibration curve at split 40

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Isovaleric acid (SPLIT 40)

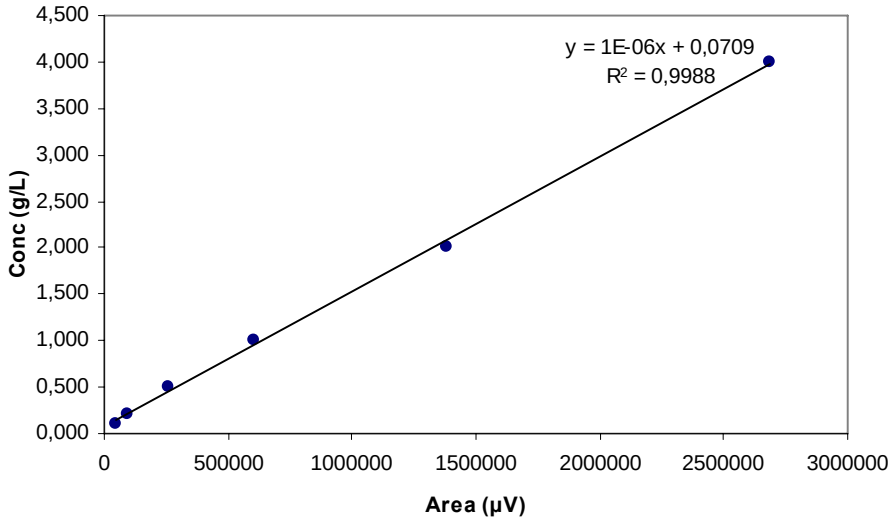

Figure 16 Isovaleric acid calibration curve at split 40

Conc (g/L)				Area			Calculated	Rel error
	SD	Average	%RSD	1	2	3	Conc (g/L)	(%)
0,100	1300,22	47401,0	2,74	47760	48484	45959	0,140	39,78
0,200	3479,64	94207,0	3,69	90659	97614	94348	0,208	3,89
0,500	9072,39	259196,7	3,50	250992	268940	257658	0,448	10,50
1,000	21259,37	607220,3	3,50	618202	620743	582716	0,953	4,68
2,000	88569,22	1380189,7	6,42	1419191	1442565	1278813	2,076	3,81
4,000	58812,11	2687352,0	2,19	2662603	2644959	2754494	3,976	0,61
			MEAN 3,67					

Table 18 Data of peak areas of isovaleric acid calibration curve at split 40

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Valeric acid (SPLIT 40)

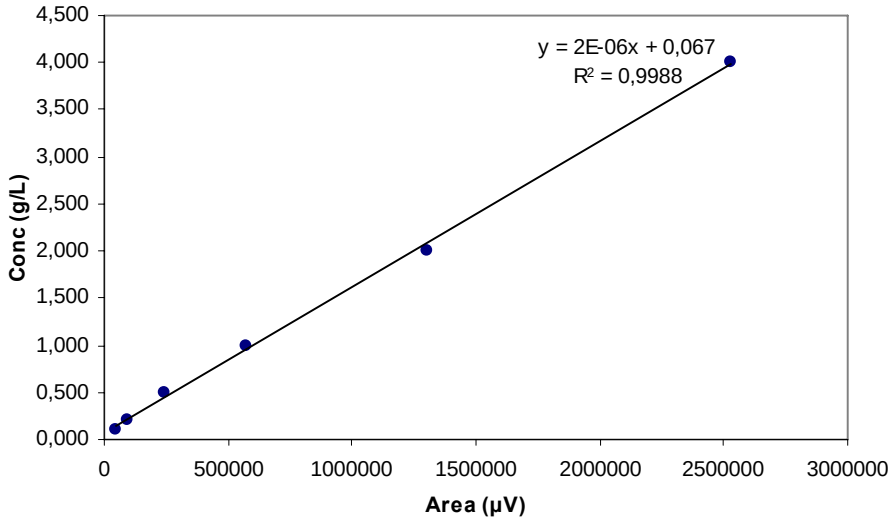

Figure 17 Valeric acid calibration curve at split 40

Conc (g/L)				Area			Calculated	Rel error
	SD	Average	%RSD	1	2	3	Conc (g/L)	(%)
0,100	1350,76	47813,3	2,83	48089	49005	46346	0,141	40,93
0,200	2931,02	91434,7	3,21	88536	94397	91371	0,208	4,18
0,500	7579,61	246270,3	3,08	240483	254850	243478	0,448	10,46
1,000	16615,91	571193,0	2,91	580234	581328	552017	0,950	5,01
2,000	81205,89	1300938,3	6,24	1342086	1353334	1207395	2,078	3,89
4,000	52790,36	2528517,3	2,09	2496823	2499271	2589458	3,975	0,62
			MEAN 3,39					

Table 19 Data of peak areas of valeric acid calibration curve at split 40

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

8.1.2.Data of analysis of liquid samples from compartment II

Table 20 Data of analysis of liquid samples from compartment II. Calculated concentrations and statistics.

Compound	Calculated concentrations (g/L)						
	Mean conc (g/L)	SD	%RSD	1	2	3	4
acetic	0,02615	4,8747E-04	1,86	0,02661	0,02648	0,02597	0,02555
propionic	0,02345	5,1628E-04	2,20	0,02298	0,02402	0,02376	0,02306

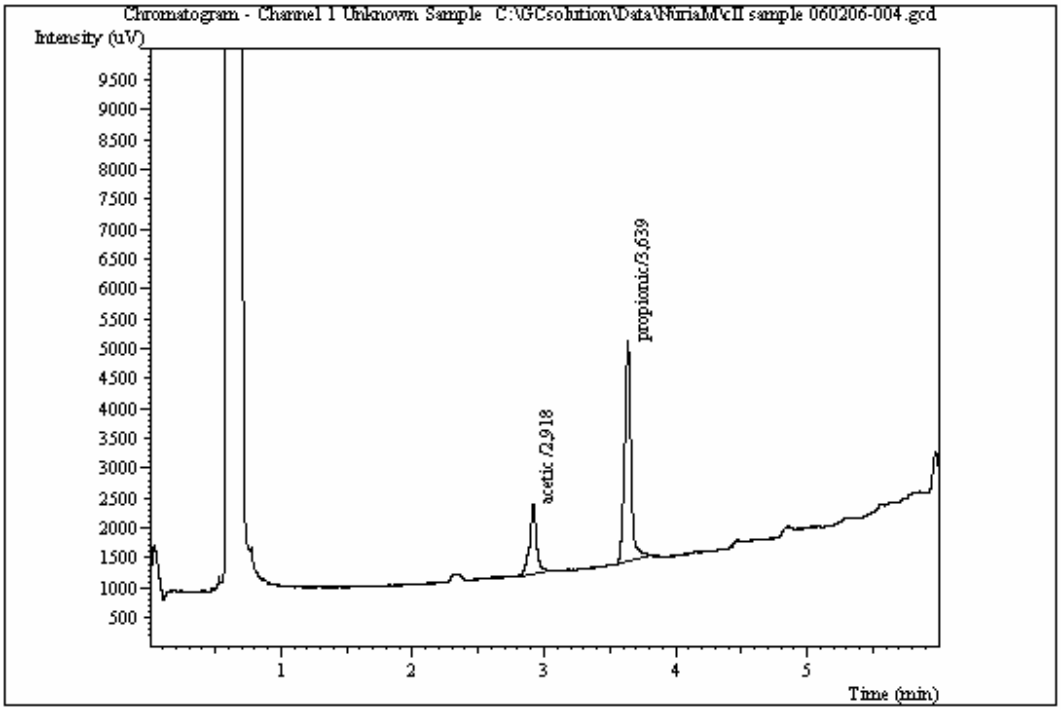

Figure 19 Example of chromatogram from the analysis of liquid samples from CII

8.1.3.Data of on-line analysis of VFA

Table 21 Data of on-line analysis of acetic acid, propionic acid and butyric acid. Calculated concentrations and statistics.

Compound	Mean conc (g/L)	Rel error (%)	SD	%RSD	Calculated concentrations (g/L)					
					1	2	3	4	5	6
acetic	0,1001	0,1433	5,1545E-03	5,15	0,09957	0,09631	0,10781	0,10186	0,09298	0,10233
propionic	0,1015	1,4983	4,9933E-03	4,92	0,10214	0,09612	0,10705	0,10336	0,0948	0,10552
butyric	0,1031	3,1033	4,1287E-03	4,00	0,10355	0,09779	0,10525	0,10454	0,09878	0,10871

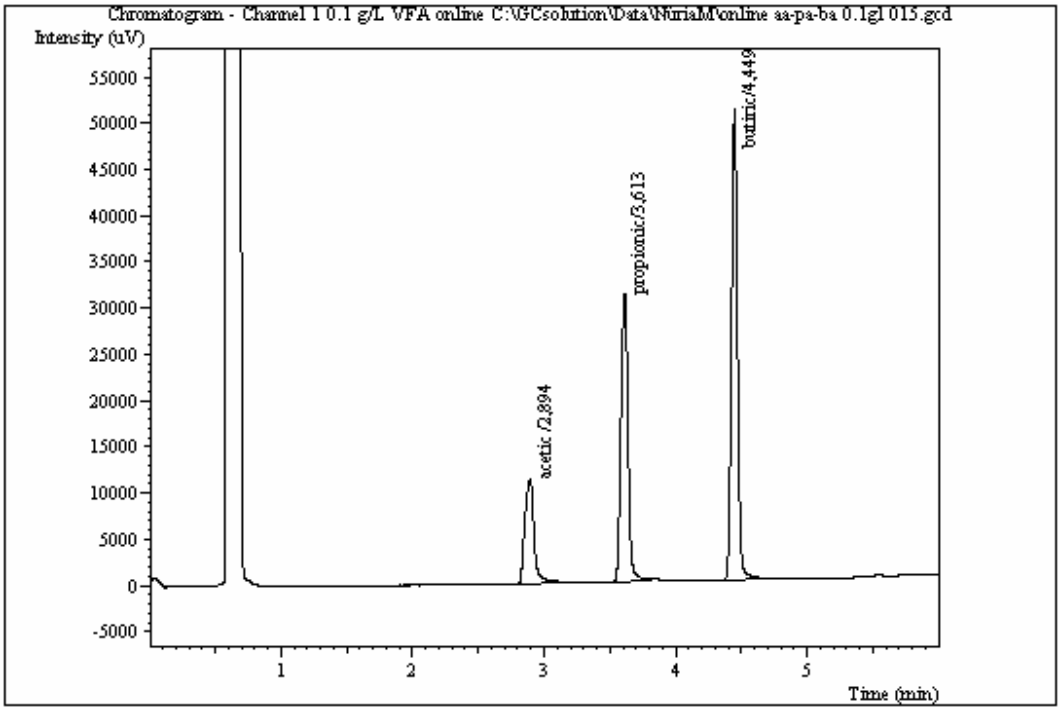

Figure 20 Example of chromatogram from the on-line analysis

8.2. Calculations for the determination of the ratio volume/distance and dead time

Experimental determination of the loop volume

For the experimental determination a glass was connected to the loop and filled with approximately 200ml of MilliQ. The glass was weighted before and after filling the loop with the MilliQ water. Result of experimental loop volume is in table 20.

Table 22 Experimental determination of the liquid loop volume. Weight of MilliQ water that fits into the loop.

Weight (g)	Test 1	Test 2
Glass before filling the loop with MilliQ water	311.4	287.0
Glass after filling the loop with MilliQ water	300.6	276.3
Difference	10.8	10.7
Volume of liquid loop (mL)	10.75 (±0.05)	

Theoretical determination of the loop volume

For the theoretical determination of the loop volume values of length and diameter of the tubing needed were used. For the theoretical determination of the flow cell there was no data, therefore it had to be determined experimentally. This determination was done by the same procedure explained above, used for the experimental determination of the loop volume. Results of the flow cell volume and the theoretical value of loop volume are in table 21 and 22 respectively.

Table 23 Experimental determination of the flow cell volume.

Weight (g)	Test 1
Glass before filling the loop with MilliQ water	294.1
Glass after filling the loop with MilliQ water. Not including the flow cell in the loop	286.2
Difference	7.9
Volume of flow cell (mL)	10.75 (±0.05) – 7.9 = 2.85(±0.05)

Table 24 Theoretical determination of the liquid loop volume.

	Pump tubing	Teflon® tubing	Flow Cell
Lenght (mm)	410	3210	-
Diameter (mm)	2.06	1.59	-
Volume ($V = \pi r^2 \cdot L$) (mL)	1.367	6.374	2.85(±0.05)
Total Volume (mL)		10.59(±0.05)	

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Determination of the ratio volume/distance

The following calculations were made for the calculation of the volume/distance ratio:

$$\frac{V_{TT}}{L_{TT}} = \frac{6.374}{3.21} = 1.98567\left(\frac{mL}{m}\right)$$

Where V_{TT} is the Teflon® tubing volume in "mL" and L_{TT} is the Teflon® tubing in "m".

To this ratio the constant values V_{FC} and V_{PT} must be added:

$$V(mL) = 1.98567\left(\frac{mL}{m}\right) \cdot L(m) + V_{FC}(mL) + V_{PT}(mL) \qquad \text{eq 1}$$

where V is the loop volume in "mL", L is the length in "m" of the loop excepting the pump tubing length used, V_{FC} is the experimental volume in "mL" of the flow cell and V_{PT} is the volume in "mL" corresponding to the pump tubing. The constant value V_{FC} is the following:

$$V_{FC} = 2.85(\pm 0.05)mL$$

V_{PT} depends on the inner diameter and length of the pump tubing used. In this case V_{PT} it is the following:

$$V_{PT} = 1.37mL$$

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

8.3. Original offer for the on-line analysis of gas phase application

IZASA

BARCELONA 13.02.2006 C/C: 137699
PRESUPUESTO Nº: 1000012109 MA
Nº VALE :
Nº AVISO : 2000176603

U.A.B.
FACULTAD DE CIENCIAS
DPTO. INGENIERIA QUIMICA
CAMPUS UNIVERSIT. BELLATERRA
08193 BELLATERRA
Att. : Nuria Martinez
Depto.: Ingenieria Química

PRESUPUESTO DE REPARACIÓN
MARCA Shimadzu europa gmbh (cromat.)
APARATO 641221-47700-34 GC-2010 AF
Nº SERIE 1324001448

ANOMALIAS
Modificar sistema GC para inyección automática de muestra gaseosa por loop.

TRABAJOS A REALIZAR
Instalación de válvula Valco 6 ports y 2 posiciones, tubo de 1/16", para inyectar muestra gaseosa a temperatura ambiente en sistema GC2010, adaptando conexiones neumáticas, control eléctrico y software- métodos, a fin de poder integrarla en un sistema ON LINE.

MATERIAL A UTILIZAR	CANTIDAD	PRECIO/U	TOTAL	% IVA/IGIC
6 PORT 2POS VALVE, STD ELECTRI	1	4.645,80	4.645,80	16,00
8PIN RELAY CABLE	1	264,64	264,64	16,00
Adaptador tubo 2mm a 1/16"	2	37,41	74,82	16,00
1/16" STAINLESS STEEL LOOP 1 m	1	146,75	146,75	16,00
TUBING SUS 316 1,6X0,8 MM , 2	1	52,63	52,63	16,00

MANO DE OBRA Y DESPLAZAMIENTO	CANTIDAD	PRECIO/U	TOTAL	% IVA/IGIC
TIEMPO EMPLEADO	2	174,41	348,82	16,00
TIEMPO DESPLAZAMIENTO	1,500	146,39	219,59	16,00

BASE IMPONIBLE	%	% IVA/IGIC
4.959,53	16,00	793,52
TOTAL	EUR	5.753,05

Queda excluída de este presupuesto la solución de cualquier anomalía no descrita en el mismo.

La validez de este presupuesto es de 040 días a partir de la fecha de su expedición.

Los precios indicados, solamente se aplicarán si la reparación la realiza integramente el Servicio Técnico de Izasa, S.A.

Las reparaciones efectuadas por nuestro Servicio de Asistencia Técnica, tienen una garantía de 090 días a partir de la fecha en que se haya finalizado. La cobertura de la garantía corresponde a la reparación efectuada, tanto en materiales sustituídos como en mano de obra y desplazamiento empleados y no a otras posibles anomalías que podrían presentar iguales o parecidos síntomas.

1 / 2

IZASA, S.A. Tecnología y Servicio
Ramón Turró, 112-118 - 08005 BARCELONA
Teléfono (+ 34) 93 401 01 27 - Fax: (+ 34) 93 401 03 30
Inscrita en el Registro Mercantil de Barcelona en hoja nº 56.158, folio 142, tomo 5284, libro 4590, sección 2ª de sociedades N.I.F. A-28114742

IZASA

BARCELONA 13.02.2006 C/C: 137699
PRESUPUESTO Nº: 1000012109 MA
Nº VALE :
Nº AVISO : 2000176603

N O T A
La refencia indicada 557E2C6WE es una: Válvula VALCO 6 port 2 pos valve, std.electric/4" 1/16", 4mm 225°C/ 400psi gas, N60/E.

En caso de conformidad con este presupuesto, rogamos nos envíen por escrito la aceptación del mismo.

Aceptado el presupuesto

IZASA, S.A. SERVICIO TECNICO CENTRAL Ramón Turró, 112-118 08005 BARCELONA

Francisco Reguant
DIVISION SERVICIO TECNICO

Sello y firma
Cliente

BELLATERRA, ____ de __________________ de _____

2 / 2

IZASA, S.A. Tecnología y Servicio
Ramón Turró, 112-118 - 08005 BARCELONA
Teléfono (+ 34) 93 401 01 27 - Fax: (+ 34) 93 401 03 30
Inscrita en el Registro Mercantil de Barcelona en hoja nº 56.158, folio 142, tomo 5284, libro 4590, sección 2ª de sociedades N.I.F. A-28114742

8.4. Technical specifications of equipment

GC-2010 Specifications:

Column oven	
Dimensions (mm) :	280(W) × 280 (H) × 175 (D)
Volume (L) :	13.7
Range of temperature :	Room temperature +4°C to 450°C -50°C to 450°C (When liquid carbon dioxide gas is used.)
Accuracy of temperature :	±1% (K) (Calibrated at 0.01°C)
Deviation of temperature :	Within 2°C (on a 200mm diameter column holder)
Stability of temperature :	Within ±0.05°C
Temperature coefficient :	0.01°C/°C
Range of linear temperature increase:	(in power voltage 100 VAC) 40°C/min up to 200°C 15°C/min up to 350°C 7°C/min up to 450°C (in power voltage 230 VAC) 70°C/min up to 200°C 50°C/min up to 350°C 35°C/min up to 450°C
Cooling speed :	Approximately 6 minutes cooling from 450°C to 50°C.
Overheat protection :	Programmable up to 470°C (A fixed circuit provides protection at 500°C)
Temperature program	
Program ramps :	20 ramps in total (Heating and cooling available)
Setting :	0.1°C increments
Program setting :	-250 to 250°C/min, 0.01°C/min increments
Total time of total program :	Up to 9999.99 minutes
Injection port	
Range of temperature :	Up to 450°C
Temperature setting :	0.1°C increments
Overheat protection :	Programmable up to 470°C
Injection unit :	Split/Splitless injection, Direct injection
Detector	
○ Hydrogen flame ionisation detector (FID)	
Range of temperature :	Up to 450°C, 0.1°C increments
Overheat protection :	Programmable up to 470°C
Minimum detection :	3pg C/s
Dynamic range :	107
Jet material :	Fused quartz
Time constant :	4 ms to 2 s selectable
Auxiliary heated zone	
AUX3 to AUX5 : Available (optional)	
Carrier gas flow control unit	
○ Split/Splitless mode	
Range :	0 to 970 kPa (The maximum pressure limit is the primary pressure minus 10 kPa.) 0.1 kPa increments

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Program ramps :	7 ramps possible
Program rate :	-400 to 400 kPa/min, 0.01 kPa/min increments
Split rate setting :	0 to 9999.9, 0.1 increments

○ Direct injection mode

Pressure mode

Range :	0 to 970 kPa (The maximum pressure limit is the primary pressure minus 10 kPa.) 0.1 kPa increments
Program ramps :	7 ramps possible
Program rate :	-400 to 400 kPa/min

Flow rate mode

Range :	0 to 1200 ml/min (When primary pressure is 980 kPa)
Program ramps :	7 ramps possible
Program rate :	-400 to 400 ml/min/min 0.1 ml/min/min increments

Detector gas flow controller

Range :	0 ~ 1200 ml/min (Air), 0.1 ml/min increments 0 ~ 200 ml/min (H2) 0 ~ 100 ml/min (Makeup He)
Program ramps :	7 ramps possible
Program rate :	-400 to 400 ml/min/min, 0.01 ml/min/min increments

Display

Back-light LCD 240 × 320 dot, 16 lines

The display can be switched between Japanese and English.

Dimensions, weight and power supply

Dimensions (mm) :	515 (W) × 440 (H) × 530 (D) mm
Weight :	30 kg
Power supply :	100 VAC (standard model and FID detector), 1800 VA, 50/60Hz 230 VAC (standard model with FID detector), 2600 VA, 50/60Hz

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

9. Comments

Hereunder the comments from revision 1 and 2 are annexed for additional information. Comments belonging to revision 1 are marked as ESA (1) and UAB (1) and comments belonging to revision 2 are marked as ESA (2) and UAB (2).

Page/paragraph	Comment
3/3.1.2	**ESA (1):** Maximum concentration of VFA in CII liquid input: We expect an amount around 5 g/L total VFA, 80% of the total VFA being acetic acid. If we estimate the average molar molecular weight around 65g/Mol for an average formula with 2.3 C atoms, then the corresponding C concentration in CII liquid input will be around 2gC/l. **ESA (2):** Please reflect this comment in the TN. **ESA (1):** If we understand properly, you have prepared a stock standard solution with 10 g/l of 6 various VFA, i.e. one standard solution with a total concentration of 60g/L VFA, is it correct? Then, you have diluted this solution to reach 0.01, 0.025…up to 4g/L of each VFA, i.e. 0.01X6=0.06 g/L …up to 4*6 =24 g/L total VFA, is it correct? **UAB (1):** Yes. **ESA (1):** If this is correct, then, according to our calculations (see attached table), we are working in the range of 0.032 up to 12.6 g C/L in your standard solutions. Our questions/remarks are: - Don't you think the dilutions you have performed (from 10g/L down to 0.01g/L, i.e. up to 1000X dilution) are challenging the accuracy of the concentrations? - **UAB (1):** Yes, in fact I made another stock solution containing 0.5g/L of each VFA to make the following solutions containing: 0.010, 0.025, 0.075, 0.1 and 0.2g/L of each VFA. Paragraph has been corrected to: "*Two stock standard solutions of a mixture of VFA at concentrations of 0.5g/L and 10g/L of each compound were prepared in MilliQ water. Final solutions of the following concentrations: 0.01, 0.025, 0.075, 0.1, 0.2 and 0.4, 0.5, 1, 2, 3, 4g/L of each compound were also prepared with MilliQ water from stock solutions of 0.5g/L and 10g/L, respectively.*" **ESA (1):** If our assumptions are correct, then we are not in the calibration range you mention (0.01 to 2 gC/L); please clarify. **UAB (1):** In the GC, VFA are separated and analysed one by one. If you expect 2gC/L of total VFA in the sample, there must be less

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

	than 2gC/L of each VFA, and the sum of all of them will result in 2gC/L. Therefore, each VFA will be in a possible range of 0.01 to 2gC/L. This is the range that has been used for calibration. If all VFA are put in the same vial it does not affect the result, because in the GC they will be separated. **ESA (2):** If we understand correctly, you wanted to check concentrations of each VFA individually up to 2gC/L, and you did it with a mixture of VFA, as you assume having a mono-VFA or a multi-VFA solution does not interfere with the quality of your calibration? **UAB (2):** Yes. In fact, that is why a mixture of VFA can be analysed in a real sample, because GC is capable of separating each VFA individually and analyse it one by one, regardless the number of different VFA's that the sample contain or whether they are at the same concentration or not. Under this basic, standard samples are prepared with a mixture of VFA's. They could have been prepared with one VFA individually. But this does not affect the analysis. **ESA (2):** If this is correct, please state it clearly in the TN. **UAB (2):** The following paragraph has been added: "*GC is capable of separating each VFA and analyse them one by one, regardless the number of different VFA's that the sample contain or whether they are at the same concentration or not. Under this basic, standard samples are prepared with a mixture of VFA's. Each standard sample could have been prepared with one VFA, but the number of components per sample does not affect the analysis.*" **ESA (2):** However, one remark: it is not expected to have all VFA at the same concentration in the " real" liquid phase. It could be then extremely relevant to calibrate your GC with a synthetic solution containing VFAs at a respective concentration which is more representative of your study case. **UAB (2):** This is not going to change the results because VFA are separated and analysed one by one in the GC depending on each VFA calibration curve, regardless if they are at the same concentration or not.
5/3.3.2	**ESA (1):** Why do we have a conc. Range of 25-400 mg/L whereas your dilutions correspond to 500 mg/L? **UAB (1):** Because we made a mistake. There is another standard solution of 400mg/L of each VFA I did not mention in the document. This paragraph has been corrected in paragraph 3.1.2, page 3.
6/3.3.3	**ESA (1):** Please correct RSD instead of RDS. **UAB (1):** Corrected.

	Can you comment the range of 0.3 to 16% RSD with regards to the average RSD values that are around 3 to 6 in table 2. **UAB (1):** Reproducibility of samples was studied buy a number of standard samples, that where analysed in triplicate. The RSD values are calculated for each sample. These values range from 0.3 to 16%, the highest RSD corresponding to the lower concentrations for acetic acid and propionic acid at split 10 (the specific data for these are provided in "8. Appendix" tables 8 and 9). In addition, an AVERAGE RSD was calculated by averaging all RSD values of a given VFA. These AVERAGE RSD are reported in table 2, and range between 3 and 6%, depending on the specific VFA. Paragraph 3.3.3 is rephrased to: "*Precision was studied by measuring the reproducibility of peak areas. Reproducibility was measured by calculating the relative standard deviation (RSD) of peak areas for three repeated analysis of each sample. Results of RSD of individual samples of all different concentrations range from 0.3 to 16%. The highest RSD values correspond to the lowest acetic and propionic acids concentrations analysed at a split ratio of 10. In addition, average values of RDS of each VFA were calculated from all the RSD values for a given VFA. These averages RSD are represented in table 2, and range from 3 to 6%.*" **ESA (1):** The analysis at split ratio 10 is more accurate ONLY for concentrations > 200 mg/L, please rephrase. **UAB (1):** Rephrased in paragraph 3.3.3 as it is shown below. **ESA (1):** Again, how do we have values for 400 mg/L with a standard solution at 500 mg/L? **UAB (1):** This has been corrected in paragraph number 3.3.2 page 5 to: "*For concentration ranges 0.025g/L-0.4g/L (25, 75, 100, 200 and 400 mg/L) and 0.1g/L-4g/L (0.1, 0.5, 1, 2 and 4g/L) split 10 and split 40 were used respectively. Correlation factors (R) were calculated for each compound at each split ratio (table 2).*" **ESA (1):** You mention the relative error at split ratio 40 for a concentration of 100 mg/L. This cannot be found in table 3. **UAB (1):** This is in "8. Appendix" from table 12 to 19. **ESA (1):** The optimal range of concentration used in the analysis at split ratio 40 must be from 200 to 4000 mg/L : this statement is not consistent with your previous comments, as we are more accurate at split ratio 10 for 200 and 400 mg/L. Please clarify. **UAB (1):** Although the analysis of concentrations ranging from 200 to 400mg/L is more accurate at split ratio 10, concentrations from 200mg/L to 500mg/L can be analysed at split 40, because the error

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

	is acceptable (see table 3). **ESA (1):** Please add references to your appendixes. **UAB (1):** Paragraph 3.3.3 in page 6 has been corrected to: "*It is remarkable that at split ratio 40 the relative error calculated for the lowest concentration (100mg/L) is considerably high (see section "8 Appendix" tables 12 to 19). Therefore, the optimal range of concentration used in the analysis of VFA at split 40 must be from 200 to 4000mg/L of compound. Although the analysis of concentrations ranging from 200 to 400mg/L is more accurate at split ratio 10, concentrations from 200mg/L to 500mg/L can be also analysed at split 40, because the error is acceptable (see table 3 below).*" **ESA (1):** Can you comment the results you have obtained? After an optimisation process we could obtain good results. **ESA (2):** Please detail and insert your conclusions in the TN **ESA (1):** According to your expectations? **UAB (1):** Yes. **ESA (1):** Consistency with supplier's information? **UAB (1):** Yes. **ESA (2):** Please detail a bit and include this information in the TN. **UAB (2):** A new paragraph: "3.4 Conclusions" has been added to this section.
7/4.2	**ESA (1):** Table 4: the RSD of 1.86% you have obtained is not consistent with the 6% mentioned in table 2, please clarify. **UAB (1):** The explanation is the same of point 6/3.3.3. 6% is the AVERAGE RSD value for acetic acid at split 10. However, individual RSD values range from 0.6 to 16% (see "8.Appendix" table 8).Therefore the individual value of RSD 1.86% is consistent with the AVERAGE value of 6%, because it is in the range of individual values.
8/5.2.1	**ESA (1):** Do you use solutions with ONE VFA or with a mixture of them, each of them being at a concentration of 0.1 g/L? **UAB (1):** Yes, it is a mixture, each of them at a concentration of 0.1g/L. **ESA (2):** Please clarify it in the TN (mixture of three VFA?) **UAB (2):** Sentence rephrased to: *"Standard solution of a mixture of three VFA at 0.1g/L each compound was analysed on-line using the flow cell 1."*
8/5.2.2	**ESA (1):** The equation that defines….please rephrase your sentence. **UAB (1):** Paragraph has been modified to: "*To estimate the*

	Volume/Distance ratio we calculate the loop volume as a function of distance D (see equation 1 below). Once the loop volume has been determined by equation 1, dead time of flow cell ($T_{d,FC}$) can be determined with the loop volume previously calculated and the volumetric flow used."
9/determination of the ratio vol/distance	**ESA (1):** Last sentence; you mention a relative error of 2% whereas 1.5 is mentioned in table 6, please clarify. **UAB (1):** 1.5% is round up to 2% because it is an error value. **ESA (2):** Please clarify in the TN. **UAB (2):** The following note has been added: *"[1] The value shown in table 6, 1.5%, is round up to 2% because it is an error value."*
10	**ESA (1):** You mention T=1.2+/- 0.4 min. Do you mean that, as Q is varying from 5 to 10 ml/min, T will vary from 0.8 to 1.6 min? **UAB (1):** Yes **ESA (1):** If this is correct, please rephrase your sentence the meaning of 'tentative interval of dead time' being confusing. **UAB (1):** Sentence rephrased to: "*If we take an interval of possible values of Q from 5 to 10mL/min, the interval of dead time will be the following:"*

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP