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Report

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SPME as a tool in WEA – CONCAWE Contribution to OSPAR Demonstration Project 2005-2006

Final Report on Measuring Potentially Bioaccumulative Substances in Effluents: Interlaboratory Study Workshop and Review

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Foreword

OSPAR Convention for the Protection of the Marine Environment of the North East Atlantic formed a Hazardous Substances Committee (HSC) to facilitate the implementation of the OSPAR Strategy with regard to Hazardous Substances. This strategy includes the development of programmes and measures to identify, prioritize, monitor and control (i.e., to prevent and/or reduce and/or eliminate) the emissions, discharges and losses of hazardous substances that reach, or could reach, the marine environment.

Within the HSC, an Intersessional Expert Group (IEG) on Whole Effluent Assessment (WEA) was formed to develop and execute a work programme on the topic of 'Persistence and Bioaccumulation'. A subdivision of the IEG, Product Team II, 'Bioaccumulation Methodology', is focusing on comparison of extraction and bioaccumulation estimation methods. The current study, funded by CONCAWE and coordinated by the Netherlands Institute for Fisheries Research (RIVO), examined two methods: the Swedish "Extractable Gas-chromatographic Organic Material" liquid-liquid extraction (EGOM-LLE) and biomimetic solid-phase microextraction (SPME), in order to assess their relative merits and shortcomings for assessing potentially bioaccumulatable substances in effluents.

This document represents a compilation of various data and deliverables from the study programme. An executive summary is followed by the presentation of data generated in an interlaboratory study of effluents assessed using both EGOM-LLE and biomimetic SPME methods (Part 1). The proceedings of a workshop at which the results of the studies were discussed is presented in Part 2.

Further information is given the appendices, which include a detailed initial review and comparison of the two analytical methods (pre-laboratory study), the analytical protocols used for the EGOM-LLE and biomimetic SPME methods, and the chromatograms and report information from the laboratories that participated in the ring test. The appendices were supplied to the IEG and are available at RIVO but have not been circulated to OSPAR Hazardous Substances Committee (HSC).

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Executive Summary

Two extraction methods to screen and assess whole effluents for potentially bioaccumulating substances (PBS) being considered by the Intersessional Expert Group on Whole Effluent Assessment, Hazardous Substances Committee of OSPAR were examined in this study programme. Both methods were tested in parallel on 5 different samples, including STP and refinery effluents, as part of a ring test (Part 1). Five laboratories from 3 European countries and the US participated in the ring test. A literature review of the methods (Appendix 1) acts as a background document describing and comparing the two methods: the conventional liquid-liquid extraction (LLE) method using the Swedish 'EGOM' approach[1] and the solid-phase microextraction (SPME) performed according to the 'biomimetic' approach. Detailed protocols for both methods were prepared for the ring test (Appendices 2 and 3). A final workshop attended by participants in the ring test and members of the IEG on WEA was organised to discuss the results, the output of which is described in Part 2.

The limits of detection of PBS in effluents using SPME in this study were between 1 and 1.5 mmol/L poly(dimethylsiloxane) (PDMS), with blank values typically <0.4 mmol/L PDMS. Blanks were lower (ca. factor 10) than the values achieved for demineralized water in the SPME method. For EGOM-LLE, the limit of detection (LOD) is around 0.2 mg organic carbon/L effluent (three times a typical blank value). Blank EGOM-LLE values varied in this study between 0.085 and 0.127 mg OC/L. Although the different units make direct comparison of LODs difficult, we can say that all laboratories were able to detect PBS in all solutions and effluents provided using the SPME method, whereas the EGOM-LLE method required all laboratories to measure very close to limits of detection, and in many cases, no PBS could be identified and quantified. Generally, the SPME method could clearly distinguish a complex refinery effluent and a low-level sewage treatment plant effluent or low-level spiked water. The EGOM-LLE method could distinguish between refinery and sewage treatment plant effluents when laboratories could quantify the amounts. Both methods were comparable in repeatability and reproducibility.

The interlaboratory exercise and workshop brought forth a number of technical aspects of performing EGOM-LLE and biomimetic SPME. Both techniques use similar gas chromatographic-flame ionization detection (GC-FID) methodology for determination of the potentially bioaccumulating substances (PBS). However some principle technical advantages of SPME over EGOM-LLE include: fewer extraction steps, smaller volumes of effluent and organic solvents needed, significantly more efficient in terms of resources and time needed to assess effluents (ca. 50% less was the experience in the ring-test) and its potential for automation (already implemented by ExxonMobil, see Appendix 7). Furthermore, the current SPME protocol as used in the ring-test showed significant improvements in background signals over previous experience.

Besides comparisons of the methods in terms of technical issues, it is important to note that each method uses a significantly different and distinct extraction approach. The EGOM-LLE method is a more exhaustive extraction than biomimetic SPME, meaning that bioavailability or differences in bioconcentration factors are not taken into account in the EGOM-LLE method. In the context of a "B" (bioaccumulation) evaluation of effluents, for assessing effluents on a mass basis, (exhaustive-PBS) then EGOM-LLE is a useful technique, because chemicals sorbed to organic carbon or particulate matter may also be extracted with EGOM-LLE (although extraction efficiency will depend on the strength of this binding). In particular if there is concern about an effluent and further work is required, EGOM-LLE could be seen as one of the tools supporting further investigation. Some additional method development (minor adaptations to protocol) may be desirable, as suggested at the workshop. Within WEA and for a "B" screening of effluents and for highlighting those that need further investigation, non-exhaustive biomimetic SPME is the favoured technique because this approach takes into account bioavailability and the fiber's

[1] EGOM stands for Extractable Gas-chromatographic Organic Matter

accumulation patterns simulate bioconcentration in biota. This has an added advantage of making it easier to relate this PBS to narcotic toxicity, which may be observed in toxicity tests if PBS is high enough.

In conclusion, in order to select effluents of concern in a WEA of "B", OSPAR requires a screening method that:
can detect and discriminate between low to high levels of potentially bioaccumulating substances (PBS) in effluents;
is reproducible and repeatable; and which can provide data that are meaningful in assessing what could potentially be accumulating in organisms. This report supplies experimental data, background theory and information from literature, a discussion of applications and limitations of the two methods.

List of Abbreviations

AOC adsorbable organic carbon
AOX adsorbable organic halogen
BCF bioconcentration factor
DOC dissolved organic carbon
EGOM extractable gas-chromatographic organic material
EOC extractable organic carbon
EOX extractable organic halogen
GC-FID gas chromatography-flame ionisation detection
GC-MS gas chromatography-mass spectrometry
HPLC high performance liquid chromatography
K_{ow} octanol-water partition coefficient
$K_{PDMS\text{-}water}$ PDMS-water partition coefficient
$K_{SPM\text{-}water}$ SPM-water partition coefficient
LLE liquid-liquid extraction
LOD limit of detection
PBS potentially bioaccumulatable substances
PDMS poly(dimethylsiloxane)
POC purgeable organic carbon
POX purgeable organic halogen
pp-LFER polyparameter linear free energy relationship
RMR relative molar response
RSD relative standard deviation
SOC suspended organic carbon
SPE solid phase extraction
SPM suspended particulate matter
SPME solid-phase microextraction
TBR_{est} total body residue, estimate
TOC total organic carbon
VOC volatile organic carbon
VOX volatile organic halogen
WEA whole effluent assessment
XAD (polymeric resin beads that scavenge organic compounds)

Part 1. Interlaboratory trial SPME and EGOM-LLE of water and whole effluents

1.0 Overview of interlaboratory exercise

The interlaboratory exercise was primarily performed to help increase proficiency of the SPME analysis in laboratories participating in the interlaboratory study and to compare this method to the EGOM-LLE method. An overview and review of both methods is provided in Appendix 1. The approach chosen to assess both methods was to prepare a number of samples ranging from simple to complex, i.e. distilled water, distilled water spiked with a small chemical set, effluent, and effluent spiked with the same chemical set as for distilled water and to have different participant laboratories perform EGOM-LLE and SPME on each. Participating laboratories included: RIZA and RIVO (the Netherlands), VITO (Belgium), UFZ (Germany) and ExxonMobil (USA). ExxonMobil performed no EGOM-LLE but did perform the SPME both manually and using an automated system. Protocols for the SPME methods (Appendix 2) and the EGOM-LLE method (Appendix 3) were prepared and sent to participants for use in the interlaboratory exercise.

In addition, using the SPME method, an uptake curve of a low level effluent in the SPME fibers was made. A repeatability test was also performed (three fibers together in one sample bottle versus in different sample bottles).

1.1 Preparing Samples

The samples sent were sent to the participants of the interlaboratory exercise in 1-L glass bottles. The solutions included:
a) Demineralised water
b) A "low level" sewage treatment plant effluent
c) Demineralised water spiked with mixture
d) A "low level" sewage treatment plant effluent spiked with mixture
e) A complex refinery effluent

An effluent sample from a sewage treatment plant in Velsen, The Netherlands was collected as low-level effluent; Shell kindly provided a complex refinery effluent. Solutions of the test set were prepared using a generator column for the least water-soluble components to ensure that these compounds were completely dissolved, as well as the more water soluble components to be spiked. The set of chemicals used for spiking spanned a range of log K_{ow} values and include characteristics such as volatile and ionisable chemicals in order to determine the limits of the methods for PBS analysis of effluents (Table 1-1). Spiked water was used to 'dilute' the Velsen STP effluent 1:1 since chemicals could not be spiked directly to the effluent itself due to the necessity of using a generator column for the highly insoluble compounds.

Table 1-1. Compounds for spiking exercise in demineralised water and 'low level' effluent with partition coefficients for octanol and PDMS to water. Log K_{ow} values include calculated and estimated values. Log $K_{PDMS\text{-}water}$ values are either measured values from the literature or calculated from the log K_{ow} with the equation of Mayer *et al.* (2000). Maximum water solubilities, S_w max, are experimental data from literature except musk xylene and tetrachloroaniline, which were calculated with Wskowwin version 1.41 (US EPA).

Compound	log $K_{PDMS\text{-}water}$	S_w max (mg/l)	Comment	log K_{ow}
Quinoline	0.9*	6110	slightly polar	2.0
2,3-Dichlorophenol	2.2*	3600	polar	2.8
1,2-Dichlorobenzene	2.2	150	nonpolar	3.4
Pentane	2.5	38	nonpolar, very volatile	3.5
1,2,3-Trichlorobenzene	3.1	18	nonpolar	4.0
Musk xylene	3.4*	0.82	nonpolar	4.5
2,3,4,5-Tetrachloroaniline	3.2	9.5	polar	4.5
Fluoranthene	4.1	0.26	Nonpolar	5.3
Hexachlorobenzene	4.8	0.005	Nonpolar	5.7
p,p'-DDE	5.9	0.0055	nonpolar	7.0

1.2 Results and Discussion of Interlaboratory Exercise

The chromatograms and individual reports of each laboratory are included in Appendices 4-8.

EGOM-LLE

Four laboratories provided results for EGOM-LLE (Table 1-2). Clear problems with detection limits were encountered by two of the four laboratories. The two laboratories that did report results were measuring near limits of detection. Blank EGOM-LLE values varied in this study between 0.085 and 0.127 mg organic carbon/L. The limit of detection for this method is around 0.2 mg organic carbon/L effluent (three times a typical blank value). The EGOM-LLE method was able to distinguish between the refinery effluent and lower level effluents. However, it is not possible to distinguish between demineralised water and the STP effluent due to detection problems. The relative standard deviations (RSDs) of this method are generally quite low (≤24 % for the laboratories that were consistently able to detect organic carbon in the effluents (Table 1-3). Serious emulsion problems were reported which may be alleviated with some adaptation to the method protocol, for instance using a different solvent than cyclohexane (e.g. dichloromethane). This is also discussed in Part 2.

Table 1-2. Concentrations of PBS (mg organic C/L effluent) in test solutions per laboratory measured by the EGOM-LLE method. ND is not detected. (LOD ca. 0.2 mg organic C/L)

Effluent type	UFZ	VITO	RIZA	RIVO
Water	ND	0.133	0.305	ND
	ND	0.136	0.378	ND
	ND	0.143	0.272	ND
STP	ND	NR	0.272	ND
	ND	0.129	0.288	ND
	ND	0.149	0.261	ND
spiked water	ND	0.348	0.289	ND
	ND	0.462	0.291	ND
	ND	0.460	0.250	ND
spiked STP	ND	0.247	0.264	ND
	ND	0.192	0.281	ND
	ND	0.220	0.398	ND
refinery	ND	2.30	3.861	ND
	0.667	2.23	4.383	ND
	1.27	2.24	4.372	ND
blank		0.085 (min) 0.116 (max)	0.127	

Table 1-3. Summary of PBS concentrations determined by EGOM-LLE (mg organic C/L effluent) with RSD in parentheses. ND is not detected.

Effluent type	UFZ	VITO	RIZA	RIVO
Water	ND	0.14 (3.7%)	0.31 (24%)	ND
STP	ND	0.14 (10%)	0.27 (5%)	ND
spiked water	ND	0.42 (15%)	0.27 (10%)	ND
spiked STP	ND	0.22 (13%)	0.31 (23%)	ND
Refinery	0.97 (44%)	2.26 (1.7%)	4.12 (8.8%)	ND

SPME

Five laboratories provided results (Table 1-4). ExxonMobil provided additional data on automated extractions using a 30 µm PDMS fiber exposed to the solution on the autosampler just prior to injection (Table 1-4). The limits of detection of PBS in effluents using SPME in this study were between 1 and 1.5 mmol/L PDMS, with blank values typically <0.4 mmol/L PDMS. Blanks were lower (ca. factor 10) than the values achieved for demineralised water in the SPME method. As with the EGOM-LLE method, it was difficult to measure differences in PBS between a low level STP effluent and the demineralised water. Generally, the low-to-high PBS trends observed were similar to the EGOM-LLE.

Reproducibility and repeatability. The literature indicates reproducibility and repeatability can be as high for normal SPME extractions of single chemicals as for normal liquid injections onto a GC. The introduction of matrix (as in real effluent samples) will affect the system, but does not necessarily have to hinder the analysis or reproducibility. In an experiment addressing repeatability/reproducibility, three fibers were exposed in a single 1-L glass bottle to compare the RSD to results obtained in three separate smaller bottles. This has not been standard procedure for commercial fibers but there has been experience with multiple disposable fibers being exposed simultaneously to the same solution at Utrecht University (IRAS) for example.

The three-in-one fibers gave peak areas of 4755, 4655, 4236. The average is 4549 and the RSD 6%, which is very low, as low as most liquid injections. However, very similar results were also achieved in fibers exposed separately to the same effluent: 4960, 4660 (third fiber broken before analysis). From this data, it can be concluded that fibers exposed together or separately will give the same results and will not significantly improve or worsen the repeatability. With this result arises the idea of multiple fibers per sample bottle, which has a few advantages over performing three extractions of separate bottles (fewer sample bottles, extraction possible in same bottle sample is delivered in, extra fiber can be included to act as back up for possible breakage of fibers, fewer magnetic stirrers occupied per fiber, etc.). A criterium for multiple fibers per sample bottle is that PDMS:water volume ratio is conserved to avoid significant depletion.

As can be seen in the ExxonMobil data, results from automated fiber exposures are very repeatable, although these were performed in duplicate instead of triplicate as the manual protocol prescribed (Table 1-5). Some problems with detection limits are seen, as only the manual method appears to be able to detect any of PBS for the first two test samples. Very low level effluents tend to have higher RSDs (Table 1-5).

Uptake curve. An uptake curve of the STP effluent was made to yield important information about the time to equilibrium in more complex solutions (Fig 1-1). From this and other work performed at RIZA (p. 24 in Kienhuis 2004, Appendix 1 reference), it can be concluded that uptake in the fiber continues after 24 hours. The advantages of a 24 hour exposure period using the current manual injection method are described in Part 2. Furthermore, it is clear that a large proportion of the total mixture is taken up in the 24 hour period. Disadvantages of lengthening the fiber exposure period include changes (resulting in transformation of chemicals) that occur in the effluent sample itself.

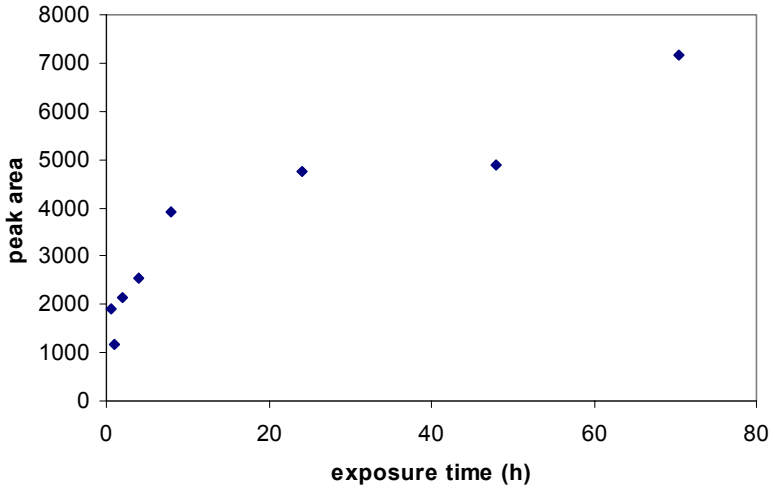

Figure 1-1. Uptake curve of PBS from STP effluent in SPME fibers (100 µm PDMS) under conditions of constant stirring at room temperature.

Table 1-4. Concentrations of PBS (mmol/L PDMS or µmol/ml PDMS) in test solutions per laboratory measured using the biomimetic SPME method. *data measured using 30 µm PDMS fibers according to ExxonMobil automated method. ND is not detected.

Effluent type	UFZ	VITO	RIZA	RIVO	ExxonMobil
water	2.49 1.76 1.87	32.62 29.07 14.08	1.56 3.09 2.30	6.69 8.48 6.76	ND* 0.527 0.982
STP	2.22 1.49 1.61	5.92 4.96 3.69	3.05 1.83 2.10	4.85 4.99 4.96	ND*
spiked water	5.93 6.46 5.80	6.31 9.78 8.30	14.11 14.60 9.83	4.48 4.23 6.44	7.08* 7.27* 8.59 10.6
spiked STP	3.36 2.67 3.26	6.29 6.00 5.26	4.68 5.70 5.32	5.76 4.15 3.93	3.43* 3.42*
refinery	27.6 24.3 21.3	33.23 38.75 33.81	23.32 10.39 42.42	28.7 56.1 52.0	22.4* 21.9* 29.3 28.7
blank	1.14 0.33 0.57	0.22 0.18 0.22	0.16	0.30	

Table 1-5. Summary of PBS concentrations determined by SPME (mmol/L PDMS or µmol/ml PDMS) with RSD. *data measured using 30 µm PDMS fibers according to ExxonMobil automated method. ND is not detected.

Effluent type	UFZ	VITO	RIZA	RIVO	ExxonMobil
Water	2.04 (19.3%)	25.26 (39%)	2.32 (33%)	7.31 (13.9%)	ND* 0.75 (43%)
STP	1.72 (22.1%)	4.85 (23%)	2.33 (27%)	4.93 (1.5%)	ND
spiked water	6.06 (5.8%)	8.13 (21%)	12.85 (20%)	5.05 (24.0%)	7.18 (1.9%)* 9.60 (14.8*)
spiked STP	3.10 (12.0%)	5.85 (9%)	5.23 (9.9%)	4.61 (21.7%)	3.43 (0.21%)*
Refinery	24.4 (12.9%)	35.26 (9%)	25.38 (64%)	45.6 (32.4%)	22.2 (1.6%)* 29.0 (1.46%)

This interlaboratory exercise brought forth a number of technical aspects of performing EGOM-LLE and biomimetic SPME (see also Part 2 of this report for further reporting on detailed discussion points handled during the workshop). Both techniques use similar gas chromatographic-flame ionization detection (GC-FID) methodology for determination of the potentially bioaccumulating substances (PBS). However some technical advantages of SPME over EGOM-LLE confirmed during the ring-test include: fewer extraction steps, smaller volumes of effluent and organic solvents needed, significantly more efficient in terms of resources and time needed to assess effluents (ca. 50% less was the experience in the ring-test) and its potential for automation to measure "B" in effluents, which has been demonstrated by ExxonMobil, see also Appendix 7). The current protocol, as used in the ring-test, for SPME showed significant improvements in the background over previous experience.

1.3 Discriminatory power of SPME and LLE

Although the different units make direct comparison of LODs difficult, we can say that all laboratories were able to detect PBS in all solutions and effluents provided using the SPME method, whereas the EGOM-LLE method required all laboratories to measure very close to limits of detection, and in many cases, no PBS could be identified and quantified. Generally, the SPME method could clearly distinguish a complex refinery effluent and a low-level sewage treatment plant effluent or low-level spiked water. The EGOM-LLE method could distinguish between refinery and sewage treatment plant effluents when laboratories could quantify the amounts. Both methods were comparable in repeatability and reproducibility.
As these are screening methods, they are not designed to maximize discriminatory power but give a quick answer to the "B" question. An example of how to interpret SPME results could be that <5 mmol/L PDMS represents very low level of PBS (clean effluents of no concern); 5-20 mmol/L PDMS low level PBS effluent; >20 mmol/L PDMS high level PBS effluent of concern; >40 mmol/L PDMS – acute narcotic toxicity expected based on this level of PBS (cf. data Parkerton *et al.* 2000).

1.4 Applicability for WEA

In order to select effluents of concern for "B" in a WEA, OSPAR requires a screening method that:

- can detect and discriminate between low and high (potentially toxic) levels of potentially bioaccumulating substances (PBS) in effluents;
- is reproducible and repeatable;
- and which can provide data that are meaningful in assessing what could potentially be accumulating in organisms.

Both methods could discriminate between different effluents, provided they were not too close to detection limits. In general, it is straightforward to determine the difference between a low level effluent that will not contribute to enough "B" to elicit narcotic toxicity, and higher level effluents approaching the "B" range where narcotic toxicity may start to take effect. The reproducibility of the EGOM-LLE test is high and the SPME method has shown improvement in this regard compared to SPME studies in the past. For this type of broad screening method requiring integration of the sum total peak areas, the reproducibility is acceptable. The laboratories show good agreement in the values reported with some exceptions, as mentioned above and also discussed in Part 2.

Besides considering the performance of the methods, it is important to note that each method uses a significantly different and distinct extraction approach. The EGOM-LLE method is a more exhaustive extraction than biomimetic SPME, meaning that bioavailability or differences in bioconcentration factors are not taken into account in the EGOM-LLE method (see Appendix 1). In the context of a "B" (bioaccumulation) evaluation of effluents, for assessing effluents on a mass basis, (exhaustive-PBS) then EGOM-LLE is a useful technique, because chemicals sorbed to organic carbon or particulate matter may also be extracted with EGOM-LLE (although extraction efficiency will depend on the strength of this binding). In particular if there is concern about an effluent and further work is required, EGOM-LLE should be seen as one of the tools that could be used. Some additional method development (minor adaptations to protocol) may be desirable, as suggested at the workshop. For screening effluents and for highlighting those that need to be further investigated, non-exhaustive bioimimetic SPME is the favoured technique as this approach takes into account bioavailability and the fiber's accumulation patterns simulate that in biota. This has an added advantage of making it easier to relate this PBS to narcotic toxicity, which may be observed in toxicity tests if PBS is high enough. It is recommended to continue with the implementation of the SPME method as a primary tool for assessing "B" in whole effluent assessment, with the EGOM-LLE technique in the optional toolbox.

Part 2. Biomimetic SPME/EGOM-LLE in WEA Workshop Output Reporting

26 January 2005 RIVO, IJmuiden 10am-4pm

This chapter describes the output of the workshop with participants of the ring test described above of the biomimetic SPME and EGOM-LLE methods (protocols in Appendices 2 & 3).

2.0 Summary & Conclusions of Workshop

In order to select effluents of concern in a WEA of "B", OSPAR requires a screening method that:

a) can detect and discriminate between low to high levels of potentially bioaccumulating substances (PBS) in effluents
b) is reproducible and repeatable
c) provide data that are meaningful in assessing what could potentially be accumulating in organisms.

Both methods were able to identify a high PBS level complex refinery effluent. However, EGOM-LLE showed a large number of nondetects in the low level effluents in certain laboratories. All the data were near the limit of detection, and therefore the results may be less reliable. All laboratories were able to distinguish between the different low level effluents using the SPME method. Blanks were lower (ca. factor 10) than the values achieved for demineralized water in the SPME method. Both methods were comparable in repeatability and reproducibility. The variation between triplicate measurements was generally lower for the LLE extractions however than for SPME, with some exceptions. The SPME variation between triplicates is improved in comparison to results of ring tests in the past. It can also be concluded that the patterns observed in the different chromatograms for the SPME extractions are very similar among the different laboratories. It is known from previous research with SPME fibers including those with PDMS coatings that the patterns observed in SPME fibers and biota (before any biotransformation) are similar (see Review in Appendix 1). The EGOM-LLE method is not designed to reflect the pattern of chemicals in biota as biomimetic SPME is, and therefore for point c, SPME would be a method of choice. However, EGOM-LLE would be more useful than biomimetic SPME for determination of total emissions.

2.1 Workshop Objectives

- Discuss SPME/EGOM-LLE round robin exercise
- Comment on the ease/resource
- Produce a brief report of the findings
- Discuss the applicability

2.2 Workshop Agenda

1. Welcome & introduction of workshop attendees
2. Background: OSPAR work programme/CONCAWE support
3. WEA in OSPAR
4. Workshop Objectives
5. Overview of SPME and EGOM-LLE methods
6. Ring test results – SPME & LLE
7. Uptake curve and repeatability tests results
8. Discussions & recommendations for further development

9. Brief summary of workshop output - discussion for production - who and when
10. SETAC poster - brief discussion re content

2.3 Participants

Workshop participants	email address
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Tom Parkerton (ExxonMobil, USA)	Thomas.f.parkerton@exxonmobil.com

2.4 Background of the OSPAR Work Programme

The study under discussion at the workshop was conducted under auspices of the Intersessional Expert Group (IEG) with Marijke Ferdinandy as the chairperson. The IEG would need to make a recommendation for a 2006 work programme to the HSE, which next meets at the end of April. In order to be discussed at that meeting documents needed to circulated mid-March. It was noted that CONCAWE were providing RIVO with financial support to conduct the ring-test and carryout the literature review (Appendix 1).

2.5 Summary of EGOM-LLE Discussion Points

- **Protocol.** Except evaporation until dryness the EGOM-LLE method was followed according to the original protocol from Sweden.
- **Low MW analytes.** A large solvent peak at the beginning of the chromatogram masks the presence of low molecular weight compounds extracted from the effluents with LLE. This limits the range of compounds which are visible to approximately those chemicals with log K_{ow}>3.
- **Emulsion formation** was a problem for at least three laboratories in the LLE method. RIZA noted that emulsion formation was not pH –dependent. It is unclear what the emulsion consists of. In extractions of biota, this can be protein where compounds may or may not be present in significant amounts. In the LLE extracts, it is unclear if the losses of compounds due to the emulsions were significant.
- **Blanks** measured for this method were between 0.085 and 0.127 mg C/L effluent. The values for effluents were very close to these blank values (0.2 mg C/L could be a reasonable limit of detection, being 3 times the average blank value). Limits of

detection prevented two of the four laboratories from attaining detects for with the EGOM-LLE "PBS" method. However, it should be borne in mind that these effluents were not selected primarily for the testing of LLE (LLE method was added to the study after it had been designed for SPME). Any further work on EGOM-LLE might include selection of different effluent composition for testing.

- **Solvent choice.** Some of the difficulties observed (*e.g.* emulsion formation) may be alleviated were a different solvent used. For example dichloromethane is known to be less prone to the formation of emulsions when used for extracting purposes than cyclohexane. However, as the protocol was used as originally received and an investigation of alternative extraction solvents were not within the scope of this study. RIZA noted that the polarity of the extraction solvent is a factor known to impact the efficiency of the extraction. Cyclohexane was chosen because of the original desire to combine the LLE approach with strategies to determine chlorinated contaminants with GC-ECD for example – in which case a halogenated solvent such as dichloromethane would not be suitable; however, this solvent is considered better for extracting a wide range of contaminants (polar and nonpolar). Some peak splitting problems were also reported.
- **Variability.** It is notable that the variability is low for the complex refinery effluent (Effluent E).
- The EGOM-LLE method is a new method for the participating laboratories; results may improve with more experience.
- **Extraction of dissolved phase with SPM present.** VITO will be performing the LLE method on five effluent samples in the near future and it was discussed what phase should be measured, effluent including suspended solids or not. The suggestion to make a mass balance of the whole effluent, vs. separate analysis of SPM and centrifuged water phase was considered to be too difficult for such a rough screening procedure. This was also the experience in the LOES project.

2.6 Summary of SPME Discussion Points

- **Blank fiber** values were generally low, between 0.16 (min) and 1.14 (max), but typically <0.4 mmol/L PDMS. A limit of detection for the SPME method would be expected to be between 1 and 1.5 mmol/L PDMS for most series of analyses.
- **Background SPME values in deminieralized water.** The extraction of demineralized water was high for VITO (>14 mmol/L PDMS) and for RIVO (between 6 and 9 mmol/L PDMS) for reasons that are not known.
- **Identification of complex effluent.** SPME results from all laboratories showed a major difference between the STP effluent and the complex refinery effluent. The levels detected in the complex refinery effluent were in the range expected for such an effluent.
- A trend showing that Effluent C is higher than D for three laboratories was noted.
- **Discriminating power** of SPME as a screening method for PBS can be viewed as *e.g.*

  <5 mmol/L PDMS very low level of PBS (clean effluent)

  5-20 mmol/L PDMS low level PBS effluent

  >20 mmol/L PDMS high level PBS effluent

  >40 mmol/L PDMS – narcotic toxicity expected based on this level of PBS (cf. data Parkerton *et al.*)
  One participant suggested that the SPME should be more sensitive than a toxicity test.

However the broad conclusion is that there are trends in terms of differences between background blanks, clean water, a low level STP effluent and the complex refinery effluent.

– **Chromatogram Patterns.** An important observation is that the patterns appearing in chromatograms are similar for all the laboratories (cf previous chapter and Appendices 4-8).

2.7 Quality Control in SPME analysis

A discussion about some of the factors that impact the reproducibility in SPME yielded the following points;

– While no systematic variance was observed in SPME fiber performance, it is important to examine fibers before using them for visible damage to the coating or breakage of the fiber. Fibers have a limited lifetime. Often they are broken off the devices before reaching the stage that the polymer performs badly. An alternative to reusable SPME fibers are disposable fibers. These are discussed as something to keep in mind for the long term. A recent paper by Hafka (university of Amsterdam) was cited. Disposable fibers are known from a large number of papers published by researchers at Utrecht University as well.
– RIZA has used standard solutions in the past to test fiber performance. A report from 2003 was later circulated to workshop participants from RIZA with details of this and other aspects of the analysis.
– In the current study, VITO measured a blank fiber with GC-FID directly prior to exposure to the test solution, achieving a blank for every fiber measured. Their experience indicated that this value was always low and relatively constant at about 0.2mmol/L fiber.
– VITO will circulate electronically their information on fiber performance to workshop participants.
– RIVO no longer measures a blank for every fiber after experience has shown that the fiber blanks are consistently low.
– Calibration reference was mentioned as a standard sample which could be measured in each series to test the performance in each series of analyses.

2.8 Repeatability

The results of a test in which three fibers were tested in a single 1 litre bottle were presented. Important fact was that the ratio of fiber volume:water volume was similar to that in three separate 250-ml bottles which each had one fiber in them. The repeatability of three fibres in one bottle was high, and the idea was raised as to whether or not it might be desirable for repeatability and to provide back up fibers in the case of accidental breakage. The volume of PDMS:volume of water ratio in the original protocol should be maintained. In addition, fewer magnetic stirrers would be needed and it would be unnecessary to divide a single sample over three separate bottles for a triplicate measurement. VITO may consider this method again in upcoming SPME trials.

2.9 Uptake curves

24 hours is a very practical time period for exposure duration because of the need to start exposures of fibers to the samples in the same sequence as they are measured. This means when one fiber exposure begins on day 0, it will be measured at the same time of day on day 1. An exposure of *e.g.* 6 hours would mean that the fibers must be exposed starting very early

in the morning and measured until very late in the day in order to measure a reasonable number of fibers per day.
RIZA has also produced uptake curves (cf. RIZA reports and Exxon data being circulated).
General conclusion is that there may not be equilibrium in the fiber for all compounds in the effluent after 24 hours. Is this a true stumbling block? The uptake on the fiber after the 24-hour exposure period should serve as a trigger mechanism in this PBS screening method. It was agreed, however, that given that the SPME method would be one tool to screen effluents for further work, that this would not limit its applicability.
A general point about equilibrium is that often in fresh effluents themselves, there will not be an equilibrium between the dissolved and sorbed phases for any number of chemicals in the mixture. In addition, the mixture may change considerably with time, as has been demonstrated by various toxicity tests performed at different points in time on the same effluent, showing large differences (including reduction in) toxicity. This fact points to the need to limit the SPME exposure time to something practical which gives enough information to fulfill the trigger mechanism requirements for this screening method. Extension of the exposure time would bring with it other disadvantages.
A theoretical estimate of the percentage of equilibrium can be determined using known uptake kinetic data for the 100 µm PDMS fiber.

2.10 Comparing LLE and SPME units

Although the goal of the workshop was to focus on the technical issues of the methods, to understand the meaning of the units used for both methods, a slide was shown for a theoretical effluent, which is also mentioned in the review paper written as part of this programme (Leslie and Leonards, 2004, Appendix 1).
From this example, it is clear that there can be differences between what mg C/L effluent means and mmol/L PDMS means. The patterns of compounds in the extractions with PDMS done in a non-exhausive (non-depletive) manner are expected to be different than the patterns those found with LLE. The main difference is because LLE extracts compounds more or less in the same pattern as they are present in the water phase. If SPM is present in the effluent, some of the chemicals may be extracted during the LLE to some degree, augmenting the signals for these chemicals that are extracted from SPM.
In SPME, the pattern depends not only on the dissolved concentration in water, but also on the potential to bioconcentrate (and hydrophobicity). Therefore, it is clear that chemicals that are present at a given dissolved aqueous concentrations but which have high potential to bioconcentrate due to their hydrophobic nature will be represented more strongly in the chromatograms than e.g. compounds at the same dissolved aqueous concentrations but which are less hydrophobic.

2.11 Other technical issues

FID and bias of large molecules
Is the use of the FID as detector in the analysis of PBS giving extra bias to the heavy components in the chromatogram? This question arises due to the fact that FID signal response is higher the larger the number of carbon atoms in the molecule. One approach, suggested by RIZA was that chromatograms can be divided into sections and this can be examined. Whether this would be helpful, is uncertain. Within a strategy of screening, followed by further investigation it is probable that other more specific detectors e.g. MS would be used.

Effect of Temperature
The effect of changing laboratory and/or sample temperature during the exposure of SPME fibers was discussed. Many laboratories did not wait till the effluent samples had warmed up to room temperature before beginning the SPME exposures. This may have a slightly noticeable impact on the level of uptake at the end of the 24 h period for at some of the chemicals in the mixture.

The temperature of the effluent has an effect on a) the partition coefficient and b) the kinetics of uptake and thus the time to equilibrium. The relationship between the partition coefficient and temperature is described by the following equation: log $K_{PDMS\text{-}water}$ = -ΔG / R**T** + constant, where ΔG is the change in free energy, R is the gas constant, T is temperature. If $K_{PDMS\text{-}water}$ >1 then increasing the temperature will not only lower time to equilibrium but it also lowers the $K_{PDMS\text{-}water}$.

It was agreed that the protocol should give guidance on this point. If available, climate controlled room-temperature spaces (20 °C) should be used for fiber exposures. However, the *K* and thus the amount on the fiber at 15 or 25 °C is not expected to be noticably different than what would be observed at 20 °C in this screening method. But should temperatures in laboratories get to be 30 °C and higher, as can be the case in the summer, the *K* may be effectively reduced by about 10% (or conversely increased by ca. 10% should the temperature go down to 10 °C). At 40 °C we can expect the K to be roughly 20% higher than at 20 °C. These estimates are based on calculations using data for uptake of methanphetamine by 100 µm PDMS fibers in Pawliszyn (1997).

Quantification

Integration was performed without much difficulty by the different participating laboratories (between C8 and C38). There was discussion about at what level (on the y-axis of the chromatogram) the integration of the peak signals should start in chromatograms where the baseline is variable.

The use of the shorter columns in this ring test, showed a major improvement with the background observed in the chromatograms. Although RIVO suggested even shorter columns might further improve the situation, the workshop participants agreed that the current protocol was fit for purpose. There was also a possibility for overloading shorter columns, which would need further investigation. RIZA showed some chromatograms which were much more difficult from old runs with long columns used in earlier protocols. RIVO suggested to even try an even shorter column. (Some overloading of the column may occur if the column coating is too thin however). This could be tested.

RIZA demonstrated how they conducted integration (cf RIZA reports) using a macro after export of the raw data to Excel to calculate areas under the curve.

Correcting for blanks yes or no. This did not appear to have much support, given that the blanks were much lower than clean water extractions.

The question arose if the standard deviations have actually gone down since previous SPME studies, now that integration is easier. Particularly ExxonMobil data had very low variation. There may be clues in how the method is performed at ExxonMobil as to why this variation is low. ExxonMobil also has a lot of experience with the method and have developed an automated version of it.

Solvent injections of single compounds have low variability compared to SPME injections of complex mixtures.

2.5 mmol/L PDMS is generally the largest amount of variation between results of different laboratories in the present study.

Sources of variation in SPME analyses

Sources of variation in the triplicate SPME analyses were suggested. All possible contributions to variation are listed here: SPM on fiber (giving the fiber a tick after several minutes of stirring will remove SPM that may have collected on fiber), baseline changes among triplicates, stirring stopping during exposure, storage of samples (Sedimentation), temperature, physical damage to fibers.

Fiber holders

The use of fiber holders during exposure of fibers to effluents for the 24 hour period is *not necessary.* RIZA even injects without the fiber holder, although the exact fiber injection depth must be manually measured each time a fiber is injected. RIVO uses a holder for injection only, removing the fiber from the effluent sample and assembling it quickly in the holder. The use of the holder is convenient because it easily indicates to which depth the fiber must be injected to

be in the middle of the SPME inlet liner. This was demonstrated to other workshop participants at the end of the day.

FID flame

RIZA and RIVO have the same FID machine and there are some problems with the flame going out very temporarily immediately following injection in some cases. The effect on the signal is unknown. VITO (using a similar injection temperature) did not experience this problem. RIZA noted that building in a retention gap (used at RIZA for mineral oil analyses) may help this problem. E.g. a wide bore injection cap (ca. 1 m) as buffer. VITO noted that if injector temperature exceeded 250 degrees they got poor blanks due to septum (or fiber) bleeding.

Thinner fibers

Thin disposable fibers have been applied in various studies reported in the literature. They may have some potential for future WEA work but it was agreed that there is no current need for direct development. One drawback is that an autosampler is needed, and this is not standard laboratory equipment. Furthermore, some expertise needs to be developed in handling the thin disposable fibers. The volume or thickness of the PDMS phase should not have any influence on the concentration of the compounds at equilibrium. Thinner fibers only have faster kinetics. The volume of the water phase (sample) must remain high relative to the fiber phase volume in order to keep depletion to a minimum for biomimetic SPME.

Storage time and conservation of samples

These are general problems that affect other types of analyses (including bioassays) as well. In these tests, the storage method was based on Aquasense (Amsterdam) generally uses for storage of samples prior to toxicity tests, which requires samples to be stored frozen if not measured within 14 days. Otherwise, the normal storage temperature is 4 degrees. Volatiles are commonly lost during storage, and reduction in toxicity after storage is not uncommon.

Fiber volume

It was noted that 100 µm PDMS fiber volume is incorrectly reported in some SPME literature (e.g. a Pawlizsyn SPME book and in some peer-reviewed articles). Some participants used a different PDMS volume. Correct volume is 660 µL, as calculated by the formula:

$$\text{Vol of fiber} = PI*10*(0.155*0.155-0.055*0.055) = 660\ \mu l$$

(Length 10 mm; PDMS thickness 0.100 mm; Silica core diameter 0.110 mm)

External standard

The 2,3-dimethylnapthalene concentration can be reduced. Comment that as in protocol, it is too high for a thin column.

2.12 Resource Time for SPME and EGOM-LLE

For SPME, fibers can be measured on Tuesday-Friday when starting exposures on a Monday (4 injection days in a work week).

In general, about twice as many samples can be measured with SPME per week than EGOM-LLE.

RIZA 48 extractions (16 samples) in 5-day period. LLE ca. 20 extractions in 5 days (ca. 7 samples), blanks not included. VITO does 4 fibers/day but was limited by using fiber holders for each individual fiber during the exposure period. VITO can do 7 SPME injections per day. Standard solvent injections are done at night. VITO commented that extraction time for LLE was too long. Also some troubleshooting time is required for LLE when emulsions form.

2.13 Applicability of methods for assessment of “B” (bioaccumulation)

Whole effluent assessment (WEA) comes into the OSPAR Hazardous Substances Strategy for the assessment of PBT (persistence, bioaccumulation and toxicity). In the case of B, complex effluents exhibiting a large PBS value may be flagged. Selected effluents of concern will be further examined and measures taken at point sources in an effort to reduce emissions and discharges of potentially hazardous chemicals.

The Swedish EGOM-LLE method as followed requires some additional development should it be used for assessing whole effluents (cf. Appendix 1).
The biomimetic SPME method is inherently more suitable for assessing B. Plus there have been improvements to the method in this study over previous studies in which the method was tested. Continued development efforts and trials are resulting in increased expertise and better results. For laboratories to implement any new analytical method it requires a large amount of experience and development before the method is running well.

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Appendices

The appendices have been circulated to the IEG and are available from RIVO. They have not been circulated to the HSE.

Appendix 1. Review of SPME and LLE methods

Comparison of EGOM liquid-liquid extraction and biomimetic SPME for evaluating potentially bioaccumulatable substances (PBS) in effluents.

A1.0 Abstract

An overview of two extraction methods to screen and assess whole effluents being considered by the Intersessional Expert Group on Whole Effluent Assessment, Hazardous Substances Committee of OSPAR is presented. The conventional liquid-liquid extraction (LLE) approach using the 'EGOM' method is compared to solid-phase microextraction (SPME) performed according to the 'biomimetic' approach. The main differences between the methods are that LLE is a more exhaustive extraction than biomimetic SPME, meaning that bioavailability or differences in bioconcentration factors are not taken into account. Both screening methods as applied in the OSPAR group employ the same analytical detection method, GC-FID, with integration of the full scan chromatogram as a measure of the total injected amount of chemical. The chromatogram of an LLE extract estimates the pattern of chemicals in the effluent including both the freely dissolved fraction in the aqueous phase and the fraction (typically large) that is sorbed to organic matter in the effluent. SPME chromatograms present estimations of the patterns of chemicals as they would be present in biota (before possible metabolisation). The SPME method integrates bioavailability and bioaccumulation potential of the chemicals in a complex effluent into the extraction and measurement and is a measure of PBS that is more mechanistically similar to bioconcentration. LLE requires extra fractionation steps in order to roughly quantify the contributions of chemicals with different bioconcentration factors. The decision of which method to apply depends on whether an exhaustive or non-exhaustive extraction can better answer the research question.

A1.1 Introduction

OSPAR regards whole effluent assessment (WEA) to have added value for flagging complex effluents that could cause adverse effects in the marine environment because of the toxic, persistent or bioaccumulative chemicals introduced via the effluents. WEA is particularly useful for complex effluents, as it has been found that the more extensively an effluent is treated, the more the toxicity and bioaccumulation tendency present in the original effluent decreases (Gerritsen *et al.* 2004). Since toxicity can sometimes not be explained by chemical assessment alone, toxicity testing complements chemical approaches. OSPAR supports a multi-test approach to WEA, accompanied by guidelines for the selection of appropriate tests depending on the situation and effluent. Screening for the presence of potentially bioaccumulatable compounds makes hazard assessment of whole effluents more comprehensive (Grothe *et al.* 1996; de Maagd 2000).

One of the purposes of WEA in OSPAR is to screen effluents for bioaccumulation potential. In order to select appropriate tests to answer specific questions with WEA, a good understanding of available methods is required. Any bioaccumulation method used in WEA must be robust and validated for general quality control parameters (*e.g.* repeatability, reproducibility) but method users must also be confident it provides a parameter relevant to the issue of bioaccumulation potential.

A1.2 In this review

To support decision-making about bioaccumulation methodologies in WEA, a comparison is made below between an 'EGOM' liquid-liquid extraction method and an SPME method to assess the potentially bioaccumulatable substances in whole effluents. The methods are described and compared, outlining technical and practical differences and similarities between methods and

the limitations of each. The possibility of overestimation or underestimation of PBS concentrations that could be found in biota as determined by both methods is also presented, and the selectivity and sensitivity of the methods is compared. How the methods address the bioavailability issue is considered, as well as their suitability as a 'bioaccumulation test'. This is also discussed in light of the existing information on the relationship between PBS measured by each method and measured body burden values. Knowing more about this relationship could lead to the determination of a 'trigger' value so that effluents can be screened and, based on the PBS value, selected for further evaluation. The possibility of developing synthetic quality standard for method users to be able to test their systems with is discussed. Some guidelines that are useful for selecting the most appropriate method under given circumstances are presented.

A1.3 Bioaccumulation vs. bioconcentration

It is useful to briefly note the definitions of bioaccumulation and bioconcentration for this discussion of WEA methods. The term bioaccumulation is sometimes used interchangeably with bioconcentration, but the difference between the two lies in the manner of uptake from the medium of the exposed organism. Bioconcentration generally refers to uptake from the water phase, and bioaccumulation refers to uptake via all routes combined, including water and ingestion of food or suspended particles. For chemicals with log K_{ow}<5, there is no practical difference between bioconcentration and bioaccumulation (Belfroid *et al.* 1996). For chemicals with log K_{ow}>5, bioaccumulation due to uptake by ingestion may exceed bioconcentration alone. This is because of the slower kinetics of elimination to water of these very hydrophobic chemicals. It is possible that they are actively taken up through feeding at a faster rate than they can be (passively) eliminated, preventing organisms from reaching a steady state concentration according to the BCF (a steady state ratio of the concentrations in the organism and in the surrounding water).

A1.4 What is PBS?

Potentially bioaccumulatable substances or 'PBS' in effluents generally refers to the organic compounds that have hydrophobic properties enabling them to bioaccumulate in exposed biota. A sum parameter for the complex mixtures of chemicals found in effluents is practical because it is not feasible to assess bioaccumulation in effluents by measuring individual chemicals (de Maagd 2000). Sometimes PBS refers to the total extractable fraction of chemicals in an effluent, but can also be used to refer to a biomimetic[1] extractable fraction of chemicals. The sum parameters for PBS in these cases are fundamentally different, and can be applied to answer different questions. As discussed below, the former approach is an exhaustive extraction; the latter is dependent on the hydrophobicity and amount of the chemicals present, as occurs with bioconcentration.

Because the type of PBS determined depends on the method applied, we might speak of exhaustive-PBS and biomimetic-PBS. The different methods to determine PBS also result in different units and quantitative definitions of this sum parameter. The 'EGOM' LLE method defines PBS as the mg of C (organic matter) per litre of effluent that is extractable in cyclohexane and detectable with GC-FID. PBS is further defined in the EGOM method as the organic material that elutes in the HPLC fraction with retention times after a standard compound that has a log K_{ow} 3. If biomimetic SPME is used, the PBS is expressed as mmol/L polymer coating. In this case, the substances in the PBS measurement are weighted according to their partition coefficients to the hydrophobic phase (which is a surrogate for biota).

Biomimetic SPME has been used in the past to estimate total body residues (TBR_{est}). This is done by translating mmol/L polymer coating to mmol/kg biotic lipid by calibrating the concentrations in biota to a particular polymer coating (Verhaar *et al.* 1996, van Loon *et al.* 1996; Verbruggen *et al.* 2000; Leslie *et al.* 2002a,b; van der Wal *et al.* 2004). The fibers

[1] Biomimetic = mimicry of accumulation patterns in biota

mimic the bioconcentration process (passive uptake of chemicals from the water phase) but not the biomagnification process (uptake from food and water), which can occur in the food chain with highly hydrophobic chemicals, e.g. with log K_{ow}>5. The advantage of the units of moles is that the total molar concentration in biotic membranes is directly related to the effect of narcotic toxicity, which complex chemical mixtures often contribute to.
It is a goal of the work of OSPAR to in eventually define 'trigger' values of PBS in effluents, thus identifying those effluents that require further evaluation. The selection of these values will depend on the correlation between PBS and bioaccumulation and toxicity testing in biota. Methods to determine PBS which are supported by a mechanistic approach are more easily interpreted than empirical approaches because of the greater uncertainty in when and how the empirical approach can be applied, and under which criteria it works.

A1.5 Bioavailability in bioaccumulation tests

Taking bioavailability of chemicals in an effluent into account is essential for comparing chemical assessment to the results of the toxicity tests that form a major part of WEA. Emissions from industrial effluents must be monitored by exhaustive extractions as well, in order to prevent large amounts of particulate matter with a high chemical load from entering the environment unchecked. Emissions of contaminated SPM can lead to the creation of contaminated sediments. This is another subject, and we limit the discussion here to WEA in relation to bioaccumulation potential.
Non-exhaustive extractions that take bioavailability into account remain invaluable in predicting toxicity in the water phase and explaining results of WEA toxicity testing[1]. This is because exposure is often significantly limited by reduced bioavailability due to extra sorbent phases in the effluent. Bioavailability refers to the degree to which chemicals are available for uptake by biota, and is dependent on physical, chemical and biological properties of the organism-environment system (Hamelink *et al.* 1994). In effluents, the uptake can be direct from the water phase, but depending on the conditions controlling their fugacity, chemicals in the effluent can also desorb from a sorbent phase (*e.g.* SPM) into the water phase either surrounding the organism or in its gut or gill for example, and become be taken up.

> *"Bioavailability is possibly the most important factor in determining the extent to which a contaminant in water or sediment will enter the food chain and accumulate in biological tissues." (OSPAR 2000)*

The significance of bioavailability in the environmental sciences arises from the observation that the total external concentration or the total 'dose' given regularly does not explain toxicological observations or predict true exposure as well as the bioavailable fraction. Bioavailability is a qualitative term at best, since all measurements of bioavailability are approximations of one sort or the other. As many papers have previously reported, even different organisms experience exposure and bioavailability differently because of characteristics such as different behaviour, feeding habits and surface area to volume ratios (*e.g.* Borgå *et al.* 2004). But for effluent screening purposes, subtle differences between different organisms and a surrogate extraction phase for the organism are acceptable, since an exact bioavailability study for a particular species in a particular habitat is not the goal.

A1.6 Extraction of effluent samples

Extraction of environmental samples such as effluents requires an understanding of the behaviour of the analytes in an effluent when they come into contact with the extraction phase. Different extraction phases have different selectivities, the volume of the extraction phase can determine if the extraction is exhaustive or non-exhaustive, and the properties and dimensions

[1] Accumulation in exposed biota is itself a non-exhaustive extraction.

of the extraction phase and the nature of the contact between extraction phase and sample determine the dynamics of the extraction of organic compounds.

Many methods have been developed to assess organic compounds in water. Well-known sum parameters include for example total, dissolved or suspended organic carbon (TOC, DOC or SOC). Other parameters are defined in terms of the isolation method volatile organic carbon (VOC), purgeable organic carbon (POC), extractable organic carbon (EOC), adsorbable organic carbon (AOC), or a combination of isolation and analysis method, such as extractable gas-chromatographic organic material (EGOM), discussed further in this paper. Because some organic carbon is more toxic than others, other fractions containing halogens have been defined. These fractions are considered more toxic than DOC for example, and include volatile organic halogen (VOX), purgeable organic halogen (POX) adsorbable organic halogen (AOX) or extractable organic halogen (EOX).

In the case of assessing bioaccumulation potential, a general problem with the above-mentioned sum parameters is that the relationship between the sum parameter and bioaccumulation, or toxicity for that matter, is not clear (de Maagd 2000). In some cases these parameters such as EOX or AOX are very poorly correlated to bioaccumulation and toxicity (Craig *et al.* 1990). This is because of the lack of mechanistic similarities between an AOX extraction and the process of uptake by biota. Using methods that account for the difference in bioconcentration potential of compounds of differing hydrophobicities helps give a better estimate of the actual bioaccumulation potential of complex mixtures in effluents. This estimate can in turn be interpreted in terms of baseline toxicity potential. Also, depending on the research question, bioavailability may play an important role in modifying toxicity, and is important to account for when testing whole effluents with suspended particulate matter (SPM).

With the growing variety of demands being made on extraction methods, considerable progress is currently being made in extraction technologies, as international conferences dedicated exclusively to advances in techniques (*e.g.* ExTech) attest to. In the case of extraction of water samples, many different techniques are available. LLE and dynamic headspace (purge and trap technique) are among the most common for determining volatile organic chemicals in water, but solid-phase extraction, solid-phase microextraction, Single-drop liquid extraction, liquid-phase microextraction, and various membrane extraction techniques (Jönsson and Mathiasson 2000; Chimuka *et al.* 2004) are also being developed and widely applied.

Two methods will be discussed here, the EGOM-liquid-liquid extraction and solid-phase microextraction. Both are methods of interest to the OSPAR IEG/WEA for use in determining the amount of PBS in effluent samples.

Table A1-1. Overview of steps in procedure for SPME, EGOM liquid-liquid extraction method described by Hynning 1996 (long and short versions). EGOM = extractable gas chromatographic organic material.

Biomimetic SPME	EGOM LLE *(shortened version RIZA)*	EGOM LLE
250 ml effluent sample (perform in triplicate)	300 – 1000 ml effluent sample	500 to 1000 ml effluent sample
Option to adjust sample pH to pH of effluent receiving water	Extract with 50 ml cyclohexane	Adjust sample pH to acidic or basic
Add stir bar and screw on cap	Place (in separatory funnel) on shaker 2 h	Extract 3 times with hexane/ t-butyl methyl ether (7:3)
Insert fiber	Repeat extraction	Add NaCl and centrifuge 10 min, 1000 *g* if emulsions occur
Stir 24 h on magnetic stirrer	Adjust sample pH to 10	Dry extract with Na_2SO_4
Remove fiber	Extract twice more with 50 ml cyclohexane	Concentrate to ca. 5 ml volume, take to dryness under nitrogen and weigh (gravimetric method)
Inject in a GC-FID	Combine 4 extracts and concentrate by rotary evaporation under nitrogen to dryness in a weighed vial	Separation by semi-preparative HPLC (UV detection)
Inject external calibration standard, 2,3-dimethylnaphthalene	Redissolve and inject in GC-FID	Inject 8 OECD reference compounds
Integrate area under curve for "PBS" (mM)	Inject external calibration standard, eicosane (n-C_{20} H_{42})	Collect fractions for log K_{ow} <3, 3-5 and >5
	Integrate area under the curve "EGOM"	Derivatise each fraction (3 different derivitisations)
	Dissolve extract in methanol and fractionate with HPLC on C18 column	Silica gel (40 x 6 mm) for clean up using different solvents (5 ml)
	Calibrate with compounds of known K_{ow}	Inject derivatised samples (1-2 µl) in GC-MS
	Collect fractions log K_{ow} <3, 3-5 and >5 and concentrate, dilute with 0.5 M HCl and extract with cyclohexane.	Quantify the various fractions by direct weighing or by GC-FID and integration of the relevant areas on the chromatogram.
	Inject fractions in GC-FID as before	Quantify unknown compounds using squalene as empirical standard for calculating the concentrations of EGOM.
		Quantify compounds successfully identified by GC-MS with suitable surrogate standards.

A1.7 Liquid-liquid extraction method

Liquid-liquid extraction (LLE) or 'solvent extraction' is a conventional approach to preparation of aqueous samples for chemical analysis. It is the method of choice described in most of the official protocols for extraction of aqueous samples (*e.g.* EPA protocols, etc.). An organic solvent that is immiscible with water is brought into contact with the sample, so that compounds in the sample are able to partition between the water phase and the solvent phase. Hydrophobic compounds that have the potential to accumulate in biota also have favourable solvent phase-water partition coefficients.

The efficiency with which chemicals are extracted depends on the nature of the extraction solvent. Nonpolar solvents will extract both nonpolar and polar chemicals due to attractive van der Waals interactions between chemicals in the effluent and solvent molecules. However, trace analysis of polar compounds extracted by nonpolar solvents sometimes have poorer recoveries if one compares it to the recovery when extracting with octanol, which can form H-bonds with polar solutes but not with nonpolar solutes. This makes it seem like a poorer extraction recovery of polar compounds in nonpolar solvents, although this depends largely on using K_{ow} as a reference. Goss and Schwarzenbach have presented interesting discourses on this subject (*e.g.* Goss and Schwarzenbach 2003).

Polar solvents or solvents mixed with polar compounds will extract both nonpolar and polar chemicals, but may be more efficient in extracting polar chemicals than nonpolar solvents if favourable H-bond interactions between analyte and solvent occur. Often the pH of the effluent is adjusted to neutralise acidic or basic components in the complex mixture making them more readily extractable by a nonpolar extraction solvent. Emulsion formation is an important problem in LLE, and therefore if possible, a solvent should be chosen that minimises this effect, *i.e.* a solvent that is as insoluble in water as possible. When emulsions occur in LLE, centrifugation is often applied to separate the phases.

On the other hand, it is of great importance that the solvent comes into contact with the water during extraction, for which a variety of techniques have been applied (manual shaking, stirring, ultrasonic vibrations). Shaking is a good option, but for an exhaustive extraction, one study showed that the shaking time needed can vary anywhere from 30 s for 90-100% recovery of compounds that are extremely soluble in the extraction solvent, to several tens of minutes for compounds with less favourable partition coefficients (Burgasser and Calaruoto, 1979). In addition to lengthening the extraction times, multiple extractions and pH adjustments are also used to improve the recoveries of the extraction.

In liquid-liquid extraction of trace analytes a concentration step is almost always necessary, in which the extract volume must be reduced by evaporation. Using a large volume of the extraction solvent leads to a better recovery but the concentration factor decreases, and therefore a minimum of extraction solvent is warranted. Losses of the more volatile compounds in the extract occur in the evaporation process, and any contamination present in extraction solvents leads to higher background in the concentrated extracts. The solvent used for the extraction must be of the highest possible purity for this reason. Blanks and recoveries are therefore important to correct for in LLE.

Effluents often contain a significant amount of SPM, which is usually defined as the particulate matter that is retained on a 0.45-μm filter. This SPM is often first filtered or centrifuged out of the effluent before LLE is performed, with the SPM fraction being analysed separately (Lacorte *et al.* 2003). However, filtration is not a simple process and perfect separation of SPM from the freely-dissolved fraction presents some complications (Hermens *et al.* 1992).

Performing SPM extractions - separately from the water phase - results in a pattern of concentrations that should reflect the accumulation patterns in biota. This is because SPM is also a hydrophobic phase to which the chemicals in the mixture partition to according to similar principles as bioconcentration. An LLE of an effluent without a filtration or centrifugation step to remove SPM is an extraction of the freely-dissolved fraction plus the SPM. The water phase concentrations will give a very different pattern than the SPM phase, or biota for that matter. This is illustrated for a hypothetical effluent with a complex mixture of chemicals from log K_{ow} 1 to 7 with a SPM content of 5 g/L (Figure 1). The contribution of freely-dissolved chemicals to the total extract will disturb the pattern resulting from SPM alone (Figure 1), and not necessarily

reflect the patterns in biota. This effect is strongest for chemicals with log K_{ow}<4 due to the strong partitioning from the water phase to organic matter, so the effect on the relevance to a bioaccumulation parameter will depend on the specific character of the effluent in question. The weakness of LLE of whole effluent samples is that it is not well-related to the bioaccumulation parameter.

Thus, in theory, the extraction of pure SPM reflects the pattern of bioaccumulation better than the extraction of the combination of water plus SPM. Should SPM be extracted along with water in a simple LLE, it should be known how efficient the extraction of the SPM is. Recovery calculations are useful not only because extraction efficiencies may vary considerably from chemical to chemical for a given solvent, but also because of the differences in efficiency due to the degree and type of binding of the analytes to the SPM present. Some SPM will be more easily extracted than others.

In many LLE applications, a clean-up is performed on the extract, such as a silica gel column, before injection. These procedures also lead to losses of chemical analytes, which should be checked by recovery experiments. The more steps in the sample preparation the more chances there are for losses of chemicals. The drawbacks of LLE are widely known. First of all, there are the large amounts of costly, high grade solvents used, which according to the Montreal Protocol on substances that deplete the ozone layer, should be reduced due to environmental and occupational hazards. Also the formation of emulsions and the loss of volatile and semivolatile compounds during evaporation of the solvent are often cited as major drawbacks of LLE methods.

EGOM-LLE Method

The OSPAR IEG/WEA group is interested in the 'EGOM' LLE method (Adolfsson-Erici, and Wahlberg 1992; Hynning 1996), which is a measure of the extractable gas-chromatographic organic matter in an effluent sample. This method has been developed and is used in Sweden for the separation, identification and quantification of components of industrial effluents with bioconcentration potential (Hynning 1996, Tarkpea *et al.* 1998). In its original form, it begins with extraction with a mixture of hexane and *t*-butyl methyl ether, followed by HPLC fractionation into three different K_{ow} ranges. The use of this solvent system appeared to increase the fraction of chemicals with log K_{ow}<3 compared to cyclohexane. Those with log K_{ow}>3 are derivatised and a clean up with silica gel column are performed before analysis and identification with GC-MS. GC-FID of the fractions is performed to quantify identified compounds (Table 1).

The method is multi-stage and tedious but does provide a lot of information. For screening effluents for bioaccumulation potential, it is not necessary in the first step to identify compounds with GC-MS. Therefore members of the IEG/WEA group focusing on bioaccumulation methodologies have chosen a shortened version of the original EGOM method.

The shortened method (Table 1) is performed as follows. Approximately 1 L of an effluent sample is extracted twice with 50 ml cyclohexane in a separatory funnel, which is placed on a shaker for 2 h for each extraction. Following this, the pH of the effluent sample is adjusted to ≥10 and two more extractions with 50 ml cyclohexane are performed as before. The four extracts (total ca. 200 ml) are transferred to a flask for rotary evaporation under a gentle stream of nitrogen. The final extract is evaporated to dryness in a weighed vial.

The residue is then dissolved in an appropriate solvent and injected in a gas chromatograph with a capillary column (*e.g.* J&W Scientific DB5, 30 m long, 0.32 mm ID, 0.25 μm film thickness,) with flame ionisation detection. The temperature programme used begins at 40°C and increases to 320°C. The area under the curve of the chromatogram is integrated between retention times for n-C_{10} to n-C_{40}. This area is quantified by using an external standard, eicosane (n-$C_{20}H_{42}$), which has a number of carbons in between n-C_{10} to n-C40. Because the FID is sensitive to number of carbon atoms, a substance with an average number of C atoms in the range being investigated is in theory a good choice of calibration standard for quantifying the amount of C injected. A validation and evaluation of the uncertainty involved in using this external standard should be performed and published. Otherwise, a gravimetric procedure for the quantification has been applied. The result, called the 'EGOM' is expressed as mg organic C/L. This value represents the total cyclohexane extractable amount of organic material.

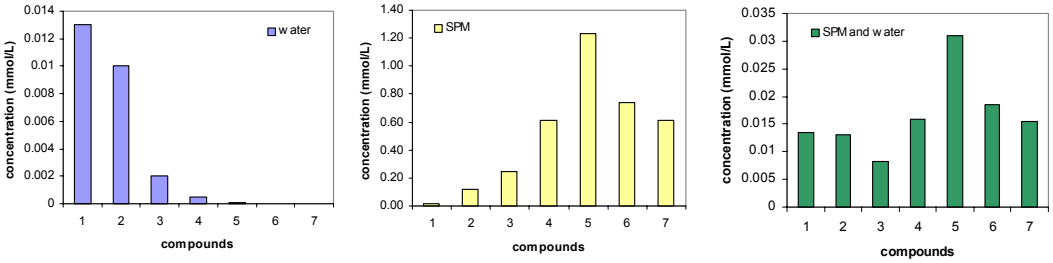

Figure A1-1. Illustration of the patterns of concentrations of compounds with log K_{ow} from 1 to 7 (x axis) in the freely-dissolved aqueous phase (left), in the suspended particulate matter phase (middle) and the total concentration per volume sample when SPM and water phase are combined, as in EGOM-LLE (right). Hypothetical effluent sample with assumed SPM concentration of 5 g/L. Example of $K_{SPM\text{-}water}$ taken from Karickhoff *et al.* (1979). The water concentrations of compounds 5, 6 and 7 are 0.1, 0.006 and 0.0005 µM respectively.

In order to determine to what extent the EGOM in the effluent sample could contribute to bioaccumulation based on lipophilic properties (*i.e.* PBS), an HPLC fractionation is performed. For this, the extract is dissolved in methanol and fractionated with HPLC on a C18 column (*e.g.* HyPurity C18 5 µ, 250 x 4.6 m, Thermo Hypersil) eluted with a gradient from 30% phosphate buffer pH 2.5 in methanol to 100% methanol. To be able to collect different fractions of the injected sample, compounds of known K_{ow} are injected. Then the fractions with log K_{ow} <3, 3 to 5 and >5 are collected, concentrated, diluted with 0.5 M HCl and solvent is changed to cyclohexane. These extracts are then injected into a GC-FID as before. The fractions representing different ranges of log K_{ow} are quantified using an external standard. In some cases, the quantification is performed gravimetrically.
The potentially bioaccumulating fraction of the EGOM is defined by this method as the contribution of chemicals with K_{ow} >1000 (*i.e* the log K_{ow}<3 fraction is excluded). This is not to say however that chemicals in the excluded fraction do not bioaccumulate. The BCF of chemicals in this K_{ow} range predict that the concentration in organisms can be up to 1000 times higher in organism lipid than in the effluent. In theory, it is possible that an effluent contain a large enough loading of these lower K_{ow} chemicals to cause narcotic toxicity through uptake and accumulation in lipid cell membranes. This method does not take the bioavailability of the PBS into account (M. Adolfsson-Erici, pers. comm.). This is also clear considering the extraction steps in the methodology: an exhaustive extraction is done of the whole effluent.

A1.8 Biomimetic solid-phase microextraction method

In the mid 1990's a partitioning-based methodology for the determination of potentially bioaccumulating substances (PBS) in whole effluents was developed, called biomimetic solid-phase microextraction (SPME). It is a sorbent-based extraction method, similar to solid-phase extraction, but solventless and with a very small amount of sorbent phase compared to the sample volume. The term "biomimetic extraction" is used to distinguish this method from other types of analytical extraction methods which are primarily exhaustive extractions, such as liquid-liquid extractions, Soxhlet, SPE, XAD and, etc.
Based on the pioneering work by Verhaar *et al.* (1995) and Van Loon *et al.* (1996), a biomimetic extraction uses a hydrophobic absorptive phase to simulate bioconcentration by exposing the surrogate phase to a sample, *e.g.* effluent, and measuring the total molar concentration using minimal separation gas chromatography.
Such a sum parameter is considered useful for screening of effluents for chemicals with potential to accumulate in biota. It also provides a unique type of information unavailable from the total extraction methods mentioned above, because it mimics (nonmetabolizing) biota in the uptake pattern. Just as with biotic lipids, SPME fibers absorb the chemicals, weighted according to their hydrophobicity. This gives information on the overall potential bioconcentration of a complex mixture of chemicals in a sample with vastly different bioconcentration factors in a single step.
This method also takes bioavailability of the chemicals in the sample into account, which is considered an added advantage, particularly in the many cases where bioavailability plays a major role in governing toxicity.
The surrogate (fiber), just as biota, takes up chemicals from a sample by passive diffusion between the water phase and the hydrophobic phase, until steady state is reached. This sum parameter is more directly related to steady state total body residues (in non-metabolizing organisms) than many other methods (Figure 2). The PBS parameter is therefore also used to predict the likelihood of baseline toxicity in exposed organisms (Verhaar *et al.*, Van Loon *et al.* 1996; Verbruggen *et al.* 2000; Parkerton *et al.* 2000; Leslie *et al.* 2002a, 2003; Van der Wal *et al.* 2004), a nonspecific type of toxicity for which concentration addition applies (Könemann 1981).

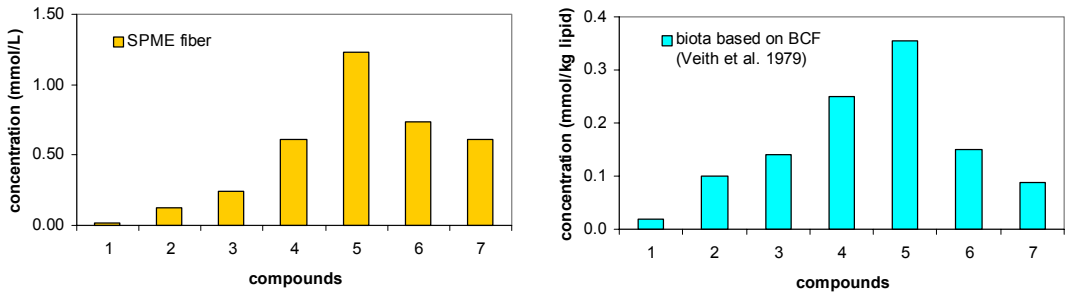

Figure A1-2. Illustration of the patterns of concentrations of compounds with log K_{ow} from 1 to 7 (x axis) in the PDMS coating of an SPME fiber at equilibrium (log $K_{PDMS\text{-}water}$ = 1.00 * log K_{ow} – 0.91 for 17 nonpolar compounds from Mayer *et al.* 2000) (left) and steady state concentrations in biota based on BCF estimates made from a relationship of BCF (of fish) to K_{ow} for 55 chemicals of various classes: log BCF = 0.85 * log K_{ow} – 0.70 (Veith *et al.* 1979) (right). The hypothetical effluent is identical to that in Figure 1.

This PBS determination is a solventless method without the need for clean-up steps, making it in principle a simple, fast, green procedure using a minimum of environmental laboratory equipment (Table 1). It can be viewed as a quantitative screening method for PBS to complement already existing chemical testing and bioassays in performing whole effluent assessments.

SPME fibers are exposed to whole effluents, with agitation, for 24 h. The polymer coating on the fibers is poly(dimethylsiloxane) or PDMS, a viscous liquid. Chemicals partition to and absorb into the PDMS. Per effluent sample, SPME fiber measurements are performed in triplicate. The fibers are removed from the effluent solution and injected into a gas chromatograph (GC) equipped with a flame ionization detector (FID). The column used is short and the temperature programme of the GC is adjusted so that the chemicals reach the detector within a short time span to minimize separation of separate peaks (Verhaar *et al.* 1995 and Van Loon *et al.* 1996), in contrast to most standard chromatographic methods. This adjustment enables easier determination of total area under the curve, which is quantified with a liquid injection of a standard with an average response factor. This enables molar quantification of the signal to within a factor of about 2 of the actual molar concentration.

The most suitable external standard for the FID method determined to date is 2,3-dimethylnaphthalene (Parkerton *et al.* 2001; Leonards 2001). Leonards tested this compound against 50 organic compounds representing various retention times, C-atom content and different chemical classes (phenols, ethers, phthalates and alkanes). The molar response of the chemicals injected in the GC-FID was linearly related to the number of C-atoms. This was a similar result to that published by Parkerton *et al.* (2001). 2,3-Dimethylnaphthalene had an average molar response compared to this large set of chemicals, *i.e.* a relative molar response (RMR) of 1.00 Therefore, using it as an external standard to quantify the signal of the total chromatogram will give a very close estimate (within a factor of about 2) of the moles of organic chemicals present.
There are two extreme cases where the estimate will be less accurate. One is when the effluent contains only compounds with a very high relative molar response (*e.g.* C38 alkane, with a RMR of 2.97). Calibrating the total molar concentration of an effluent with chemicals primarily of this RMR with 2,3-dimethylnaphthalene would result in an overestimation of the molar concentration by a factor of 3. In the case that an effluent had predominantly chemicals with a low RMR, such as 2-chlorophenol (RMR 0.51), the calibration with 2,3-dimethylnaphthalene will underestimate the molar concentration by factor 2.
These uncertainties seem very acceptable in light of the fact that most effluents are rather complex mixtures, consisting of compounds with a variety of RMRs. This would act to average out the RMR, making the calibration quite reliable. Furthermore, the RSD of the analysis of 2,3-dimethylnaphthalene (n=5) was 3.6% and satisfies the RSD criterion of 5%. The result of Leonards' 2001 study was to recommend the GC-FID standard 2,3-dimethylnaphthalene (40 mg/L in ethyl acetate).

Table A1-2. Summary of comparison of various aspects of LLE and SPME methods. a) practical aspects, b) Analytical details, c) Type of information and QC, d) Practical pitfalls or inconveniences of methods.

a) Practical aspects

	Biomimetic SPME	**EGOM LLE, short (RIZA)**	**EGOM LLE, long (from Hynning 1996)**
Green chemistry	Yes	No	No
Amount of solvent used	-	200 ml	600 ml
Health hazard to technician	No	cyclohexane safer than hexane	Yes, hexane and t-butyl methyl ether
Test organisms used?	No	No	No
Effluent sample volume for one replicate	250 ml	300 - 1000 ml	500 – 1000 ml
Steps in analysis	4-5	11-12	14, but may be shortened
Technician hours for 1 series (8 analyses)	8-12	24-32	>32
Hours from start to finish per series	48	48	>48
Analytic equipment needed	Magnetic stirrer SPME fibers GC-FID	GC-FID	HPLC-UV detector Centrifuge GC-MS GC-FID
Chemical consumables	Buffer to adjust pH (optional) Dimethylnaphthalene (extn. std) SPME fibers *(commercial 100um last 100-150 runs, but thinner disposable PDMS fibers are also convenient)*	HCl or NaOH to adjust pH Cyclohexane eicosane (extn. std) Surrogate standards for recovery	HCl or NaOH to adjust pH, Solvents, Na_2SO_4 Salt, Nitrogen OECD ref compounds (8), Derivitisation chemicals, Silica gel, Surrogate standards for GC-MS
Automation possible?	Yes, esp. using thinner fibers (require no stirring) with a thermal desorption autosampler	No, emulsion formation a problem.	Some steps automation possible.
Amount of laboratory waste produced per series besides extracted samples	Sample bottles 6-10 ml solvent disposable microextraction fibers, if used	Sample bottles Solvent 600 ml Salts Silica gel	Sample bottles Solvents Salts Silica gel
Pieces glassware/smp	1	5	5

b) Analytical details

	Biomimetic SPME	**EGOM LLE, short** (RIZA)	**EGOM LLE, long** (from Hynning 1996)
Integration of GC-FID chromatogram necessary?	yes	Yes	Yes
Chemicals measured by analytical detection technique	Using GC-FID, compounds contributing to the total signal have a bp of ≤400°C (GC requirement) and contain C atoms (necessary for FID).	Same as for SPME.	Same as for SPME.
Extraction hydrophobic phase	PDMS (viscous liquid)	Cyclohexane	hexane/ t-butyl methoyl ether (7:3)
Chemical classes (selectivity of extraction)	Nonpolar organics Polar hydrophobic Nonionic	Nonpolar organics Polar hydrophobic Nonionic	Nonpolar organics Polar hydrophobic Nonionic
Fraction of chemicals extracted	Freely dissolved in hydrophobicity-dependent manner	Freely dissolved plus some of the fraction bound to particles present in the effluent	Near exhaustive extraction of bound and unbound fractions
Bioavailability taken into account	Yes	No	No
Recovery experiments necessary?	No	Yes: low extraction efficiencies, losses due to evaporation	Yes, same as short method and because clean-up steps introduce losses.
Surrogate standards necessary?	No	Yes	Yes
Effect of sample matrix (*e.g.* DOM, SPM) on analysis	No matrix extracted and injected	Cyclohexane-extractable matrix injected.	Yes, interferences possible. Suspended solids may be filtered as pre-treatment.
Qualitative or Quantitative PBS result	Quantitative	Semi-quantitative	Semi-quantitative
Units of Result	mmol/L PDMS	mg organic C/L, of which the PBS is define as EGOM with K_{ow} > 1000.	
Reference value	0.5 mM for surface waters (example: Marker Lake, NL Leonards & van Barneveld, 2002)	0.1 mg organic C/L for acceptable content in wastewater discharge (OSPAR IEG WEA Report, 2004)	

c) Type of information and QC

	Biomimetic SPME	**EGOM LLE, short** (RIZA)	**EGOM LLE, long** (from Hynning 1996)
Trigger value, GC-FID method?	Unknown	Unknown	Unknown
Information provided	TBR_{est} of complex mixture measurable by GC-FID based on similarity of hydrophobic phases PDMS–biotic lipids	Estimate of cyclohexane extractable fraction of organic material "EGOM"	Estimate of hexane/ t-butyl methyl ether (7:3) extractable fraction of organic material "EGOM"
PBS type	Bioimimetic-PBS	Exhaustive-PBS	Exhaustive-PBS
Over/underestimation of bioavailable fraction?	Reasonable estimation	Overestimation	Overestimation
Over/underestimation of bioaccumulatable fraction?	Overestimation due to fact that metabolism is unaccounted for	Overestimation (exhaustive extraction and no metabolism). Underestimation due to <100% recoveries	
Relevance to bioaccumulation sum parameter	Yes	No, bioavailability not taken into account.	
Derive TBR_{est} from sum parameter?	Yes	No	No
Ease of communication of results	High	High	High
Work needed for large scale implementation?	Additional validation as predictor of body residues of organisms	Additional validation as predictor of body residues of organisms (or biotic lipids)	
Stability of detector response	Usu. good for FID. *(Can be checked if autosampler is used – with 1 ul of standard per tube with disposable fiber.)*	Usu. good for FID.	Usu. good for FID. Can vary (10%) for GC-MS
Level of validation as bioaccumulation (and toxicity) indicator	Medium (see text)	Low (see text)	
Detection limits	Ca. 1 mM PDMS	Unknown	
Blanks	Ca. 0.5 mM PDMS	Unknown	Unknown
Reproducibility	Unknown	Unknown	Unknown
Repeatability	2-60%	Unknown	Unknown
Recovery	High (no pretreat-ment) Equilibrium conditions should be met.	Log K_{ow} < 3: 7.4% (spiked sample) Log K_{ow} 3-5: 22.1% Log K_{ow} >5: 1.5% (Hynning 1996)	

d) Practical pitfalls or inconveniences of methods

Biomimetic SPME	**EGOM LLE, short** (RIZA)	**EGOM LLE, long** (from Hynning 1996)
○ Magnetic stirrers have different stirring powers ○ Nonequilibrium measurements ○ Autosamplers for thermal desorption of disposable SPME fibers not standard laboratory apparatus ○ Must be calibrated to toxicity depending on detector used (*i.e.* FID vs. MS)	○ Emulsions ○ Not easy to automate ○ High solvent usage ○ Recovery unknown ○ Water and SPM concentrations combined so no direct estimation of total PBS or bioaccumulation patterns in biota	○ Emulsions ○ Procedure long and tedious ○ Makes demands on many laboratory machines. ○ High solvent usage ○ No direct estimation of PBS patterns in biota ○ Recovery unknown

e) Advantages of each method for bioaccumulation assessment

Biomimetic SPME	**EGOM LLE, short** (RIZA)	**EGOM LLE, long** (from Hynning 1996)
○ Bioavailability taken into account ○ Relevant to bioaccumulation sum parameter ○ Fast cheap and automatable ○ Green chemistry ○ Useful as a research tool for uptake rate studies ○ Useful as a standardized surrogate organism	○ Good experience with method in Sweden ○ Classic method which is easy to explain to laboratory personnel ○ Useful as a standardized method to replace organisms	○ Potential for in depth effluent assessment including identification of ○ Good experience with method in Sweden ○ Classic method which is easy to explain to laboratory personnel ○ Useful as a standardized method to replace organisms

A1.9 Comparison of EGOM-LLE and biomimetic SPME

As can be seen in the above tables, the main similarity of the two methods begins at the moment of injection in the GC-FID. The area under the full scan chromatogram is integrated. Each method chooses a different standard, although in the EGOM-LLE method, eicosane may be substituted by the external standard 2,3-dimethylnaphthalene (from SPME method). The the choice of 2,3-dimethylnaphthalene as external standard (with an RMR of 1.00 compared to 50 other chemicals of different classes) makes it possible to express the result in units of moles instead of g C. This is convenient if the result is to eventually be translated to total body residue estimates or (narcotic) toxicity levels which depend on total molar concentration accumulated in biota. More work has been published on this last point for biomimetic SPME (Verbruggen *et al.,* Parkerton *et al.*, de Maagd, Leslie *et al* 2002a,b.) than for EGOM. Tarkpea *et al.* (1998) have reported a correlation between Microtox test results with the total EGOM (all values of log K_{ow}), but not with the EGOM-PBS (*i.e.* log K_{ow} >3) fraction (Table 2c). Niether method takes biotrans-

formation into account, which is convenient from the point of view that all organisms biotransform at their own rate and with their own selectivity. This makes the either type of PBS value standardised for a simple organism that doesn't have highly evolved or efficient metabolic machinery.
The main difference between the two methods, as stated above and in Table 2, is that EGOM-LLE is exhaustive and biomimetic SPME is not, meaning that the former does not take bioavailability into account. The effect of presence and type of sample matrix has more of an impact on the EGOM-LLE method than on the SPME method. Because the methods in the exact forms as they are as described here have not been extensively tested, it is difficult to compare the level of quality assurance for each. However, some information and insight can be gained from previous work on very similar LLE methods and similar biomimetic SPME methods using slightly different measurement parameters or using SPME fibers with polyacrylate polymers. For example, Mayer *et al.* (2000) published data on the determination of individual compounds between log K_{ow} 4.47 and 7.51 in PDMS-coated SPME fibers with RSDs <3%.

A1.10 When to choose EGOM-LLE and when to choose SPME?

The choice of methods to determine bioaccumulation potential depends on the exact research question (Figure 3). If the PBS results must be correlated to the results of toxicity tests on the same whole effluents, then a method that takes bioavailability of the chemicals into account is expected to show the greatest correlations to the toxicity tests, since bioavailability is often a major factor in modifying toxicity. Effluents are also known to contain SPM, which can drastically reduce bioavailability.
As mentioned above, it is important for regulators to have good information on the total loading in the effluent for the purpose of controlling total emissions. This requires an exhaustive extraction of the effluent, possibly with separation of the SPM from the aqueous phase to determine the loading of the particulate matter that may sediment out when emitted, and what will be emitted to the water column of receiving waters. For this, biomimetic SPME is not the appropriate method. LLE or near-exhaustive applications of SPME (different fiber polymer to effluent volume ratio) are excellent alternatives. The recovery problem must still be dealt with in applying the LLE approach since evaporation of extracts must take place.
When bioavailability is taken into account, and it is desirable to produce a chromatogram with a similar pattern of chemical contributions as expected in biota (*i.e.* $K_{PDMS\text{-}water}$ or approximately BCF-weighted), the biomimetic SPME method gives the best answer. The EGOM-LLE method makes a rough distinction between chemicals that are likely to bioconcentrate by a factor of 1000 to 100,000, and those that are likely to bioconcentrate by a factor of more than 100,000. The biomimetic SPME method uses a more finely tuned scale that distinguishes for each chemical's individual partition coefficient instead of lumping them all into two large groups. Both methods may thus be implemented to assist in screening of complex industrial effluents, however it must be kept in mind the different type of information provided and how that matches with the goal of the screening.

A1.11 Relationship of PBS to body burden

When selecting methods for PBS determination, it can be important to the user that the parameter is relevant to the prediction of body burdens in real organisms. The best case is when it is not only empirically related, but that there is a known mechanistic reason for the relevance.
One may argue that the most direct way to measure of measuring body residues is to measure them in biota themselves. Determining internal residues in exposed biota gives a direct indication of bioavailability but also integrates many other species-specific and other modifying factors, such as metabolism, microhabitat, feeding behaviour, etc. However, there are major drawbacks for using measured residues in biota on a regular basis, including time and budget-consuming procedures and the fact that it is inconvenient for standardization due to high variability in biotic sample concentrations. Futhermore, complex effluents contain a large

number of unknown chemicals which are difficult to separate from the biotic matrix, making it impractical to use FID or even MS for quantification (de Maagd, unpublished data). Extrapolation is needed for predicting bioavailability from one species to the next. Testing for bioaccumulation from environmental samples in bioassays is normally only possible within a small range of test conditions (*e.g.* O_2, NH_3, salinity), which many effluent samples for example do not meet. Furthermore, there are currently large efforts to reduce the number of test organisms.

The drawbacks of measuring body residues directly have stimulated the exploration of chemical extraction techniques to estimate bioavailability based on empirical observations or mechanistic theories about bioavailability and subsequent bioaccumulation by organisms. This enables faster and cost-beneficial screenings of bioavailable substances and estimates of potential internal exposure to chemicals, complementing existing techniques. Chemical extraction methods are more easily standardized and therefore more easily implemented for risk assessments and regulations. However, it is a more difficult task to use the PBS parameter measured with EGOM-LLE or biomimetic SPME to estimate a total body burden in biota.

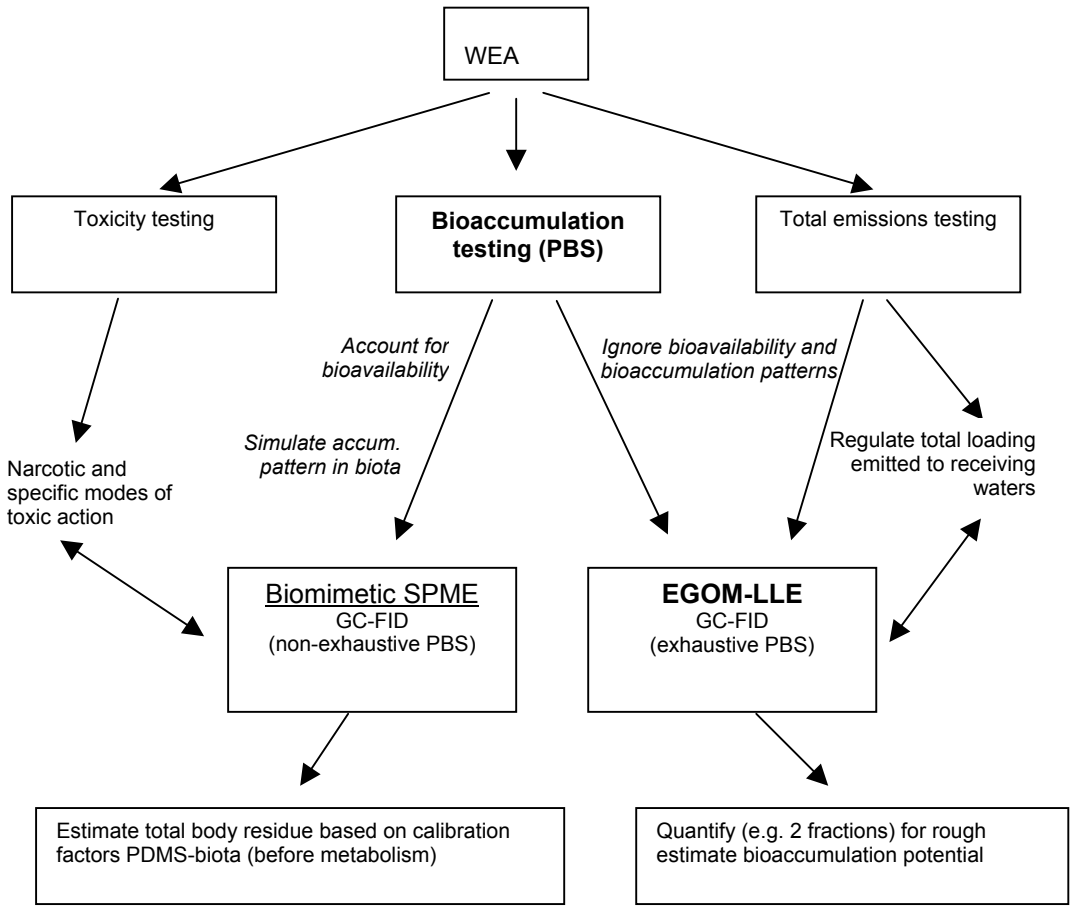

Figure A1-2. Decision-making guide for selecting appropriate bioaccumulation methodology.

A1.12 Establishing 'trigger' PBS values for effluents

For the EGOM-LLE method, some toxicity data has been generated to give an idea of some empirical relationships between the EGOM-PBS parameter and hazard for biota exposed to the effluent. However, because of the rough method of weighting the contributions based on two ranges of K_{ow}, the uncertainty of a trigger value for the bioaccumulation parameter is high. Body burdens cannot be accurately estimated from an LLE or even an extraction of SPM, as the EGOM method will be similar to if the loading of SPM is high. This is because the nature of

SPM in different effluents is not standardised, and the different degrees and types of binding of chemical analytes to the SPM will affect the efficiency of the extraction.

For estimating the total load of hydrophobic chemicals entering the receiving water the EGOM-PBS parameter is a good choice. The trigger value would then depend on the maximum acceptable loading determined by the dilution factor, conditions in the receiving water, frequency of emissions, etc. Monitoring the PBS in this way can also lead to signals of fluctuations in the emissions throughout a period of time. Trigger values may then be determined as a certain factor above a value determined to be nontoxic in assays with test organisms.
Combining the log $K_{PDMS\text{-}water}$ to log K_{ow} relationship for 17 nonpolar compounds (Mayer *et al.* 2000) with the relationship of Veith *et al.* (1979) results in a difference of about a 3 between concentrations in PDMS and biotic lipids (Figure 2). If the relationship of Veith is reliable for the compounds in the effluent in question, this could be considered a reasonable conversion factor for fiber to internal concentrations. However, it is known that the $K_{PDMS\text{-}water}$ of polar compounds is not as easy to predict based on K_{ow} as sole descriptor. This relationship is therefore expected to be more appropriate for effluents containing nonpolar hydrocarbon mixtures.

In other studies where a small mixture of halogenated compounds were measured individually with GC and electron capture detection (ECD), the relationship was closer to a factor of 10 higher in biota (lipid normalised) than in PDMS (Leslie *et al.* 2004). Parkerton and coworkers use PBS derived from biomimetic SPME measurements of water contaminated with hydrocarbon mixtures and compare these to results of toxicity tests with different organisms. Using GC-FID with dimethylnaphthalene as external calibration standard, the toxicity of the mixtures (mainly the narcotic mode of action) is directly related to the total molar concentration measured in PDMS. This is a pragmatic approach, which does not attempt to validate the relationship between body residue and concentration in PDMS for each individual compound in these extremely complex samples. The assumption that the partitioning to PDMS reflects partitioning to the test organisms is reasonable because of the nonpolar nature of the compounds in the effluent, which as mentioned above, appear to behave in a more predictable way. This is both observed in comparisons between concentrations in biota and PDMS and is also in accordance with expectations based on interactions influencing partitioning in theory.

To determine trigger values a combination of empirical and theoretical mechanistic-based information will be necessary to consider. Polyparameter linear free energy relationships (pp-LFERs) can provide insight into the variation and the causes of variation between the ratio of $K_{PDMS\text{-}water}$ and BCF for different compounds and compound classes typical of effluents (Goss and Schwarzenbach 2001; Leslie *et al.* 2003). More $K_{PDMS\text{-}water}$ data for different chemicals (generated quickly by using a PDMS-coated capillary column) would facilitate such pp-LFER analyses.

A1.13 Limitations of EGOM-LLE and biomimetic SPME

Limitations of a method depend on the research question asked and some analytical limitations. Many of these points are listed in Table 2. Below some more general limitations of both methods are discussed. In addition, Table 3 lists some sources of error that can be expected for both methods.

Relevance to bioaccumulation parameter

- Being an exhaustive extraction, EGOM-LLE does not take bioavailability into account.
- Biomimetic SPME takes bioavailabiliy into account, but does so when the extraction condition criterion of negligible depletion is met.
- Because biomimetic SPME is a sensor for uptake from the aqueous phase, it may underestimate the concentrations in organisms of chemicals (generally with log K_{ow}>5)

which could be taken up through other uptake routes at a faster rate than can be compensated for by the fugacity driving towards an equilibrium with the water phase.

- Neither method takes metabolism or growth dilution of accumulated substances into consideration. Whatever bioaccumulation potential is determined with chemical methods is a 'worst case scenario' for organisms which do not metabolise and eliminate chemicals faster than by passive diffusion, and which do not grow fast. Both methods will tend to overestimate bioaccumulation in metabolising organisms for this reason, unless the metabolites remain in the body at a 1:1 ratio with parent compounds. (In that case, it is equally able to contribute to narcotic toxicity).
- Neither method takes into account the time it takes organisms of different sizes and shapes to reach the full potential bioaccumulation from a sample. Again, bioaccumulation potential is measured with chemical methods assuming that the chemicals have had sufficient time to reach and steady state concentrations in the exposed organisms. For larger organisms, this takes longer. Although larger organisms are higher in the food chain, meaning that biomagnification can play a role. Biomagnification is often less dramatic than one might expect however. Biomagnification factors are normally not as high as bioconcentration factors.
- Interpretation of the amount of bioavailable PBS that would be measured by biomimetic SPME in receiving waters after effluent is diluted presents some complications because dilution of an effluent with more water does not lead to a linear decrease of free concentration, as it does for the total concentration (Kienhuis 2004). This is because chemicals will be released from sorbent phases in a hydrophobicity dependent way until most of the chemical is present in the water phase, and the sorbent phase is exhausted. The effect will be stronger the more hydrophobic the chemical. So many dilutions are necessary to start diluting the equilibrium aqueous phase concentration of compounds with very high SPM-water partition coefficients. Because EGOM-LLE is a total extraction, dilution of the effluent results in a linear decrease in the amount of PBS resulting from performing the method. To circumvent the application of dilution factors after the fact, biomimetic SPME could be performed on an effluent already diluted with receiving water.

From chromatogram to 'PBS'

For EGOM-LLE, a C20 is in with MS leads to the same concentration as dimethylnaphthalene with FID. Until this used to calibrate, although no published data has been found validating this choice of external standard

For biomimetic SPME, 2,3-dimethylnaphthalene has been shown to have an average molar response factor compared to a large set of different organic chemicals (n=50). For measurements with GC-MS, Verhaar *et al.* (1995) performed a similar exercise as Leonards (2001) and trichlorotoluene had a relative molar response of 1, making it a good choice for measurements if GC-MS is used. Trichlorotoluene appears to give similar PBS results as dimethylnaphthalene in the SPME method (Keinhuis 2004). However, any calibration of a complex mixture is accompanied by some uncertainty, when compared to calibrations of individual chemicals.

In both methods, the integration is an important step towards arriving at a reliable PBS result. The integration of the full chromatogram requires a baseline that is stable during the run, otherwise integration of the unseparated peaks of complex mixtures is very difficult to perform in a standardized way. This limitation must be overcome by properly adjusted temperature programmes and the use of shorter GC columns if necessary. In doing this, a balance between overloading the column and optimised minimal peak separation and fast temperature programme must be found.

Detection limits of detecting bioavailable, bioaccumulation profile mimicking PBS with biomimetic SPME is 1 mmol/L PDMS. This falls in the range of a NOEC for narcotic toxicity (Parkerton *et al.* 2001; Leslie *et al.* 2003). The SPME fiber can sense aqueous phase concentrations up to levels of saturation of the water phase with the complex mixture (Leslie, 2003). Detection limits of the EGOM-LLE method are not published.

Quality Control

- Both methods require additional validation, particularly reproducibility and repeatability needs to be established for the methods as they are to be implemented by OSPAR.

Table A1-3. Sources of error in EGOM-LLE and biomimetic SPME methods

<table>
<tr><th>EGOM-LLE</th><th>Biomimetic SPME</th></tr>
<tr><td>Recovery of compounds in LLE not equal and losses may be expected in evaporation (and clean-up) steps that cannot be corrected for without knowing the identity and volatility of the compounds in the complex effluent. Non-quantitative recovery leads to underestimation of the total PBS.</td><td>Agitation efficiency not constant and measuring in the kinetic uptake phase instead of at equilibrium</td></tr>
<tr><td>Extraction efficiency of different chemicals will be different depending on the solvent chosen and the degree of binding (or sequestration) of chemicals to sorbent phases in the whole effluent.</td><td>If non-negligible depletion does occur, the effect will be stronger in samples with little SPM compared to samples with high levels of SPM (reloading possible, as in sediment tests with large sorbent capacity in sediment acting as large ‘sink’ for chemicals).</td></tr>
<tr><td colspan="2">Inconsistent integration of full scan chromatograms – especially if the baseline is unstable – can lead to errors (overestimation or underestimation) in PBS and also to poor repeatability.</td></tr>
<tr><td colspan="2">Calibration with external standard and not a complex mixture of chemicals as found as the effluent itself introduces some error in a precise quantification of the total loading. The same would be true for calibration of full scan chromatograms of extracts of whole organisms for direct determination of total body residues.</td></tr>
<tr><td colspan="2">Peaks of compounds with very short retention times (<3 min) will not normally be integrated for the PBS parameter in the methods as they are described here. These compounds are not expected to contribute a lot to bioaccumulation potential except in the case when the amount present is so large that despite lower BCF, it still makes up a considerable part of the total body residue.</td></tr>
<tr><td rowspan="2">This method roughly lumps chemicals into two groups, log K_{ow} 3-5 and >5 and estimates bioaccumulation potential by the total amount of chemicals in these categories. Real organisms potentially bioaccumulate would do so on a much more subtly graduated scale, proportional to the K_{ow}, and including chemicals under log K_{ow} 3 as well.</td><td>The extraction efficiency will depend on the bioavailability in the sample at the time of extraction. Therefore on the degree of bioaccumulation potential represents the case in the sample bottle, and not what could be happening in the receiving water with dilution and changes in pH or salt content, since these factors all influence bioavailability and fugacity.</td></tr>
<tr><td>To translate PBS measured with PDMS fibers to estimates of total body residues in organisms before biotransformation, calibration factors are needed. Some calibration factors have been reported for PDMS and other phases, (e.g. polyacrylate) but there is uncertainty in the translation. Particularly for polar compounds, the translation is difficult due to extra H-bonding interactions between solvent and solute that are difficult to simulate perfectly with a synthetic surrogate phase for biota.</td></tr>
<tr><td></td><td>Interpretation of the amount of bioavailable PBS that would be measured by biomimetic SPME in receiving waters after effluent is diluted presents some complications because dilution of an effluent with more water does not lead to a linear decrease of free concentration, as it does for the total concentration. This is because chemicals will be released from sorbent phases in a hydrophobicity dependent way until most of the chemical is present in the water phase, and the sorbent phase is exhausted.</td></tr>
</table>

A1.14 Conclusions

- If exhaustive-PBS is desirable, then EGOM-LLE is more favourable than biomimetic SPME.
- If non-exhaustive biomimetic-PBS is desirable, bioimimetic SPME is more favourable because bioavailability is taken into account and the bioaccumulation patterns simulate that in biota, making it easier to relate this PBS to (narcotic) toxicity observed in toxicity tests.
- A method should be evaluated for usefulness not only based on the method as a whole, but also based on the usefulness of the different steps. The methods may be adjusted to include or exclude steps for optimisation and flexibility of application.

A1.15 Future research recommendations

- Development of a reference material for complex effluents in order to allow users to test their method against a standard reference material.
- More validation of extraction phase (*e.g.* PDMS) with bioaccumulation potential in biota (*e.g.* with pp-LFERs).
- Decide on the criteria for determining 'trigger' values for screening exercises and base trigger values on theoretical reasoning and empirical data.

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Appendix 2. Protocol biomimetic SPME method

PROTOCOL

Determination of potentially bioaccumulatable substances (PBS) in whole effluents using biomimetic solid-phase microextraction (SPME)

OSPAR Intersessional Expert Group (IEG) on Whole Effluent Assessment (WEA)

Interlaboratory Study

2005

Heather A. Leslie and Pim E.G. Leonards
Netherlands Institute for Fisheries Research (RIVO)

Content:

Introduction

This protocol describes a partitioning-based methodology including essential background information for the determination of potentially bioaccumulating substances (PBS) in whole effluents using biomimetic solid-phase microextraction (SPME). The term "biomimetic extraction" is used to distinguish this method from other common analytical extraction methods, such as Soxhlet, SPE, XAD and other types of SPME applications using small sample volumes (*e.g.* Langenfeld *et al.* 1996) or non-equilibrium SPME.
Based on the pioneering work by Verhaar *et al.* (1995) and Van Loon *et al.* (1996), a biomimetic extraction uses a hydrophobic absorptive phase to simulate bioconcentration by exposing the surrogate phase to a sample, *e.g.* effluent, and measuring the total molar concentration using minimal separation gas chromatography. Such a sum parameter is considered useful for screening of effluents for chemicals with potential to accumulate in biota. It also provides a unique type of information unavailable from the total extraction methods mentioned above, because it mimics (nonmetabolizing) biota in the uptake pattern. Just as with biotic lipids, SPME fibers absorb the chemicals, weighted according to their hydrophobicity. This gives information on the overall potential bioconcentration of a complex mixture of chemicals in a sample with vastly different bioconcentration factors in a single step.
This method also takes bioavailability of the chemicals in the sample into account, which is considered an added advantage, particularly in the many cases where bioavailability plays a major role in governing toxicity.
The surrogate (fiber), just as biota, takes up chemicals from a sample by passive diffusion between the water phase and the hydrophobic phase, until steady state is reached. This sum parameter is more directly related to total body residues (in non-metabolizing organisms) than many other methods. The PBS parameter is therefore also used to predict the likelihood of baseline toxicity in exposed organisms (Verhaar *et al.*, Van Loon *et al.* 1996, Verbruggen *et al.* 2000, Parkerton *et al.* 2000, Leslie *et al.* 2002, Van der Wal *et al.* 2004), a nonspecific type of toxicity for which concentration addition applies (Könemann 1981).
The PBS method described in this protocol is a solventless method without the need for clean-up steps, making it in principle a simple, fast, green procedure using a minimum of environmental laboratory equipment. It can be viewed as a quantitative screening method for PBS to complement already existing chemical testing and bioassays in performing whole effluent assessments.

Aim

This extraction method has been developed for whole effluents and other environmental samples to assess the degree to which organic chemicals present in the sample would potentially bioaccumulate in organisms. It has been designed to take bioavailability into account by performing negligible depletion extractions (nd-SPME, Verhaar *et al.* 1995 and Vaes *et al.* 1996).

Principle and synopsis of method

SPME fibers are exposed to whole effluents, with agitation, for 24 h. The polymer coating on the fibers is poly(dimethylsiloxane) or PDMS, a viscous liquid. The volume of water is large compared to the volume of PDMS (nd-SPME). Chemicals partition to and absorb into the PDMS. Per effluent sample, SPME fiber measurements are performed in triplicate. The fibers are removed from the effluent solution and injected into a gas chromatograph (GC) equipped with a flame ionization detector (FID). The column used is shorter than normal and the temperature programme of the GC is adjusted so that the chemicals reach the detector within a short time span to minimize separation of separate peaks (Verhaar *et al.* 1995 and Van Loon *et al.* 1996),

in contrast to most standard chromatographic methods. This adjustment enables easier determination of total area under the curve, which is quantified with a liquid injection of a standard with an average response factor. This enables molar quantification of the signal to within a factor of about 2 of the actual molar concentration.

Materials

For n samples:
SPME fibers (n)
Poly(dimethylsiloxane) *i.e.* PDMS fibers (Supelco, Bellafonte, CA, USA)
1 cm long
110 μm internal diameter (fused silica core)
100 μm polymer coating thickness (PDMS)
0.66 μl volume of PDMS coating (the hydrophobic phase)
SPME fiber holder (2)
An SPME fiber holder stabilizes the fiber shaft during injection and so that the fiber can be injected to the same depth each time using the scale on the fiber holder. Having access to two of these devices is convenient so that one fiber can be prepared while the other one is being injected. One device per fiber is totally unnecessary.
2,3-dimethylnaphthalene, 40 mg/l in ethylacetate
To quantify the amount of chemical that is desorbed from the fiber during analysis, an external standard in solvent is injected (1μl) three times before and after a series of fiber injections. 2,3-dimethylnaphthalene was chosen based on its average molar response for FID (Parkerton *et al.* 2001 and Leonards 2001). The average signal of these injections used, and the total molar concentration on the fiber is calculated using the total peak area of the chemicals desorbed from the fiber and the ration of moles to signal for the external standard.

250-ml glass sample bottles with PTFE lined caps with hole for fiber (n)
2-cm stir bars (n) Teflon coated,
magnetic stirrers (n)
GC-FID with short column (see below)
SPME inlet liner, diameter depends on the brand of injector. (Supelco, Bellafonte, CA, USA)
Using an inlet liner for SPME creates a high linear flow rate of the carrier gas around the fiber coating and enhances the desorption process.

GC column specifications

Column: J&W, Folsom, CA, USA, DB-1 (or similar column, such as the slightly more polar DB-5, esp. if bleeding is a problem. Using a different column will change peak order slightly but should not affect PBS parameter)
length 10 m
ID 0.25 mm
film thickness 0.1 μm

Injection depth for fibers

The correct injection depth must be determined for each injector used for analyses to ensure that the fiber is located in the middle of the liner. Note the position on the fiber holder and use the same depth for each fiber injection.

GC conditions

Fibers must be thermally desorbed in splitless mode at an injection temperature of 250 °C. A fast temperature program is used: starting at 40 °C for 2 min, followed by an increase to 320 °C at a rate of 30 °C/min. 290 °C is high enough to remove chemicals from the column that

contribute to bioaccumulation. Increasing the temperature programme to 320 °C is sufficient to get C38 off the column. (An alternative is to, after the run, allow the column temperature to increase to 310 and hold for 5 min before cooling again.)

1. Conditioning and cleaning fibers

Before using a brand new fiber, it must be conditioned for at least 2 hours at 260 °C in an injector of a GC. The condition of the fiber should be visually examined, using magnification (microscope or magnifying glass) for damage to the coating or breakage. The fiber should be measured to check its length (1 cm). The quality of polymer in Supelco fibers is generally consistent, with PDMS fibers from different batches behaving similarly in terms of partitioning properties. However, if desired, a check of a new fiber may be made by exposing it to a known solution of organic chemical at low concentration in pure water for which the partitioning kinetics are known.

Fibers that have already been used in analyses do not have to be conditioned as new fibers, but desorbed directly prior to exposure to effluent samples (10 min, 260 °C). Cleaned fibers should be added to the sample immediately.

2. Effluent sample handling

Effluent samples are taken in 1-litre bottles and an antibacterial agent (1 ml of 1 mg/ml silver nitrate) is added to prevent microbial breakdown during transport and storage before analysis.

3. Preparation of 250-ml effluent sample bottles

Effluent samples are shaken, and quickly and evenly distributed over three identical 250-ml glass bottles with a flat bottom to make it possible for a PTFE-coated stir bar (small size, 2 cm[1]) to spin constantly without jumping. Care must be taken to evenly distribute particulate matter. Any sticks or other hard objects must be removed from the effluent before transferring it into the 250 ml bottles as they will affect the total volume in the bottles, and most importantly are likely to damage the fiber during the agitation.

Add the stir bar, and leave no headspace in the extraction sample bottles (total volume will exceed 250 ml). A completely filled 1-litre bottle of effluent sample should be sufficient to fill three 250 ml bottles. The liner of the screwcap should be lined with PTFE. The screw cap should have a (small) hole in the centre through which a shaft of the SPME fiber can be pierced.

4. Exposing SPME fiber to agitated sample

A separate injection needle or old fiber shaft should be used to make the initial hole in the PTFE screwcap liner. Piercing a septum or liner for the first time with a fiber shaft may damage the fiber. Once the sample bottle is closed and there is a hole pierced in the septum, a thermally desorbed, clean SPME fiber may be inserted (may be done without the fiber holder if desired, but it is not necessary to use a holder during the exposure period). The fiber should be drawn into the protective metal shaft to protect it when not exposed to the solution. Once immerged, expose the fiber to the solution in the middle of the sample bottle. Expose the fiber once the stirring has started to avoid jumping stir bars from breaking the fiber, or start magnetic stirrer slowly. Ensure that no heat is turned on (an option on some magnetic stirrers). Some heat will be generated by the stirring, but this should be the same for each sample (use identical

[1] Using larger stir bars results in less efficient agitation. Efficiency of stirring can be compared by adding a drop of dye to water in which different sized stir bars are spinning – the effect is visible.

stirrers). If possible, the magnetic stirrers should be kept in a dark room or covered to avoid photodegradation of chemicals in the sample.

The reason for agitation (by stirring) during solid-phase microextraction to increase the rate of uptake of chemical by the fiber (Louch *et al.* 1002). It is important that all agitation conditions are kept identical among the samples being tested in order to achieve repeatable results. In principle, if magnetic stirrers are operated at maximum speed, the extraction is an equilibrium extraction. However, because it cannot be guaranteed that for all chemicals in all effluents that sample equilibrium is reached, an increase in the exposure time, especially under agitation conditions, may contribute to an extra uptake of the most hydrophobic chemicals by the fiber compared to other samples[1]. If the stirrer is shut off, or stir bar otherwise stops stirring, kinetics decrease considerably.

The SPME fiber is exposed to the effluent sample and stirred on a magnetic stirrer for 24 h (plus or minus 30 min). The exact time should be noted. Switching off the stirrer at the 24.0-hour mark and retracting the fiber while remaining in the sample bottle significantly reduces uptake on the fiber if the injection cannot be performed exactly at the 24.0-h point.

5. Fiber injections

Ensure that the correct inlet liner is in place in the injector. Should some FID systems respond unfavourably to the narrow SPME liner, a regular diameter liner could be substituted. (Regular liners seem to have little effect on the analysis of easily desorbing chemicals, but the carryover should be checked carefully for a run with slowly desorbing chemicals.)
Transport sample bottle with fiber to be injected to the GC area. Directly before injection, carefully remove the fiber from effluent samples by first pulling back the fiber in the fiber shaft before removing the fiber from the bottle. Place the fiber in a fiber holder device. Remove any debris from and dry the fiber by wiping quickly on a clean tissue to prevent water from entering the GC system. Place the fiber back in the shaft, pull it out and repeat the drying and wiping process of the fiber. Work quickly to avoid loss of chemical from fiber to air. Allow the fiber to remain in the injector for the complete run. The splitter should be closed for the rest of the run.
Inject per series (typically 8 fibers) 1 fiber (cleaned by thermal desorption) as a blank sample.

6. External standard

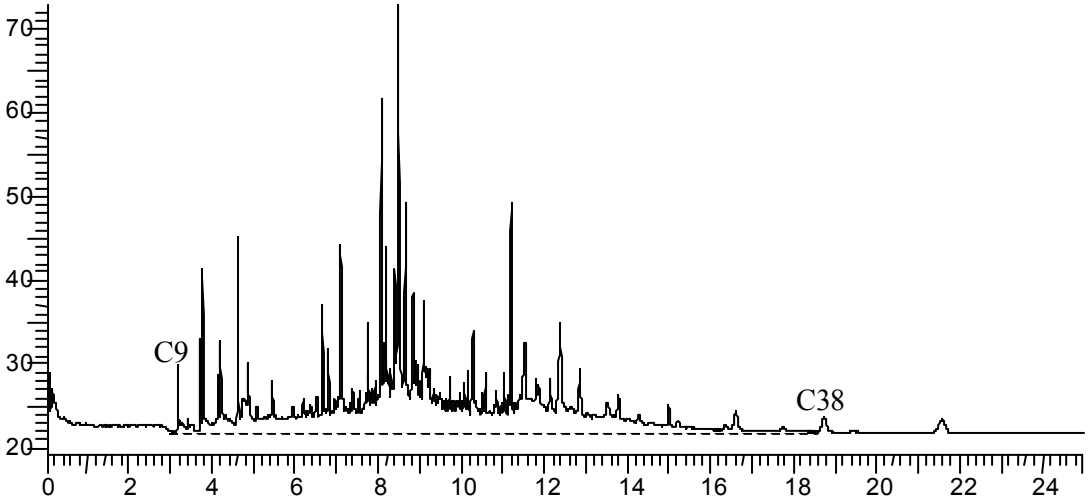
To quantify the amount of chemical that is desorbed from the fiber during analysis, an external standard, 2,3-dimethylnaphthalene (40 mg/l ethyl acetate) is injected (1 μl) three times before and after a series of fiber injections. For these injections a standard (wider) inlet liner must be used to accommodate the solvent vapour. The standard deviation of both triplicates should be less than 10%, with no significant difference ($p < 0.05$) between triplicate measurements.

7. Integration of total area under the curve

Integration should begin at ca. 3 min where the signal increases above background signal, and the end of the peak will be where the signal enters the baseline again, which will be approximately about the retention time of C38, as shown in the chromatogram below. Do not

[1] For very slowly absorbing chemicals (*e.g.* PCB153), 24 h is insufficient for such a hydrophobic chemical to reach equilibrium in pure water (Paschke *et al.* 2002). However, the presence of a matrix in the system may reduce the time to equilibrium (Oomen *et al.* 2000). In an SPME of soil slurry, the time to equilibrium for hexachlorobenzene in 15 μm fibers is considerably faster (van der Wal *et al.* 2003) than uptake from a solution in pure water (Leslie *et al.* 2002). It is unknown what the effect of a matrix is on the kinetics in typical effluents, which generally have lower amounts of suspended particulate matter than would be found in a sediment or soil slurry sample.

integrate later peaks as these can be due to bleeding of siloxanes from the fiber itself and are not relevant to the PBS parameter.



Example of integration of a chromatogram of an effluent sample.

8. Quantification of PBS sum parameter

The molar ratio for 2,3-dimethylnaphthalene is used to quantify the area under the curve for effluent samples. The PBS parameter has units of sum mmol of all chemicals per litre of PDMS (C_{fiber}). PBS (mmol/L PDMS) is calculated as the mmol of the external standard injected ($Amount_{Std}$), multiplied by total peak area of the sample (peak $area_{Sample}$), divided by average peak area of the 2,3-dimethylnaphthalene injections (peak $area_{Std}$). The volume of PDMS on these 100 µm thick fibers is 0.66 µl.[1] The PBS in units of mM (*i.e.* total molar concentration in the fiber) is calculated using the mmol injected divided by the volume of PDMS in litres.

$$C_{fiber} = Amount_{Std}\ [mmol] \times peak\ area_{Sample} / peak\ area_{Std} \times 0.66\ 10^{-6}\ L$$

9. Reporting

For each 1-litre aqueous sample (A, B, C, D and E), data is reported to RIVO. This includes:

- triplicate fiber data (mmol/L PDMS) with peak areas and final PBS data
- Peak areas of 2,3-dimethylnaphthalene injections are given for each series with the exact mmol injected.
- Blank fiber data measured in each series. The blank signal is not subtracted from the PBS values.
- Chromatograms for each fiber measurement showing start and end of integration should also be sent to RIVO.

In addition, participants are urged to report:

- any changes made to the protocol,
- specifications of solvents, instruments and columns used,
- possible difficulties encountered
- comments, suggestions

Reports may be emailed to heather.leslie@wur.nl

[1] Vol of fiber = PI*10*(0.155*0.155-0.055*0.055) = 660 µl
(Length 10 mm; PDMS thickness 0.100 mm; Silica core diameter 0.110 mm).

or sent to:
Netherlands Institute for Fisheries Research (RIVO)
c/o Dr. H. Leslie
Dept. of Environment and Food Safety
Wageningen University and Research Centre
P.O. Box 68, 1970 AB Ijmuiden, The Netherlands

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Other useful information

Dr. J. Pawliszyn has published two excellent books on SPME describing general theory and practical applications, including environmental applications. His webpage (http://www.science.uwaterloo.ca/chemistry/pawliszyn/) is a useful resource, at the University of Waterloo in Canada.

Appendix 3. Protocol EGOM-LLE method

PROTOCOL

Determination of potentially bioaccumulating substances (PBS) in whole effluents using the 'EGOM' Liquid-Liquid Extraction Method

OSPAR Intersessional Expert Group (IEG) on Whole Effluent Assessment (WEA)
Interlaboratory Study
2005

H.A. Leslie and P.E.G. Leonards
Netherlands Institute for Fisheries Research (RIVO)

CONTENTS

Introduction

This protocol describes a partitioning-based methodology including essential background information for the determination of potentially bioaccumulating substances (PBS) in whole effluents using a liquid-liquid extraction technique (LLE). Liquid-liquid extraction (LLE) or 'solvent extraction' is a conventional approach to preparation of aqueous samples for chemical analysis. It is the method described in most of the official protocols for extraction of aqueous samples (*e.g.* EPA protocols, etc.). An organic solvent that is immiscible with water is brought into contact with the sample, so that compounds in the sample are able to partition between the water phase and the solvent phase. Hydrophobic compounds that have the potential to accumulate in biota also have favourable solvent phase-water partition coefficients. The main differences between LLE and the (biomimetic) solid-phase microextraction (SPME) method is that LLE is a more exhaustive extraction, meaning that bioavailability or differences in bioconcentration factors are not taken into account.

In Sweden, the 'EGOM' LLE method has been developed to measure the 'extractable gas-chromatographic organic matter' in an effluent sample (Adolfsson-Erici, and Wahlberg 1992; Hynning 1996). This method is used in Sweden for the separation, identification and quantification of components of industrial effluents with bioconcentration potential (Hynning 1996, Tarkpea *et al.* 1998). In its original form, it begins with extraction with a mixture of hexane and *t*-butyl methyl ether, followed by HPLC fractionation into three different K_{ow} ranges. The use of this solvent system appeared to increase the fraction of chemicals with log K_{ow}<3 compared to cyclohexane. Those with log K_{ow}>3 are derivatised and a clean up with silica gel column are performed before analysis and identification with GC-MS. GC-FID of the fractions is performed to quantify identified compounds (Table 1).

The method is multi-stage and tedious but does provide a lot of information. For first-tier screening of effluents for bioaccumulation potential, it is not necessary in the first step to identify compounds with GC-MS. Therefore members of the IEG/WEA group focusing on bioaccumulation methodologies have chosen a shortened version of the original EGOM method, provided by Åke Unden (see Appendix). Some aspects have been harmonized with the SPME protocol being used in this study (Leslie, 2004), such as GC-FID conditions, and quantification based on 2,3 dimethylnaphtalene instead of eicosane. This enables a better comparison of the PBS values achieved by the SPME and EGOM-LLE methods.

The method has been tested by RIZA, and this protocol is also based on the protocol written by Paul Kienhuis (2005) of RIZA, who is acknowledged for valuable input.

EGOM summary provided by Åke Unden to the OSPAR IEG/WEA Group:

"The Determination of Potentially Bioaccumulating Substances (PBS) using the LPE method. Approximately 1 L sample was extracted twice with 50 ml cyclohexane. pH was adjusted to 10 and extraction was repeated twice with 50 ml cyclohexane. All extractions were performed in separatory funnels on a shaker for at least 2h. The four extracts were combined. The volume was reduced by rotary evaporation and finally by a gentle flow of nitrogen to dryness in a weighed vial. The residue was redissolved and an aliquot was injected on a gas chromatograph (HP5890A) with a capillary column (DB5 30m x 0.32 mm, 0.25 µm film, J&W Scientific) and flame ionization detector. The temperature of the column was programmed from 40°C to 320°C. The area under the chromatogram in the range corresponding to the normal alkanes n-C10 to n-C40 was integrated. Quantification was made using eicosane (n-C20H42) as a standard. The result is referred to as EGOM (extractable gas chromatographic organic material)."

Aim of Method

This extraction method has been developed for whole effluents and other environmental samples to screen for the presence of organic matter in the sample that could potentially bioaccumulate in organisms (range corresponding to the normal alkanes n-C10 to n-C40).

Materials

Effluent samples (300 mL per extraction, stored cool and dark in glass bottles)

Separatory funnels (*e.g.* 1 L) and shaking system

HCl (6M)

cyclohexane (*e.g.* glass distilled, 99+%, A.C.S reagent; Losses of the more volatile compounds in the extract occur in the evaporation process, and any contamination present in extraction solvents leads to higher background in the concentrated extracts. The solvent used for the extraction must be of the highest possible purity for this reason.)

NaOH (2.5 M) **(**Dissolve 50 g solid NaOH in 400 ml milliq water. Allow solution to cool to 20°C. Increase volume to 500 ml with milliQ water.)

litmus paper

flask for rotary evaporation and 60° bath

Na_2SO_4 to dry extract

nitrogen

2,3-dimethylnaphthalene, 40 mg/l in ethylacetate as external standard (store in freezer)

n-alkane standards (C9 and C38) in cyclohexane - retention time markers (store in freezer)

GC-FID with short column (ca. 10 m), GC column specifications: J&W, Folsom, CA, USA, DB-1, ID 0.25 mm, film thickness 0.1 μm (or similar column, such as the slightly more polar DB-5, esp. if bleeding is a problem. Using a different column will change peak order slightly but should not affect the EGOM parameter)

Method description

Effluent sample handling

Effluent samples are provided in 1-litre glass bottles and have been pre-treated with an antibacterial agent (1 ml of 1 mg/ml silver nitrate per litre sample) at the time of sampling. Samples are stored at 4°C in the dark. Should samples be stored more than 2 weeks before analysis, the samples should be stored frozen (in small volumes to avoid bottle breakage) for best results.

Extraction

Directly before splitting samples into aliquots for triplicate extraction and analysis, shake bottles well. To start the extraction, 300.0 ml of the effluent sample (room temperature) is acidified with 6M HCL (4 ml) to pH<2. Check pH with litmus paper by pH adjustments in this procedure. The sample is then extracted twice with 30 ml cyclohexane in a separatory funnel, which is placed on a shaker for 2 h for each extraction and then allowed to separate into two phases. Following this, the pH of the effluent sample is adjusted to ≥10 with 2.5M NaOH (4 ml) and two more extractions with 30 ml cyclohexane are performed as before. The four extracts (total ca. 120 ml) are combined and transferred to a flask for rotary evaporation (bath temperature 60 °C) to reduce the volume to 10-15 ml. The extract is dried with Na_2SO_4 and quantitatively transferred to a calibrated vial. The extract is further carefully concentrated under a gentle stream of nitrogen to a volume of 1.5-2 ml. The volume of the final extract is adjusted to 2.0 ml. If the extract is evaporated to dryness, loss of components will result; this should be avoided.

Injection in GC-FID

The extract (1 µl) is injected in a gas chromatograph with a capillary column with flame ionisation detection (GC-FID). Analysis is on a split/splitless injector in splitless mode (1 min). Injector temperature 290°C. A fast temperature program is used: starting at 40 °C for 2 min, followed by an increase to 320 °C at a rate of 30 °C/min.

Blanks Per series, a blank is run to account for background from solvents that can occur from concentrating extracts. Blanks and recoveries are therefore important to correct for in LLE. Participants are required to provide blank signals and original sample data.

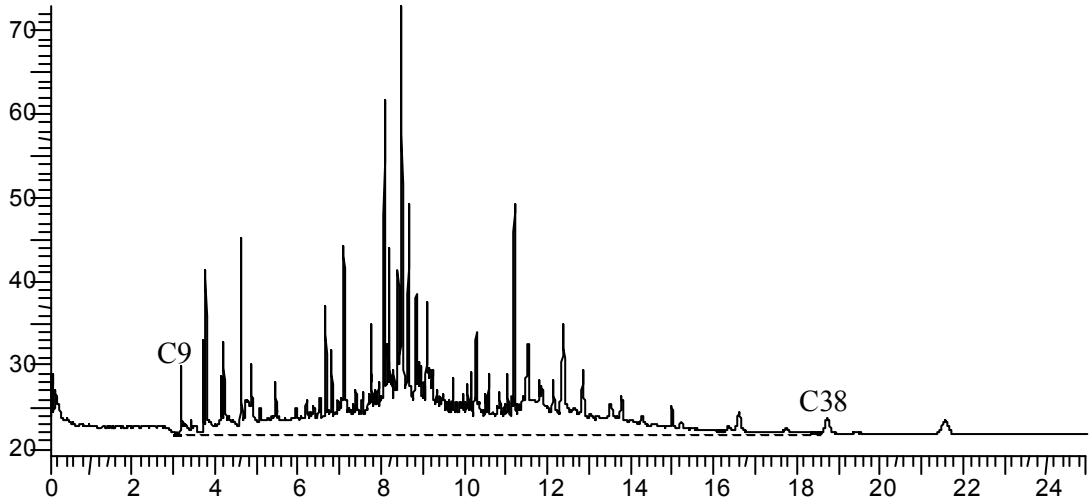
External standard

To quantify the amount of chemical that is injected, an external standard, 2,3-dimethylnaphthalene (40 mg/l ethyl acetate) is injected (1 µl) three times before and after a series of sample injections. 2,3- dimethylnaphthalene was chosen based on its average molar response for FID (Parkerton *et al*. 2001 and Leonards 2001). The average is calculated and the standard deviation of both sets of triplicates should be less than 10%, with no significant difference ($p < 0,05$) between triplicate measurements.
Because the FID is sensitive to number of carbon atoms, a substance with an average number of C atoms in the range being investigated is in theory a good choice of calibration standard for quantifying the amount of C injected.

Note: Injection of n-alkane standards C9 and C38 to determine retention times is recommended as a guide for marking start and end of integration, (see below).

Quantification of EGOM sum parameter

Integration should begin at ca. 3 min, after the solvent peak and where the signal increases above background signal, and the end of the peak will be where the signal enters the baseline again. The area under the curve of the chromatogram is easiest integrated between retention times for n-C_9 to n-C_{38}.



Example of integration of a chromatogram of an effluent sample (solvent peak not shown).

The signal for the external standard, 2,3-dimethylnaphthalene, is used to quantify the area under the curve for effluent samples. 1 µl of external standard with a concentration of 40 mg/L is injected, meaning $4*10^{-5}$ mg external standard is injected. It is assumed that the ratio of mass of organic C:signal is the same in the standard and in the samples.

Therefore, mg of C in the injected 1 µl of sample = mg of C in the injected external std/peak area external standard * peak area sample.
Because 1 µl of the sample extract with volume of 2000 µl was injected, the total mg of C in the overall sample is mg of C injected calculated above * 2000.
The original effluent sample volume extracted to 2000 µl cyclohexane was 300 ml. Therefore the above value divided by 0.300 L gives the mg of C/L effluent.
This result, the 'EGOM' value, expressed as "mg organic C/L," represents the total cyclohexane extractable amount of organic material in the sample.

EGOM (mg C/L) whole effluent =
[$amount_{extn\ sd}$ injected [mg] ÷ $peak\ area_{Extn\ Std}$ * $peak\ area_{sample\ extract}$] * 2000 /0.300 L

Reporting

For each of the 5 effluent samples (A,B,C,D and E), the following should be reported to RIVO:

- triplicate EGOM data expressed as mg organic C/L whole effluent, with peak areas and final PBS data
- peak areas of 2,3-dimethylnaphthalene injections are given for each series with the exact mg injected
- the blank data measured in each series. The blank signal is not subtracted from the EGOM values in the report.
- Chromatograms for each measurement showing start and end of integration

In addition, participants are urged to report:

- any changes made to the protocol,
- specifications of solvents, instruments and columns used,
- possible difficulties encountered
- comments, suggestions

Reports may be emailed to heather.leslie@wur.nl

or sent to:
Netherlands Institute for Fisheries Research (RIVO)
c/o Dr. H. Leslie
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Wageningen University and Research Centre
P.O. Box 68
1970 AB IJmuiden
The Netherlands

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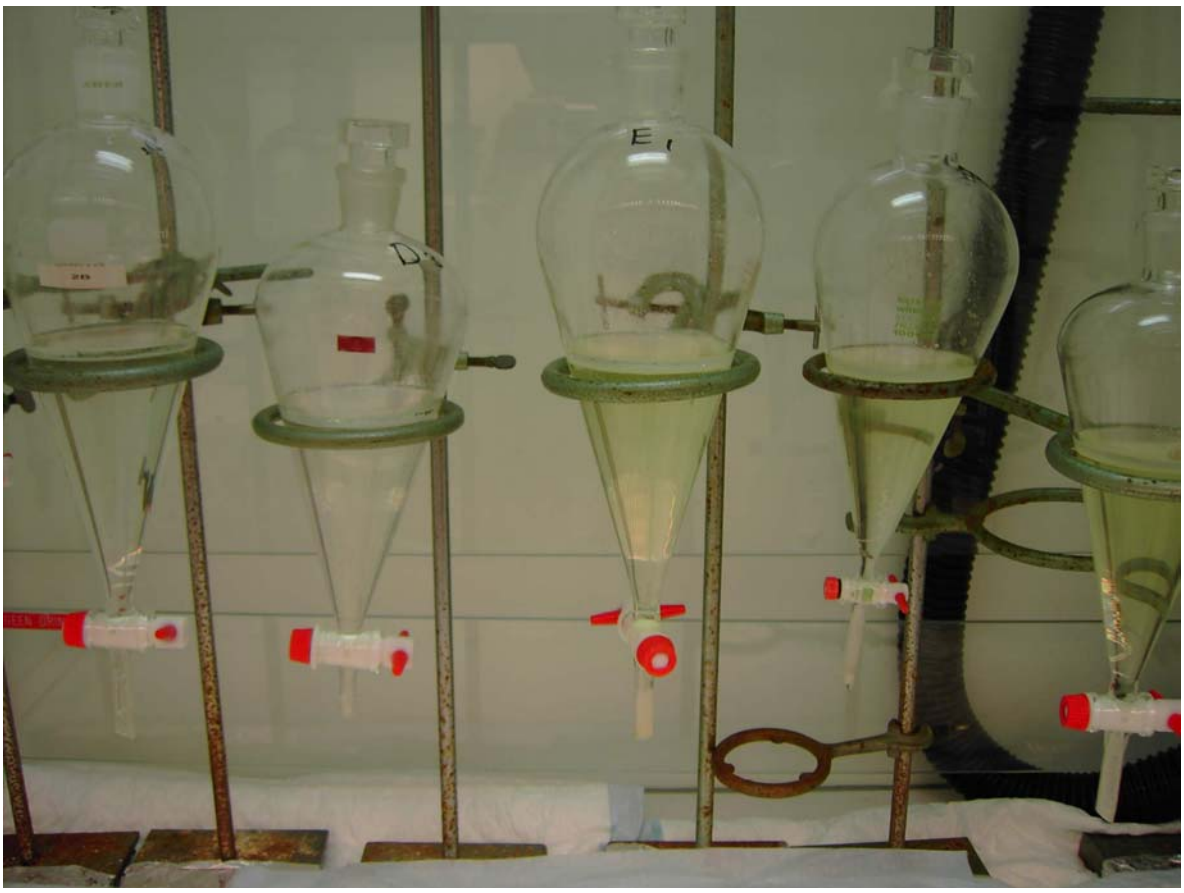
Appendix 4. Report and Chromatograms RIZA

RIZA results of the interlaboratory study of the OSPAR Intercessional Expert Group on Whole Effluent Assessment

The RIZA result of SPME and LLE analyses of effluents.

17 november 2005



RIZA results of the interlaboratory study of the OSPAR Intercessional Expert Group (IEG) on Whole Effluent Assessment (WEA)

The RIZA result of SPME and LLE analyses of effluents.

17 november 2005

Colofon

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Client(s)	RIVO; RIZA dep WIL and WIA
Dated:	17 nov 2005
Status:	Concept
Document number	

Inhoudsopgave

Abstract

This report describes the results of an interlaboratory study organized by RIVO for two methods used for Whole Effluent Assesment.
5 samples have been extracted in triplicate by means of LLE and SPME.
The results represented as chromatograms and integrated data are shown and shortly discussed.

The LLE method results in cleaner chromatograms and a somewhat lower variance. The RSD's of the triplicate results of the SPME method have a range of 10 to 60%, whereas the LLE method results in a range of 6 to 30%.

During LLE extraction a "mousse" was formed of which the composition is unknown. In how far this has influenced the extraction efficiency is questionable.

1. Introduction

Riza has received the samples and protocols from RIVO around 15 Augustus.
The samples were stored at 4°C in the dark.

The samples have first been analyzed for SPME on 7 and 8 September. The FID detector however didn't work properly, which resulted in strange baselines and unreliable results for most of the samples.
After the SPME extractions the samples were stored again in the original bottles at 4°C to be able to reanalyze the samples later on.

The LLE extraction has been performed in two series in the period of 12 to 15 September.
The extracts were stored at 4°C and analyzed on 25 October.

The second series of SPME analyses were performed on 26 and 27 October

The resulting chromatograms were translated in csv files (comma separated values) and read into an Excel spreadsheet file.
Total area's were calculated by summing the intensities between the retention times of C9 and C38 and were compared with the total area of the 2,3 DMN peak.

2. Method

The extraction protocols for both methods were strictly followed.

For the GC-FID analyses two methods were made.
TEBfib.m for the analysis of fibers
TEBsyr.m for the analyses of syringe injected samples. It was used for the reference injections of 2,3 DMN for the SPME analyses and for all LLE analyses.
The only difference between the two methods is the split time.
For **TEBsyr.m** the split time is 0.5 min and for **TEBfib.m** 16 min.

Details about the settings are given in the figures below.

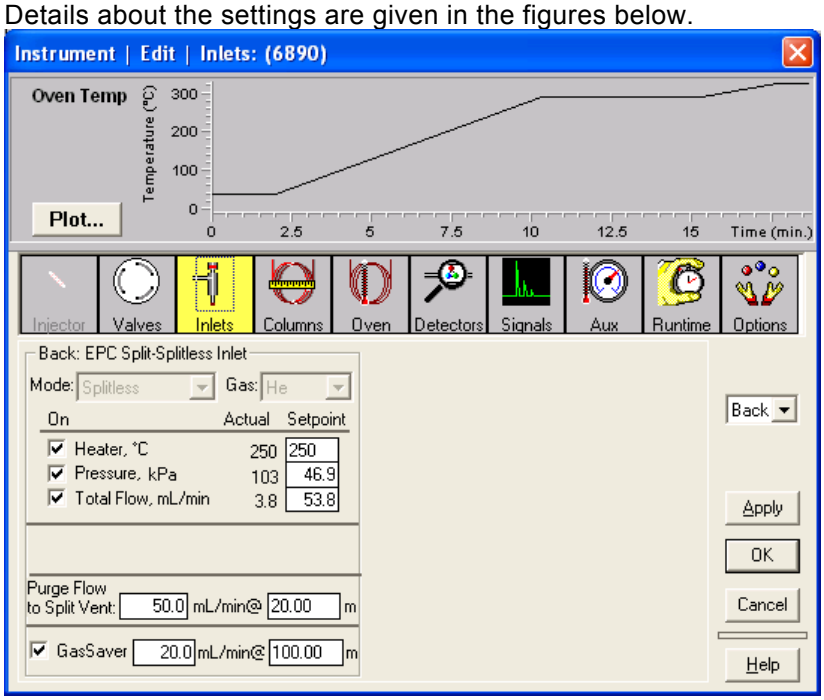

Fig 1: Inlet settings.
Note: The split time was first tested on 20 min but later on changed to 16 min.

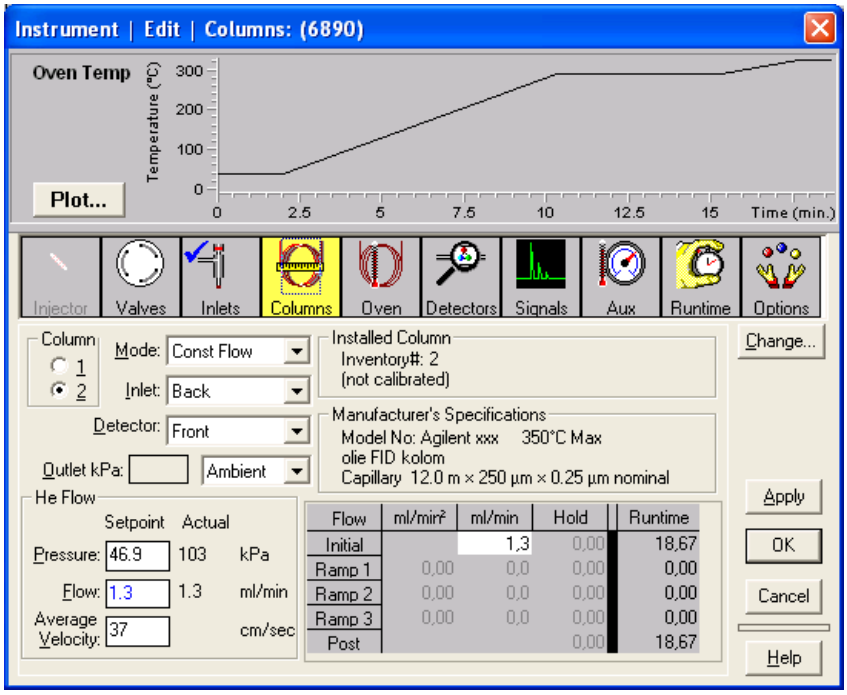

Fig 2: Columns settings.
.A 12 m DB-5MS column was used

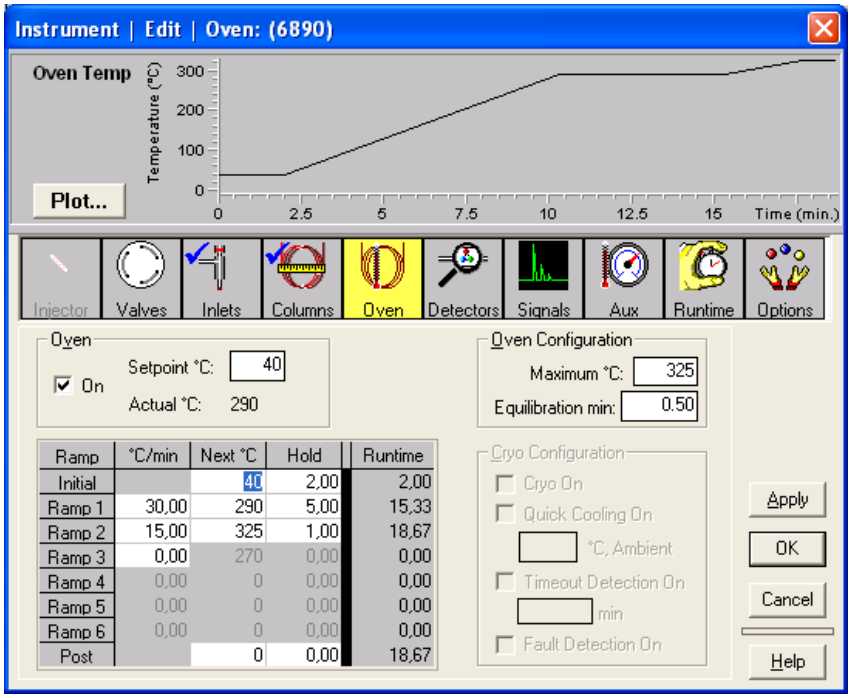

Fig 3: Oven settings.
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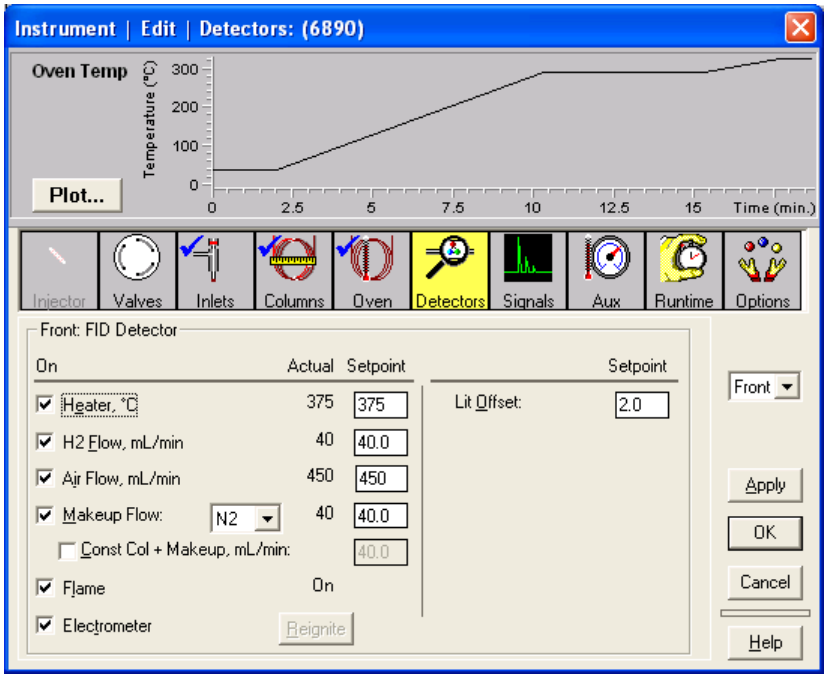


Fig 4: Detector settings.

A new stock standard of 2,3 DMN was prepared and combined with existing stock solutions of C9 and C38.
The working standard contained 40 mg/l 2,3 DMN.

Cyclohexane Baker 9258 “Baker ultra resi-analyzed” containing a minimum of 99.0% cyclohexane was used for LLE.

3. Results and Discussion

3.1 Liquid Liquid extraction.

After mixing and shaking the samples with cyclohexane for the first time a “mousse” was formed, which made it difficult to separate the samples in the separator funnel.

Fig 5: “Mousse” in the funnel.

Therefore the water part was roughly separated and the remaining “mousse” and cyclohexane collected in a 50 ml centrifuge tube.

The samples were centrifuged during 5 min at medium speed.
Most of the “mousse” was pressed together in a small band, which made it easy to transfers the cyclohexane layer to a rotary flask.
Sample E however contained so much “mousse” that the samples had to be centrifuged two times in between each extraction step.
After the first centrifugation step the cyclohexane above and in the “mousse” was remove by keeping the tube almost horizontal.
The “mousse” stayed stable and the water under it didn’t move. The cyclohexane run out of the “mousse” and was removed.

Fig 6: “Mousse” in the centrifuge tube.
.Before centrifugation

Fig 7: “Mousse” in the centrifuge tube.
.After centrifugation and removing of the cyclohexane layer above and in the mousse

During the second centrifugation step the “empty mousse” was pressed together and the remaining cyclohexane could be remove. Then the remaining cyclohexane, “mousse” and water have been moved back into the separator funnel for the next extraction step.

3.2 Analytical results LLE

In the protocol it was asked to give the results without blank subtraction. Because the calculations have been made in a spreadsheet based on csv results, it is easy to give results with and without blank subtraction.
To study the effect both data are given in Table 1 and summarized in Table 2.

File	Description Conc. in mg C/l	C9-C38	Blank subtracted C9-C38
T5101701	std DMN 40 mg/l	0,793	0,693
T5101702	std DMN 40 mg/l	0,475	0,375
T5101703	std DMN 40 mg/l	0,420	0,320
T5101704	Bl cyclohexane	0,114	0,014
T5101705	A1	0,305	0,200
T5101706	A2	0,233	0,141
T5101707	A3	0,378	0,273
T5101708	B1	0,272	0,181
T5101709	B2	0,288	0,186
T5101710	B3	0,261	0,163
T5101711	C1	0,289	0,188
T5101712	C2	0,291	0,186
T5101713	C3	0,250	0,155
T5101714	Bl cyclohexane	0,086	-0,014
T5101715	std DMN 40 mg/l	0,404	0,304
T5101716	std DMN 40 mg/l	0,423	0,323
T5101717	std DMN 40 mg/l	0,442	0,342
T5101718	Bl cyclohexane	0,078	0,002
T5101719	C1 duplo	0,222	0,145
T5101720	C2 duplo	0,235	0,156
T5101721	C3 duplo	0,205	0,133
T5101722	D1	0,264	0,180
T5101723	D2	0,281	0,205
T5101724	D3	0,398	0,328
T5101725	E1	3,861	3,781
T5101726	E2	4,383	4,306
T5101727	E3	4,372	4,298
T5101728	Bl including acid and base	0,127	0,051
T5101729	Bl cyclohexane	0,074	-0,002
T5101730	std DMN 40 mg/l	0,389	0,314
T5101731	std DMN 40 mg/l	3,003	2,927
T5101733	std DMN 40 mg/l	0,395	0,319

Table 1 Results LLE
Results in mg C/l without and with blank subtraction.

To be specific:
Without blank subtraction:
A line is drawn at the level of the smallest height of the baseline in the analysis sequence. The area above this line and between the retention times of the end of the C9 peak and the beginning of the C38 peak is used for calculations.
With blank subtraction:
The mean area of the two blanks (1 ul injection of cyclohexane analyzed by GC-FID) around a series of samples acquired in the above mentioned way is used for subtraction of that part of the series.

Conc. in mg C/l	C9-C38	Blank subtracted C9-C38
Sample A 3x		
mean	0,31	0,20
stdev	0,07	0,07
RSD%	23,73	32,48
Sample B 3x		
mean	0,27	0,18
stdev	0,01	0,01
RSD%	5,08	6,62
Sample C 3x		
mean	0,27	0,17
stdev	0,03	0,02
RSD%	10,12	13,48
Sample C duplicate 3x		
mean	0,22	0,14
stdev	0,02	0,01
RSD%	6,83	7,79
Sample D 3x		
mean	0,31	0,24
stdev	0,07	0,08
RSD%	23,29	33,41
Sample E 3x		
mean	4,12	4,04
stdev	0,36	0,37
RSD%	8,78	9,06

Table 2 Results LLE
Statistical evaluation

The extract of sample C is analyzed in duplicate to study the influence of the GC-FID analysis part of the method. The results in content and variation are quite similar.

The values shown in Table 1 for the standard injections are total areas. The areas for the 2,3 DMN peaks including a statistical evaluation is shown in table 3.

File	Area 2,3 DMN	Mean	St. dev.	RSD%
T5101701	1670331			
T5101702	1683030	1640186	63527	3,9
T5101703	1567199			
T5101715	1666124			
T5101716	1761304	1735836	61099	3,5
T5101717	1780081			
T5101730	1984057			
T5101731	2491485	2183546	270540	12,4
T5101733	2075097			
mean	1853190			
st. dev.	288694			
RSD%	16			

Table 3 Results LLE
Evaluation standard injections

The first two standard sets show a low variation and a low difference. The last standard set is higher and shows a higher variation mainly due through the high value of File T5101731. This is reflected in the total area of this standard in Table 1.
It was decided to use the values as such.

3.3 SPME

The fibers to be used were checked by a magnifying glass and it was decided to renew fibers F2, F3 and F4. The numbers of the fibers are mentioned in Table 4. The new fibers were “baked out” during 2 hours in a purged injection port at 260°C.
Before extraction each fiber was cleaned in a purged injection port at 250°C for 30 minutes. After withdrawal from the injection port the fiber was directly put into the sample.

3.4 Analytical results SPME

Table 4 shows the results of the SPME analyses

File	Description Conc in mMolair	C9-C38	Blank subtracted C9-C38
T5102501	st DMN 40 mg/l	0,91	0,68
T5102502	Bl ethylacetate	0,09	-0,15
T5102503	F1 A1	1,56	1,32
T5102504	**F2 A2**	3,09	2,86
T5102505	**F3 A3**	2,30	2,07
T5102506	**F4 B1**	3,05	2,81
T5102507	F5 B2	1,83	1,60
T5102508	F6 B3	2,10	1,86
T5102509	F7 C1	14,11	13,88
T5102510	F8 C2	14,60	14,36
T5102511	F9 C3	9,83	9,59
T5102512	st DMN	0,76	0,53
T5102513	Bl ethylacetate	0,21	-0,03
T5102514	st DMN	0,69	0,46
T5102515	st DMN	0,81	0,58
T5102516	st DMN	0,94	0,71
T5102517	Bl ethylacetate	0,41	0,17
T5102601	F1 D1	4,68	4,45
T5102602	**F2 D2**	5,70	5,47
T5102603	**F3 D3**	5,32	5,09
T5102604	**F4 E1**	23,32	23,10
T5102605	F5 E2	10,39	10,16
T5102606	F6 E3	42,42	42,19
T5102607	F7 Effluent	1,55	1,32
T5102608	F8 Effluent	1,02	0,79
T5102609	F9 Effluent	1,18	0,95
T5102610	F9 without removing	0,16	-0,07
T5102611	st DMN	0,75	0,52
T5102612	Bl ethylacetate	0,13	-0,10
T5102613	st DMN	0,74	0,51
T5102614	st DMN	0,73	0,50
T5102615	st DMN	0,74	0,51
T5102616	Bl ethylacetate	0,15	-0,08

Table 4: Results SPME
Without and with blank subtraction.
New fibers in bold.

Table 4 shows only one standard before the series. A GC series of two blanks and three standards was started the evening before the SPME analyses but the next morning the sampler gave an error message. So just for the SPME samples only one blank and a standard could be analyzed.
Together with the samples another effluent had to be analyzed in triplicate. That results also shown in Table 4 and 5.

The effect of the use of three new fibers (F2, F3 and F4) is highlighted in Table 4. It seems that the first extraction series of 25-20-05 results

in a much higher content, but this effect is not shown in the second series.

Conc in mMolair	C9-C38	Blank subtracted C9-C38
SPME sample A 3X		
mean	2,32	2,08
stdev	0,77	0,77
RSD%	33,19	36,91
SPME sample B 3X		
mean	2,33	2,09
stdev	0,64	0,64
RSD%	27,53	30,61
SPME sample C 3X		
mean	12,85	12,61
stdev	2,63	2,63
RSD%	20,43	20,81
SPME sample D 3X		
mean	5,23	5,00
stdev	0,51	0,51
RSD%	9,81	10,26
SPME sample E 3X		
mean	25,38	25,15
stdev	16,11	16,11
RSD%	63,49	64,07
SPME sample Effluent 3X		
mean	1,25	1,02
stdev	0,27	0,27
RSD%	21,89	26,80

Table 5: Results SPME
Statistical evaluation

File	area 2,3 DMN	mean	stdev	RSD%
T5102501	1674718	1674718		
T5102514	1670077			
T5102515	1743033	1697567	39661	2,3
T5102516	1679592			
T5102613	1695882			
T5102614	1659110	1670525	21996	1,3
T5102615	1656583			
mean	1682714			
st. dev.	29679			
RSD%	2			

Table 6 Results SPME
Evaluation standard injections

The standard injections show equal results with a low variance. Much better compared to the LLE standard series.

3.5 SPME - LLE

A major problem of the SPME method in previous experiments with a 30 m GC-column was the high variance of the results of triplicate analyses [Kienhuis 2004, RIZA document 2004.079X].
Therefore in this study an analysis method with a short column and a fast GC program has been tested.
The RIZA results however show no improvement. The RSD's of the triplicate results with a range of 10 to 60% are still very high (Table 4).

As an alternative the EGOM method was introduced and tested. The method uses liquid /liquid extraction with cyclohexane.
The variance of triplicate results of the method is better with RSD's of 6 to 30% (Table 2).

The chromatograms of all samples are shown in annexes A (LLE) and B (SPME). A comparison of the chromatograms shows that LLE produces cleaner chromatograms.
During extraction in the samples containing a higher amount of visible organic matter a "mousse" was formed, with the largest amount in sample E. The composition of the "mousse" is unknown

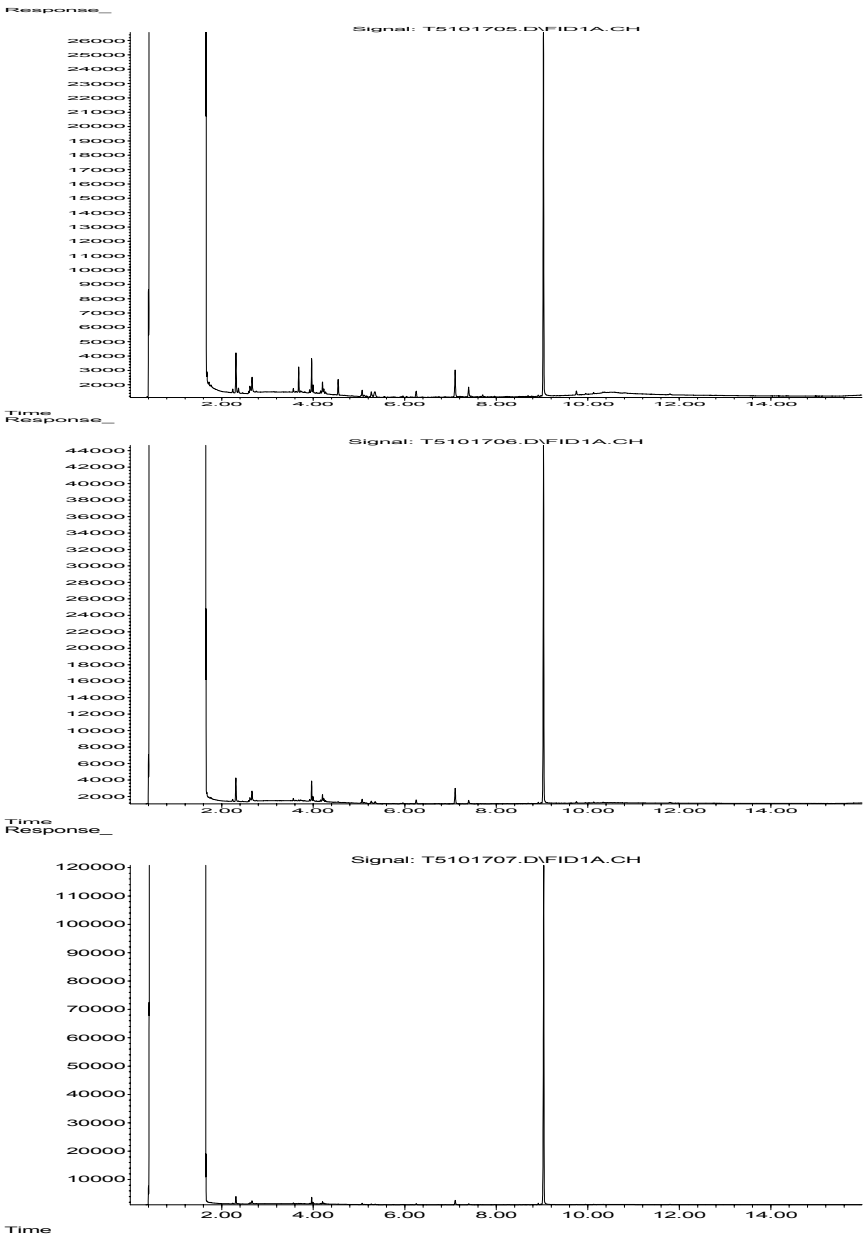
4. Conclusions

The LLE method results in cleaner chromatograms and a somewhat lower variance. The RSD's of the triplicate results of the SPME method have a range of 10 to 60%, whereas the LLE method results in a range of 6 to 30%.

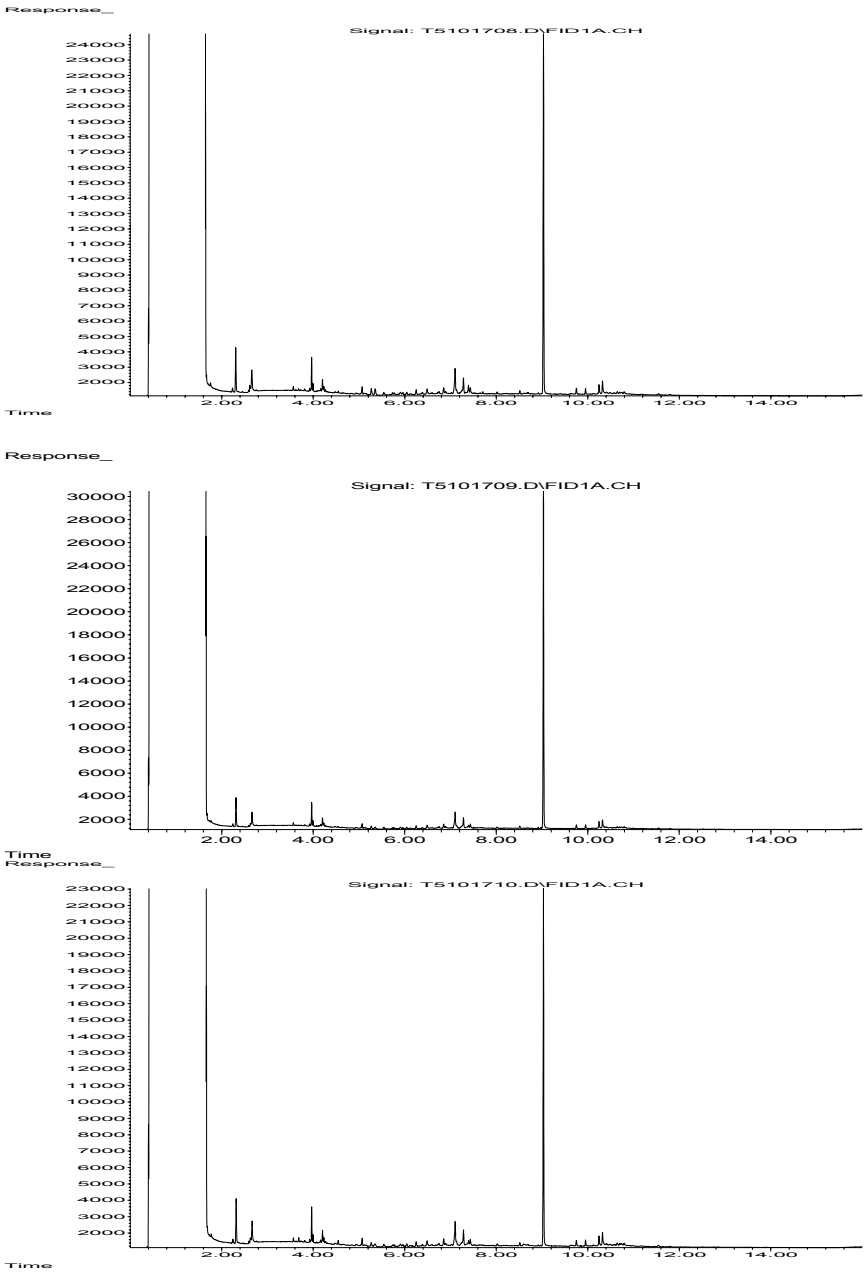
During LLE extraction a "mousse" was formed of which the composition is unknown. In how far this has influenced the extraction efficiency is questionable.

Bijlage A Chromatograms LLE

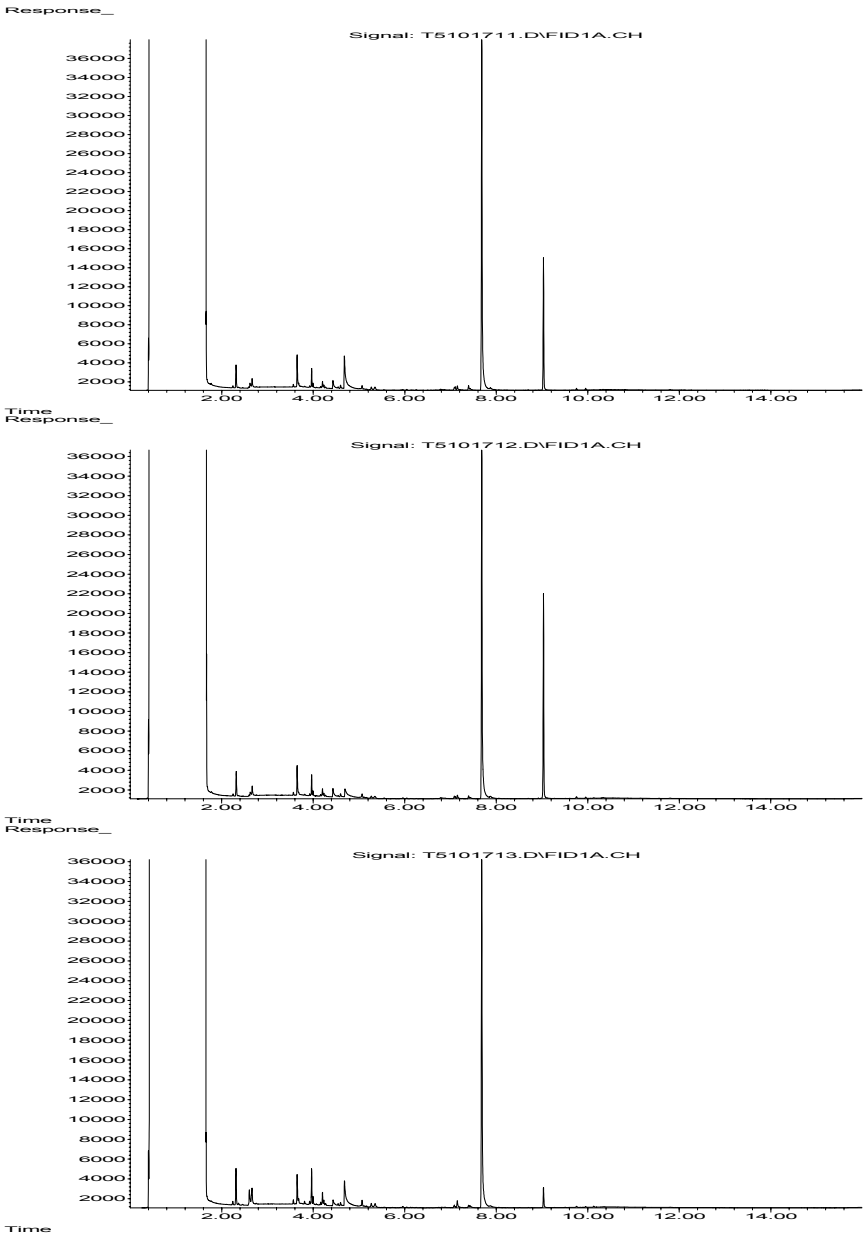
A.1 LLE - Sample A



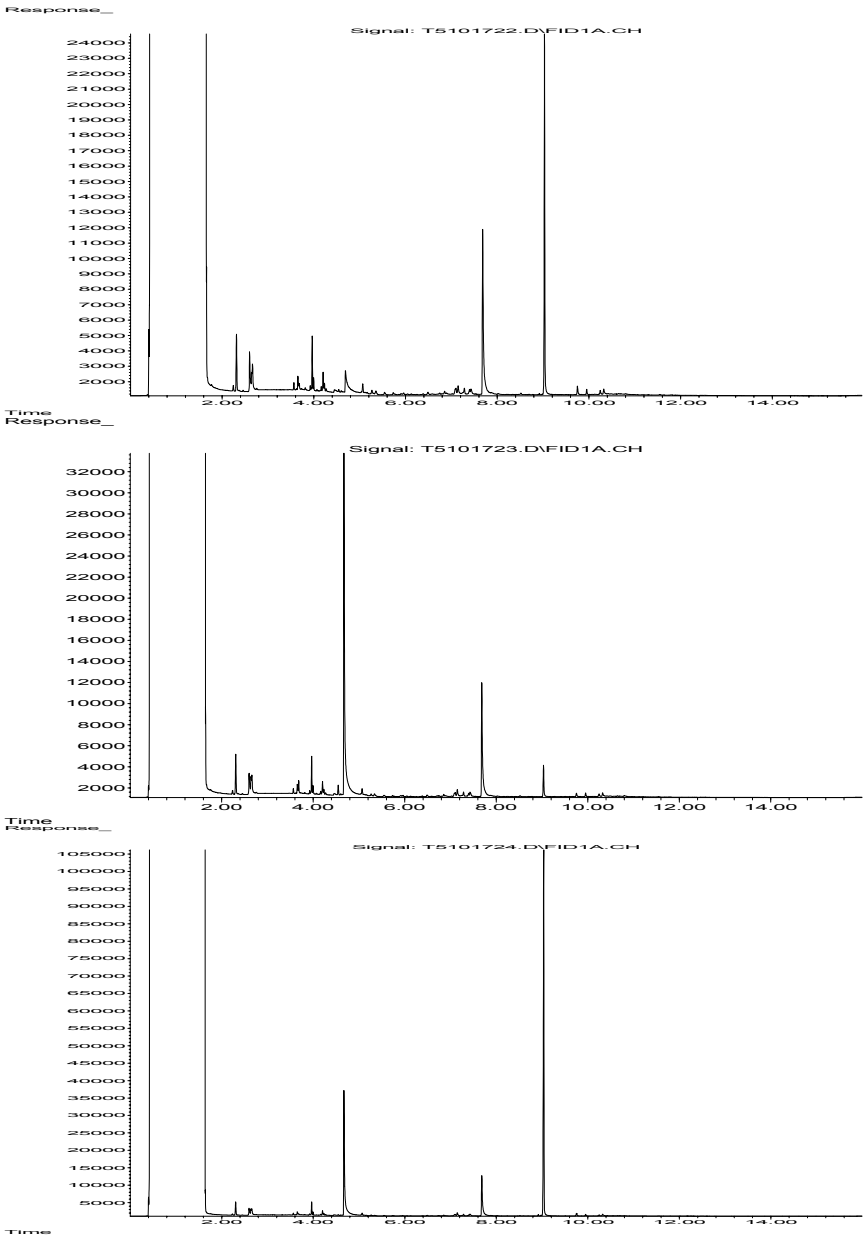
A.2 LLE - Sample B



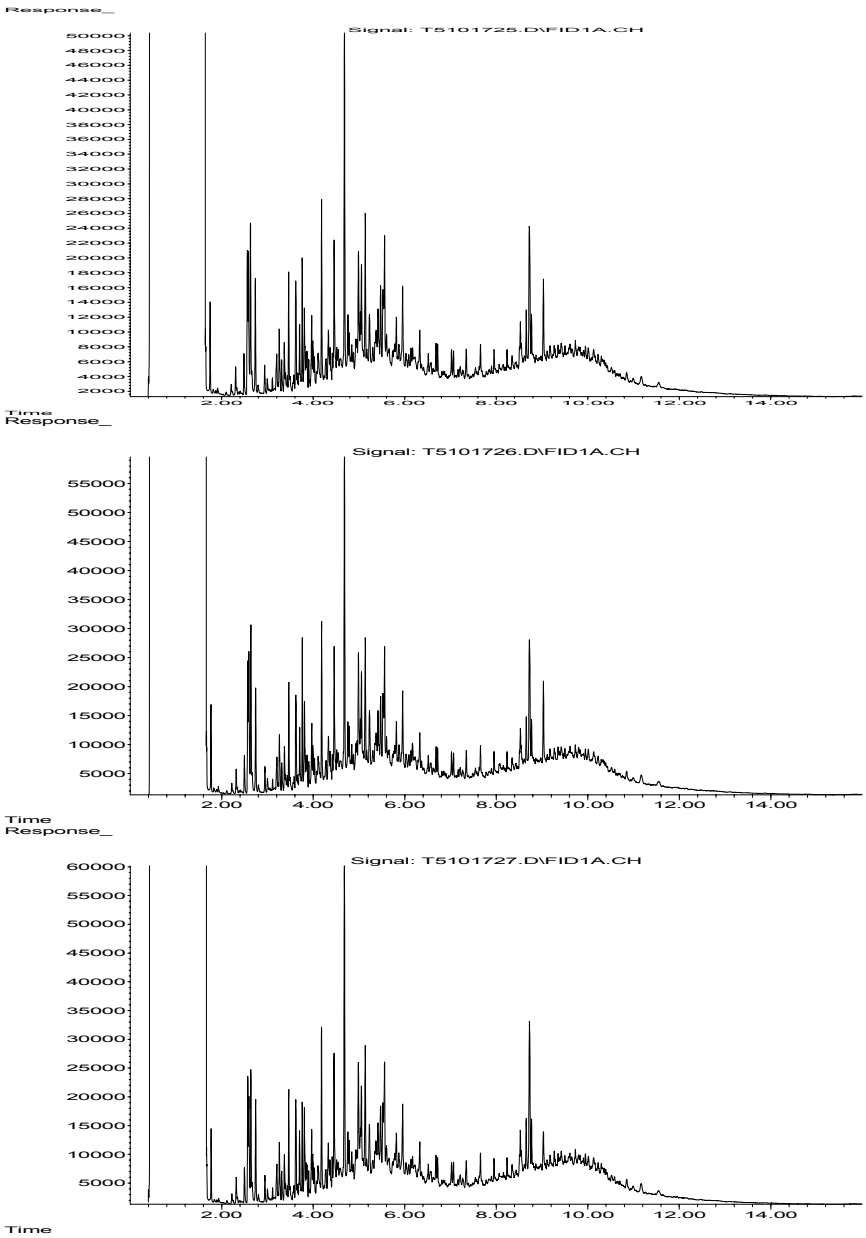
A.3 LLE – Sample C



A.4 LLE – Sample D

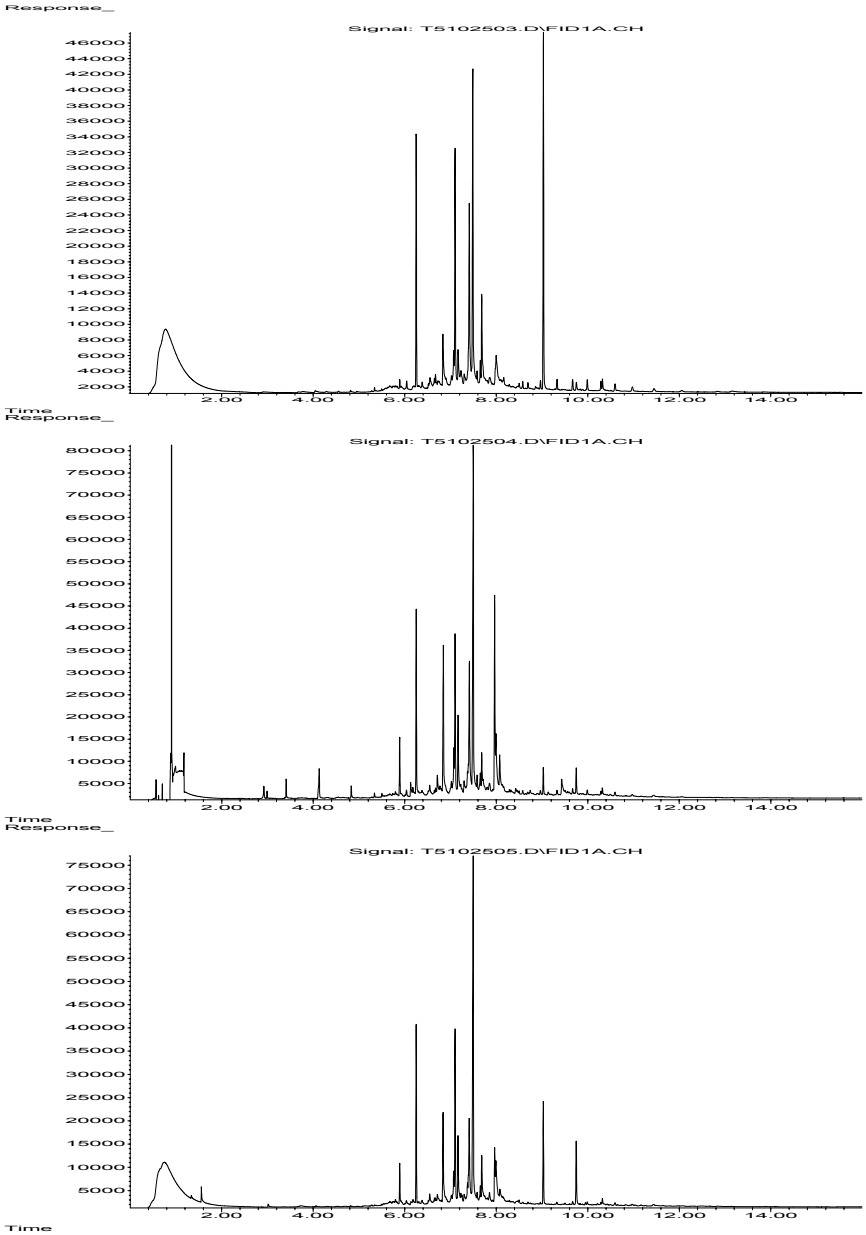


A.5 LLE – Sample E

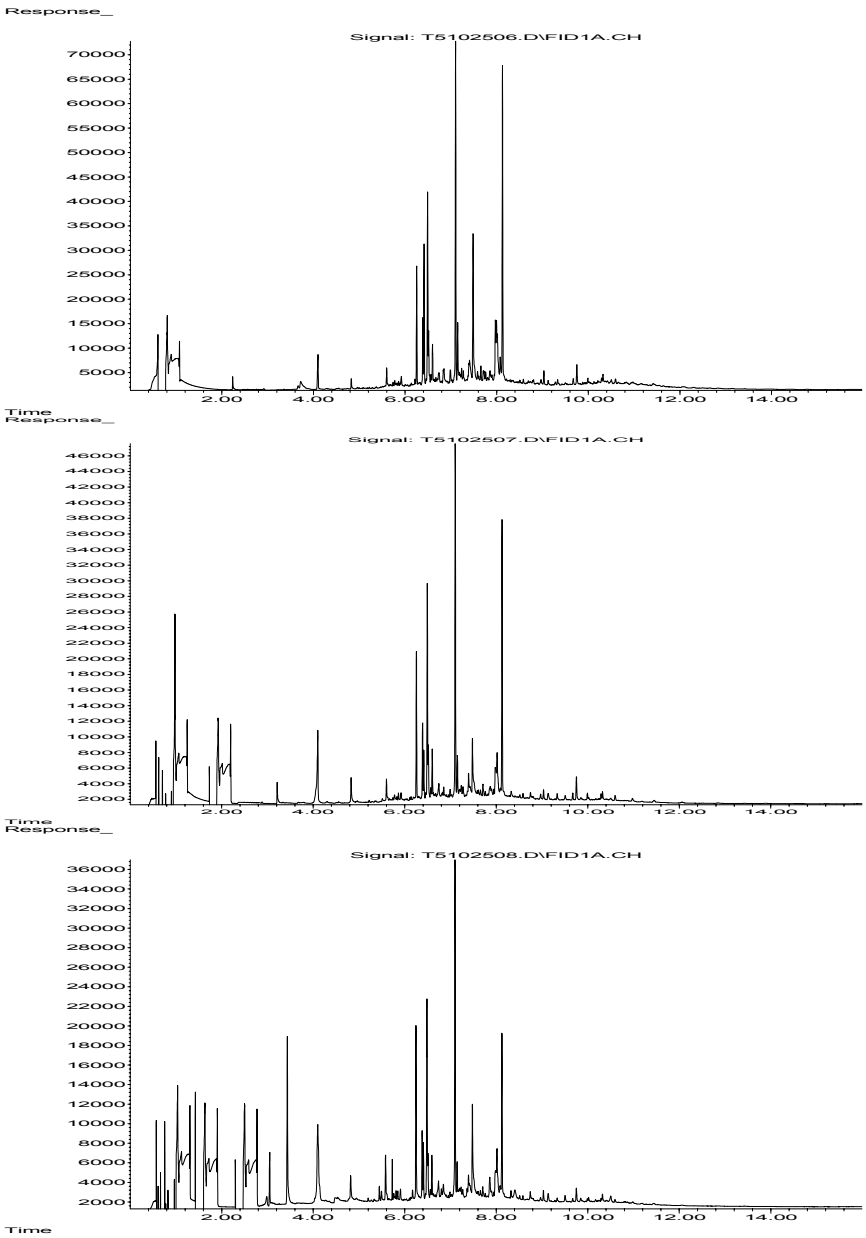


Bijlage B Chromatograms SPME

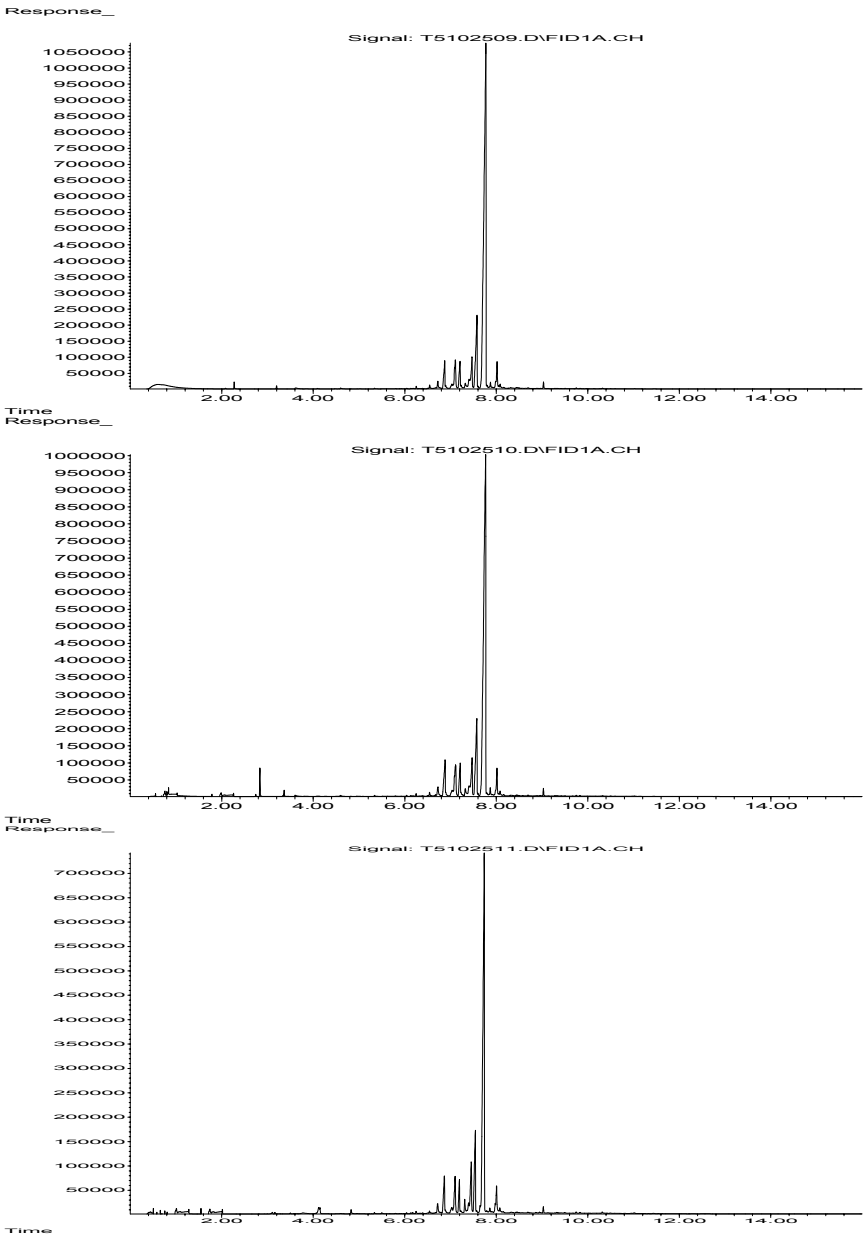
B.1 SPME – Sample A



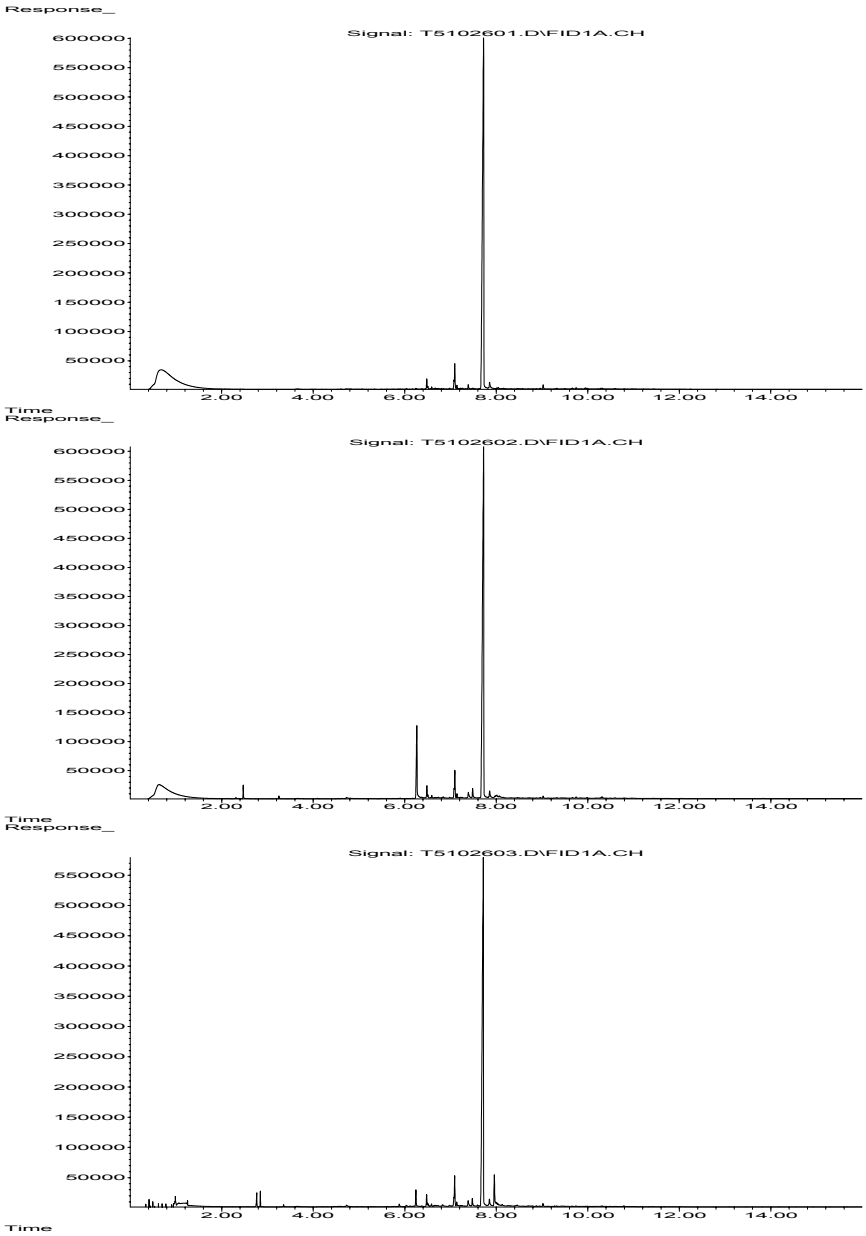
B.2 SPME – Sample B



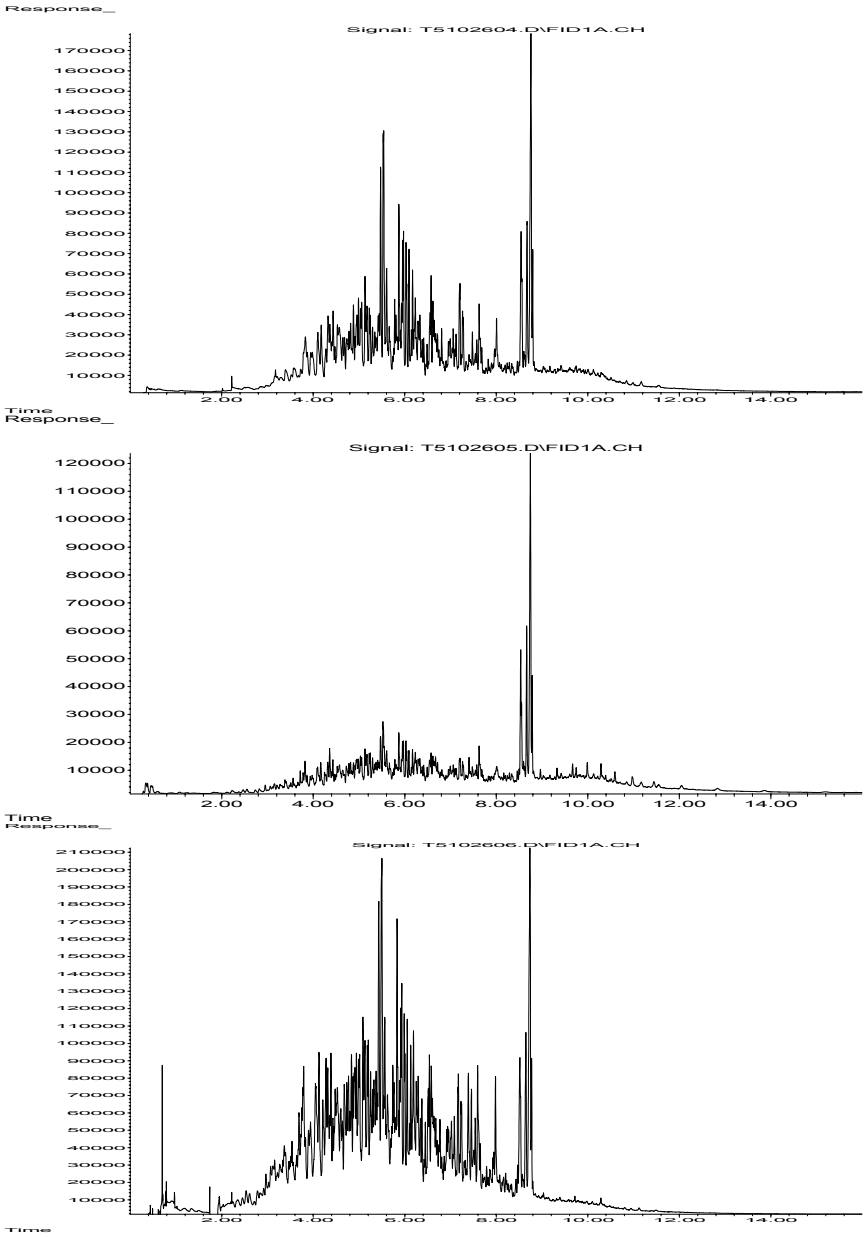
B.3 SPME – Sample C



B.4 SPME – Sample D



B.5 SPME – Sample E



Appendix 5. Report and Chromatograms VITO

OSPAR Intercessional Expert Group (IEG)
on Whole Effluent Assessment (WEA)

Determination of potentially bioaccumulating substances (PBS) in whole effluents using biomimetic solid-phase microextraction (SPME) and the 'EGOM' liquid-liquid extraction (LLE) method

An interlaboratory study

Jacobs Griet, Bertels Diane, Witters Hilda

VITO

Draft report
2005/TOX/R/0105

November 2005

TABLE OF CONTENT

1 INTRODUCTION

VITO is member of the OSPAR Intercessional Expert Group (IEG) on Whole Effluent Assessment (WEA) as a scientific expert and advisor to the Flemish Environmental Agency (VMM, Vlaamse Milieumaatschappij). As part of a strategic research project on the PBT-approach for effluents, they did volunteer to participate in the interlaboratory study of the OSPAR IEG on Whole Effluent Assessment. In this study the potentially bioaccumulating substances (PBS) are determined with two different methods, solid-phase microextraction (SPME) and liquid-liquid extraction (LLE). Performing both methods on the same samples makes it possible to make a good experimental comparison of these approaches.

The study is performed in the framework of the goals of the Bioaccumulation Methodology Product Team. The results of different laboratories measuring total molar concentrations of PBS (potentially bioaccumulatable substances) in aqueous samples using SPME and LLE of GC-measurable organic matter (EGOM-LLE) will be compared. Analysis is by GC-FID. The results and insight will provide the basis for a guidance document that can be used in effluent monitoring.

A detailed protocol for the SPME procedure and the description of the LLE method was made available to participating laboratories. Five different types of samples in 1-L glass bottles were sent to the laboratories. They include:

- one clean water sample
- one spiked water sample
- one effluent sample (low level)
- one spiked effluent sample
- one complex mixture effluent

Interlaboratory comparison of results and evaluation of methods will be performed by RIVO, The Netherlands who coordinated the study. Conclusions will be presented at the forthcoming OSPAR WEA workshop, 28-29 november 2005 in London.

2 LLE METHOD

2.1 Aim of method

This extraction method has been developed for whole effluents and other environmental samples to screen for the presence of organic matter in the sample that could potentially bioaccumulate in organisms (range corresponding to the normal alkanes n-C9 to n-C38). Method is derived from procedure described by Hyning and modified according to optimisation test at RIZA (Kienhuis, pers. communication).

2.2 Materials

- Separatory funnels 1 liter (borosilicate glass)
- Shaking system, KÖTTERMANN 4020 (max. position 10)
- Rotary vapor with 40°C bath, BÜCHI waterbath B-480 and BÜCHI rotavapor R-144
- 250 ml flask for rotary evaporation (borosilicate glass)
- GC-FID : Finnigan Trace GC ultra, Triplus autosampler, EZChrom Elite software
- GC-Column J&W DB-1, length 15 m, internal diameter 0.25 mm, film thickness 0.1 µm
- injector liner : splitless liner internal diameter 5 mm (tapered)
- analytical balance, Mettler toledo AG204
- filter paper, Whatman GF/A 125 mm diameter glas microfiber filter

2.3 Products

- HCl 32 % pro analyse, Merck
- Cyclohexane LiChrosolv, Merck
- NaOH pro analyse, Merck
- Na_2SO_4 pro analyse, Merck
- N_2 premium quality
- 2,3-dimethylnaphtalene 98%, Aldrich, used as calibration standard
- C9 (n-nonaan) > 99.8% Fluka, used as retention time marker
- C38 (n-octatriacontane) 98%, Polyscience, used as retention time marker

2.4 Method description

2.4.1 Sample treatment

The effluent samples were received at 4°C and stored in the fridge. Because the samples had to be stored for more than 2 weeks, they were splitted in triplica (small volume 300 ml) and stored frozen until analysis. The day before extraction, the samples were thawed and brought to roomtemperature. Some sample containers were broken (enlarged frozen volume) and this might give some interferences to the results (there were labels on the containers and they were marked with a pen).

2.4.2 LLE extraction

To start the extraction, 300 ml effluent sample (room temperature) was acidified with 1.5 ml 6 M HCl. The pH was checked with a pH-meter (pH<2). The sample was extracted twice with 30 ml cyclohexane in a separatory funnel, wich was placed on a shaker for 2 h for each extraction and then allowed to separate in two phases. When emulsion was formed, the cyclohexane layer was centrifuged for 5 minutes at 1500g. Following this, the pH of the effluent sample was adjusted to ≥ 10 with 4 ml 2.5 M NaOH and two more extractions with 30 ml cyclohexane were performed as before. The four extracts were combined, dried with Na_2SO_4 and, transferred to a flask for rotary evaporation (40°C, 100 rpm, 150 mbar) . The extract was reduced to a volume of 4-8 ml. The extract was quantitatively transferred to a calibrated vial and further concentrated by a gentle stream of nitrogen to a volume of 1.5-2.0 ml. The volume of the final extract was adjusted (gravimetric weighing) to approximately 1.56 g or 2.0 ml.

2.4.3 Blanks

Per series (of triplicate samples) a blank was run to account for background that can result from extraction procedure/e.g. solvents. The blank (milli-Q-water <20 µS/cm) was treated in the same way as the samples.

2.4.4 GC-FID method

2.4.4.1 Injection in GC-FID

1 µl of the extract was injected splitless (1 min) on a J&W DB-1 column (15 m). The GC-oven programme was started at 40°C for 2min, followed by an increase to 320°C (30°C/min), 6 minutes at constant temperature (run time is 18 minutes). During the analysis the column He flow was kept constant at 1 ml/min. Detection was performed with a flame ionization detector.

2.4.4.2 External standard

The calibration standard (2,3-dimethylnaphatalene in cyclohexane) was injected minimum 3 times before and after a series of sample injections. For both sets of 3 injections the mean area and the standarddeviation were calculated. The relative standarddeviation should be less than 10%, with no significant difference ($p<0.05$) between triplicate measurements.

$$s^2 = \frac{[(n_1-1)*s_1* s_1] + [(n_2-1)*s_2* s_2]}{(n_1+n_2-2)}$$

n = number of measurements n = 3 $(n_1+n_2-2) = 4$

$$t = \frac{\text{A1mean area. - A2mean area}}{\text{square root } (s^2 * (1/n_1+1/n_2))}$$

$(1/n_1+1/n_2) =$ 0.667

A mean area = mean area of 3 measurements of the calibration standard

absolute value t < t critical ?
p < 0.05
t critical = 2.776

2.4.5 Calculations and results

2.4.5.1 Sample calculation

$$\text{EGOM (mgC/l)} : \frac{\text{mg standard * area extract * µl extract}}{\text{(area standard)mean6 * volume sample (l)}}$$

mg standard = mg standard injected (1 µl of 40 ng/µl)
40/1000000 mg injected = 0.00004 mg injected
area extract = total integrated area for the extract
volume extract = volume extract in µl = weight extract in g / density cyclohexane
area standard mean 6 = mean area of the 6 injections (3 before and 3 after the samples)
volume sample = volume sample in litre = weight sample in kg

The samples and standards were measured in two series.

2.4.5.2 Serie 1 results

a. Standard

concentration ng/µl	m mol injected	area	area mean3	STDEV	RSD3 %	area mean6
40	2.56E-07	45810041 46665585 46288419	46254682	428769	0.93	45938123
40	2.56E-07	46070074 46271698 44522919	45621564	956780	2.10	

quality control : RSD < **10 % ?** OK before
OK after

p<0.05 ? s^2 = 5.50E+11
$(1/n_1+1/n_2)$ = 0.667
$s^2 * (1/n_1+1/n_2)$ = 3.66E+11
square root($s^2 * (1/n_1+1/n_2)$) = 605329

asolute value t < t critical ? OK
t = 1.046
t critical = 2.776

b. Effluent results

effluent	Weight water g	Weight extract g	Volume extract ml	area	EGOM mgC/l
blanco A	300	1.5592	2.0028	14640251	0.0851
A replica 1	300	1.5602	2.0041	22822875	0.1328
A replica 2	300	1.5598	2.0036	23320366	0.1356
A replica 3	300	1.5602	2.0041	24576022	0.1430
blanco B	300	1.5598	2.0036	16971558	0.0987
B replica 1*					
B replica 2	300	1.5600	2.0039	22094358	0.1285
B replica 3	300	1.5598	2.0036	25537728	0.1485

* B replica 1: see next table, measured in second series

2.4.5.3 Serie 2 results

a. Standard

concentration ng/µl	mmol injected	area	area mean 3	STDEV	RSD 3 %	area mean 6
40	2.56E-07	49193543 48577297 48258721	48676520	475244	1	48369411
40	2.56E-07	47702663 47211924 49272316	48062301	1076247	2	

quality control : RSD < **10 % ?** OK before
OK after

$p<0.05$? s^2 = 6.92E+11

$(1/n_1+1/n_2)$ = 0.667

$s^2 * (1/n_1+1/n_2)$ = 4.61E+11

square root($s^2 * (1/n_1+1/n_2)$) = 679256

asolute value t < t critical ? OK

t = 0.904

t critical = 2.776

b. Effluent results

effluent	Weight water g	Weight extract g	Volume extract ml	area	EGOM mgC/l
B replica 1*	150	0.7800	1.0019	34377177	0.0949
blanco C	300	1.5598	2.0036	20972785	0.1158
C replica 1	300	1.5603	2.0042	63000817	0.3481
C replica 2	300	1.5600	2.0039	83734023	0.4625
C replica 3	300	1.5601	2.0040	83265044	0.4600
blanco D	300	1.5602	2.0041	12636544	0.0698
D replica 1	300	1.5604	2.0044	44686336	0.2469
D replica 2	300	1.5597	2.0035	34831821	0.1924
D replica 3	300	1.5600	2.0039	39798117	0.2198
blanco E	300	1.5602	2.0041	19458903	0.1075
E replica 1	300	1.5598	2.0036	417064155	2.3035
E replica 2	300	1.5594	2.0031	403440113	2.2276
E replica 3	300	1.5606	2.0046	404692280	2.2363

*The sample container of this sample effluent B, replica 1 was broken and there was 150 ml of the sample left. The sample volume was reduced with a factor two, but also the final extract was 1 ml instead of 2 ml. This effluent B, replica 1 was dried over Na2SO4 twice (before and after evaporation, there were two layers visible : water and cyclohexane).

2.5 Changes made to the protocol

- acidification with 1.5 ml 6 M HCl instead of 4 ml
- the pH is controlled with a pH-meter instead of litmus paper
- centrifugation at 1500 g for 5 minutes when there was emulsion visible (effluent D and effluent E)
- the extract was dried on Na2SO4 before the evaporation step
- the cyclohexane-layers (4) were pooled together to a volume of 120 ml
- the column length is 15 m instead of 10 m (there are no 10 m DB-1 columns available)
- the injector temperature is 270°C instead of 290°C (the injector septum becomes poreus at high temperatures giving rise to interference pieks in the solvent blank chromatograms)
- the external standard 2,3-dimethylnaphtalene is made in cyclohexane instead of ethylacetate (the same solvent as the sample extracts).

2.6 Summary of LLE results

effluent	Weight water g	Weight extract g	Volume extract ml	area	EGOM mgC/l
blanco A	300	1.5592	2.0028	14640251	0.0851
A replica 1*	300	1.5602	2.0041	22822875	0.1328
A replica 2*	300	1.5598	2.0036	23320366	0.1356
A replica 3*	300	1.5602	2.0041	24576022	0.1430
blanco B	300	1.5598	2.0036	16971558	0.0987
B replica 1*					
B replica 2	300	1.5600	2.0039	22094358	0.1285
B replica 3	300	1.5598	2.0036	25537728	0.1485
blanco C	300	1.5598	2.0036	20972785	0.1158
C replica 1	300	1.5603	2.0042	63000817	0.3481
C replica 2	300	1.5600	2.0039	83734023	0.4625
C replica 3	300	1.5601	2.0040	83265044	0.4600
blanco D	300	1.5602	2.0041	12636544	0.0698
D replica 1	300	1.5604	2.0044	44686336	0.2469
D replica 2	300	1.5597	2.0035	34831821	0.1924
D replica 3	300	1.5600	2.0039	39798117	0.2198
blanco E	300	1.5602	2.0041	19458903	0.1075
E replica 1	300	1.5598	2.0036	417064155	2.3035
E replica 2	300	1.5594	2.0031	403440113	2.2276
E replica 3	300	1.5606	2.0046	404692280	2.2363

* broken bottles, result of effluent B-replica 1 is not included due to loss of sample.

3 SPME METHOD

3.1 Aim of method

This extraction method has been developed for whole effluents and other environmental samples to assess the degree to which organic chemicals present in the sample would potentially bioaccumulate in organisms. It has been designed to take bioavailability into account by performing negligible depletion extractions.

3.2 Materials

- 250 ml amber septa bottles supplied with open top poly caps with PTFE/silicone liners, (Alltech, I-chem sample container, article number : 98720)
- SPME fibers : Supelco PDMS (polydimethylsiloxane), 100 µm film thickness, 110 µm internal diameter, 0.66 µl volume PDMS coating, article number : 57300-U
- SPME fiber holders, for use with manual sampling, Supelco, article number : 57330-U
- GC-Column J&W DB-1, length 15 m, internal diameter 0.25 mm, film thickness 0.1 µm
- GC-FID : Finnigan Trace GC ultra, Triplus autosampler, EZChrom Elite software
- injector liner : splitless liner internal diameter 5 mm (tapered)

3.3 Products

- Cyclohexane LiChrosolv, Merck
- 2,3-dimethylnaphtalene 98%, Aldrich
- C9 (n-nonaan) > 99.8% Fluka
- C38 (n-octatriacontane) 98%, Polyscience

3.4 Method description

3.4.1 Sample treatment

The effluent samples were received at 4°C and stored in the fridge. Because the samples had to be stored for more than 2 weeks, they were splitted in triplica (small volume 300 ml) and stored frozen until analysis. The day before extraction, the samples were thawed and brought to roomtemperature. Some sample containers were broken (enlarged frozen volume) and this could give some interferences to the results (there were labels on the containers and they were marked with a pen).

3.4.2 SPME extraction

Effluent samples were shaken, quickly distributed over three identical 250 ml glass bottles with a flat bottom. The bottles were filled to the top (no headspace). The fibers were

conditioned in the GC-FID injector at 250°C, until a sufficient low blank signal (based on visual low background on chromatograms) was observed. Subsequently the conditioned fiber was exposed to the effluent sample and stirred on a magnetic stirrer for 24h (plus or minus 30 min). After sampling the SPME fiber was immediately desorbed in the GC-FID injector and measured.

3.4.3 Blanks

After sampling the SPME fiber was immediately desorbed in the GC-FID injector and measured (= blank). When the background was too high, the fiber was desorbed again.

3.4.4 GC-FID method

3.4.4.1 Conditioning and cleaning fibers

All the fibers were new and had to be conditioned before use. The fibers were conditioned for until the observed signal was low enough. The conditioned fiber was added to the sample immediately.

3.4.4.2 Injection in GC-FID

The fiber was placed with the fiber holder in the GC-FID splitless injector. The fiber was thermally desorbed in splitless mode at an injection temperature of 250°C for 15 minutes. A fast temperature program was used : starting at 40°C for 2 minutes, followed by an increase tot 320°C (6 minutes constant) at a rate of 30°C/min. The run time was 18 minutes. The airflow of the FID is 350 ml/min, N2 flow is 35 ml/min and the H2 flow is 35 ml/min.

3.4.4.3 External standard

The calibration standard (2,3-dimethylnaphatalene in cyclohexane) was injected min 3 times before and after a series of sample injections. For both sets of 3 injections the mean area and the standarddeviation were calculated. The relative standarddeviation should be less than 10%, with no significant difference ($p<0.05$) between triplicate measurements.

$$s^2 = \frac{[(n_1-1)*s_1* s_1] + [(n_2-1)*s_2* s_2]}{(n_1+n_2-2)}$$

n = number of measurements n = 3 $(n_1+n_2-2) = 4$

$$t = \frac{\text{A1mean area. - A2mean area}}{\text{square root } (s^2 * (1/n_1+1/n_2))}$$

$(1/n_1+1/n_2) =$ 0.667

A mean area = mean area of 3 measurements of the calibration standard

absolute value t < t critical ?
p < 0.05
t critical = 2.776

3.4.5 Calculations and results

3.4.5.1 Sample calculation

$$\text{PBS (mmol/l PDMS)} = \frac{\text{amount stand.} * \text{area sample} * 1000000}{(\text{area stand})\text{gem.} * 0.66}$$

PBS (mmol/l PDMS) = total molar concentration / liter PDMS fiber
amount stand = mmol external standard injected

standardsolution 2,3-dimethylnaftaleen in cyclohexane		
concentration :	40	ng/µl
MM 2,3,-dimethylnaphthalene :	156.23	g/mol
injected volume :	1	µl
injected amount :	2.56E-07	m mol

(aera stand)mean = mean area of the external standard (3 inj. before and 3 after samples)
area sample = total peak area sample (before C9 and after C38)
volume fiber =0.66 µl = 0.66/1000000 l

3.4.5.2 Serie 1 results

a. Standard

concentration ng/µl	mmol injected	area	area mean 3	STDEV	RSD 3 %	area mean 6
40	2.56E-07	43608231 48379329 44856400	45614653	2474279	5	44300178
40	2.56E-07	41777550 42512105 44667454	42985703	1502034	3	

quality control : RSD3 < 10 % ? OK before
OK after

p<0.05 ?

s2 = 4.18908E+12
(1/n1+1/n2) = 0.666666667
s2 * (1/n1+1/n2) = 2.79272E+12
square root(s2 * (1/n1+1/n2)) = 1671143.591

absolute value t < t critical ? OK
t = 1.57314
t critical = 2.776

b. Effluent results

effluent	Weight water g	time h	area	PBS mmol/l PDMS
A blank 1			25543531	0.22
A blank 2			20385616	0.18
A blank 3			24666680	0.22
A replica 1	251.43	24.00	947542262	8.30
A replica 2	252.46	24.00	3725348070	32.62
A replica 3	252.32	24.00	3319216532	29.07

3.4.5.3 Serie 2 results

a. Standard

concentration ng/µl	mmol injected	area	area mean 3	STDEV	RSD 3 %	area mean 6
40	2.56E-07	46384992	46465877	1722805	4	46365446
		48227700				
		44784940				
		45465647				
40	2.56E-07	47625463	46265015	1203511	3	
		45830399				
		45339182				

quality control : RSD3 < 10 % ? OK before
OK after

p<0.05 ?

s2 =	2.20825E+12
(1/n1+1/n2) =	0.666666667
s2 * (1/n1+1/n2) =	1.47216E+12
square root(s2 * (1/n1+1/n2)) =	1213327.96

absolute value t < t critical ? OK

t =	0.16555
t critical =	2.776

b. Effluent results

effluent	Weight water g	time h	area	PBS mmol/l PDMS
B blank 1			49438010	0.41
B blank 2			61521986	0.51
A blank 4			36462096	0.31
B replica 1	252.25	23.50	707500188	5.92
B replica 2	253.06	23.50	592746637	4.96
A replica 4	251.43	24.20	1683006796	14.08

3.4.5.4 Serie 3 results

a. Standard

concentration ng/µl	mmol injected	area	area mean 3	STDEV	RSD 3 %	area mean 6
40	2.56E-07	47625463 45830399 45339182	46265015	1203511	3	46075338
40	2.56E-07	45113421 44882122 47661439	45885661	1542212	3	

quality control : RSD3 < 10 % ? OK before
OK after

p<0.05 ?

s2 =	1.91343E+12
(1/n1+1/n2) =	0.666666667
s2 * (1/n1+1/n2) =	1.27562E+12
square root(s2 * (1/n1+1/n2)) =	1129432.691

absolute value t < t critical ? OK

t =	0.33588
t critical =	2.776

b. Effluent results

effluent	Weight water g	time h	area	PBS mmol/l PDMS
B blank 3			51492500	0.43
C blank 1			37644067	0.32
C blank 2			40315389	0.34
B replica 3	251.79	23.40	437807397	3.69
C replica 1	252.46	23.50	749189340	6.31
C replica 2	252.73	23.55	1161502258	9.78

3.4.5.5 Serie 4 results

a. Standard

concentration ng/µl	mmol injected	area	area mean 3	STDEV	RSD 3 %	area mean 6
40	2.56E-07	45113421 44882122 47661439	45885661	1542212	3	45981029
40	2.56E-07	47002612 45221276 46005301	46076396	892794	2	

quality control : RSD3 < 10 % ? OK before
OK after

p<0.05 ?

s2 =	1.58775E+12
(1/n1+1/n2) =	0.666666667
s2 * (1/n1+1/n2) =	1.0585E+12
square root(s2 * (1/n1+1/n2)) =	1028833.76

absolute value t < t critical ? OK

t =	-0.18539
t critical =	2.776

b. Effluent results

effluent	Weight water g	time h	area	PBS mmol/l PDMS
C blank 3			27003990	0.23
A blank 5			24435734	0.21
B blank 4			28188864	0.24
C replica 3	251.80	23.55	983880204	8.30
A replica 5	252.46	24.00	1490828905	12.58
B replica 4	252.73	24.00	355212062	3.00

3.4.5.6 Serie 5 results

a. Standard

concentration ng/µl	mmol injected	area	area mean 3	STDEV	RSD 3 %	area mean 6
40	2.56E-07	45810041 46665585 46288419	46254682	428769	1	45938123
40	2.56E-07	46070074 46271698 44522919	45621564	956780	2	

quality control : RSD3 < 10 % ? OK before
OK after

p<0.05 ?

s2 = 5.49635E+11
(1/n1+1/n2) = 0.666666667
s2 * (1/n1+1/n2) = 3.66424E+11
square root(s2 * (1/n1+1/n2)) = 605329.2979

absolute value t < t critical ? OK
t = 1.04591
t critical = 2.776

b. Effluent results

effluent	Weight water g	time h	area	PBS mmol/l PDMS
D blank 1			22922873	0.19
D blank 2			24794466	0.21
D blank 3			22897906	0.19
D replica 1	252.47	24.00	745004718	6.29
D replica 2	252.12	24.05	710397803	6.00
D replica 3	252.84	23.55	623422550	5.26

3.4.5.7 Serie 6 results

a. Standard

concentration ng/µl	mmol injected	area	area mean 3	STDEV	RSD 3 %	area mean 6
40	2.56E-07	41530077 42117887 44022842	42556935	1303089	3	43489372
40	2.56E-07	43871452 43830602 45563371	44421808	988833	2	

quality control : RSD3 < 10 % ? OK before
OK after

p<0.05 ?

s2 =	1.33792E+12
(1/n1+1/n2) =	0.666666667
s2 * (1/n1+1/n2) =	8.91944E+11
square root(s2 * (1/n1+1/n2)) =	944428.0611

absolute value t < t critical ? OK

t =	-1.97461
t critical =	2.776

b. Effluent results

effluent	Weight water g	time h	area	PBS mmol/l PDMS
E blank 1			28092559	0.25
E blank 2			13537013	0.12
E blank 3			12346290	0.11
E replica 1	254.81	24.00	3724760597	33.23
E replica 2	253.21	24.00	4343923499	38.75
E replica 3	254.09	24.00	3790266027	33.81

3.5 Changes made to the protocol

- the external standard 2,3-dimethylnaphtalene was made in cyclohexane instead of ethylacetate (the same solvent as the samples).
- the GC injector temperature used was 270°C instead of 290°C (the injector septum becomes poreus at high temperatures giving rise to interference pieks in the blank chromatograms)
- the fibers were conditioned at 250 °C just before sampling in the GC-FID injector until a sufficient low blank signal was obtained.
- the column length is 15 m instead of 10 m (there are no 10 m DB-1 columns available)

3.6 Summary of SPME results

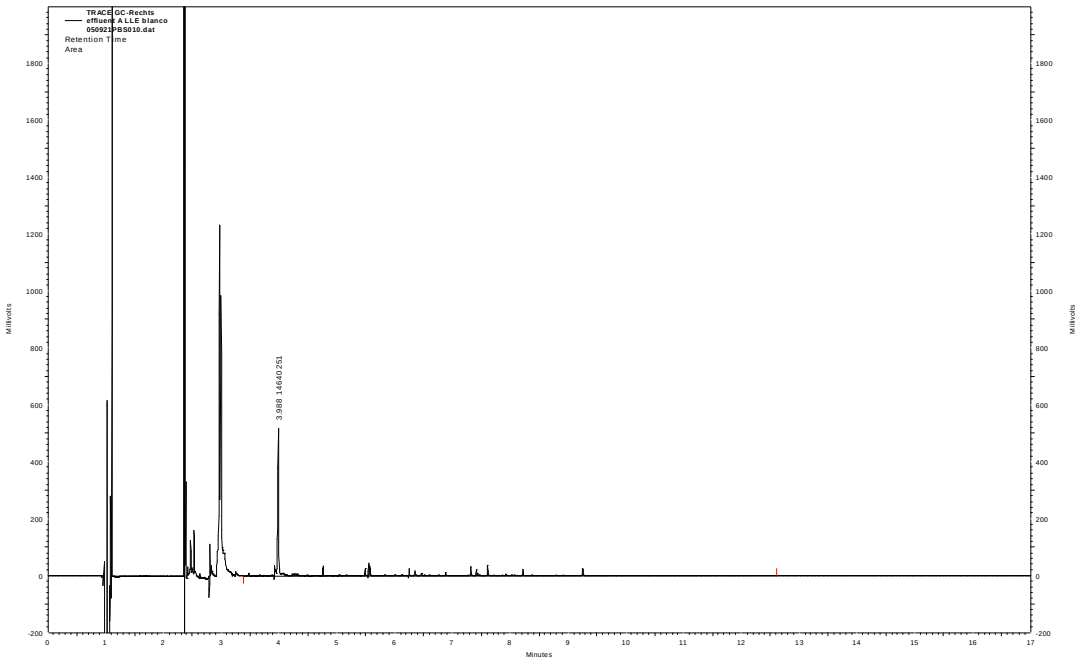
effluent	fiber nr	PBS blank fiber mmol/l PDMS	PBS effluent mmol/l PDMS	PBS mean mmol/l PDMS	RSD %
A replica 2*	2	0.18	32.62	25.26	39
A replica 3	3	0.22	29.07		
A replica 4	4	0.31	14.08		
B replica 1*	2	0.41	5.92	4.85	23
B replica 2	3	0.51	4.96		
B replica 3	2	0.43	3.69		
C replica 1	3	0.32	6.31	8.13	21
C replica 2	4	0.34	9.78		
C replica 3	2	0.23	8.30		
D replica 1*	2	0.19	6.29	5.85	9
D replica 2*	3	0.21	6.00		
D replica 3*	4	0.19	5.26		
E replica 1	2	0.25	33.23	35.26	9
E replica 2	3	0.12	38.75		
E replica 3	4	0.11	33.81		

*broken bottles

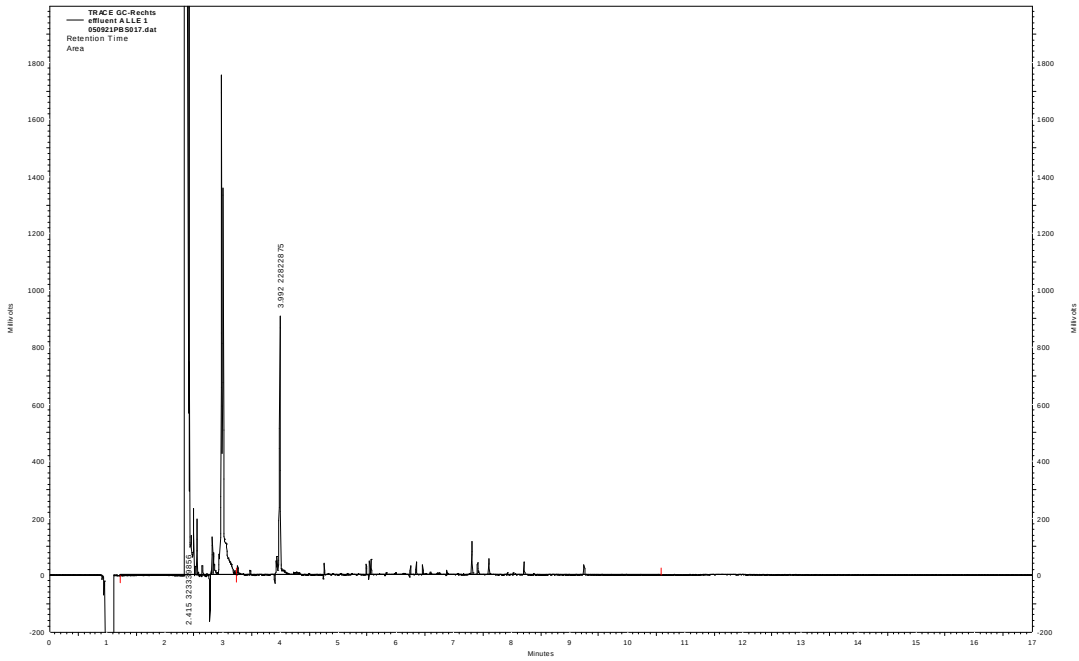
4.2 Chromatograms LLE method

4.2.1 Effluent A chromatograms

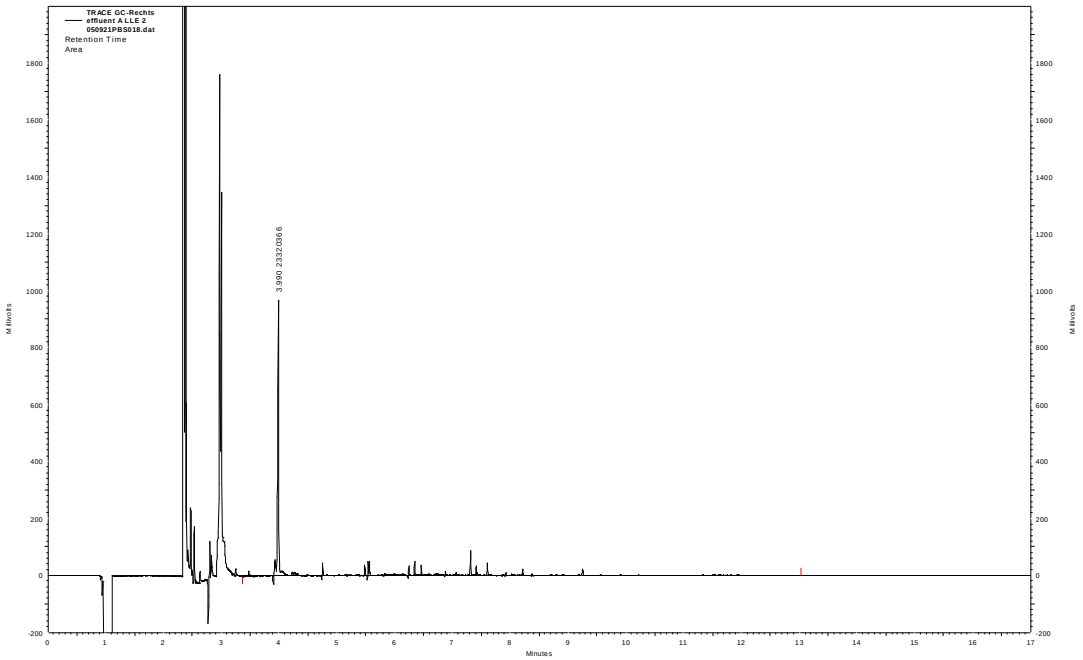
effluent A blanco LLE



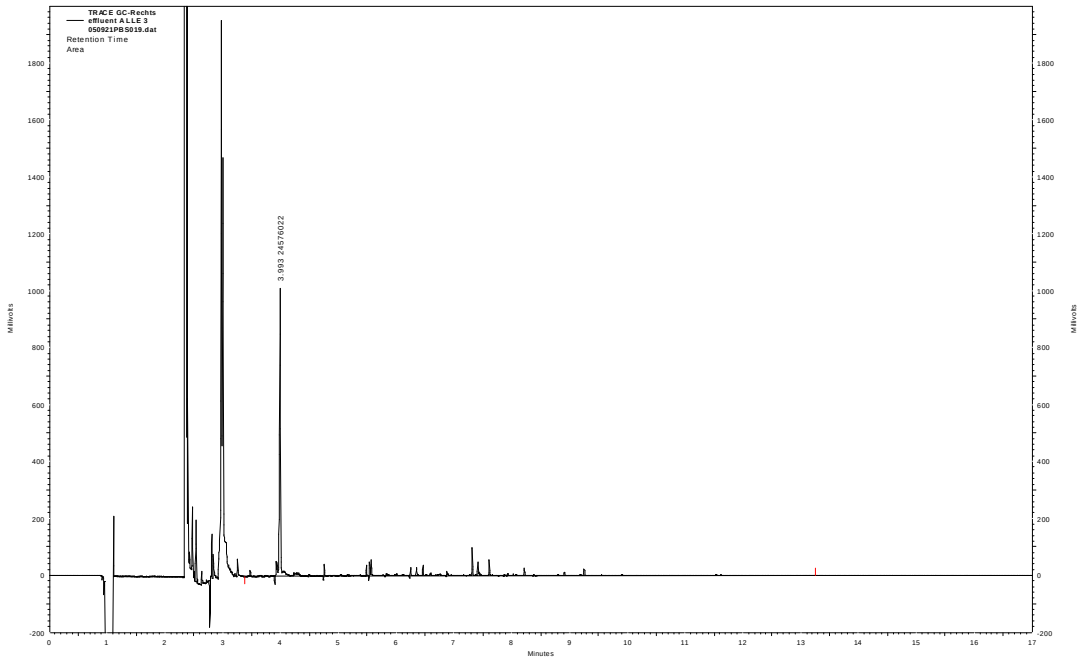
effluent A LLE 1



effluent A LLE 2

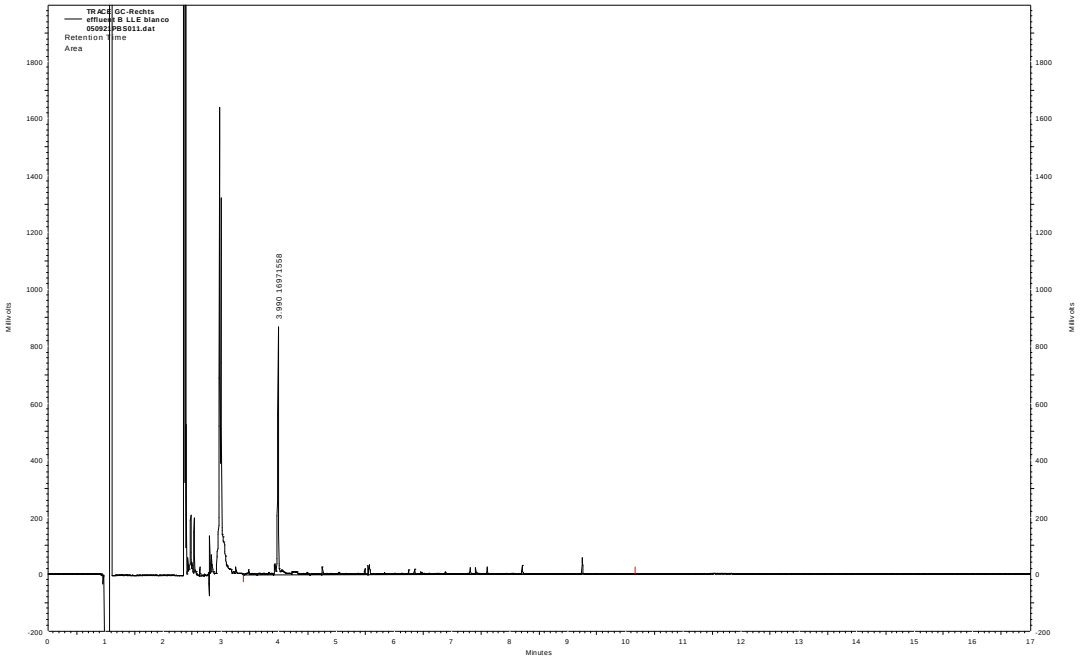


effluent A LLE 3

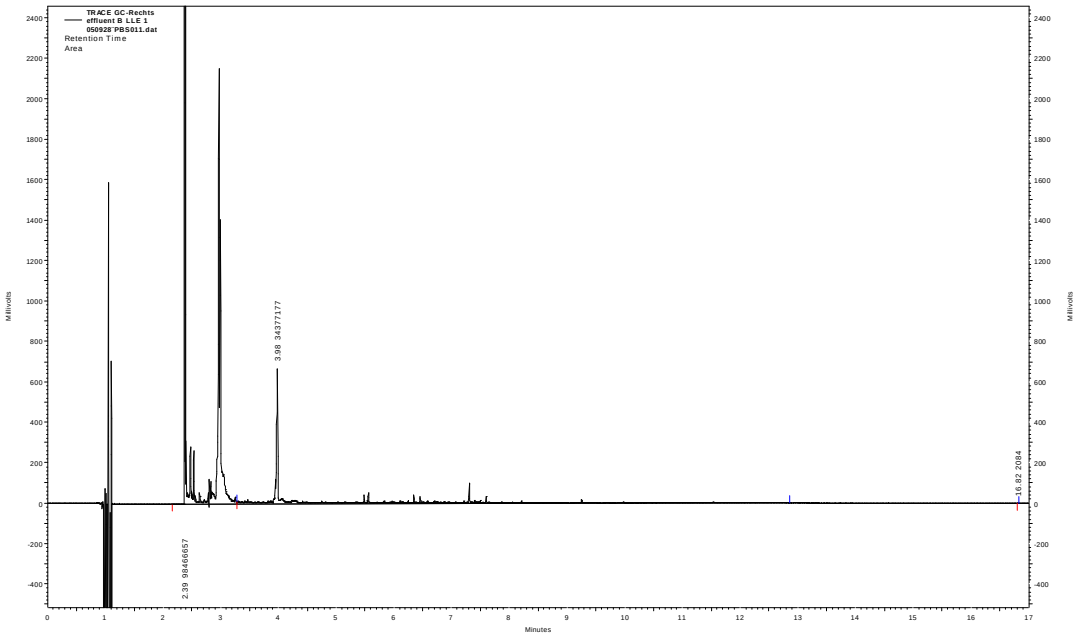


4.2.2 Effluent B chromatograms

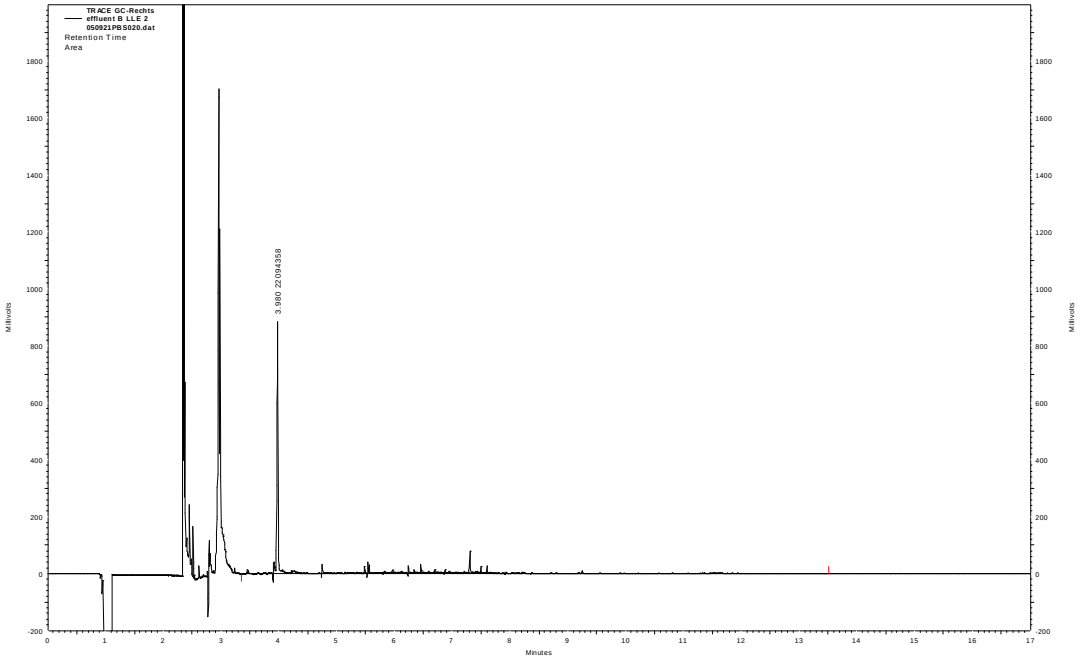
effluent B blanco LLE



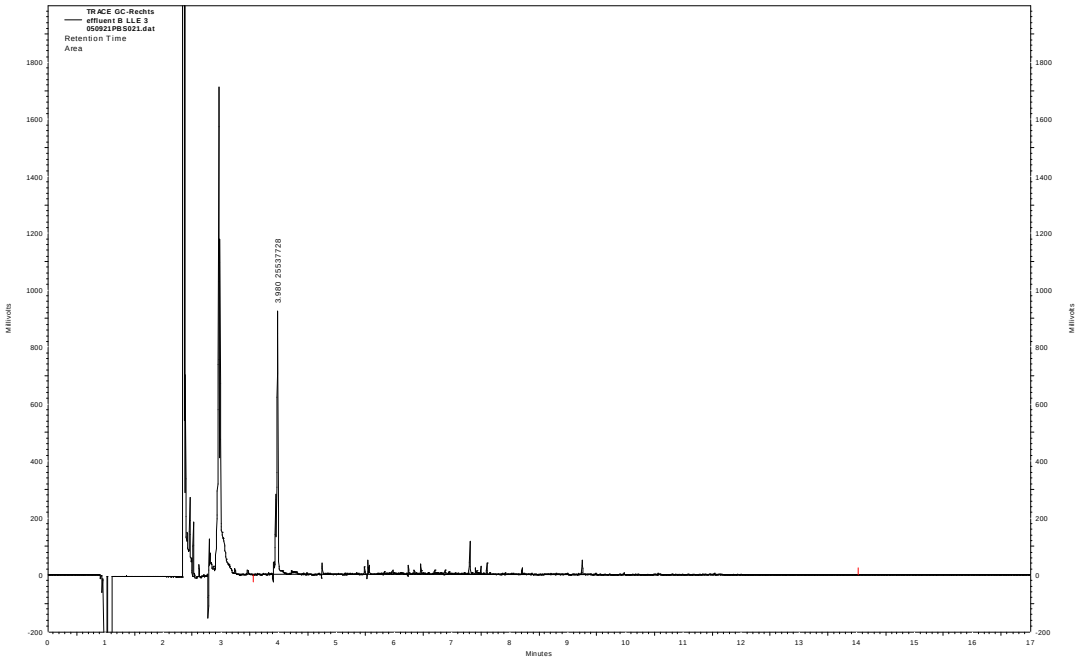
effluent B LLE1



effluent B LLE2

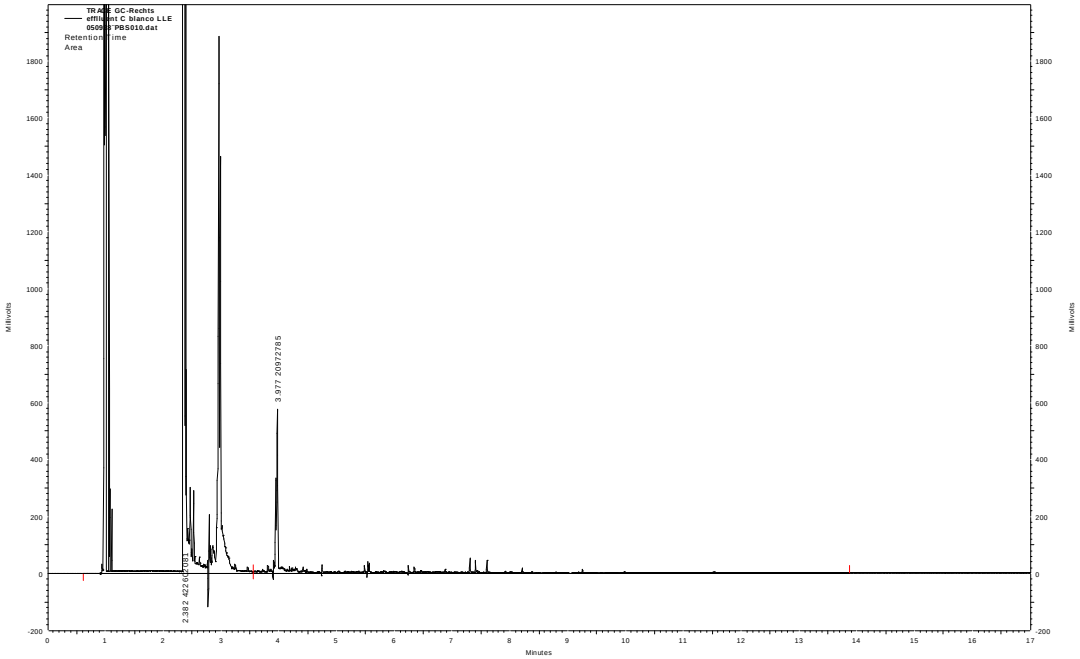


effluent B LLE3

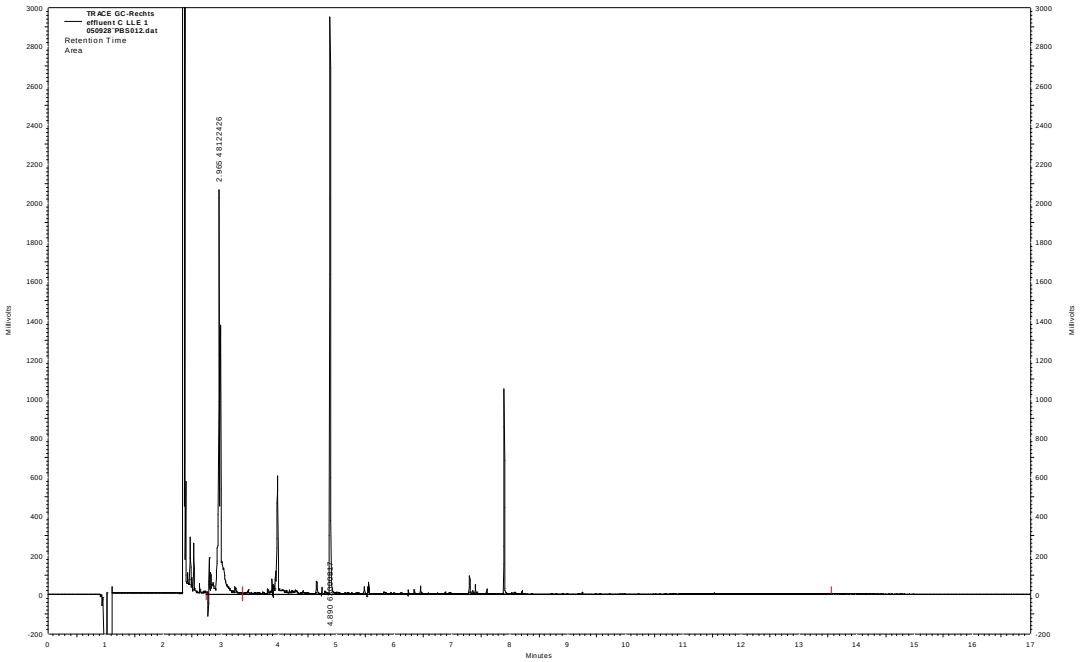


4.2.3 Effluent C Chromatograms

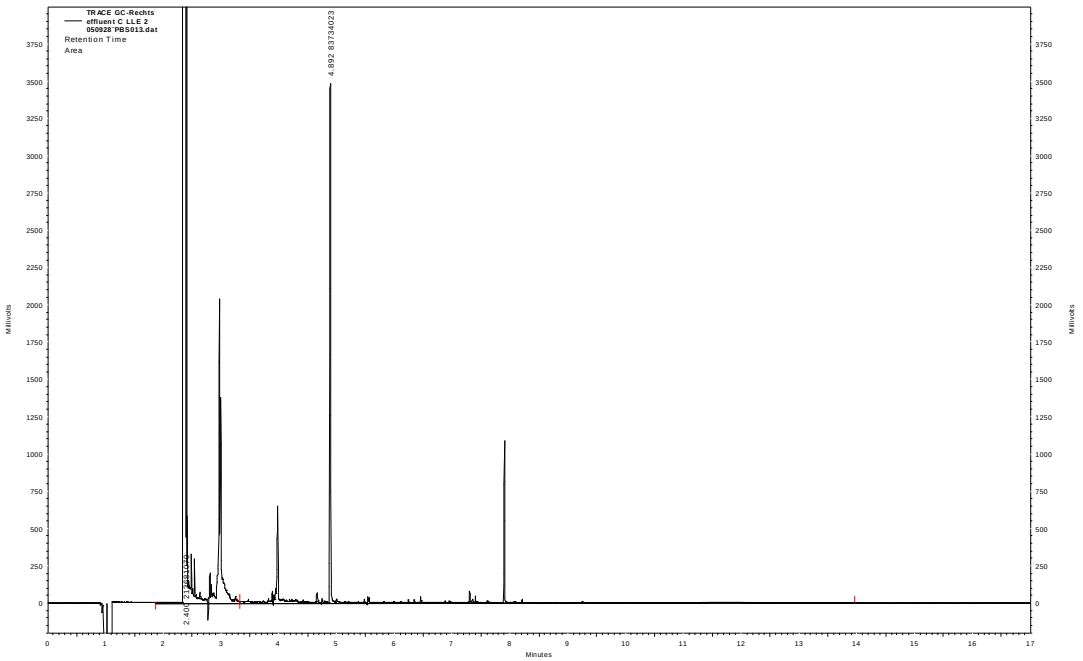
effluent C blanco LLE



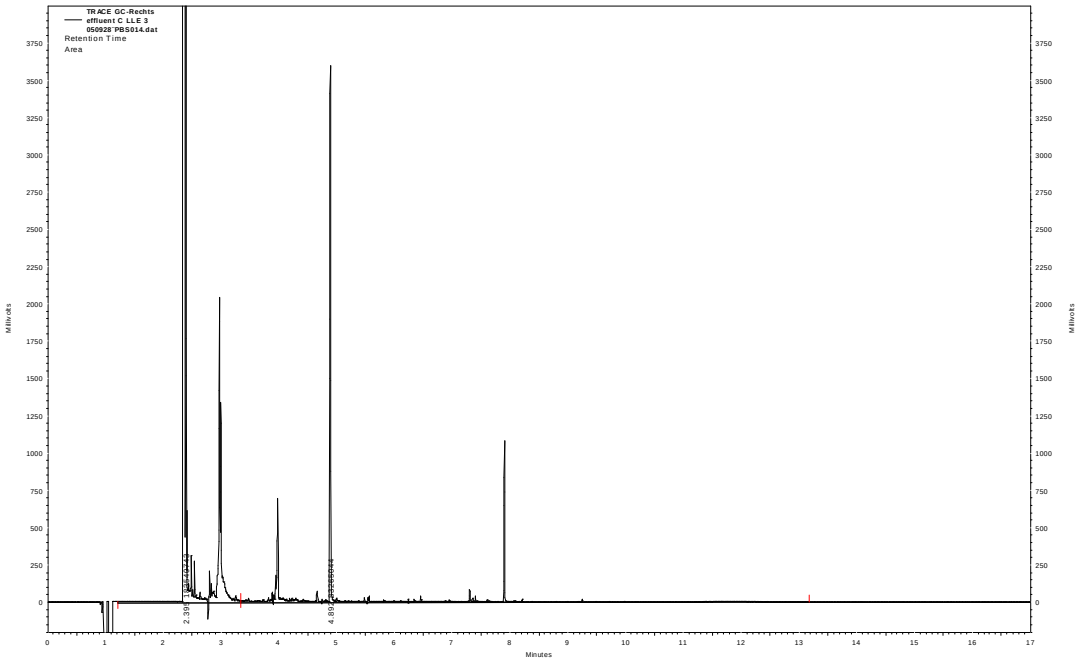
effluent C LLE 1



effluent C LLE 2

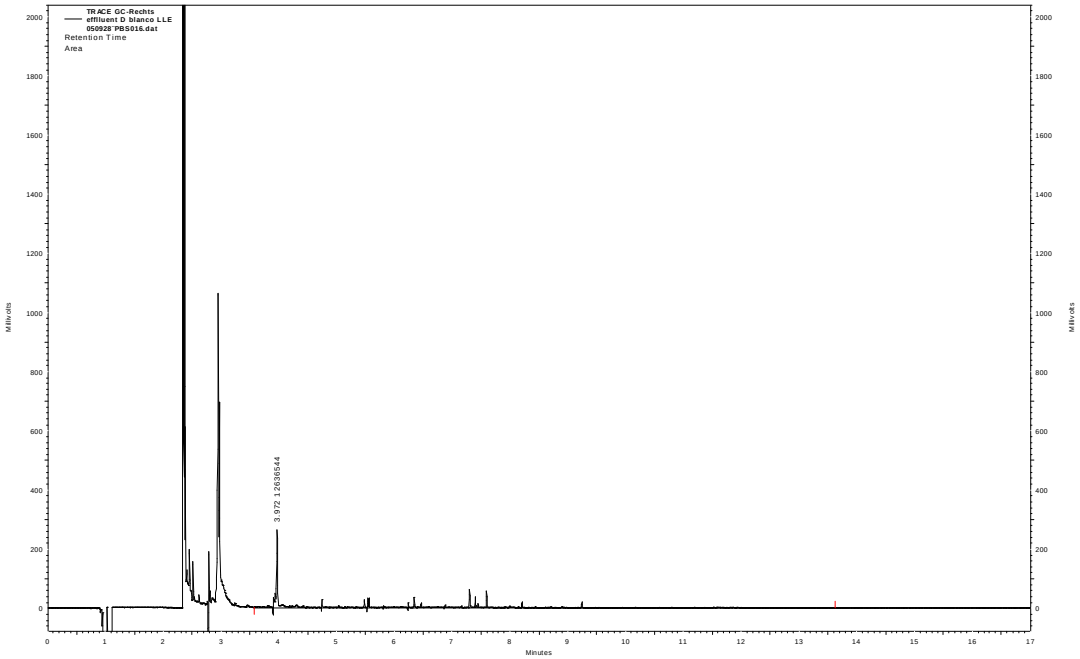


effluent C LLE 3

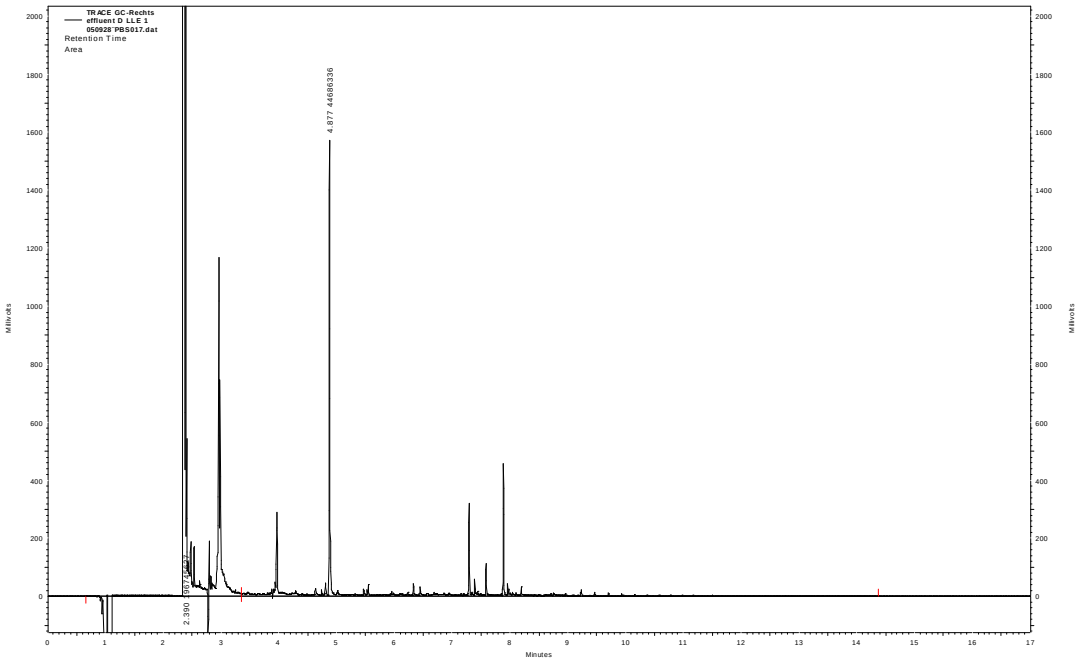


4.2.4 Effluent D Chromatograms

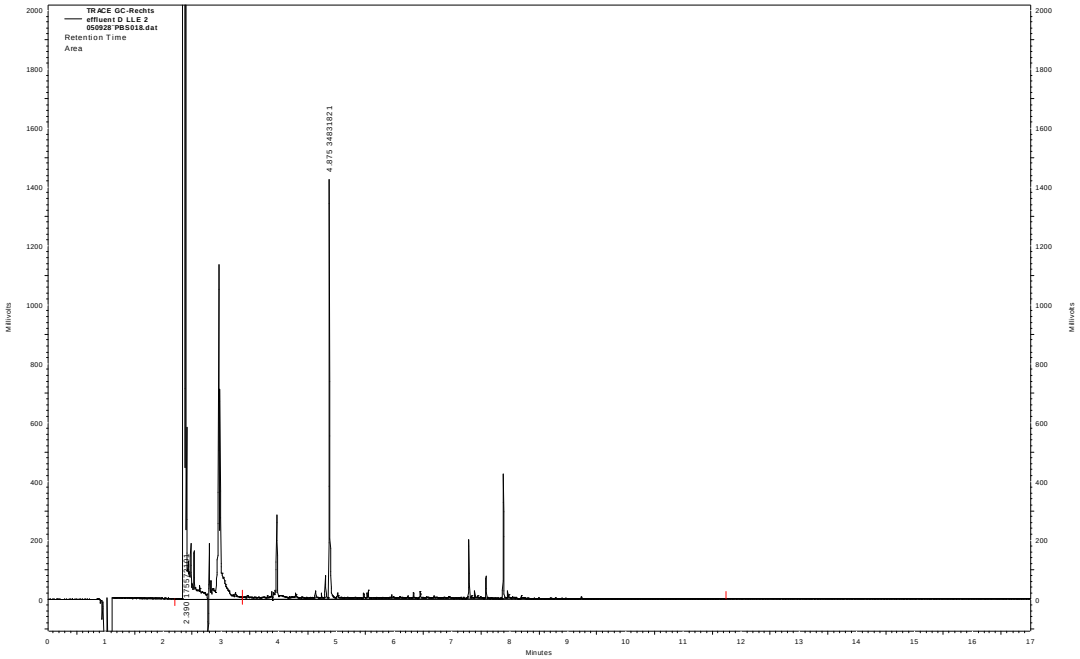
effluent D blanco LLE



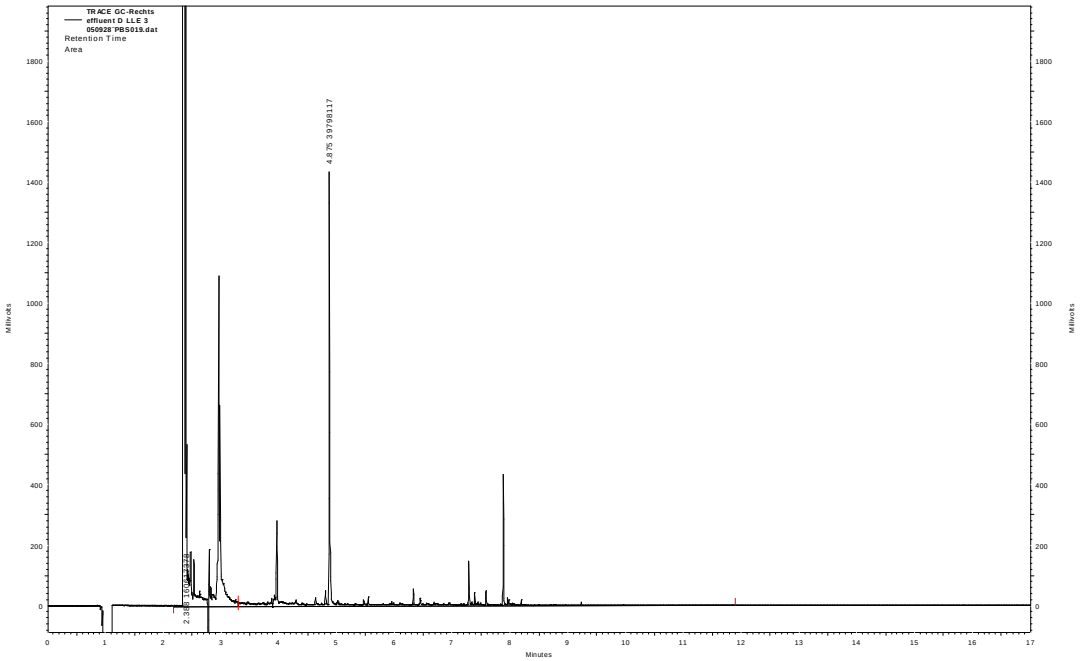
effluent D LLE 1



effluent D LLE 2

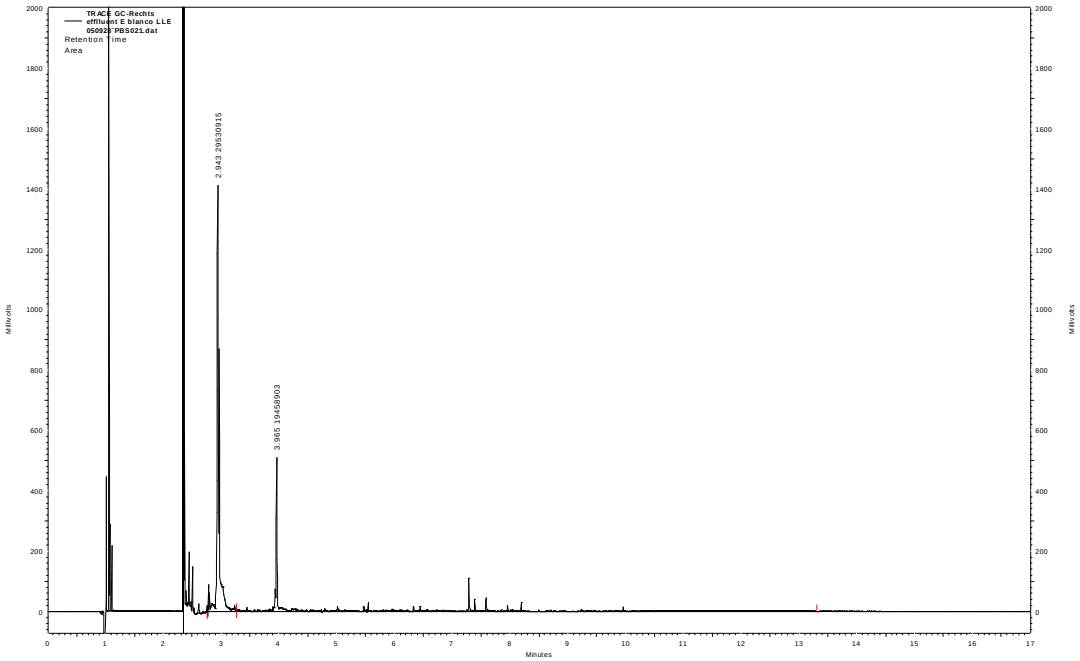


effluent D LLE 3

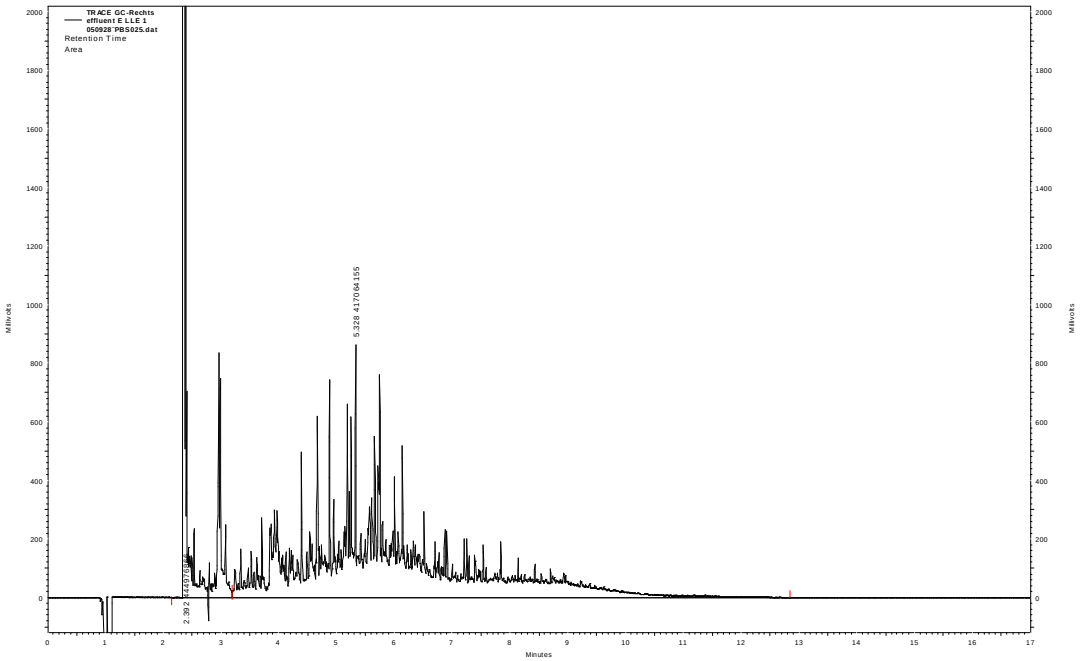


4.2.5 Effluent E Chromatograms

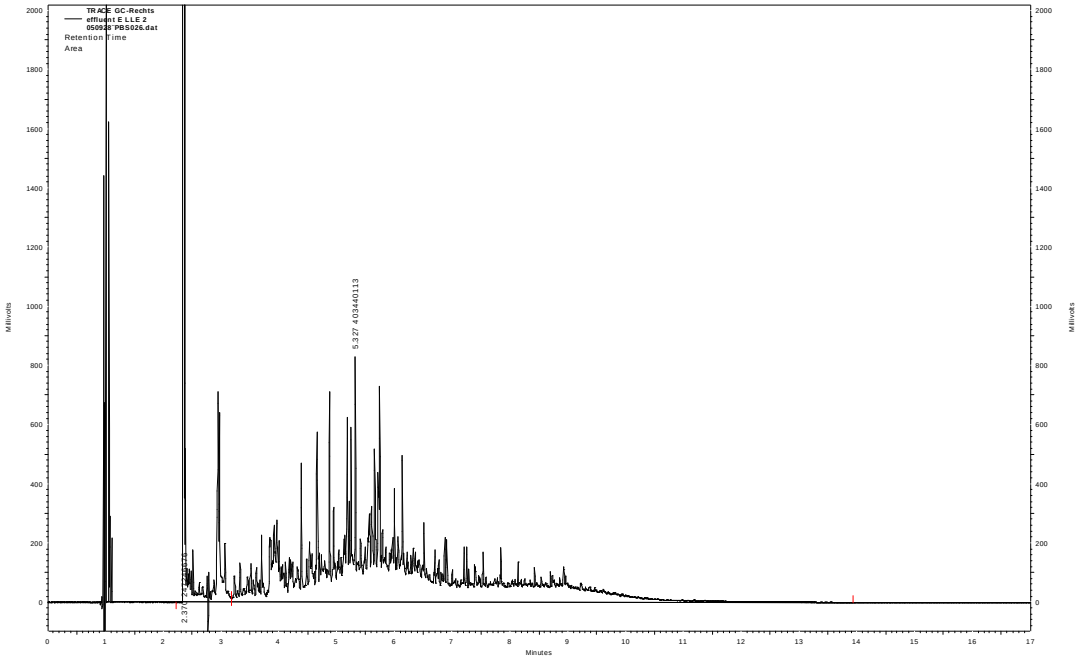
effluent E blanco LLE



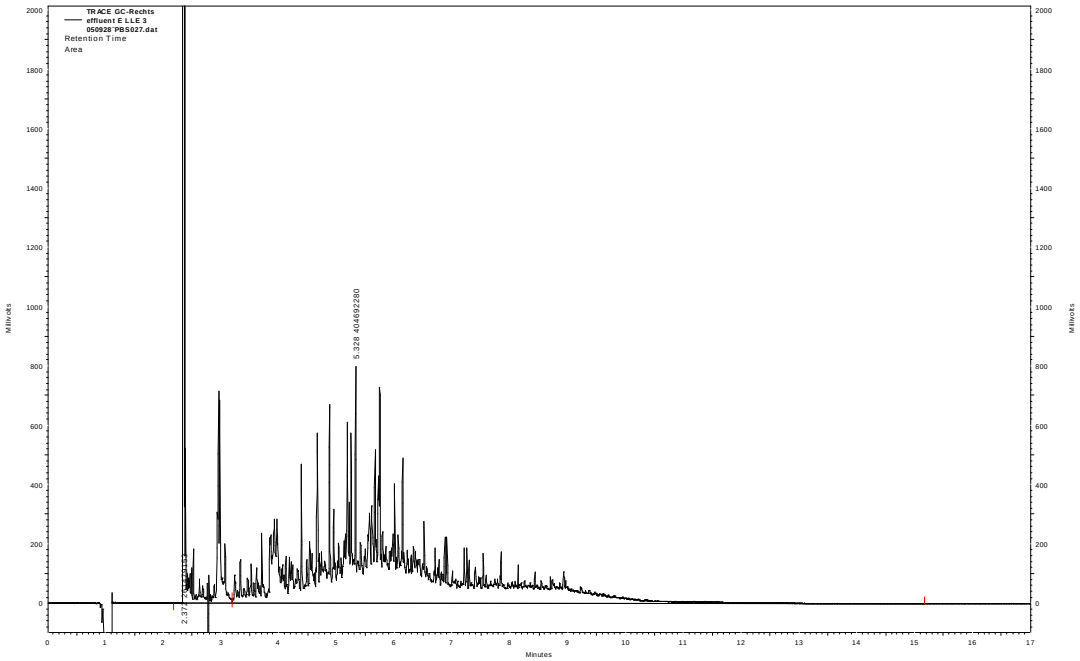
effluent E LLE 1



effluent E LLE 2



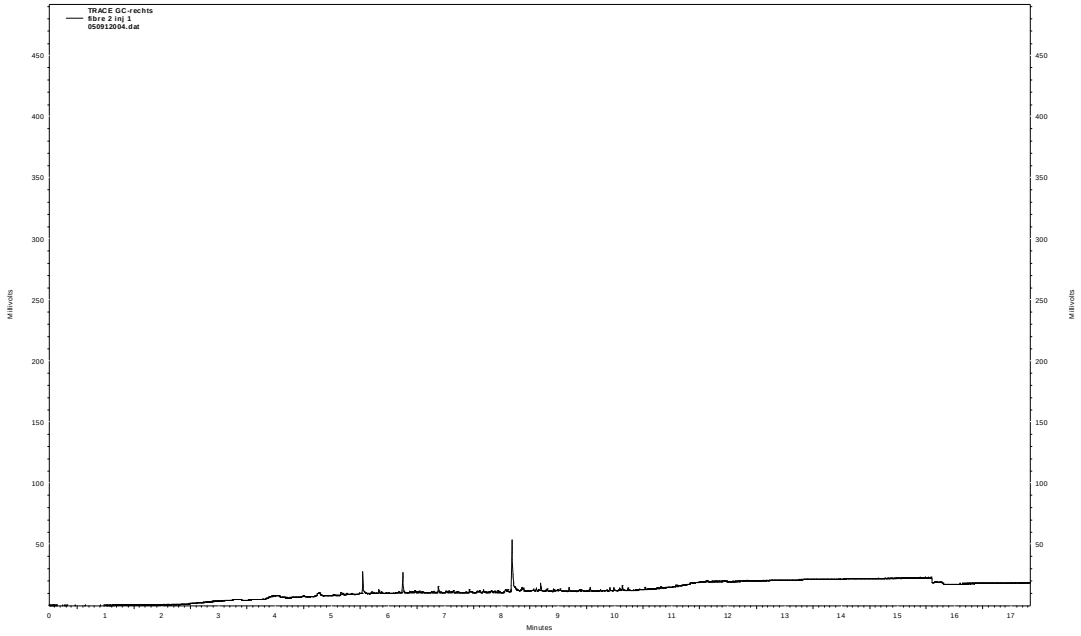
effluent E LLE 3



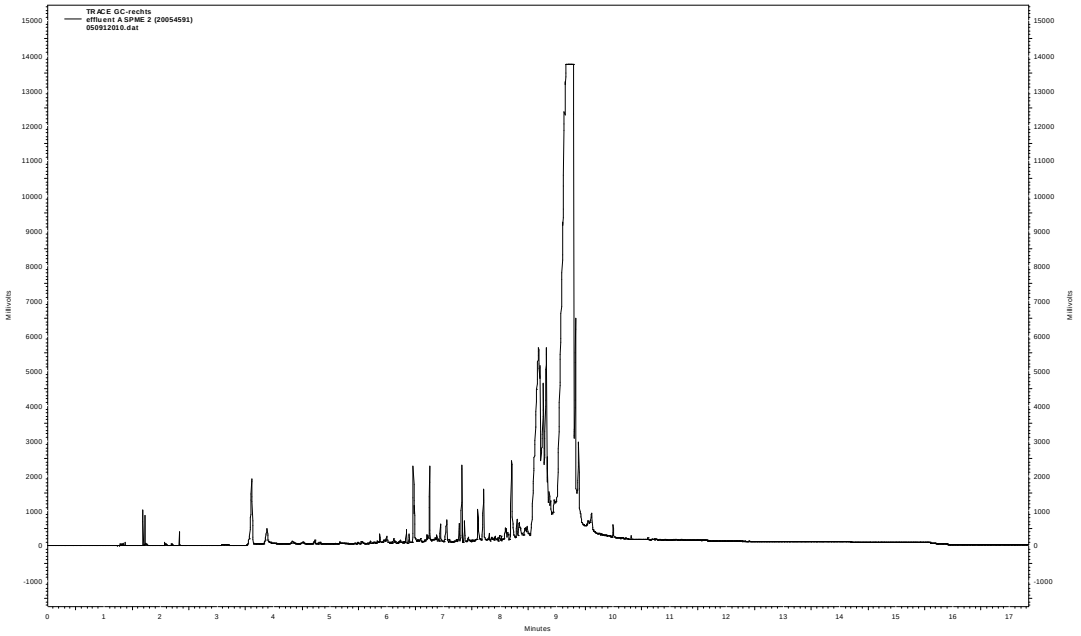
4.4 Chromatograms SPME method

4.4.1 Effluent A chromatograms

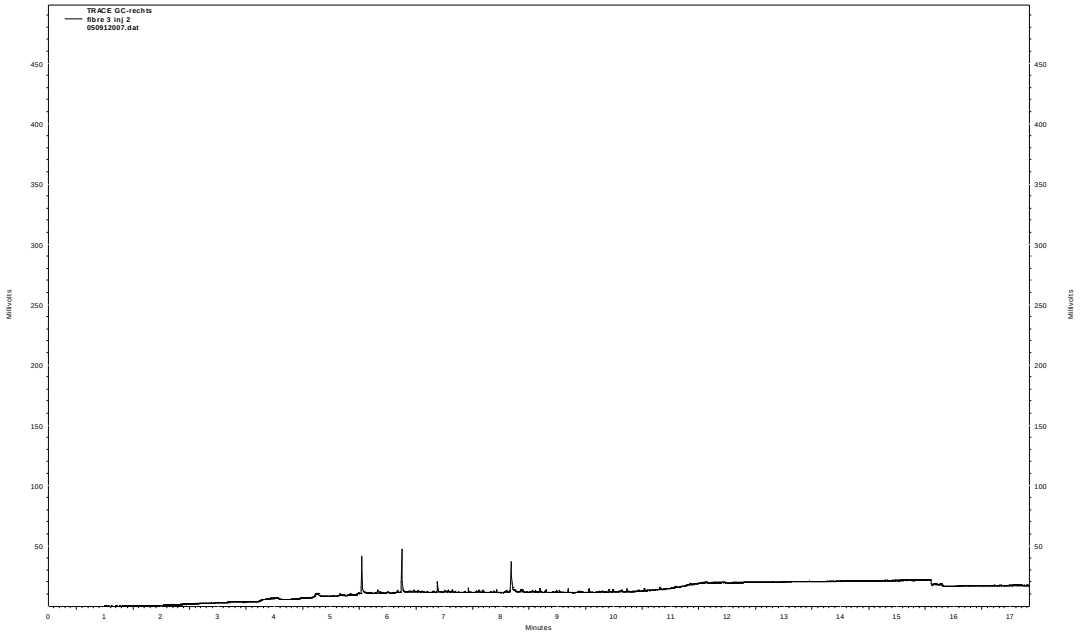
effluent A SPME 2 blanco



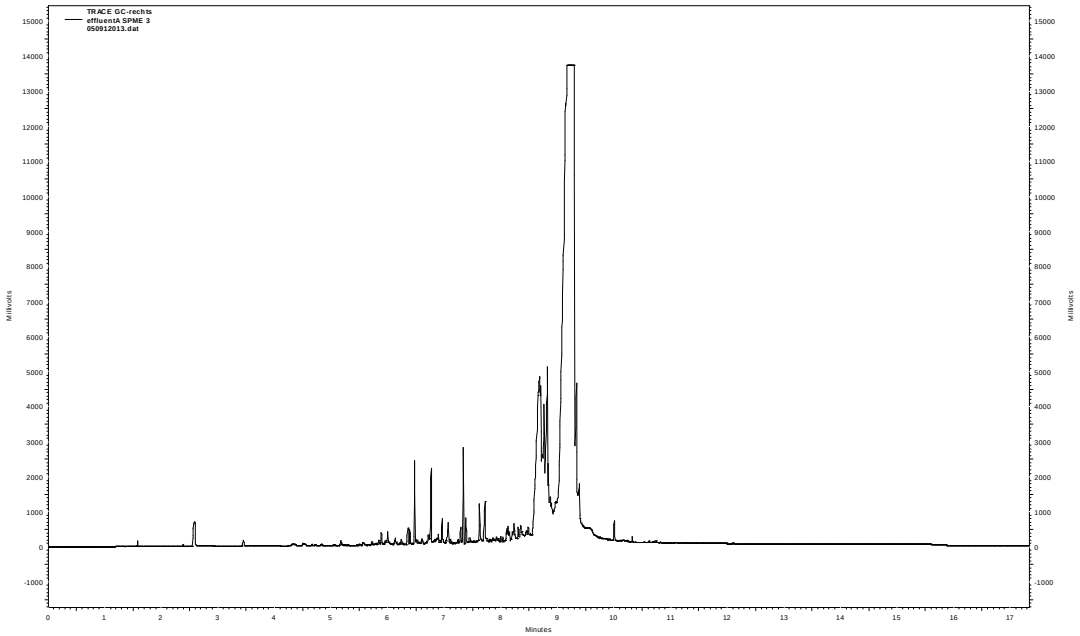
effluent A SPME 2



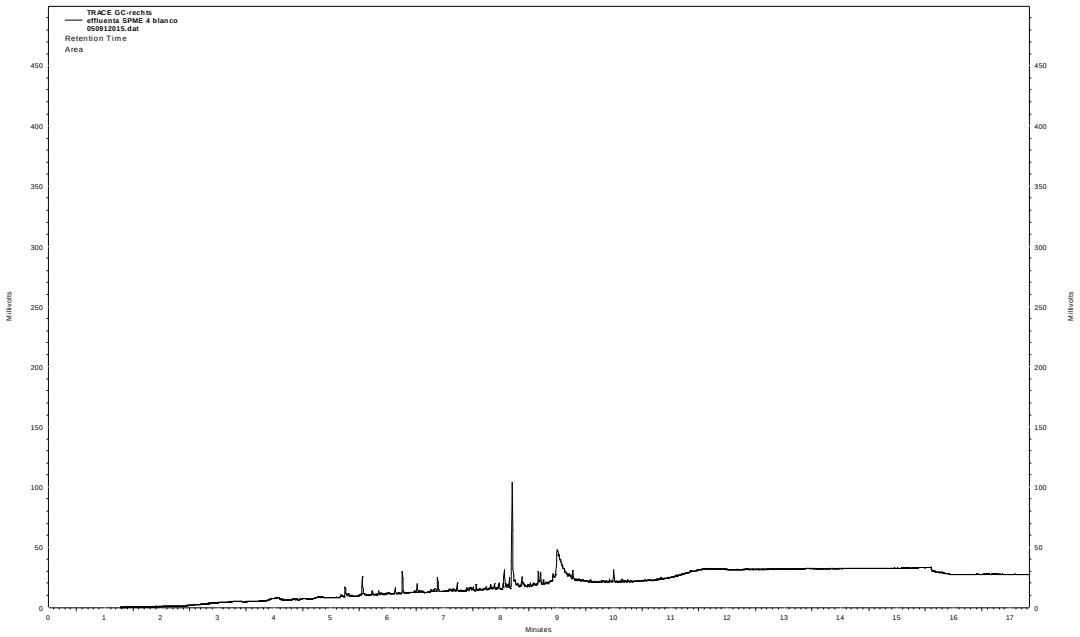
effluent A SPME 3 blanco



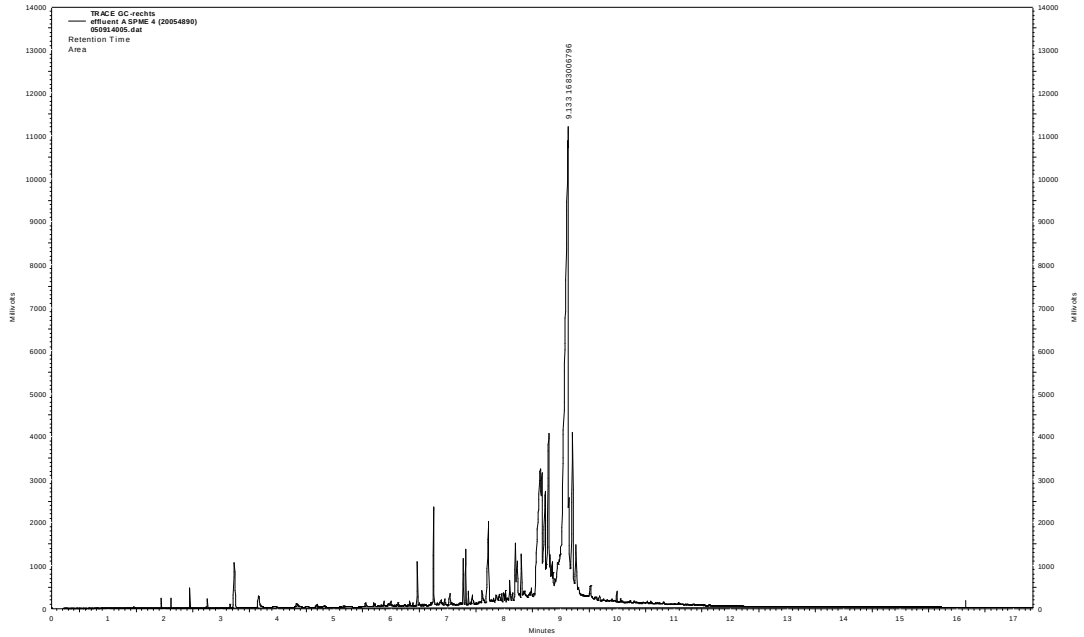
effluent A SPME 3



effluent A SPME 4 blanco

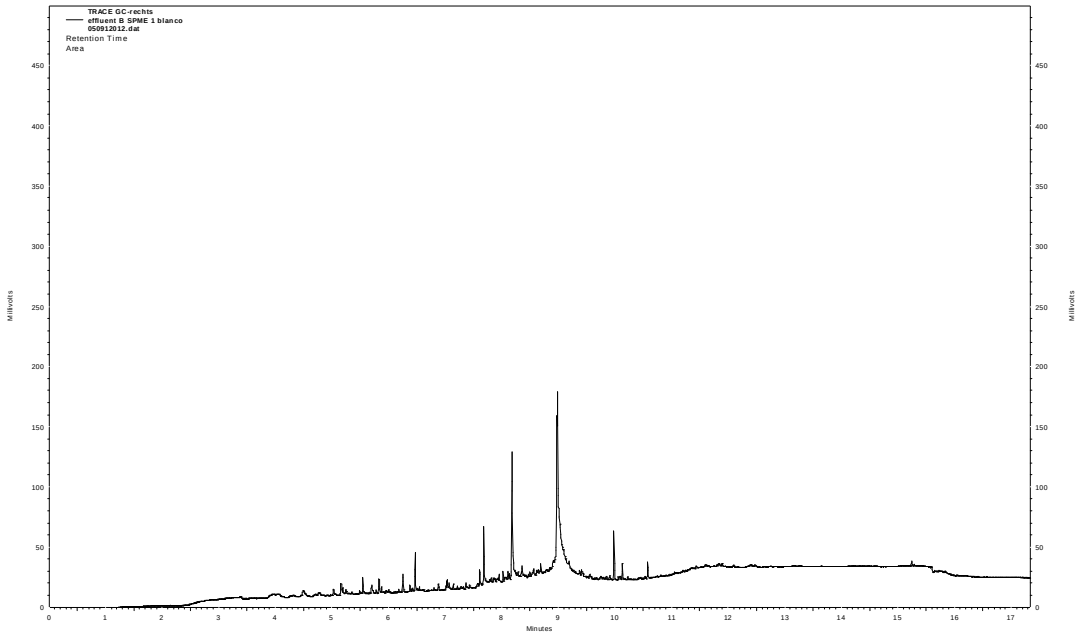


effluent A SPME 4

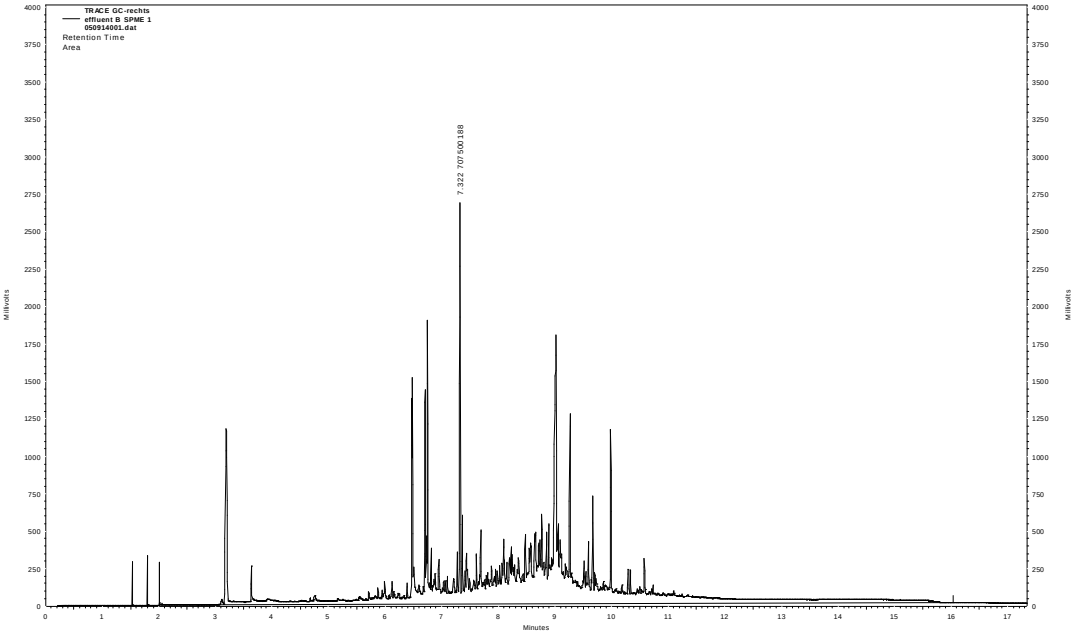


4.4.2 Effluent B chromatograms

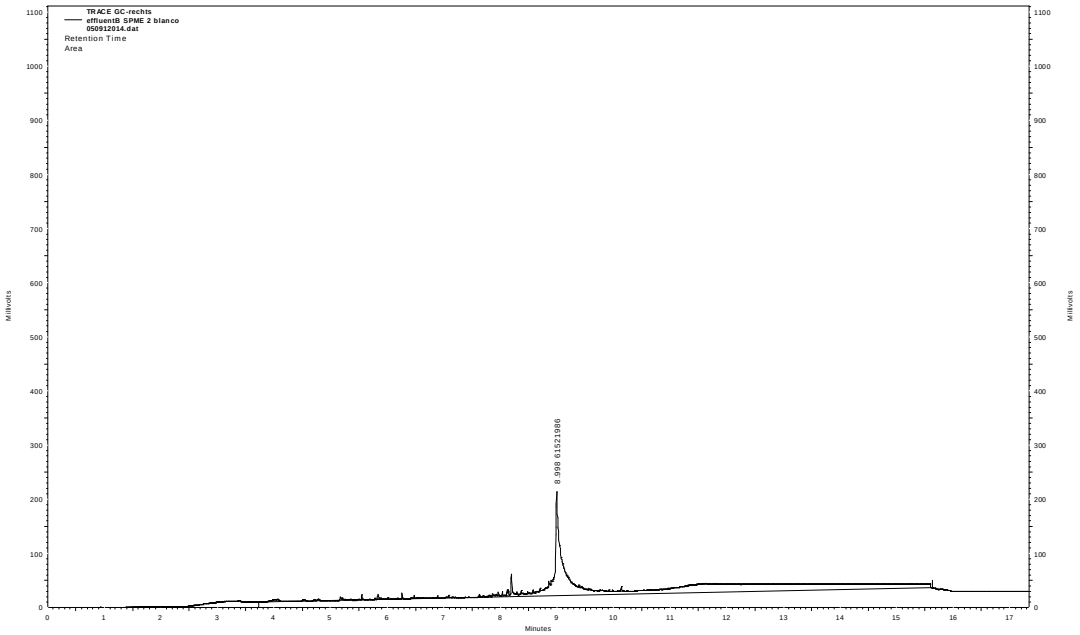
effluent B SPME 1 blanco



effluent B SPME 1



effluent B SPME 2 blanco



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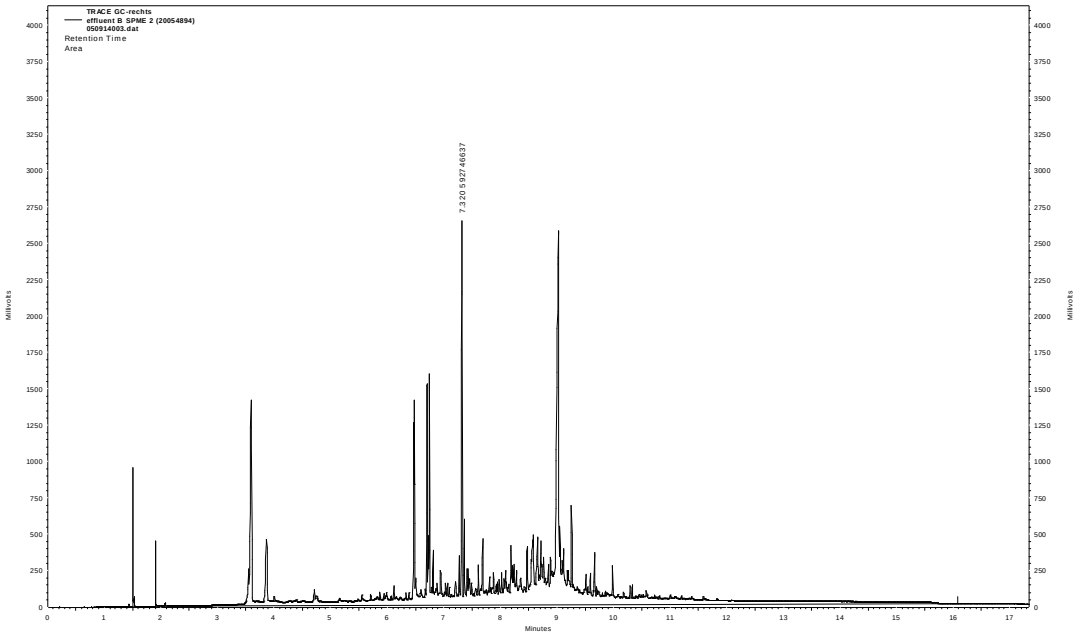

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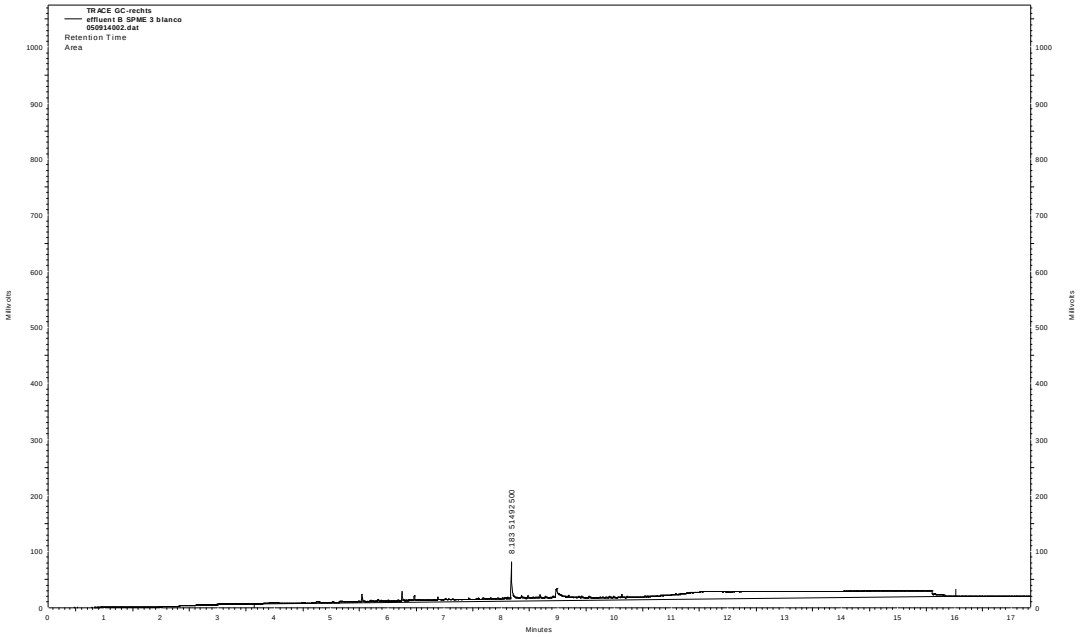
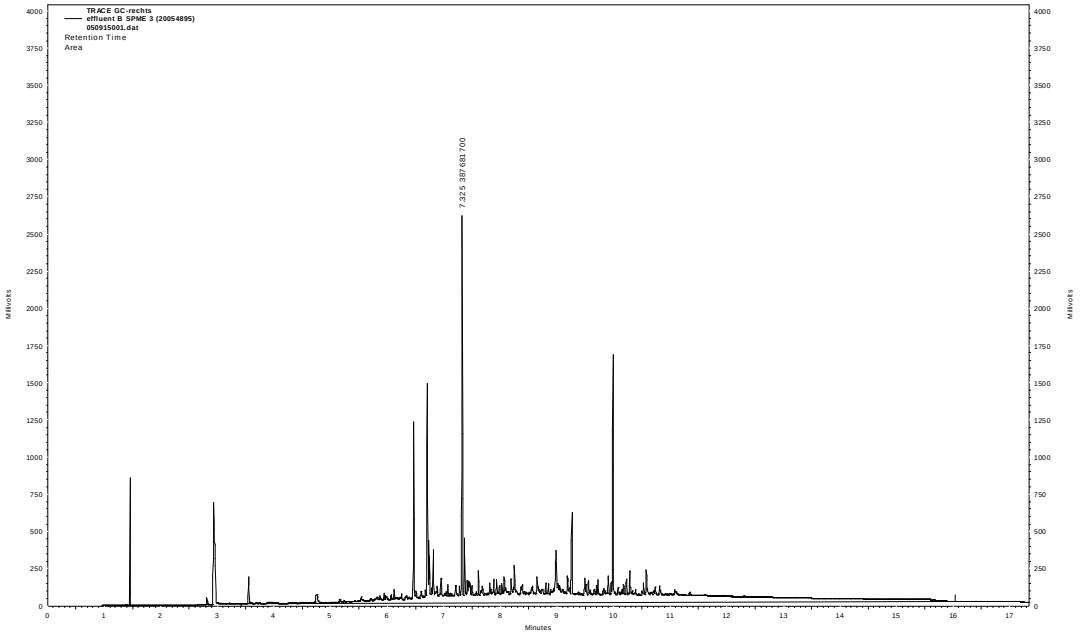
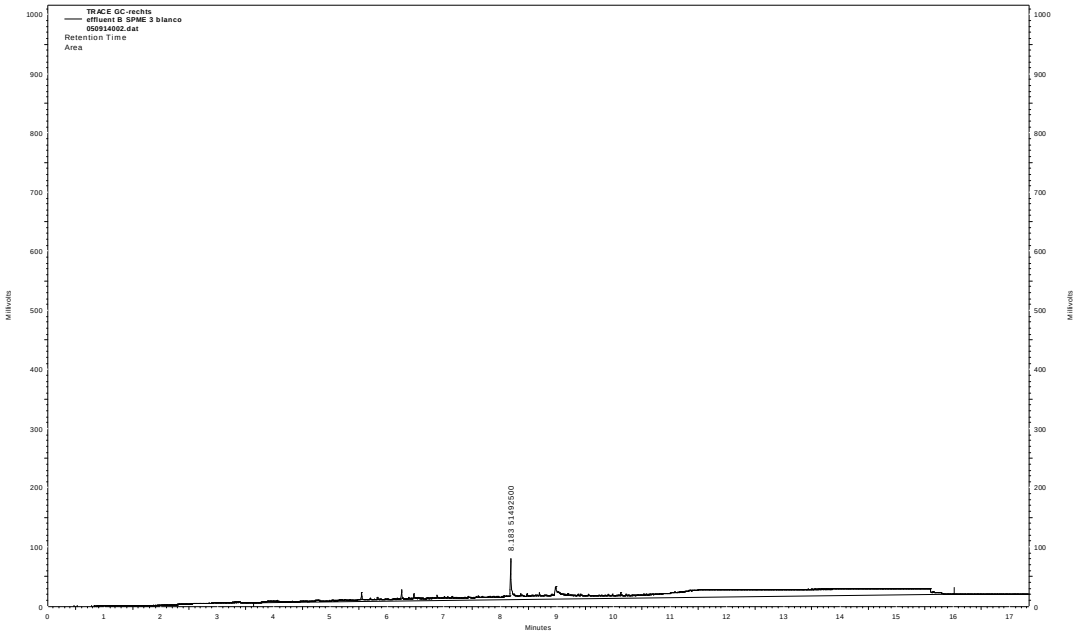


effluent B SPME 3

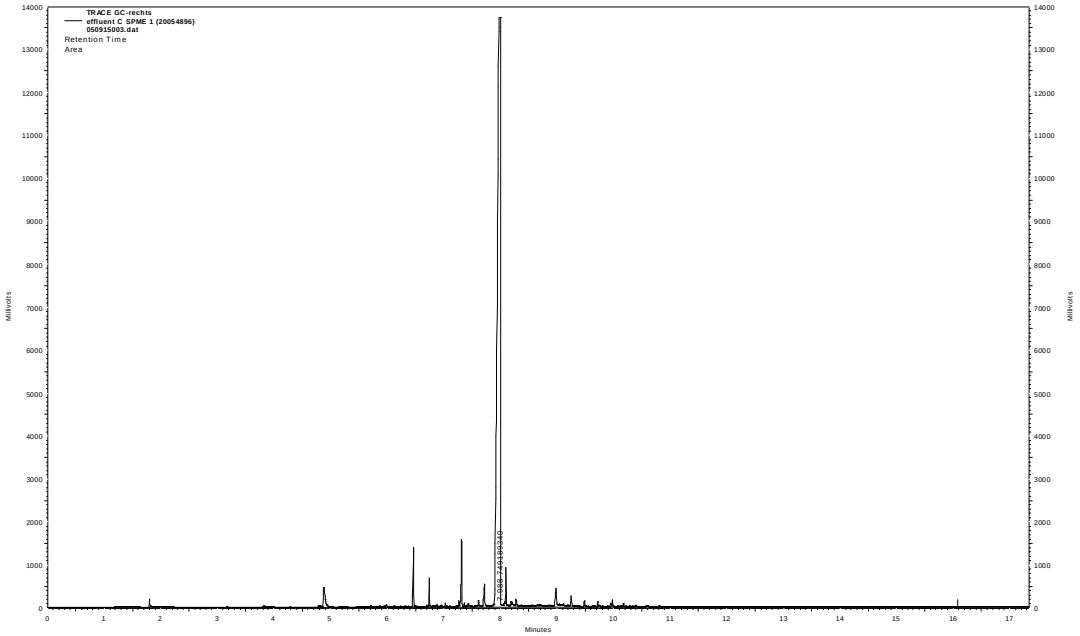


4.4.3 Effluent C chromatograms

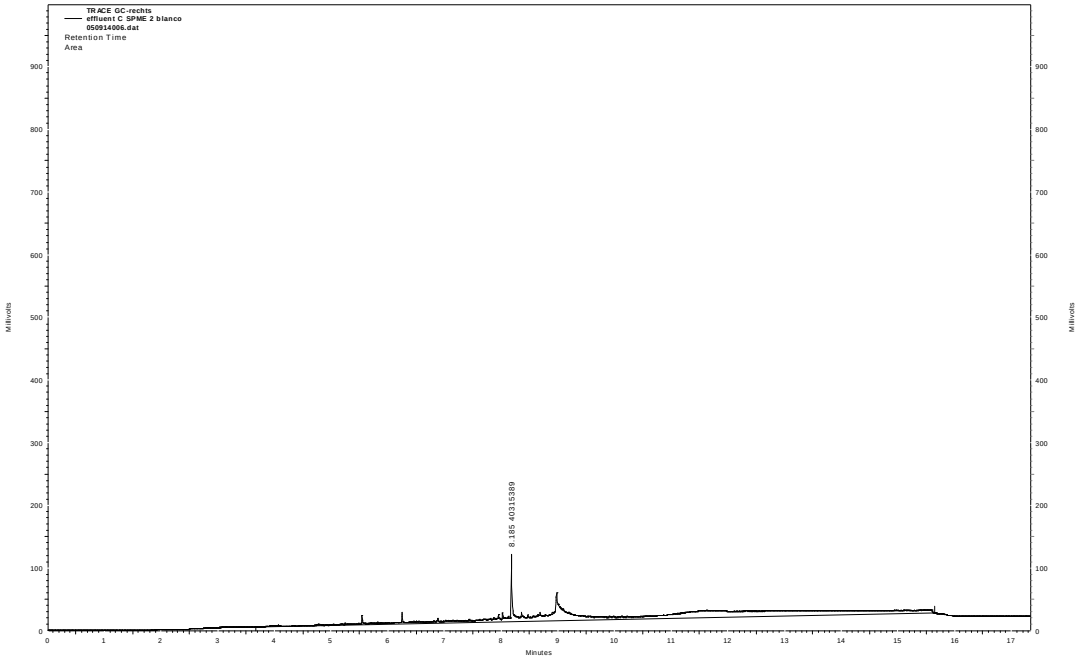
effluent C SPME 1 blanco



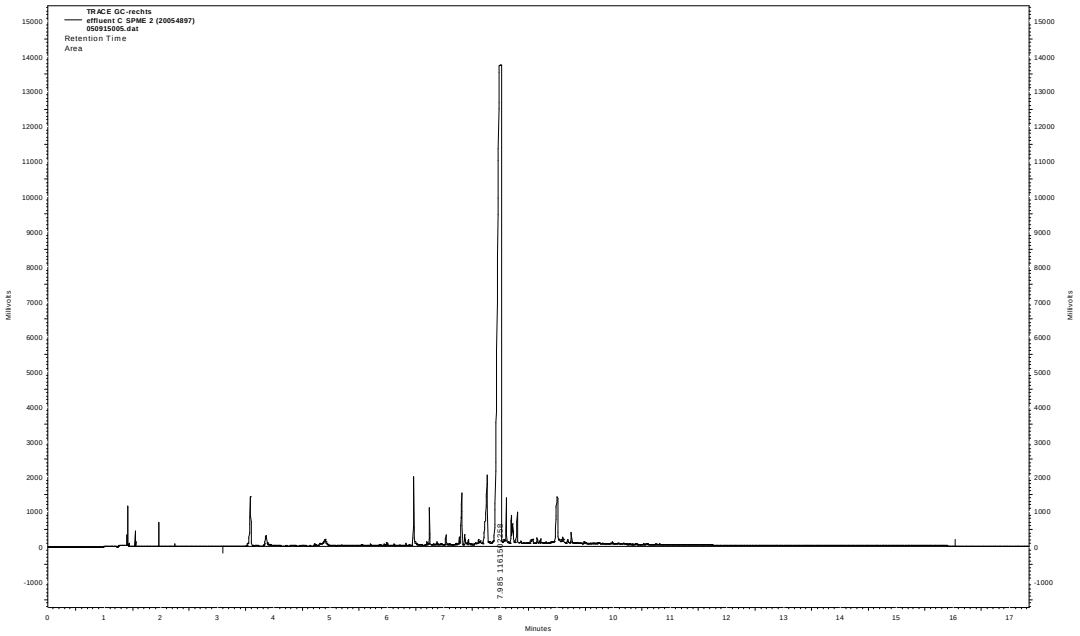
effluent C SPME 1



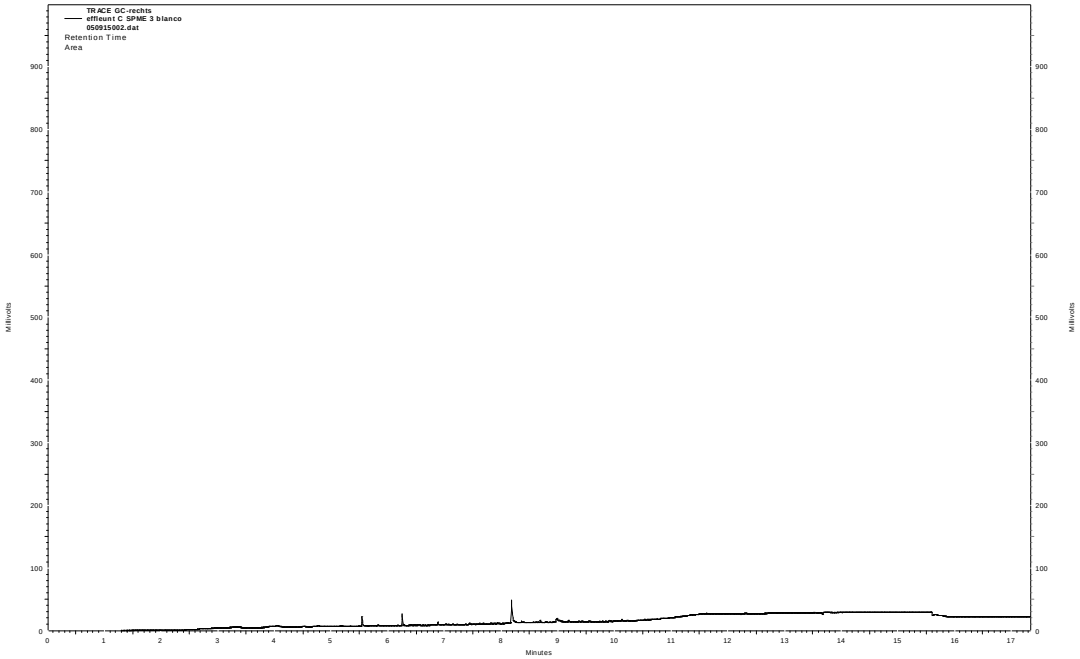
effluent C SPME 2 blanco



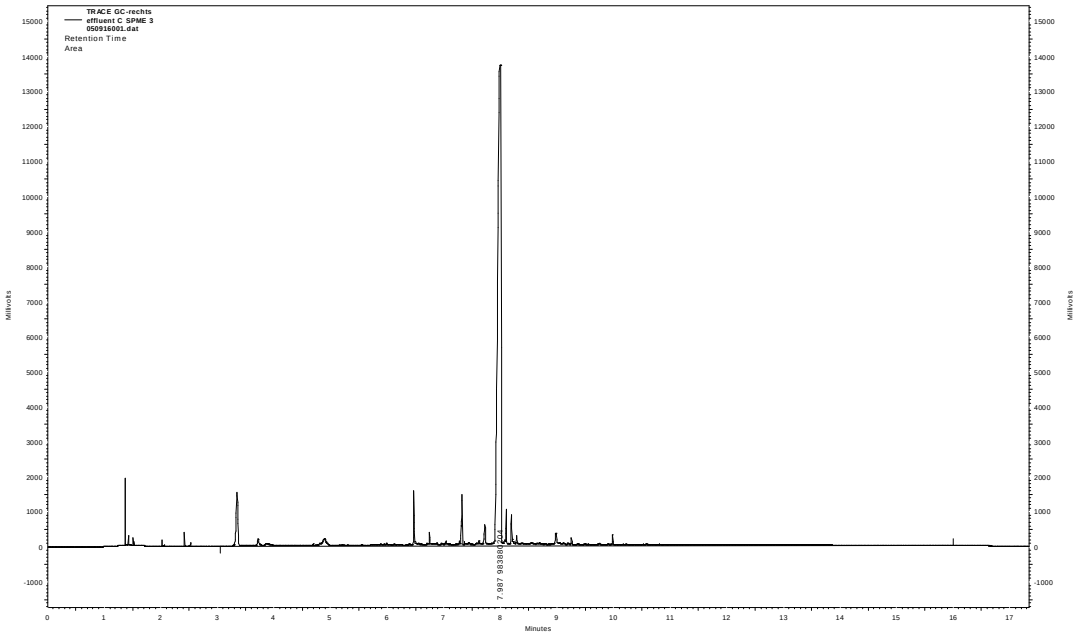
effluent C SPME 2



effluent C SPME 3 blanco

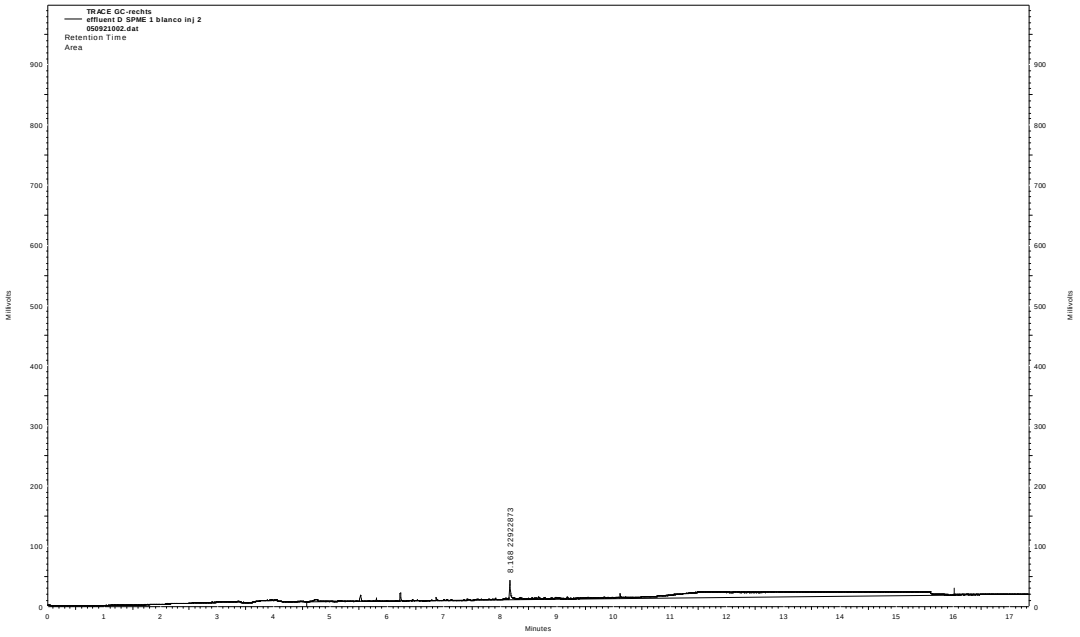


effluent C SPME 3

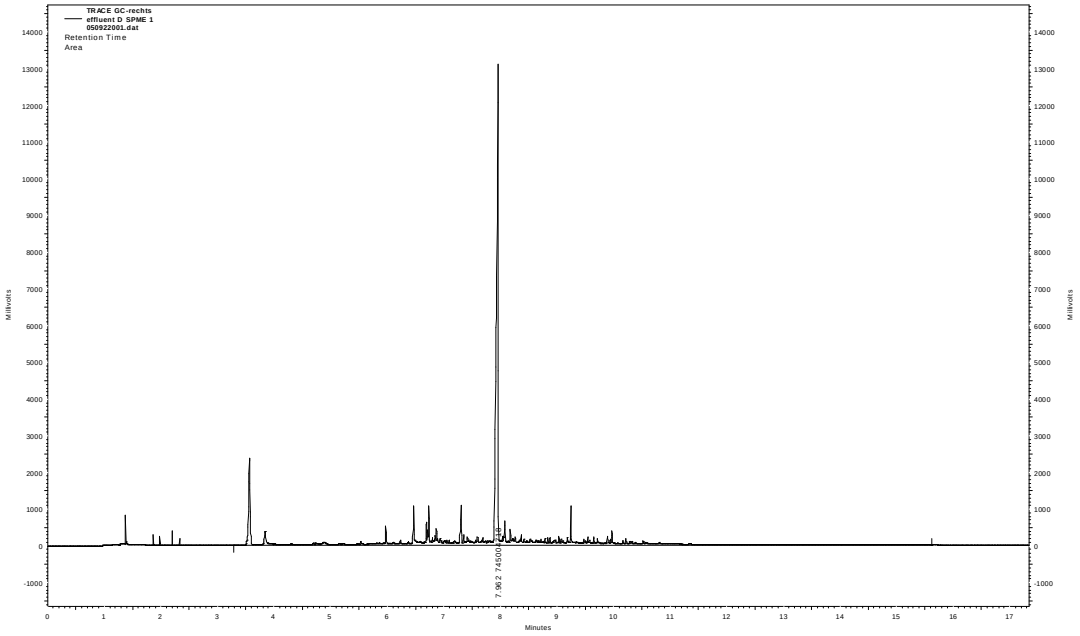


4.4.4 Effluent D chromatograms

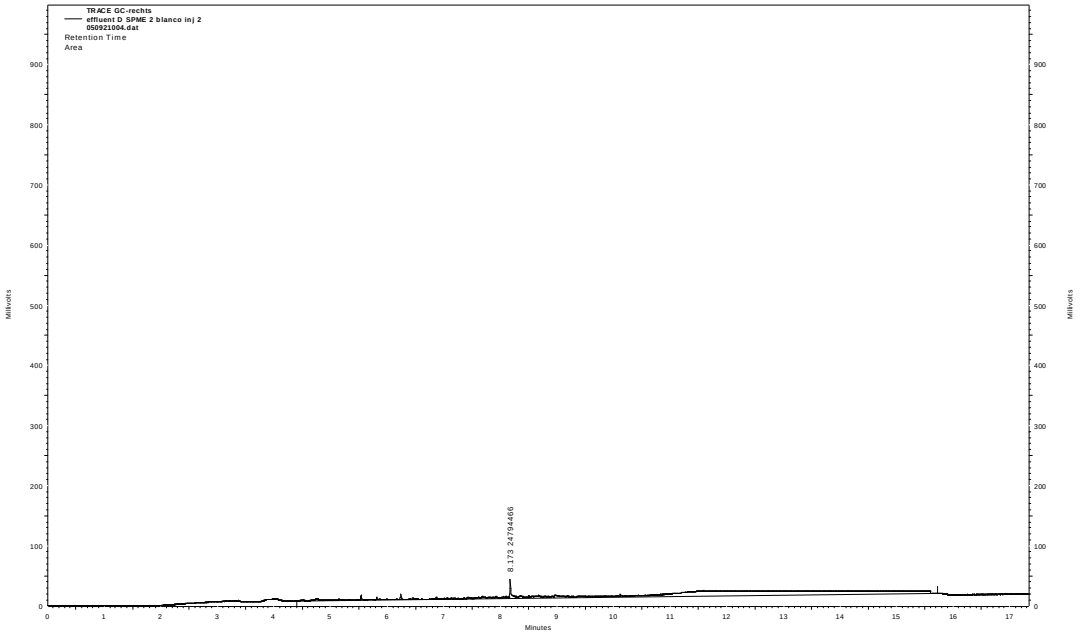
effluent D SPME 1 blanco



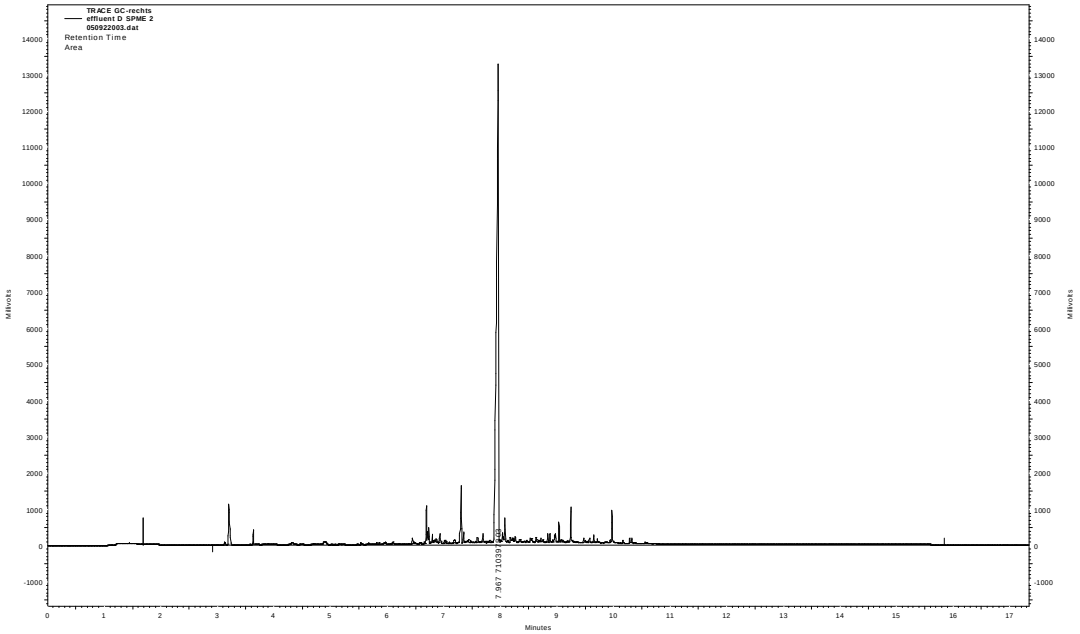
effluent D SPME 1



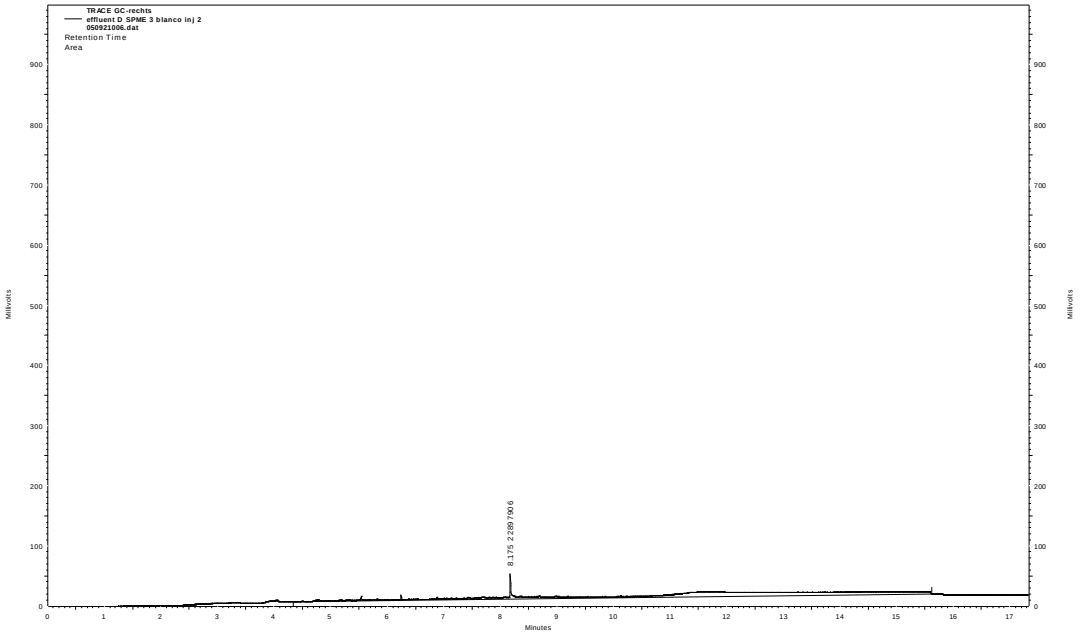
effluent D SPME 2 blanco



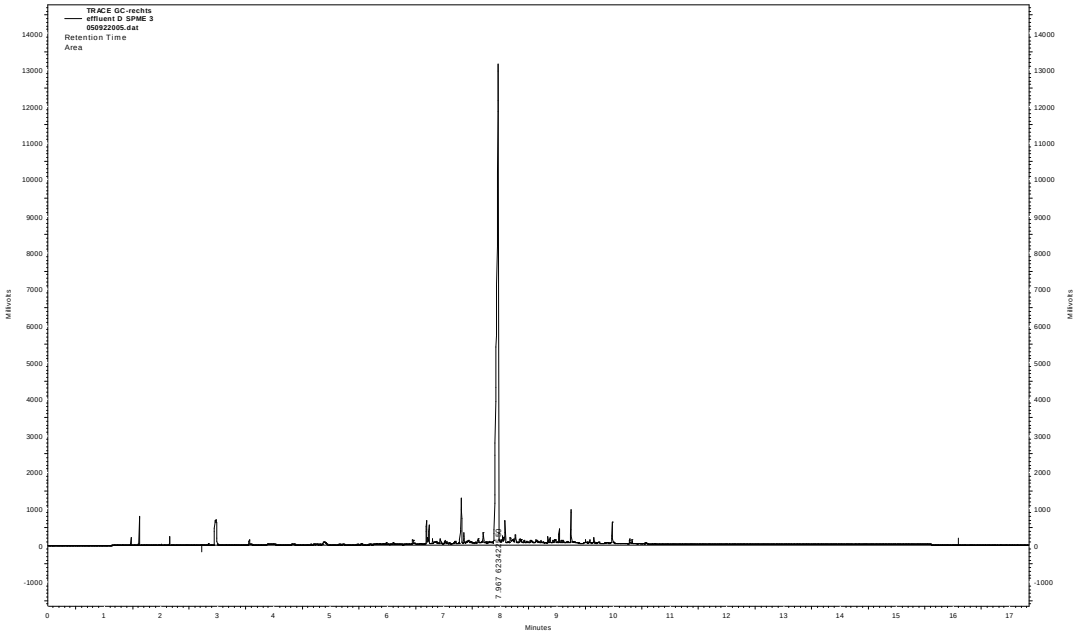
effluent D SPME 2



effluent D SPME 3 blanco

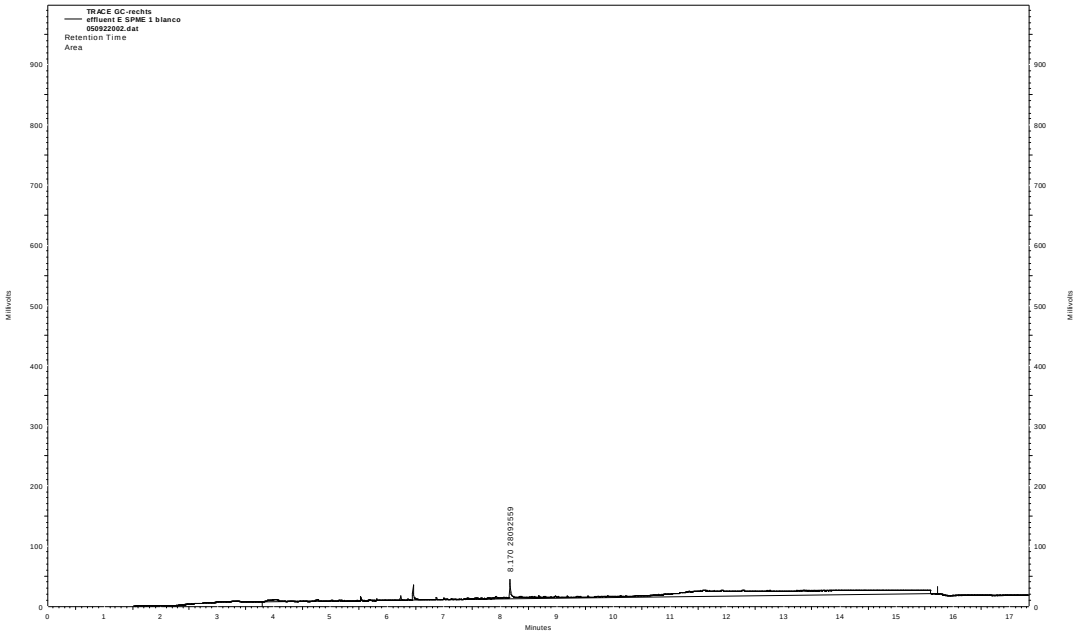


effluent D SPME 3

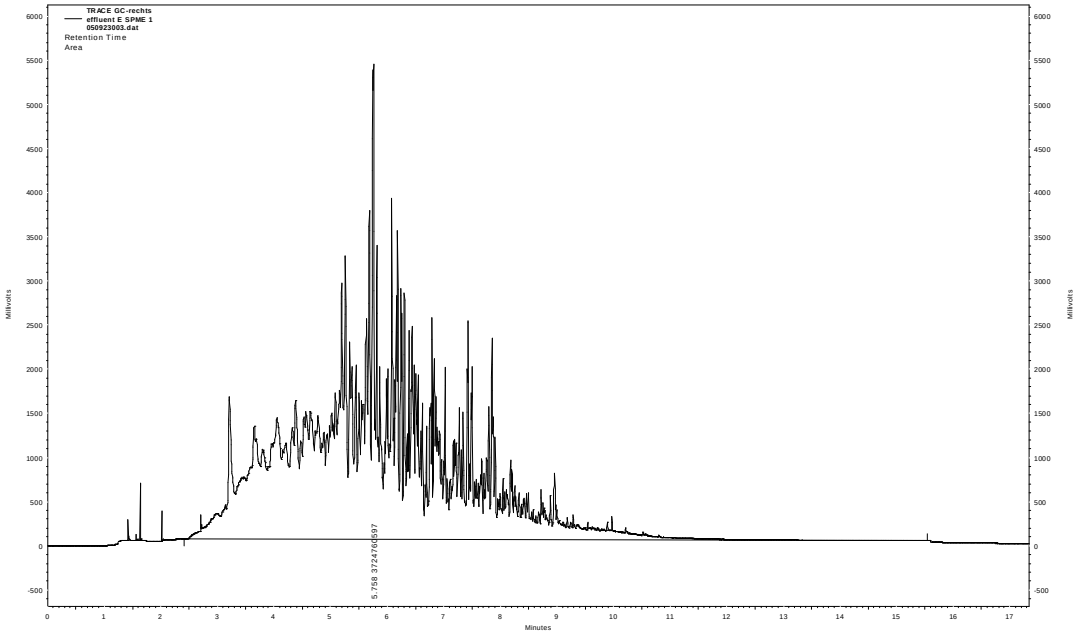


4.4.5 Effluent E chromatograms

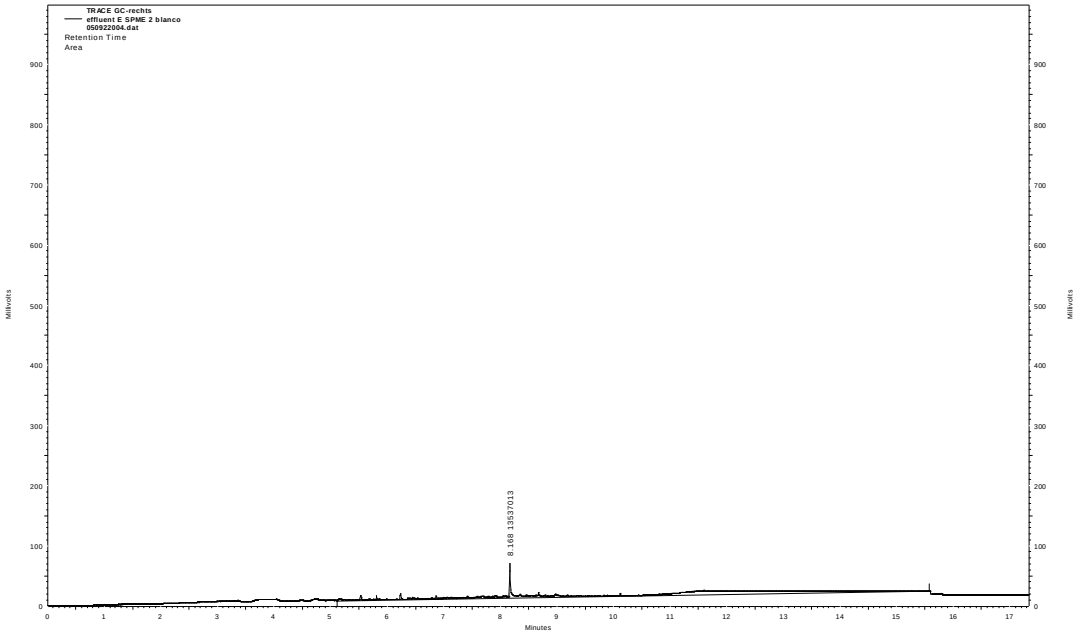
effluent E SPME 1 blanco



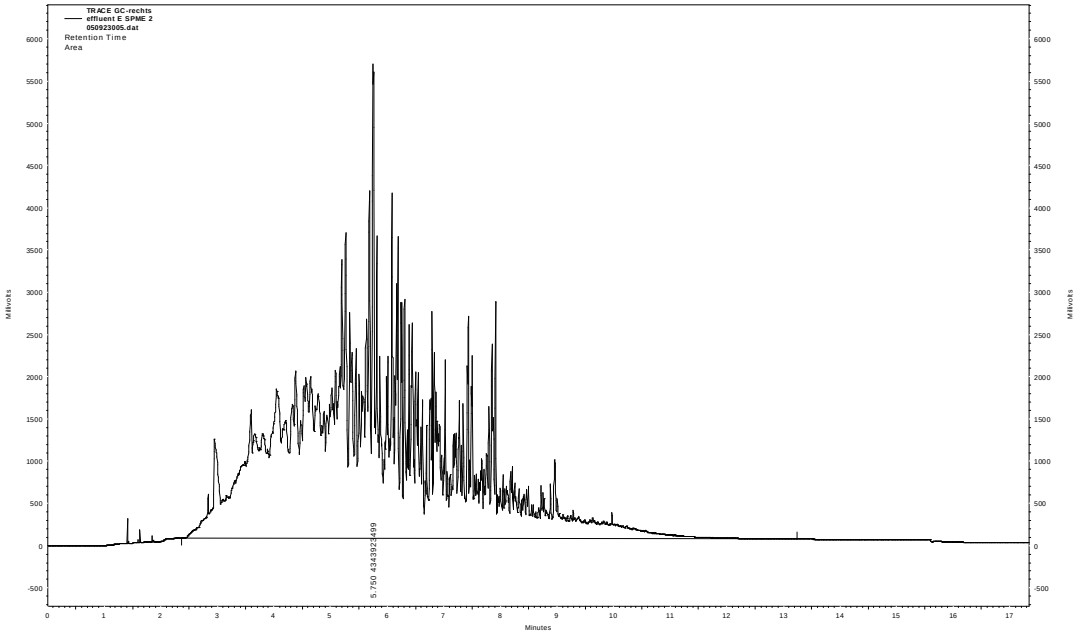
effluent E SPME 1



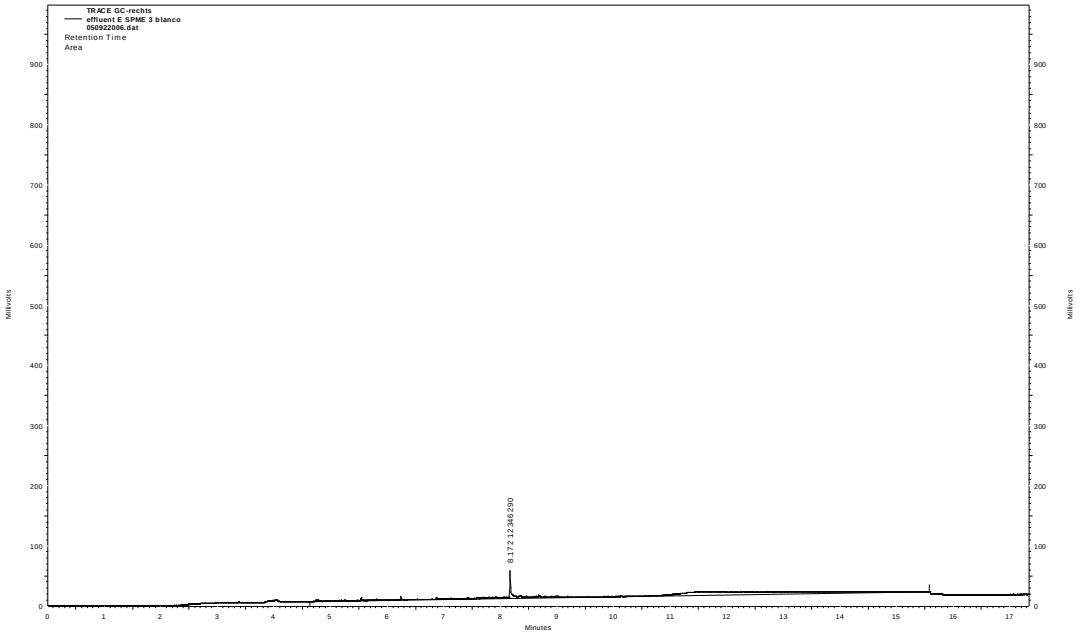
effluent E SPME 2 blanco



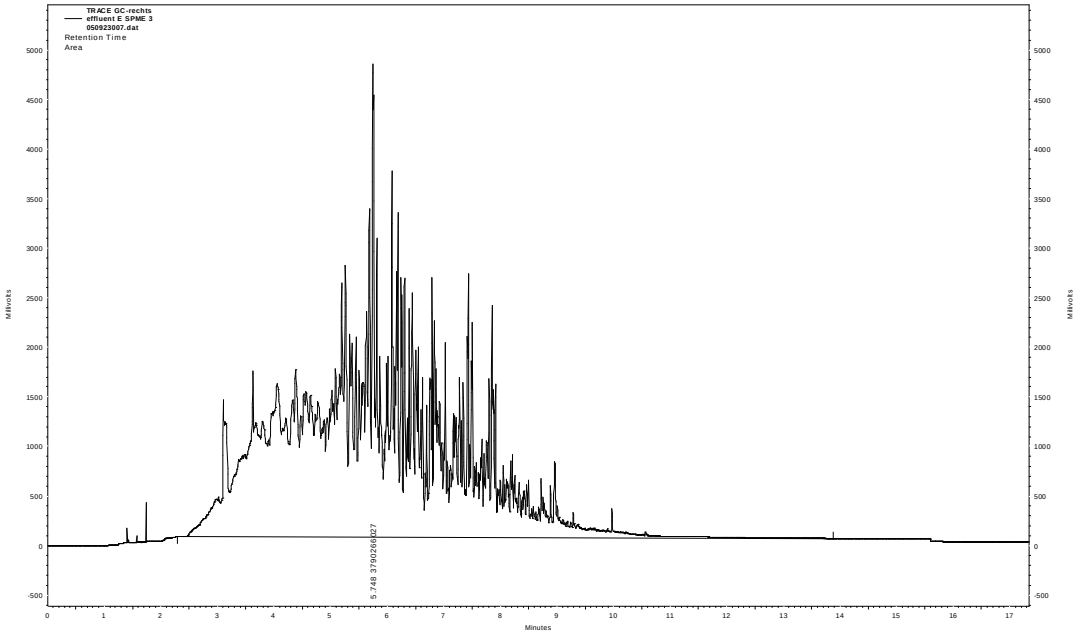
effluent E SPME 2



effluent E SPME 3 blanco



effluent E SPME 3



Appendix 6. Report and Chromatograms UFZ

Eperimental Details from Dr. A. Paschke, UFZ

Determination of potentially bioaccumulating substances (PBS)
in whole effluents using biomimetic SPME or the 'EGOM' LLE method
(according to the respective protocols redrafted by H.A. Leslie and P. E.G. Leonards)

Test Samples:
received from RIVO via parcel service on the 16 August 2005 (still in cooled state); stored at 4°C until use
Chemicals:
2,3-Dimethylnaphthalene (= DMN) (Fluka, Buchs/Switzerland)
Ethylacetate (=EtAc), SupraSolv (Merck, Darmstadt/Germany)
Cylcohexane, LiChroSolv, (Merck)
n-Octanol, reinst (Merck)
Nonane, zur Synthese (Merck)
Tetracontane, p.a. (Fluka)
HCl, p.a.(Merck)
NaOH, p.a. (Merck)
Na_2SO_4, p.a. (Merck)

SPME-Fibers: 100 µm PDMS (Supleco, Bellefonte/PA, USA); used already approx. 10 times before

pH meter: CG 841 (Schott Geräte GmbH, Hofheim/Germany)

Gas chromatorgraph
GC: CP 9001 with split/splitlesss injector and FID (Chrompack, Frankfurt a.M./Germany)
Liner: 1-mL-Insert for split/splitless injector for liquid injection; 0.25-mL for SPME fiber desorption (Chrompack)
Column: OPTIMA-1; 10 m x 0.25 mm, 0.1 µm film thickness (Macherey-Nagel, Düren/Germany)
Carrier gas: Nitrogen 5.0
Carrier gas pressure/flow: 30 kPa / 1,01 mL/min
Injektor temperature: 250°C
Injection mode: splitless over the whole gc runtime
Oven program: 50°C (2 min) ---> 30°/min ---> 310°C (15 min)
Detector temperature: 300°C

Data Evaluation

2,3- dimethylnaphthalene (=DMN)

Mol weight: 156.23 g/mol

1 µl of (100 mg DMN / 100 mL EAC = 1 mg/mL) are 6.40082E-06 mmol DMN

1 µl of (4 mg DMN / 100 mL EAC = 40 µg/mL) are 2.56033E-07 mmol DMN V(PDMS)=0.62 µL

GC-Run	Sample	Peak area	Average	DMN [g]	DMN [mol]	PBS [mmol/L]*	Calc. proof : PBS [mmol/L]
050822b	1µl DMN (1 mg/mL)	741386					
050822c	1µl DMN (1 mg/mL)	737576					
050822e	Blank Fiber 6	120869		1.65E-07	1.05622E-09	1.7036	1.7036
050822f	Blank Fiber 7	103412		1.41E-07	9.03667E-10	1.4575	1.4575
050822g	Blank Fiber 14	34023		4.64E-08	2.9731E-10	0.4795	0.4795
050822i	Blank Fiber 15	81119		1.11E-07	7.08859E-10	1.1433	1.1433
050822k	1µl DMN (1 mg/mL)	706682					
050822l	1µl DMN (1 mg/mL)	734835					
050822m	1µl DMN (1 mg/mL)	741941	732484				
050901b	1µl DMN (40 µg/mL)	24223					
050901c	1µl DMN (40 µg/mL)	40359					
050901d	1µl DMN (40 µg/mL)	23336	29306				
050901j	Fiber 6 (octanol solution)	201037		2.74E-07	1.75637E-09	2.8328	2.8328
050901k	Fiber 7"(octanol solution)	136998		1.87E-07	1.19689E-09	1.9305	1.9305
050901l	Fiber 14 (octanol solution)	140812		1.92E-07	1.23021E-09	1.9842	1.9842
050901m	Fiber 15 (octanol solution)	114792		1.57E-07	1.00288E-09	1.6176	1.6176
050907b	1µl DMN (40 µg/mL)	49120					
050907c	1µl DMN (40 µg/mL)	40939					
050907d	1µl DMN (40 µg/mL)	42988					
050907f	Fiber 6 (Sample B1)	219040		2.15E-07	1.37801E-09	2.2226	2.2226
050907g	Fiber 7"(Sample C1)	584372		5.74E-07	3.67636E-09	5.9296	5.9296
050907h	Fiber 14(Sample D1)	330730		3.25E-07	2.08067E-09	3.3559	3.3559
050907i	Fiber 15(Sample E1)	2722043		2.68E-06	1.71247E-08	27.6205	27.6205
050907k	1µl DMN (40 µg/mL)	30926					
050907l	1µl DMN (40 µg/mL)	39514	40697.4				
050908b	1µl DMN (40 µg/mL)	41726					
050908c	1µl DMN (40 µg/mL)	43691					
050908d	1µl DMN (40 µg/mL)	46679	44032				
050908h	Fiber F2 (Sample C2)	688962		6.26E-07	4.00611E-09	6.4615	6.4615
050908i	Fiber 7" (Sample D2)	284410		2.58E-07	1.65376E-09	2.6674	2.6674
050908j	Fiber 14 (Sample E2)	2591141		2.35E-06	1.50667E-08	24.3011	24.3011
050908k	Fiber 15 (Sample B2)	159016		1.44E-07	9.2463E-10	1.4913	1.4913
050912b	1µl DMN (40 µg/mL)	49234					
050912c	1µl DMN (40 µg/mL)	44695					
050912d	1µl Sample B1	n.d.***					
050912e	1µl Sample C1	n.d.***					
050912f	1µl DMN (40 µg/mL)	45128	46352.3				
050913b	1µl DMN (40 µg/mL)	43026					
050913c	1µl DMN (40 µg/mL)	36642					
050913e	Fiber F2 (Sample D3)	306136		3.16E-07	2.02141E-09	3.2603	3.2603
050913f	Fiber 7" (Sample E3)	1996531		2.06E-06	1.31831E-08	21.2630	21.2630
050913g	Fiber 14 (Sample B3)	151394		1.56E-07	9.99652E-10	1.6123	1.6123
050913h	Fiber 15 (Sample C3)	544831		5.62E-07	3.59751E-09	5.8024	5.8024
050913i	Fiber F2 (Nullwert)	31146		3.21E-08	2.05657E-10	0.3317	0.3317
050913j	Fiber 7" (Nullwert)	53448		5.51E-08	3.52916E-10	0.5692	0.5692
050913l	1µl DMN (40 µg/mL)	34690					
050913m	1µl DMN (40 µg/mL)	40743	38775.3				
050914b	1µl DMN (40 µg/mL)	39866					
050914c	1µl DMN (40 µg/mL)	43867					
050914d	1µl DMN (40 µg/mL)	38436	40723				
050914e	1µl Sample D1	n.d.***					
050914f	1µl Sample E1	n.d.***					

050914g	1µl Sample E2	101921		1.00E-07	6.40796E-10		
050914h	1µl Sample E3	171625		1.69E-07	1.07904E-09		
050914j	Fiber F2	157393		1.55E-07	9.89558E-10	1.5961	1.5961
05091b	1µl DMN (40 µg/mL)	42113					
05091c	1µl DMN (40 µg/mL)	39348	40730.5				
05091e	Fiber 15 (Sample A1)	245389		2.41E-07	1.54252E-09	2.4879	2.4879
05091f	Fiber 14 (Sample A2)	173948		1.71E-07	1.09344E-09	1.7636	1.7636
050915g	Fiber 7" (Sample A3)	184022		1.81E-07	1.15677E-09	1.8658	1.8658
050915i	1µl Sample B2	n.d.***					
050915j	1µl Sample C2	n.d.***					
050915k	1µl Sample D2	n.d.***					
050916b	1µl DMN (40 µg/mL)	44340					
050916c	1µl DMN (40 µg/mL)	44801					
050916d	1µl DMN (40 µg/mL)	45898	45013				
050916e	1µl Sample B3	n.d.***					
050916f	1µl Sample C3	n.d.***					
050919b	1µl DMN (40 µg/mL)	39244					
050919c	1µl DMN (40 µg/mL)	44044					
050919d	1µl DMN (40 µg/mL)	44848	42712				
050919e	1µl Sample D3	n.d.***					
050919f	1µl Sample A1	n.d.***					
050919g	1µl Sample A2	n.d.***					
050919h	1µl Sample A3	n.d.***					

* refers to PDMS used as extracting phase
** refers to the water sample investigated
*** n.d.= no detectable peaks and thus no integration result for the chromatogram

PBS [g/L]**
6.67E-04
1.12E-03

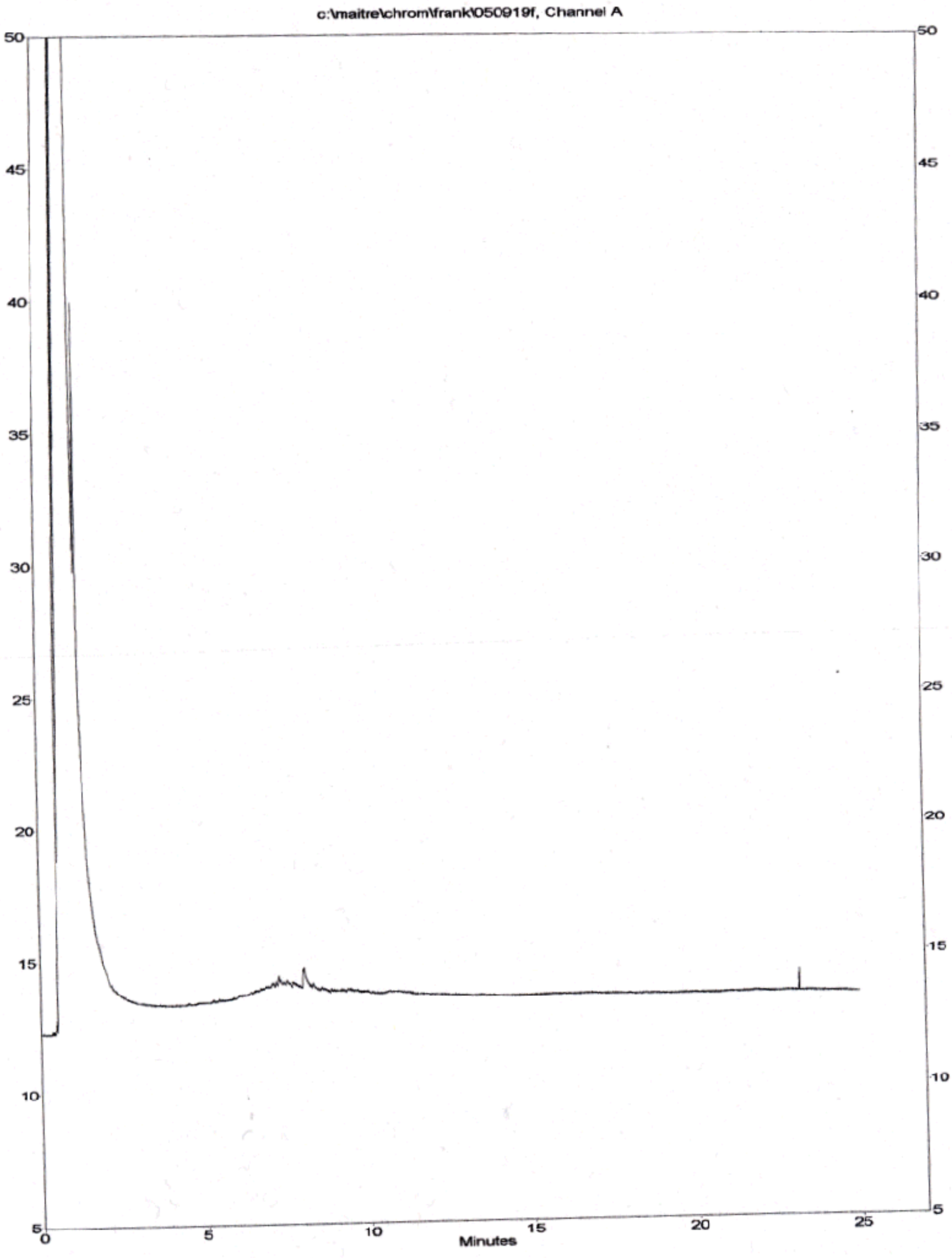
Table of Results

GC-Run	SPME-fibre uses	Sample description / Remarks	DMN-Equival.[mol]	PBS [mmol/L]*
050822e	Fiber 6	Blank (3 min exposed in gc injector)	1.05622E-09	1.70
050822f	Fiber 7	Blank (3 min exposed in gc injector)	9.03667E-10	1.46
050822g	Fiber 14	Blank (3 min exposed in gc injector)	2.9731E-10	0.48
050822i	Fiber 15	Blank (3 min exposed in gc injector)	7.08859E-10	1.14
050913i	Fiber F2	Blank (10 min exposed in gc injector)	2.05657E-10	0.33
050913j	Fiber 7"	Blank (10 min exposed in gc injector)	3.52916E-10	0.57
050901j	Fiber 6	octanol-in-water solution (830 µg/l)	1.75637E-09	2.83
050901k	Fiber 7"	octanol-in-water solution (830 µg/l)	1.19689E-09	1.93
050901l	Fiber 14	octanol-in-water solution (830 µg/l)	1.23021E-09	1.98
050901m	Fiber 15	octanol-in-water solution (830 µg/l)	1.00288E-09	1.62
050914j	Fiber F2	octanol-in-water solution (830 µg/l)	9.89558E-10	1.60
05091e	Fiber 15	A1	1.54252E-09	2.49
05091f	Fiber 14	A2	1.09344E-09	1.76
050915g	Fiber 7"	A3	1.15677E-09	1.87
050907f	Fiber 6	B1	1.37801E-09	2.22
050908k	Fiber 15	B2	9.2463E-10	1.49
050913g	Fiber 14	B3	9.99652E-10	1.61
050907g	Fiber 7"	C1	3.67636E-09	5.93
050908h	Fiber F2	C2	4.00611E-09	6.46
050913h	Fiber 15	C3	3.59751E-09	5.80
050907h	Fiber 14	D1	2.08067E-09	3.36
050908i	Fiber 7"	D2	1.65376E-09	2.67
050913e	Fiber F2	D3	2.02141E-09	3.26
050907i	Fiber 15	E1	1.71247E-08	27.62
050908j	Fiber 14	E2	1.50667E-08	24.30
050913f	Fiber 7"	E3	1.31831E-08	21.26

	Injected volume		PBS [g/L]**	mg/l
050919f	1µl	A1	n.d.***	
050919g	1µl	A2	n.d.***	
050919h	1µl	A3	n.d.***	
050912d	1µl	B1	n.d.***	
050915i	1µl	B2	n.d.***	
050916e	1µl	B3	n.d.***	
050912e	1µl	C1	n.d.***	
050915j	1µl	C2	n.d.***	
050916f	1µl	C3	n.d.***	
050914e	1µl	D1	n.d.***	
050915k	1µl	D2	n.d.***	
050919e	1µl	D3	n.d.***	
050914f	1µl	E1	n.d.***	
050914g	1µl	E2	6.40796E-10	6.67E-04 6.67E-01
050914h	1µl	E3	1.07904E-09	1.12E-03 1.12E+00

* refers to PDMS used as extracting phase
** refers to the water sample investigated
*** n.d.= no detectable peaks and thus no integration result for the chromatogram

LLE A1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pA
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35
30
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15
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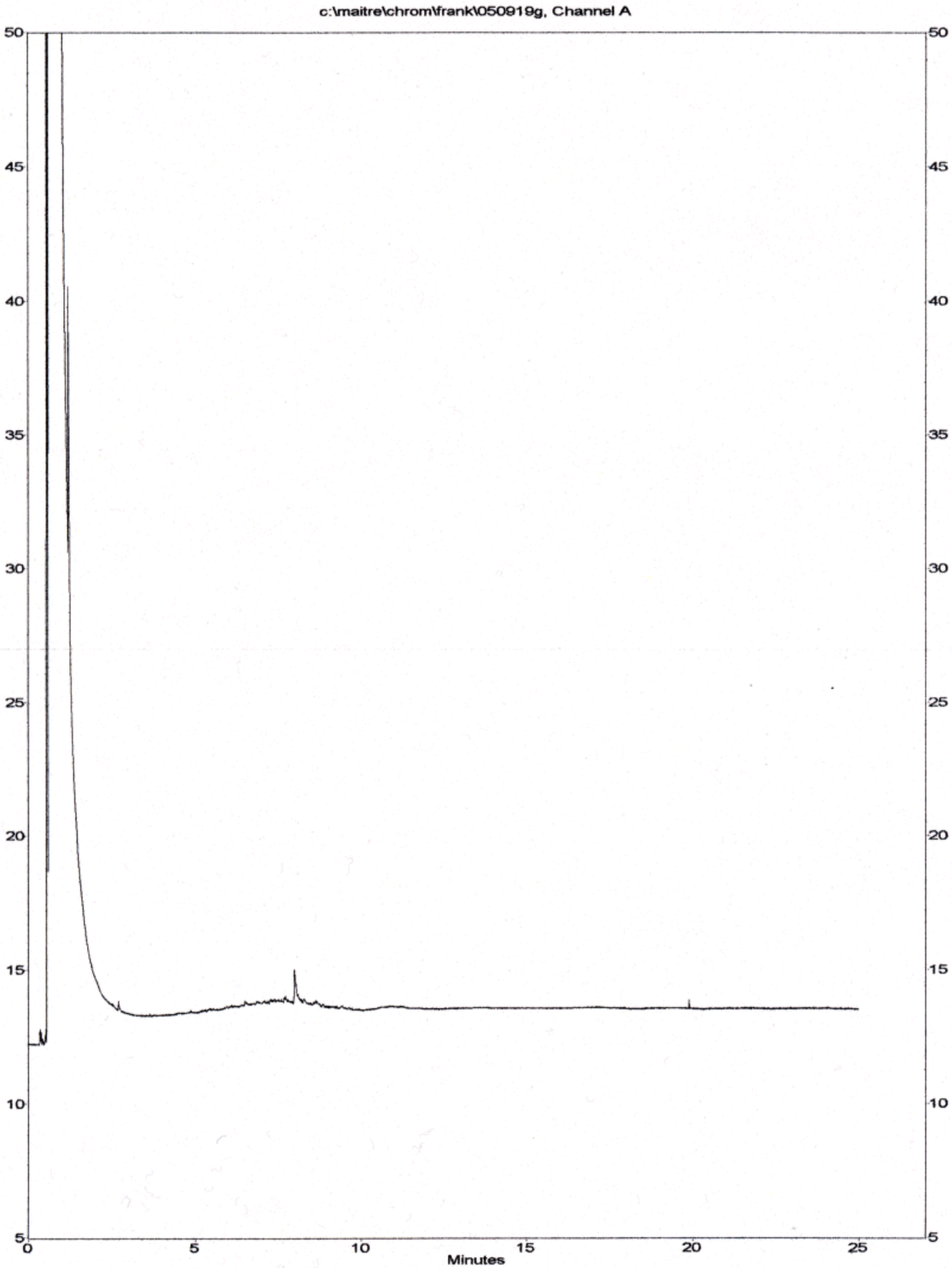
5
10
15
20
25
Minutes

LLE A2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PA
0
5
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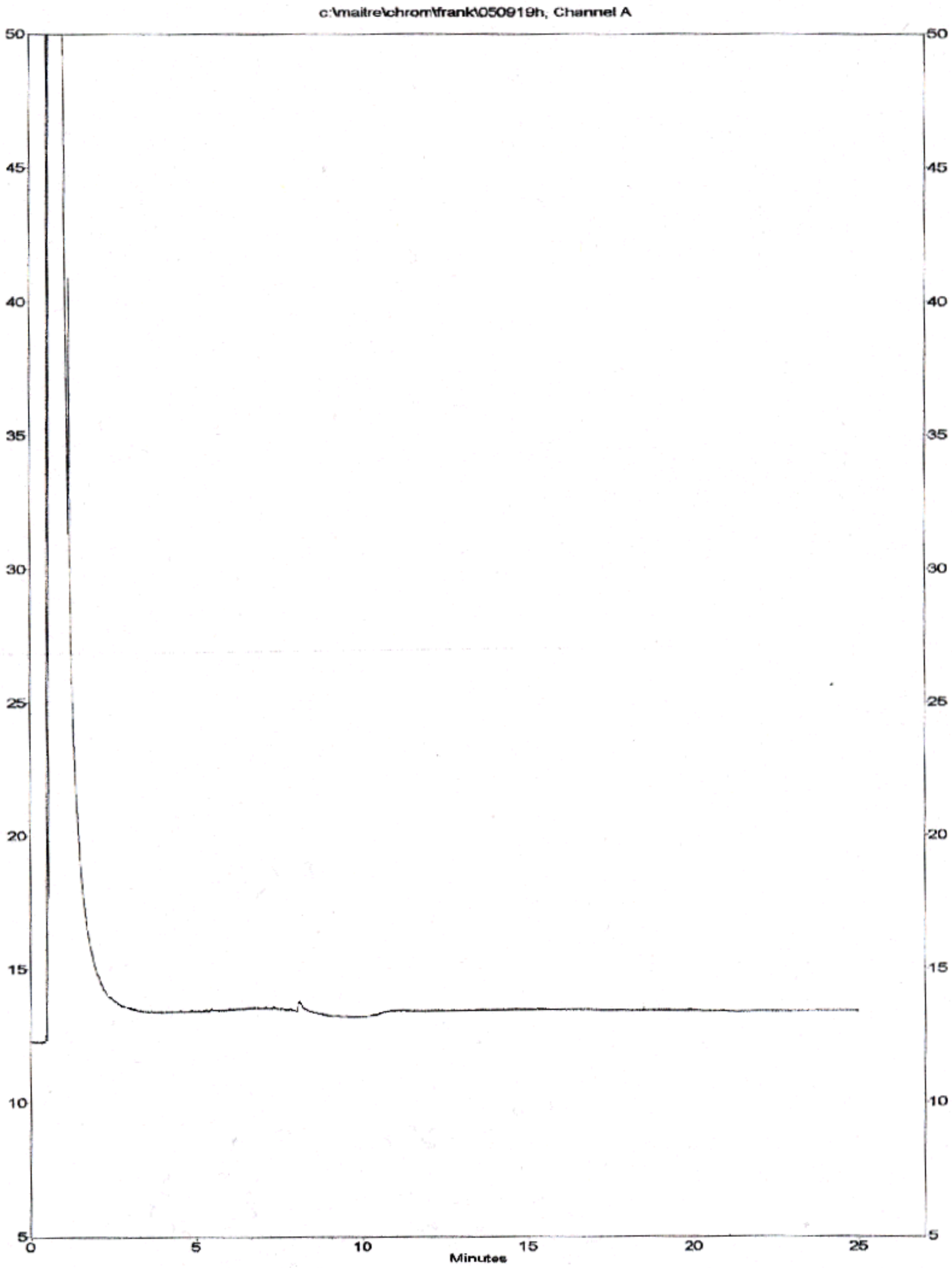
15
20
25
Minutes

LLE A3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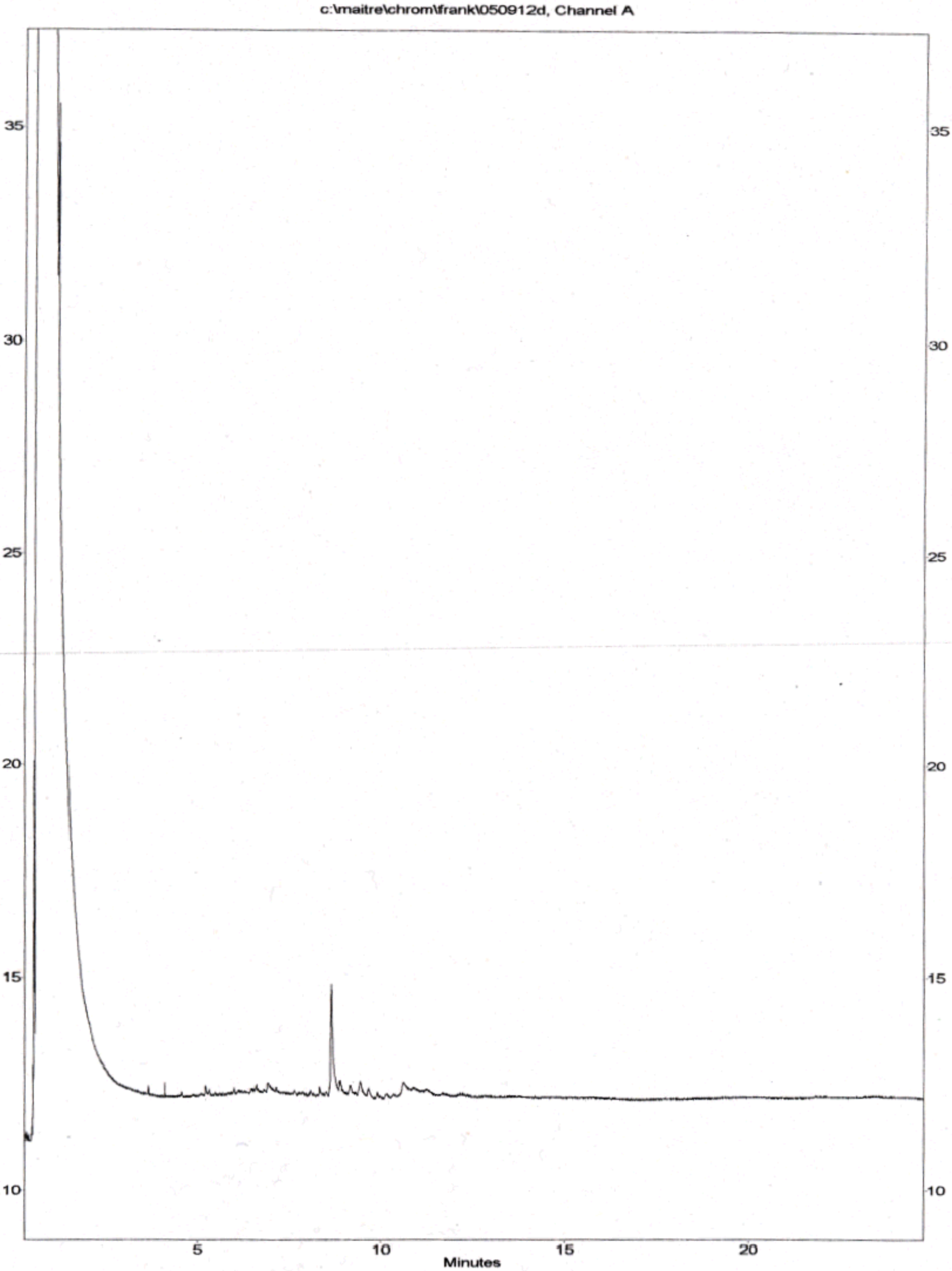
20
25
Minutes

LLE B1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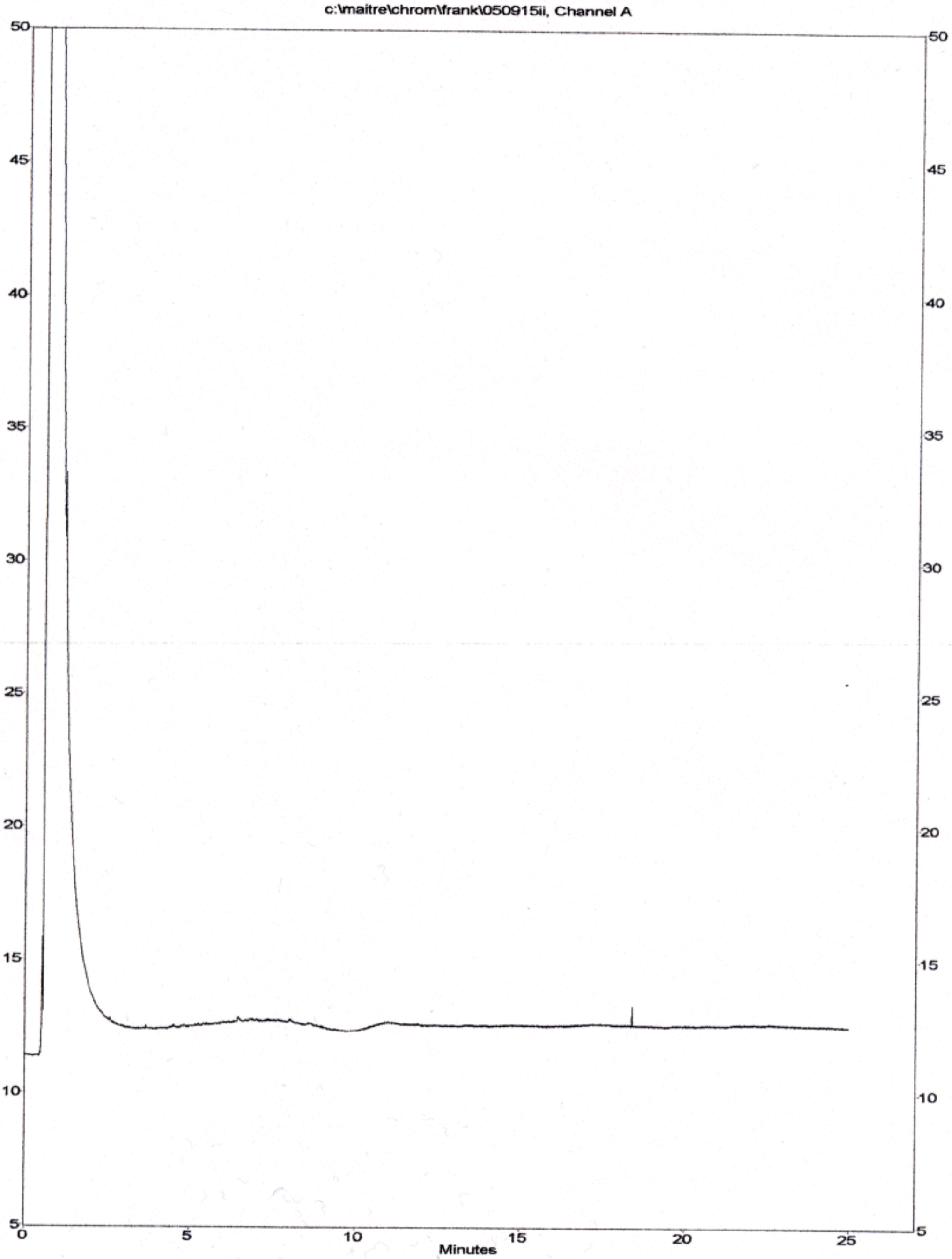
Minutes

LLE B2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10
5
pA
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5
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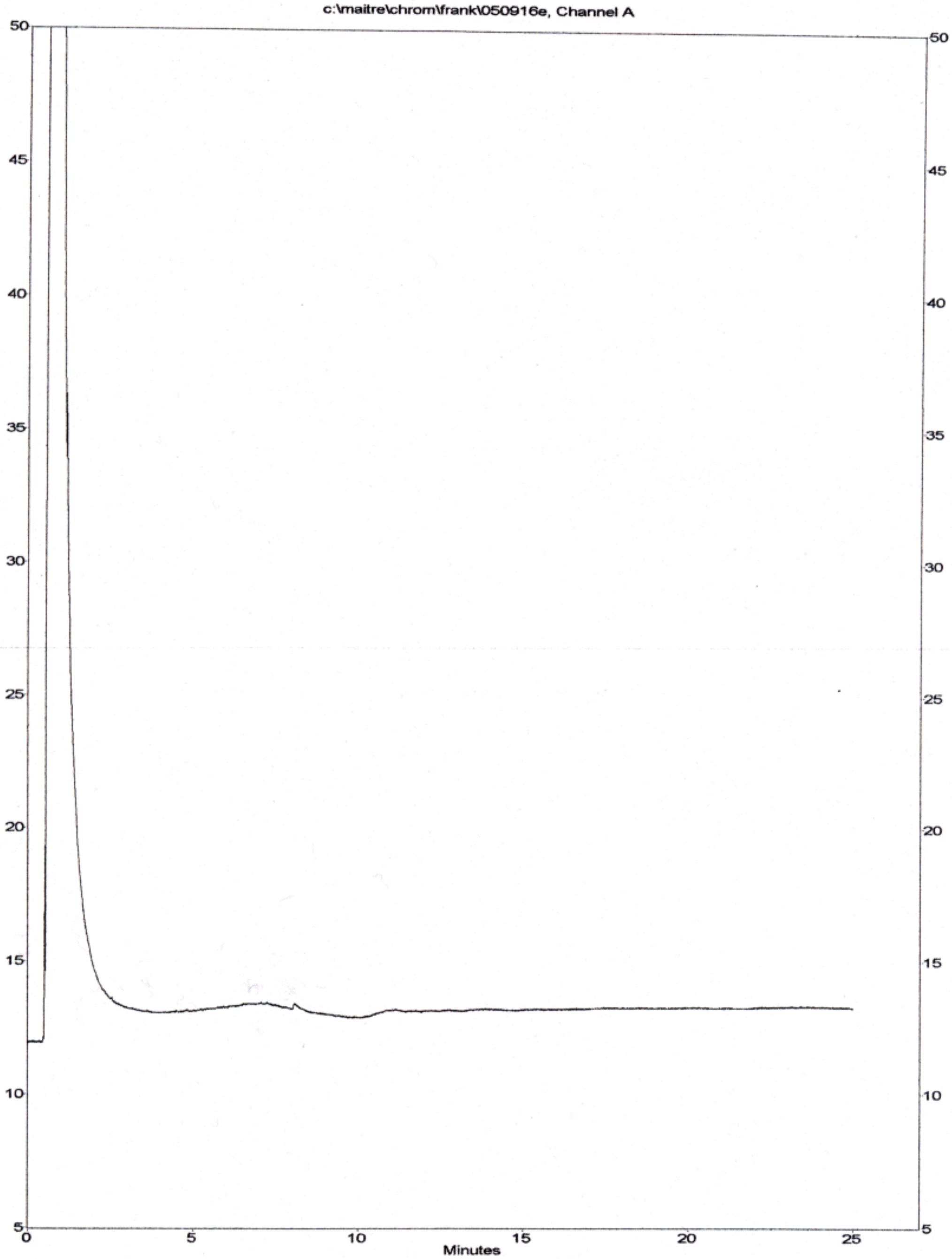
15
20
25
Minutes

LLE B3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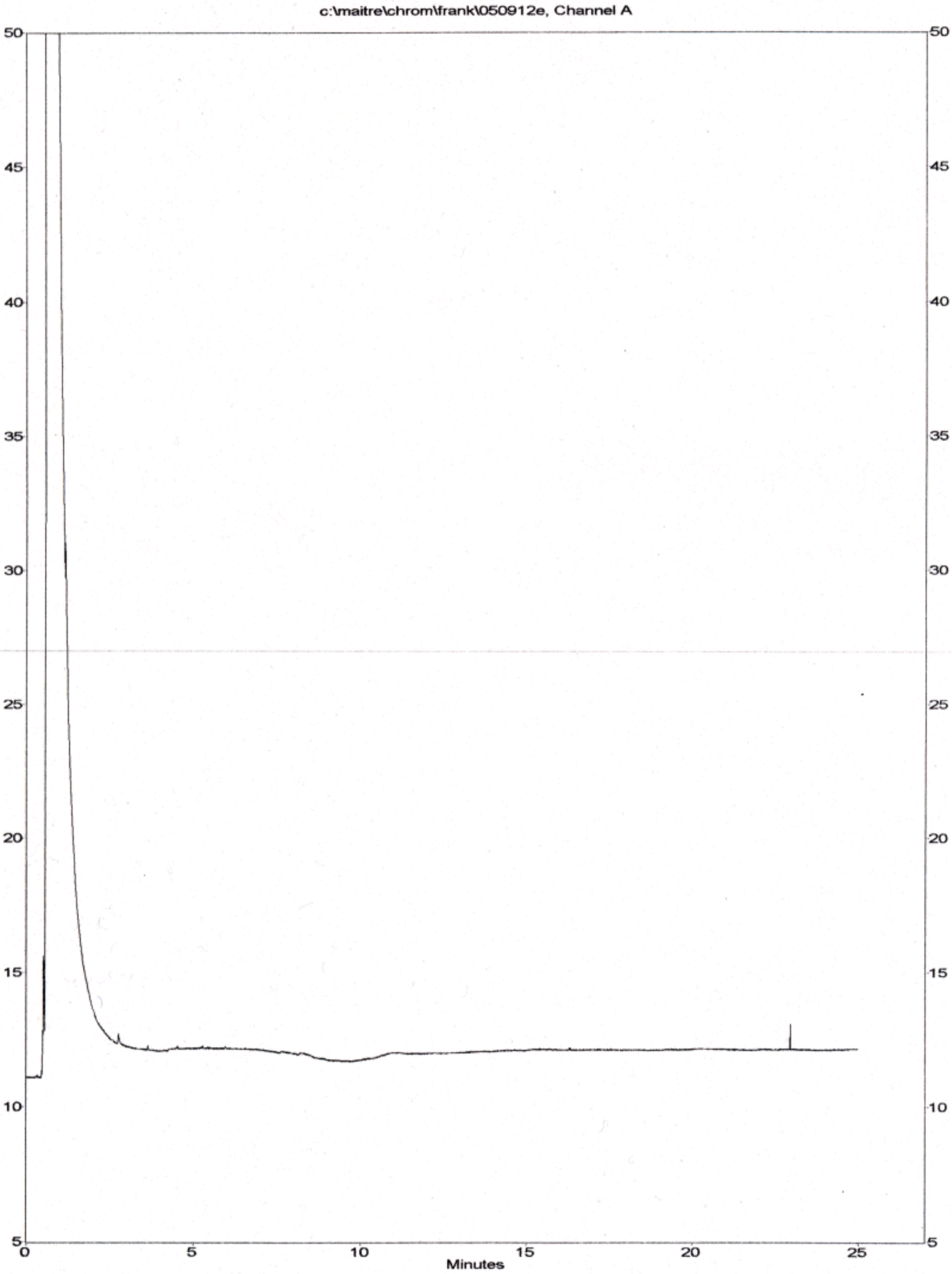
20
25
Minutes

LLE C1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20
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PA
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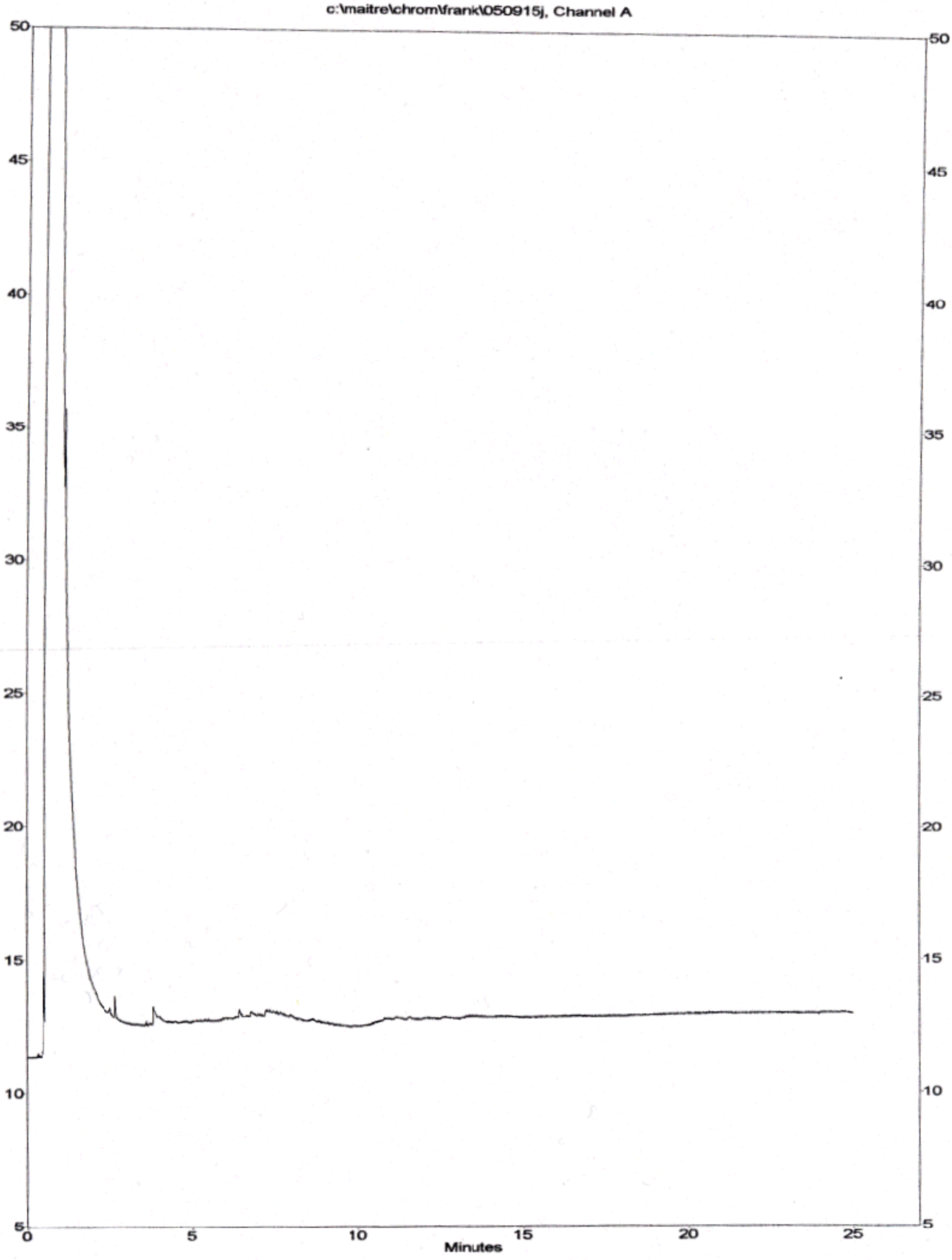
5
10
15
20
25
Minutes

LLE C2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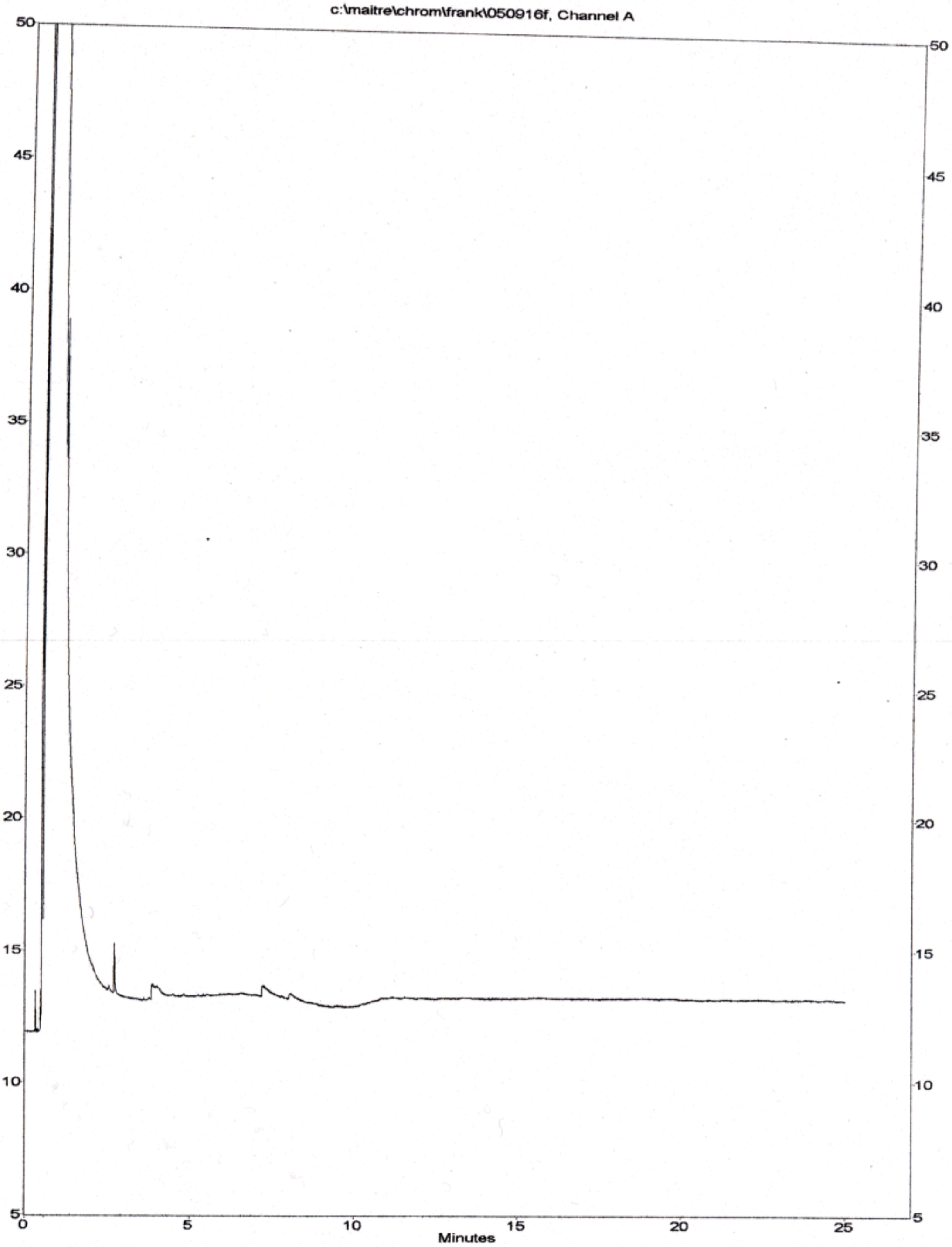
10
15
20
25
Minutes

LLE C3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20
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10
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PA
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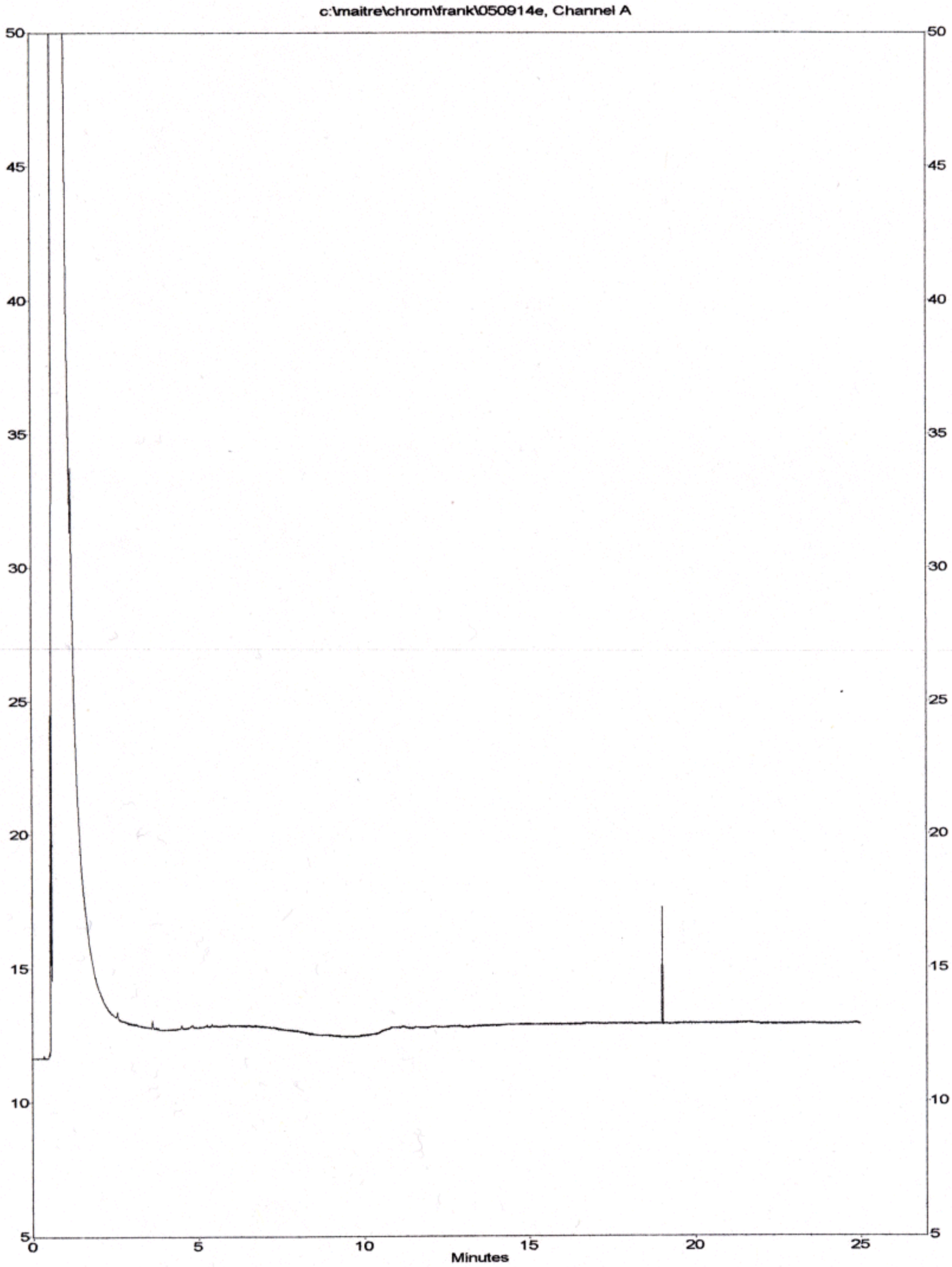
5
10
15
20
25
Minutes

LLE D1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pA
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5
10
15
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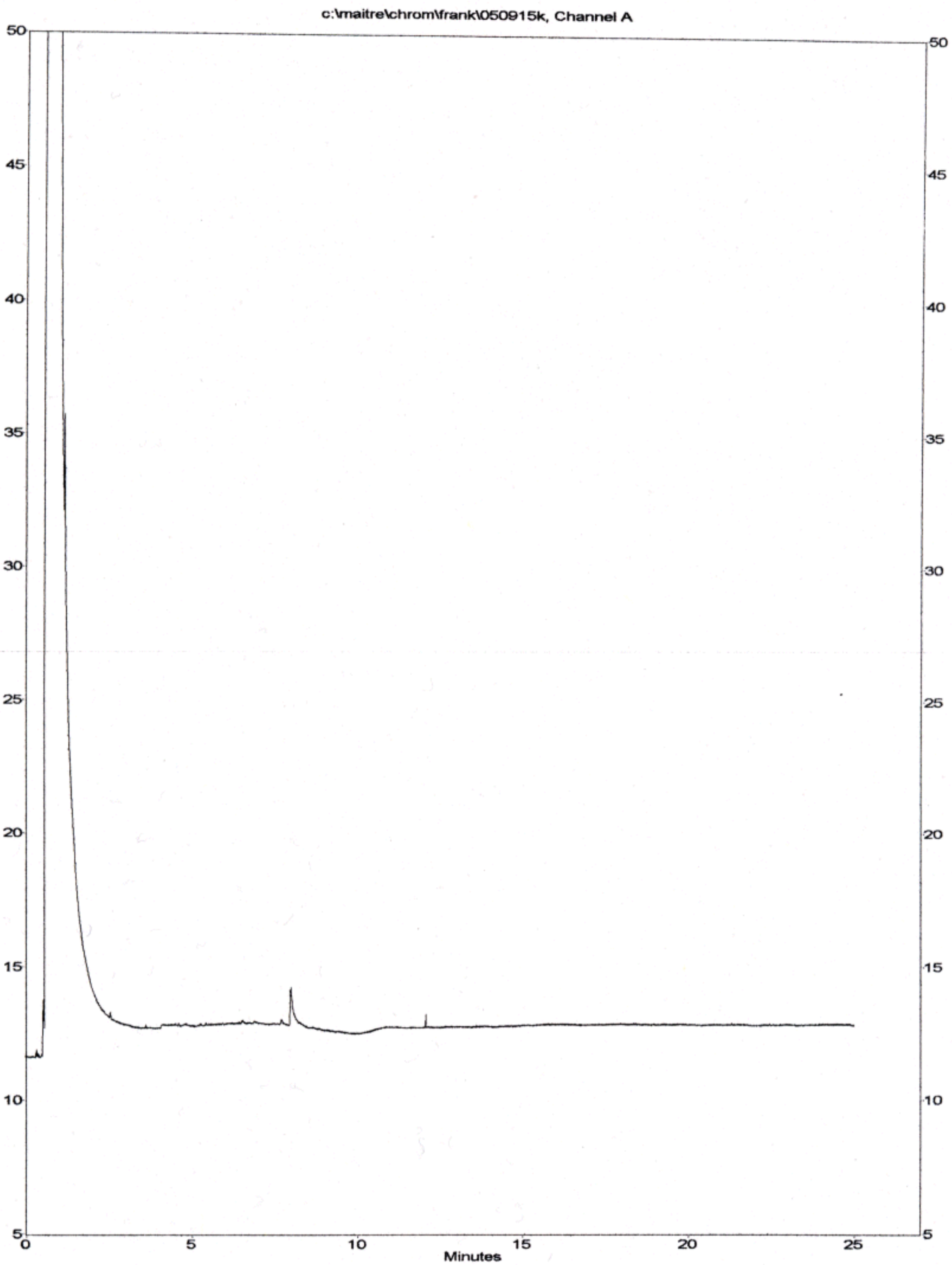
25
Minutes

LLE D2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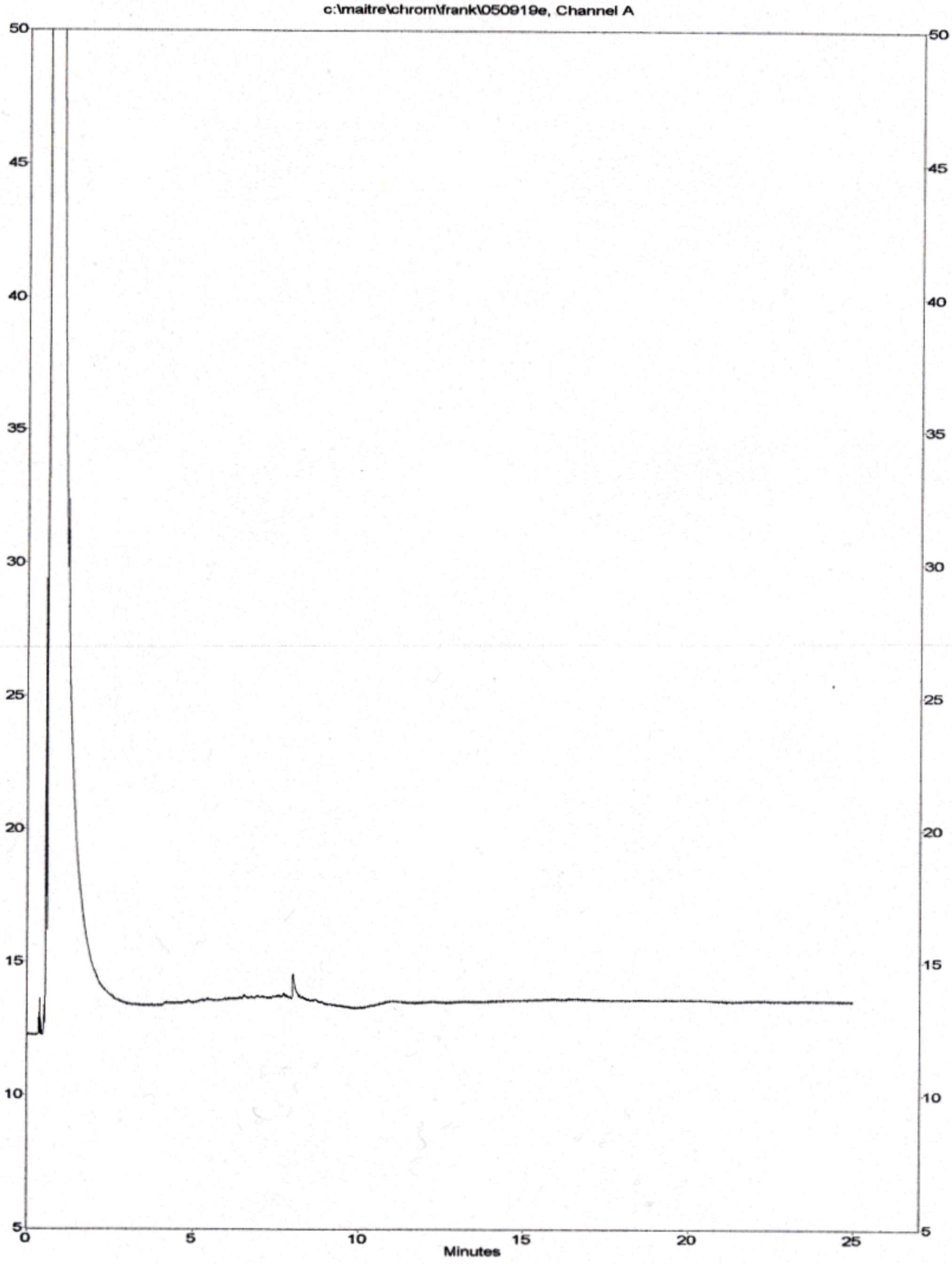
Minutes

LLE D3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20
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10
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pA
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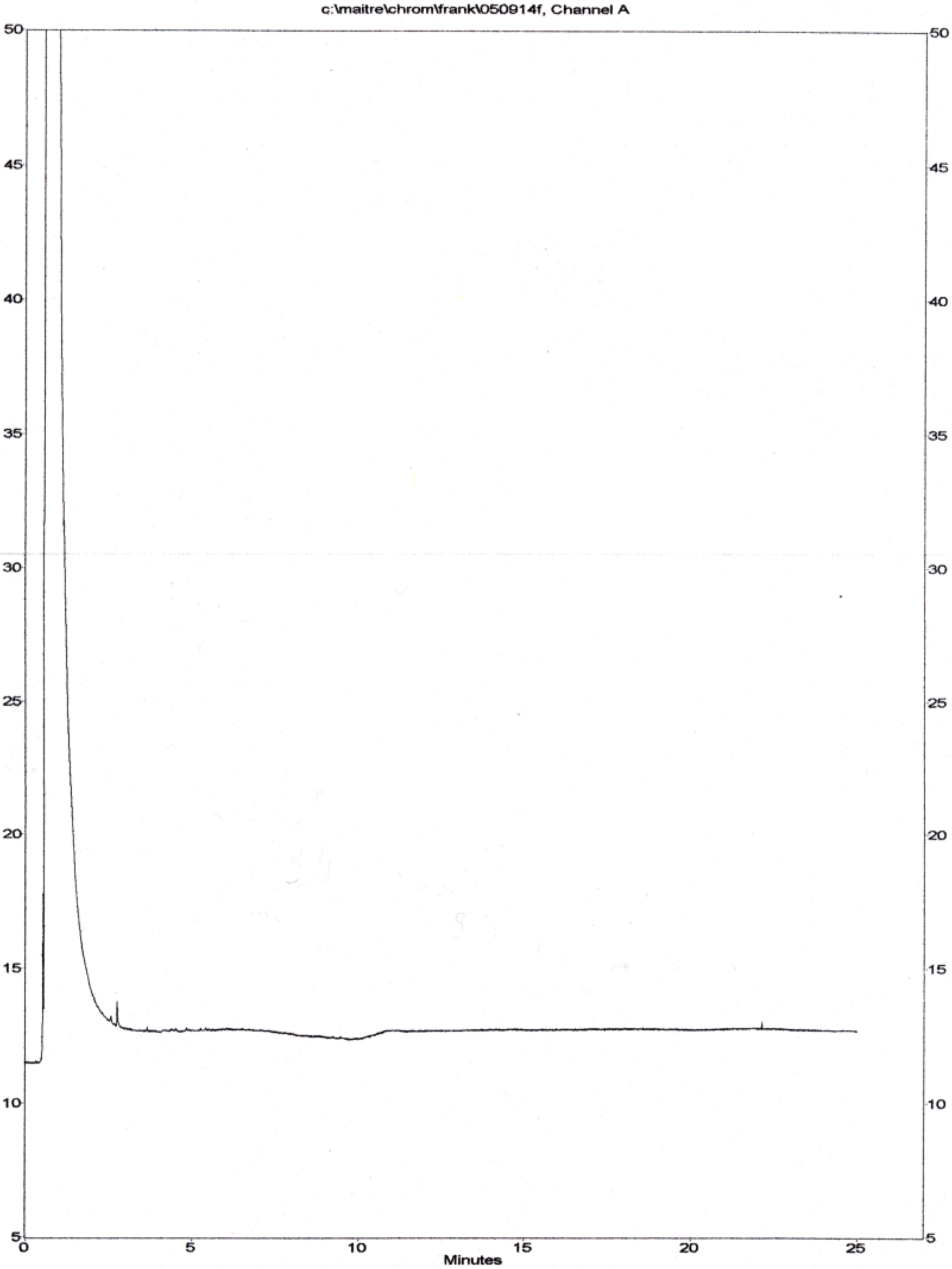
5
10
15
20
25
Minutes

LLE E1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10
5
pA
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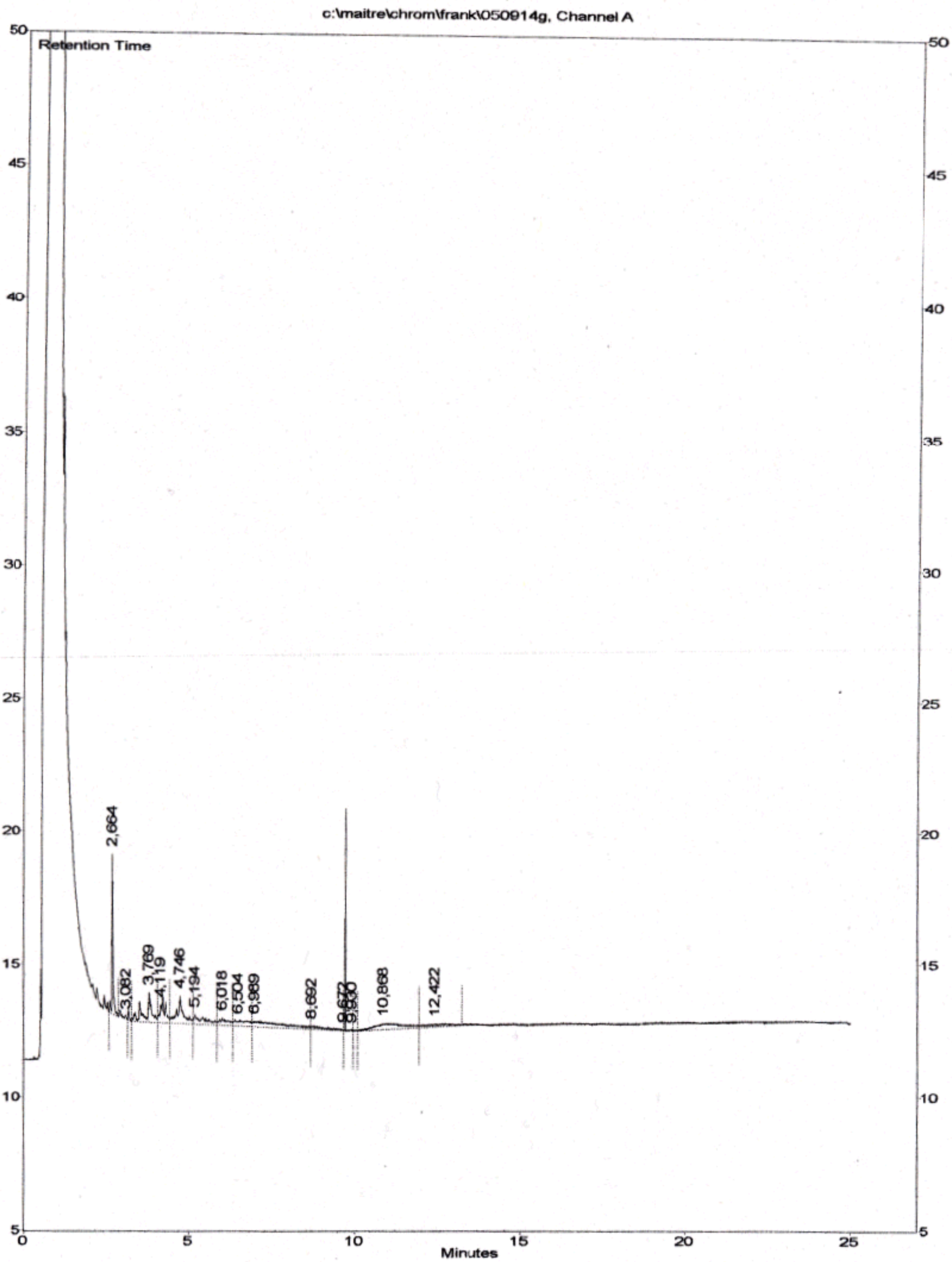
5
10
15
20
25
Minutes

LLE E2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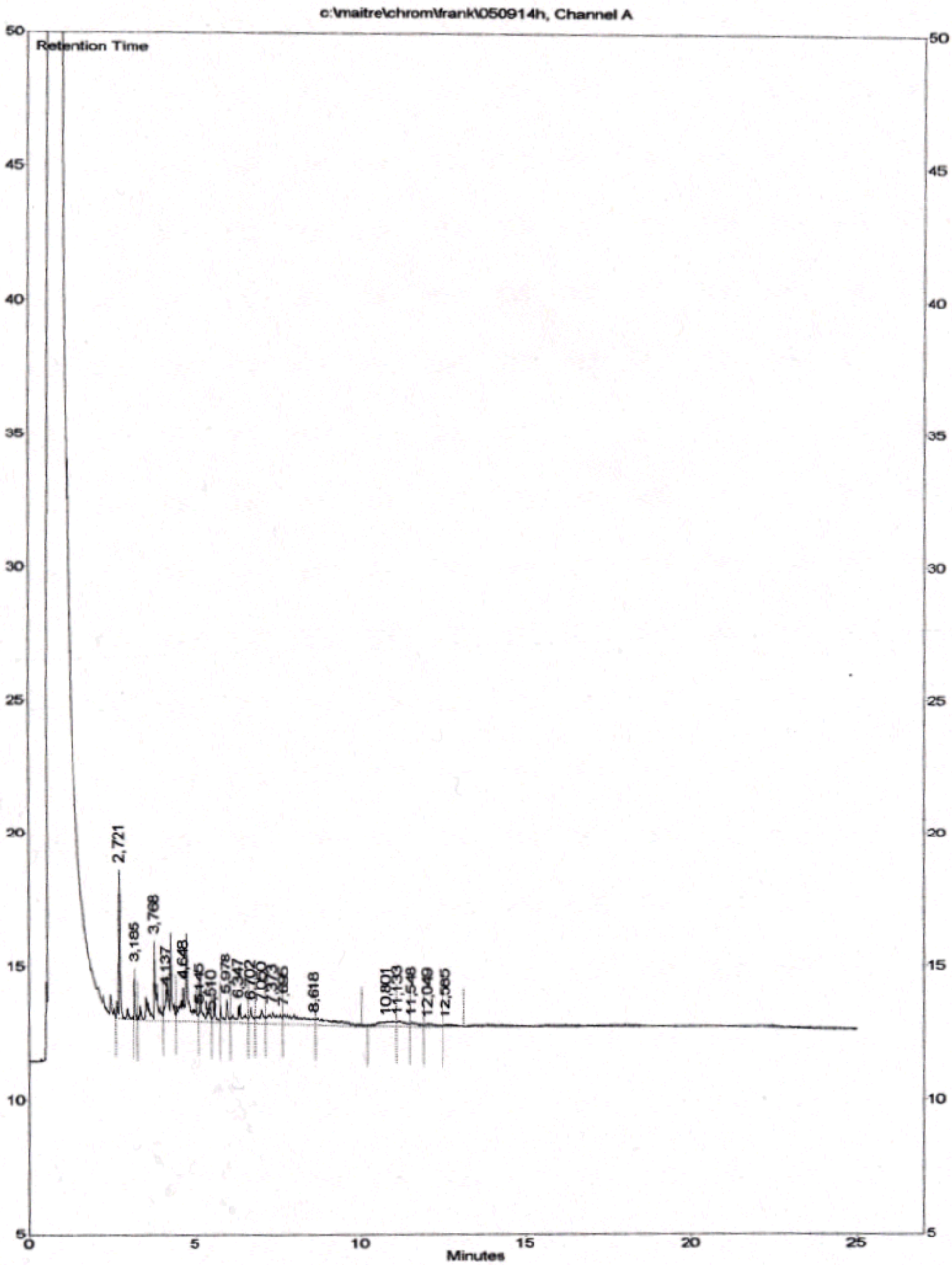
Retention Time
PA
2,664
3,082
3,769
4,119
4,746
5,194
6,018
6,504
6,989
8,692
10,868
12,422
Minutes

LLE E3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Retention Time
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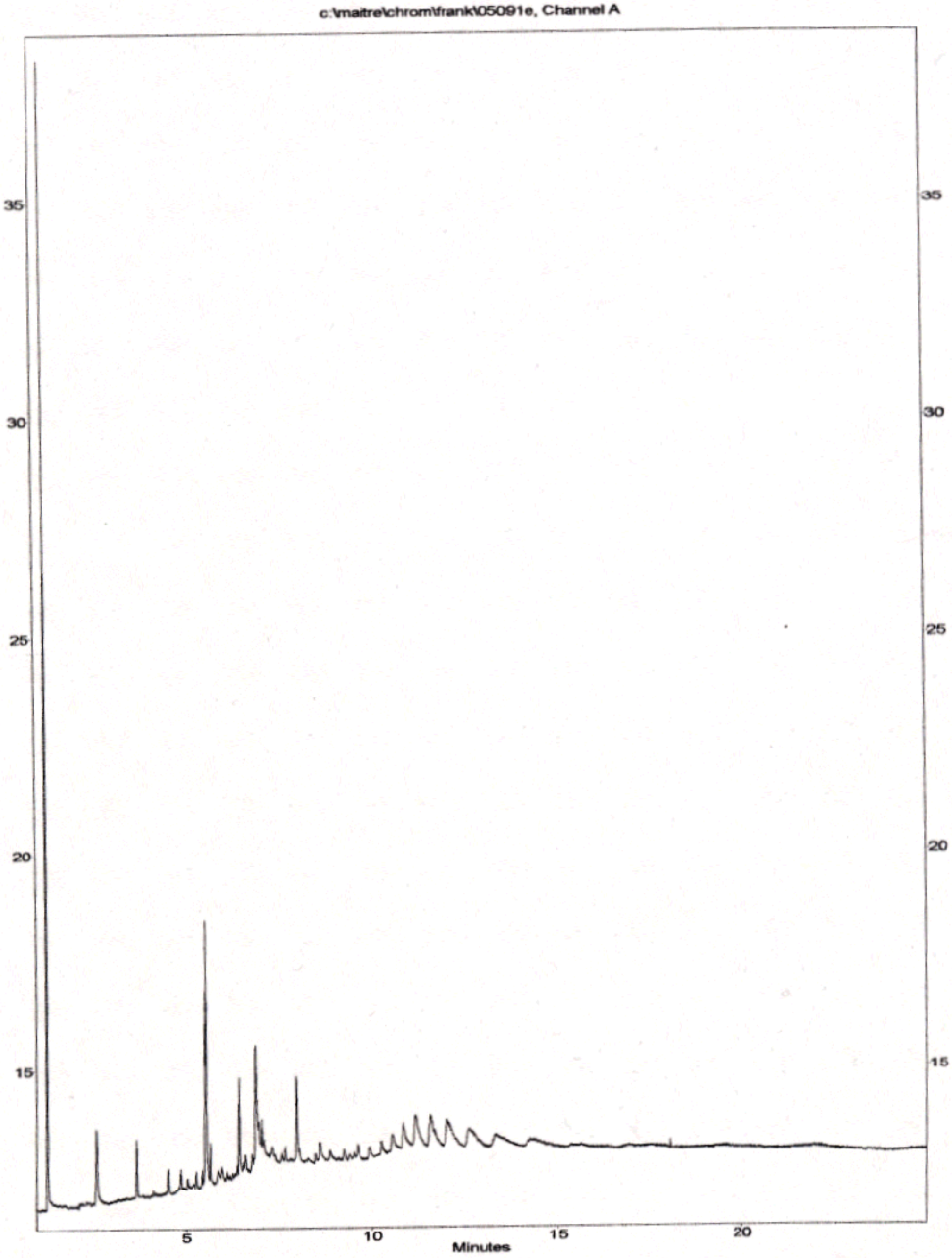
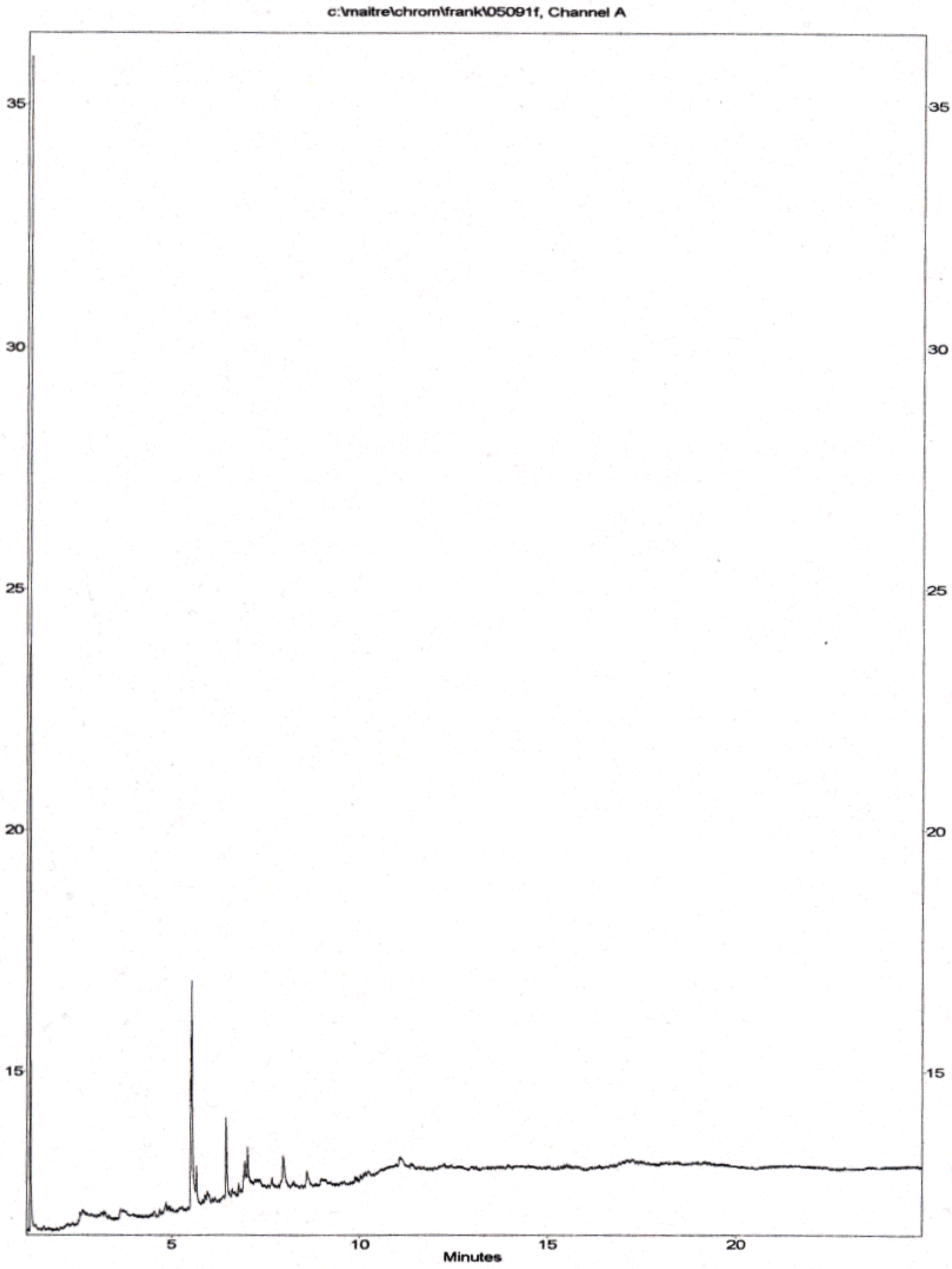
3,768
4,137
4,648
5,145
5,510
5,978
6,347
6,702
7,053
7,373
7,826
8,618
10,801
11,133
11,548
12,049
12,565
50
45
40
35
30
25
20
15
10
5
0
5
10
15
20
25
Minutes

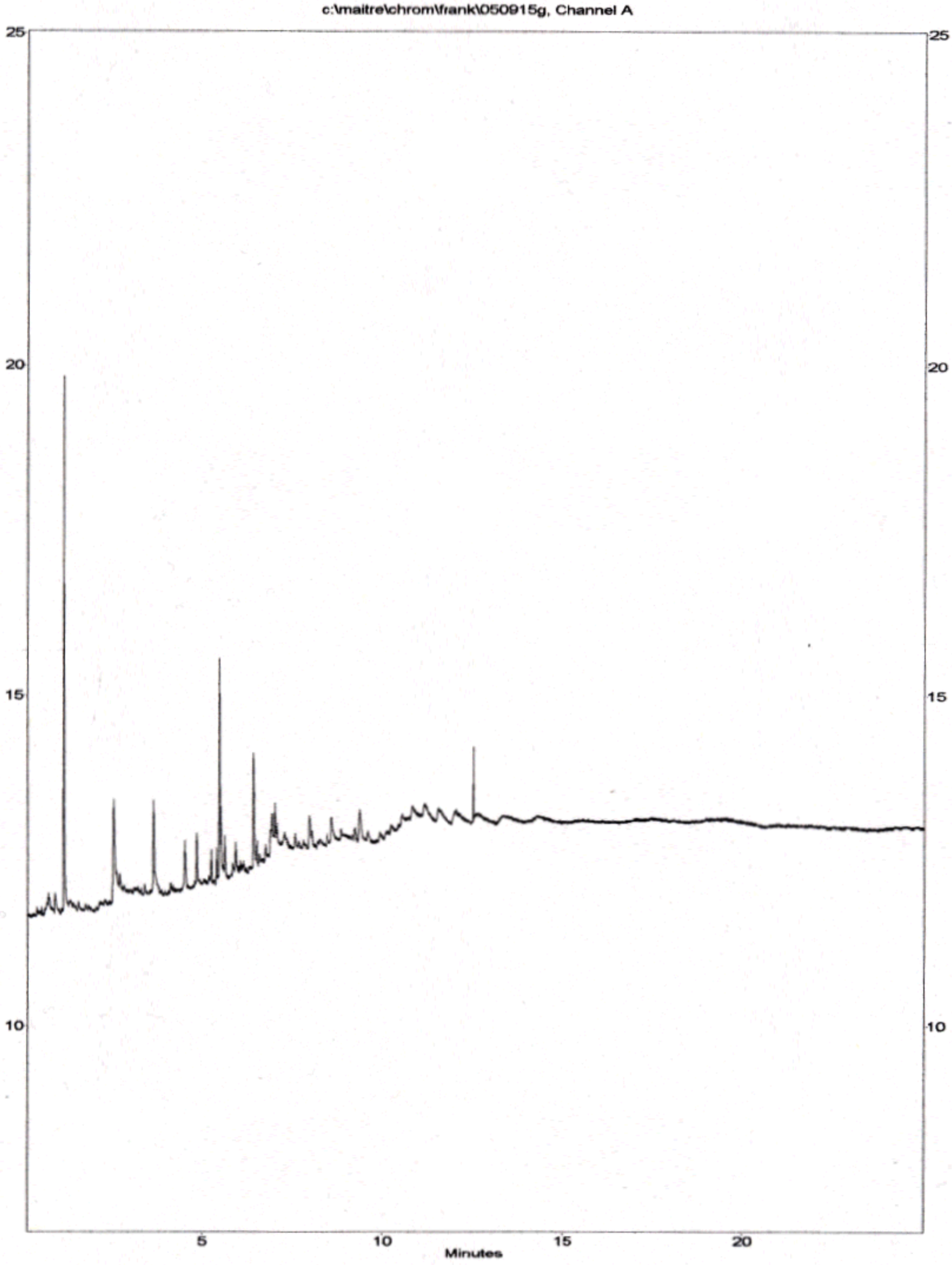
SPME A1 2.49 mmol/L



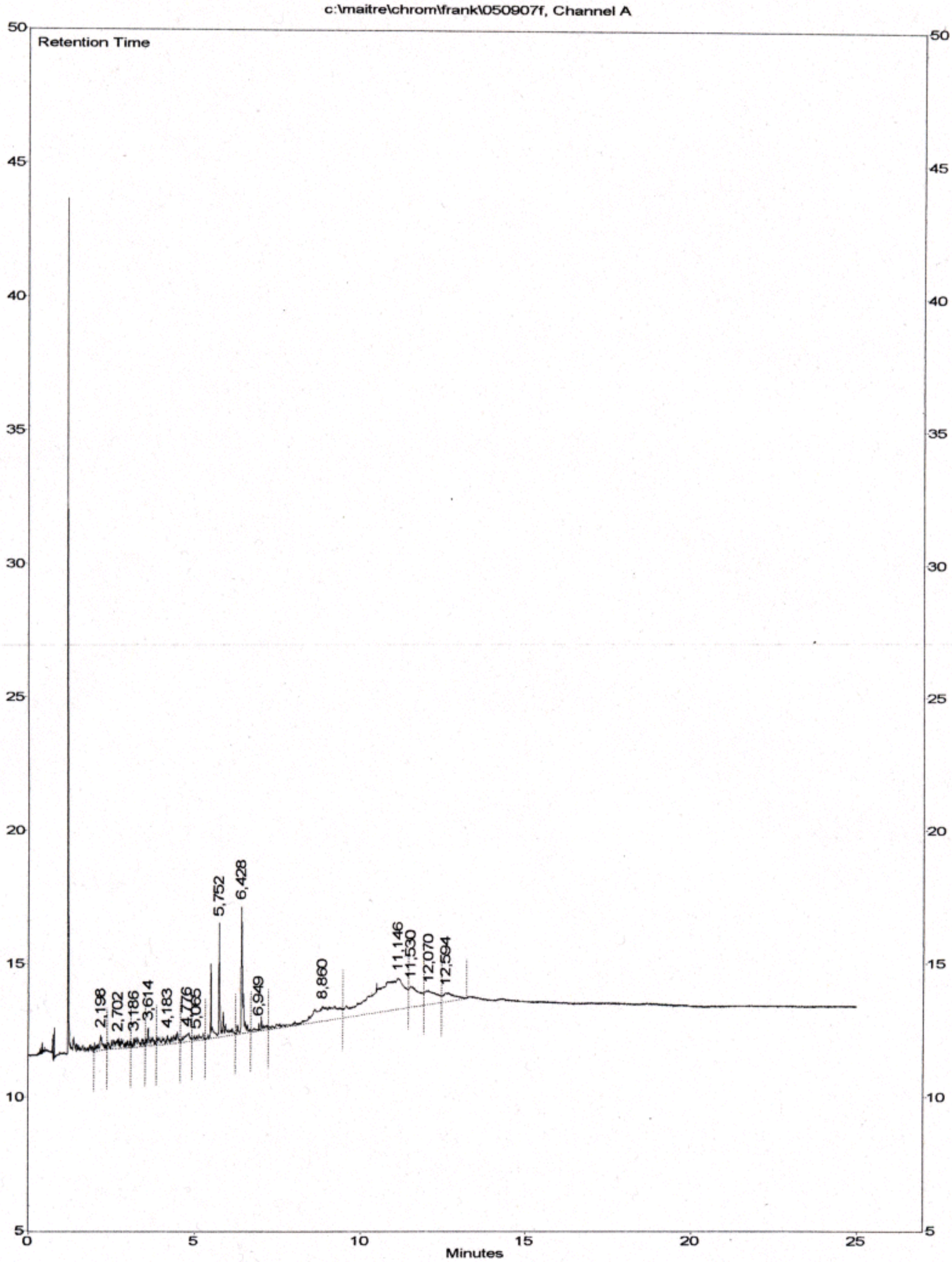
SPME A2 1.76 mmol/L



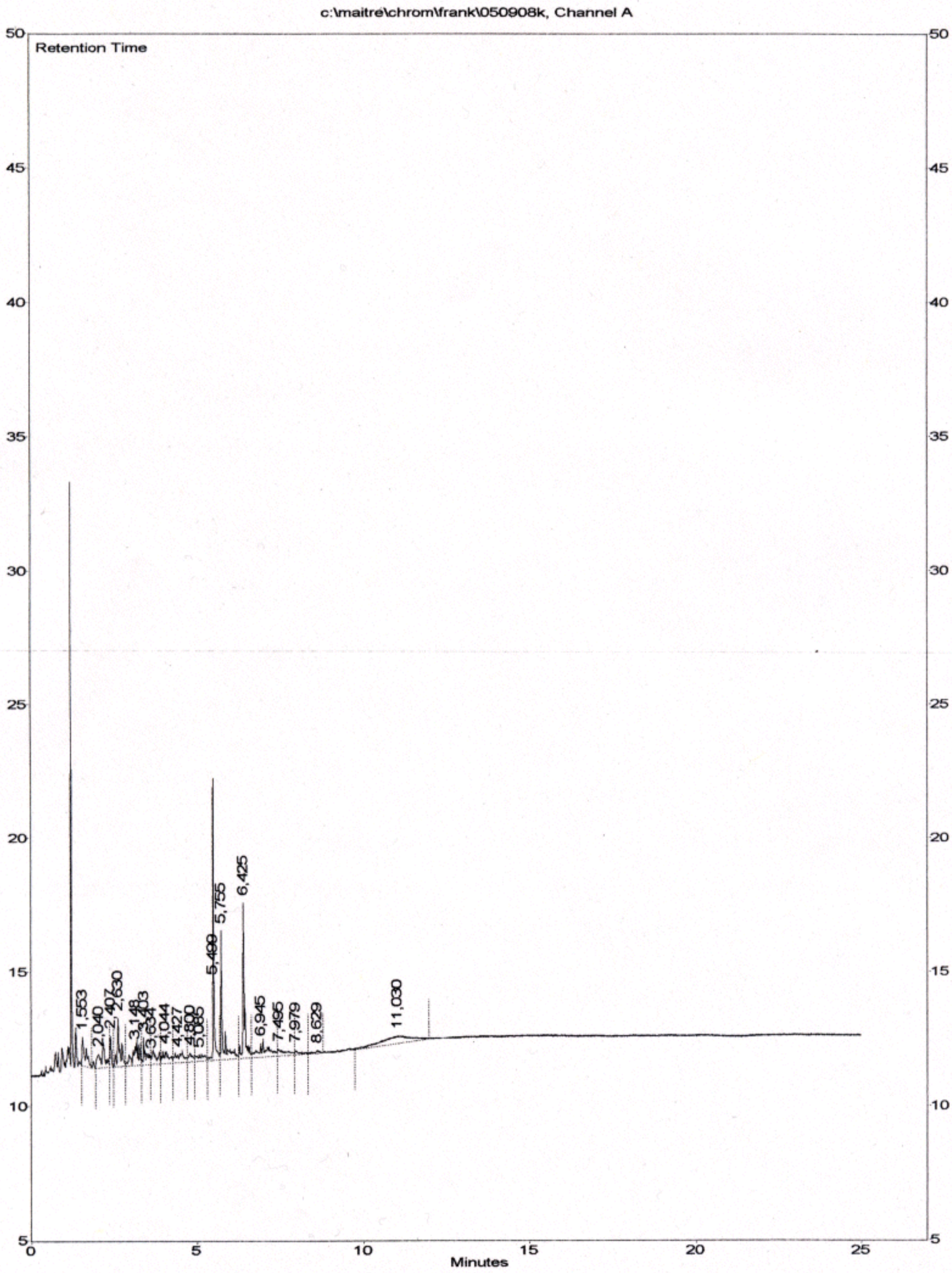
SPME A3 1.87 mmol/L



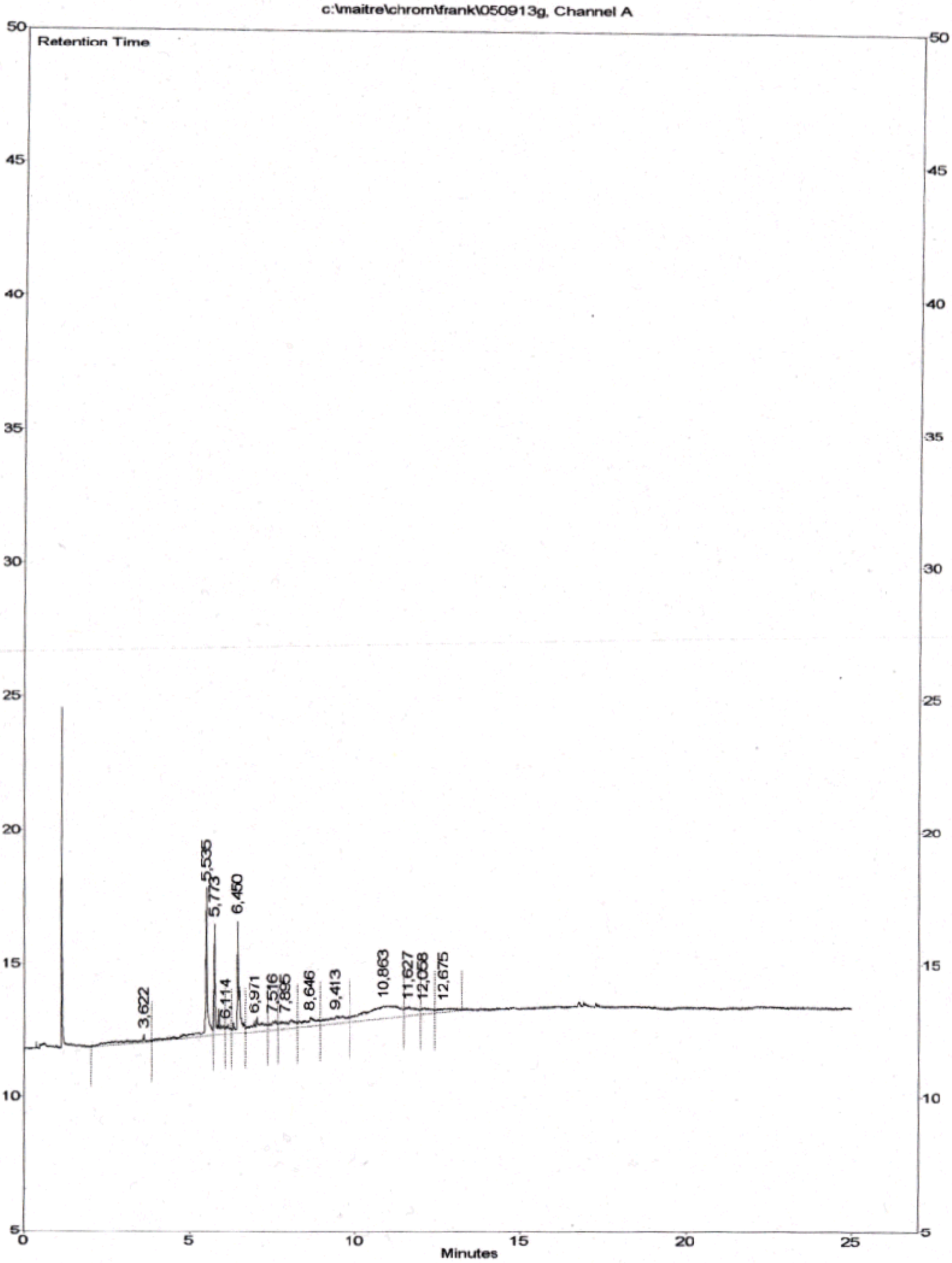
SPME B1 2.22 mmol/L



SPME B2 1.49 mmol/L



SPME B3 1.49 mmol/L



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Sample ID : Faser 6 (Probe B)
Acquired : Sep 07, 2005 12:02:05
Printed : Oct 14, 2005 11:31:57
User : System

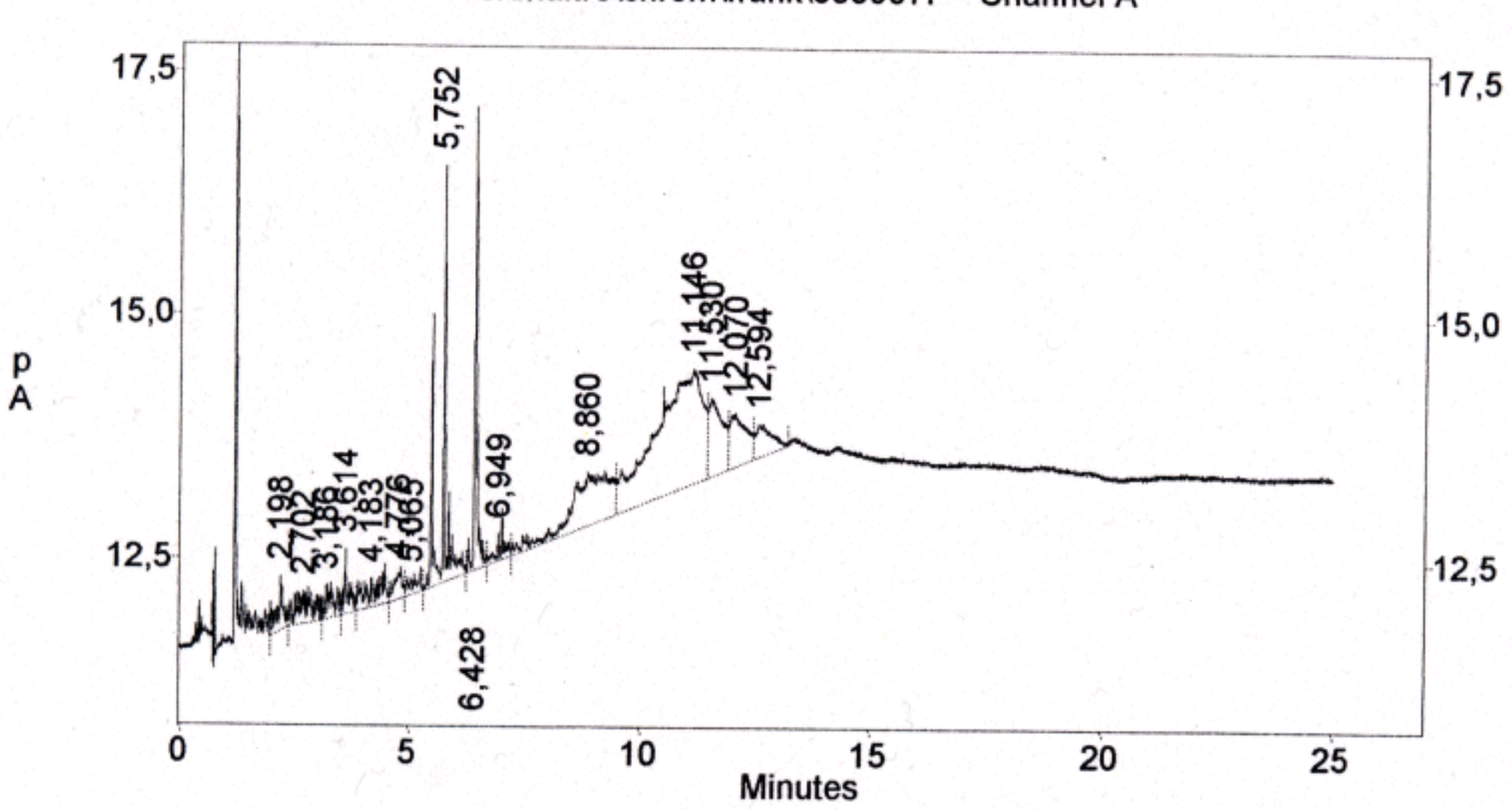
Channel A Results

Peak	Retention Time	Area
1	2,198	4265
2	2,702	6720
3	3,186	3863
4	3,614	3599
5	4,183	6818
6	4,776	3424
7	5,065	2492
8	5,752	17764
9	6,428	13943
10	6,949	3124
11	8,860	29711
12	11,146	85134
13	11,530	16666
14	12,070	13187
15	12,594	8330

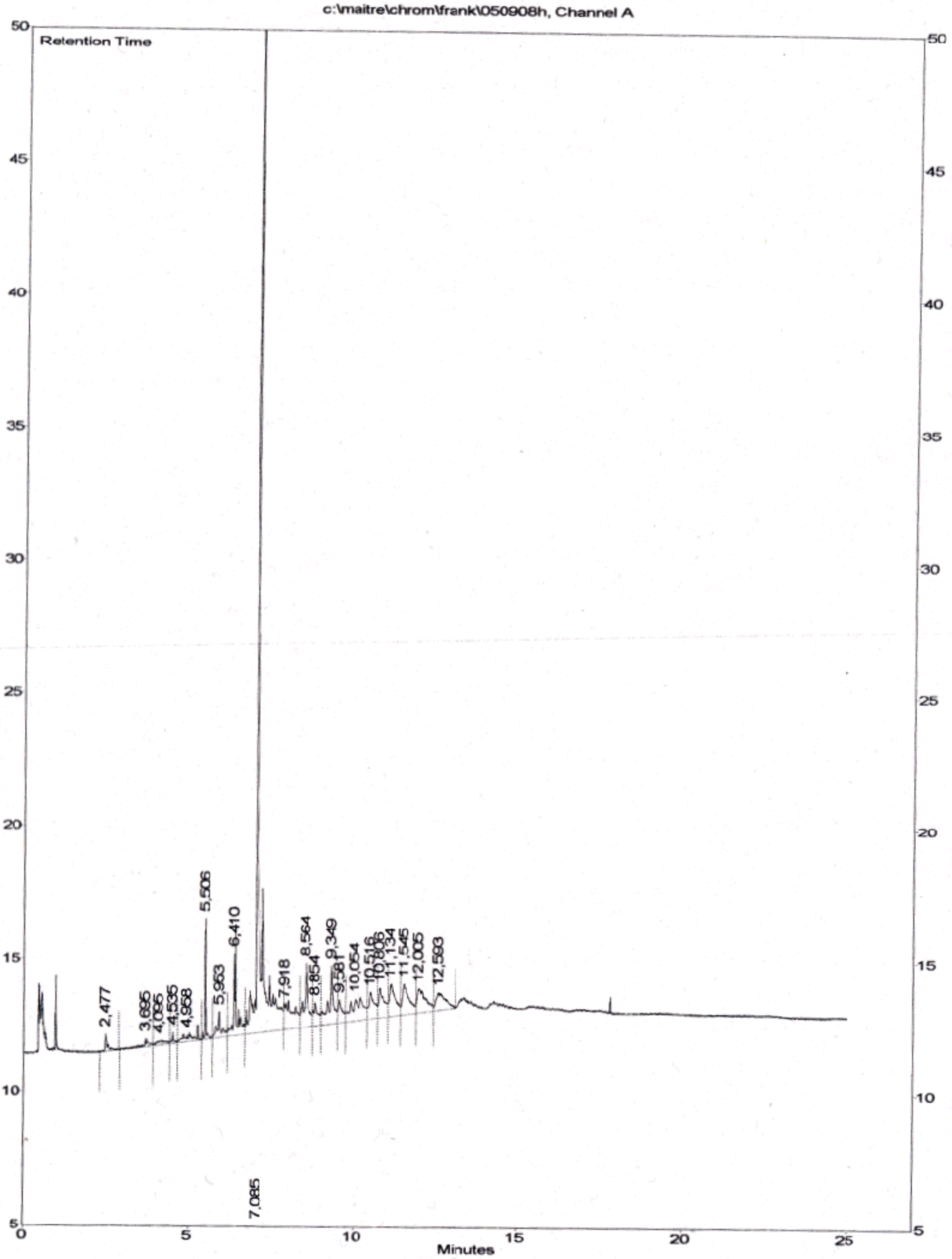
Totals :

219040

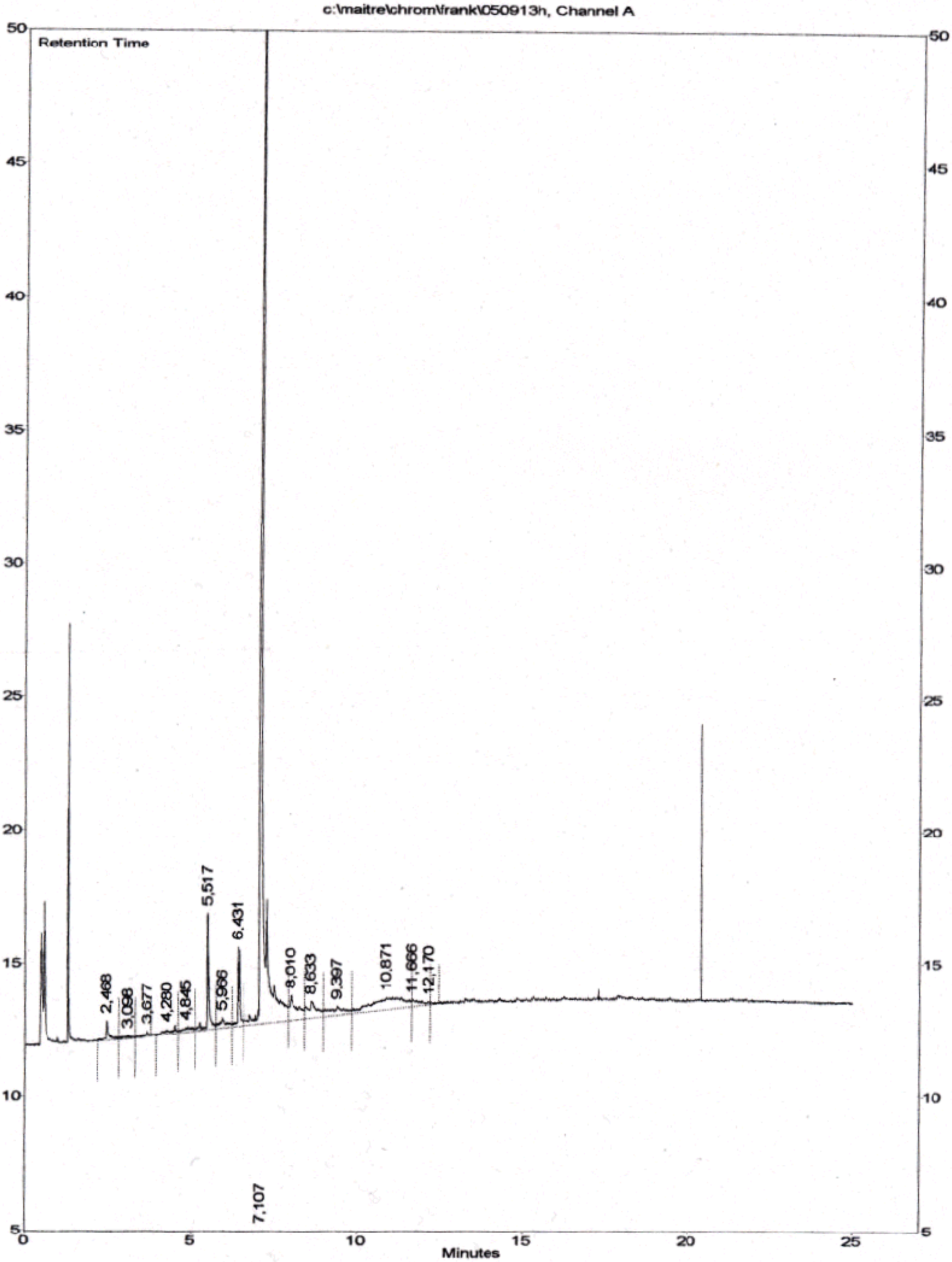
SPME C1 5.93 mmol/L



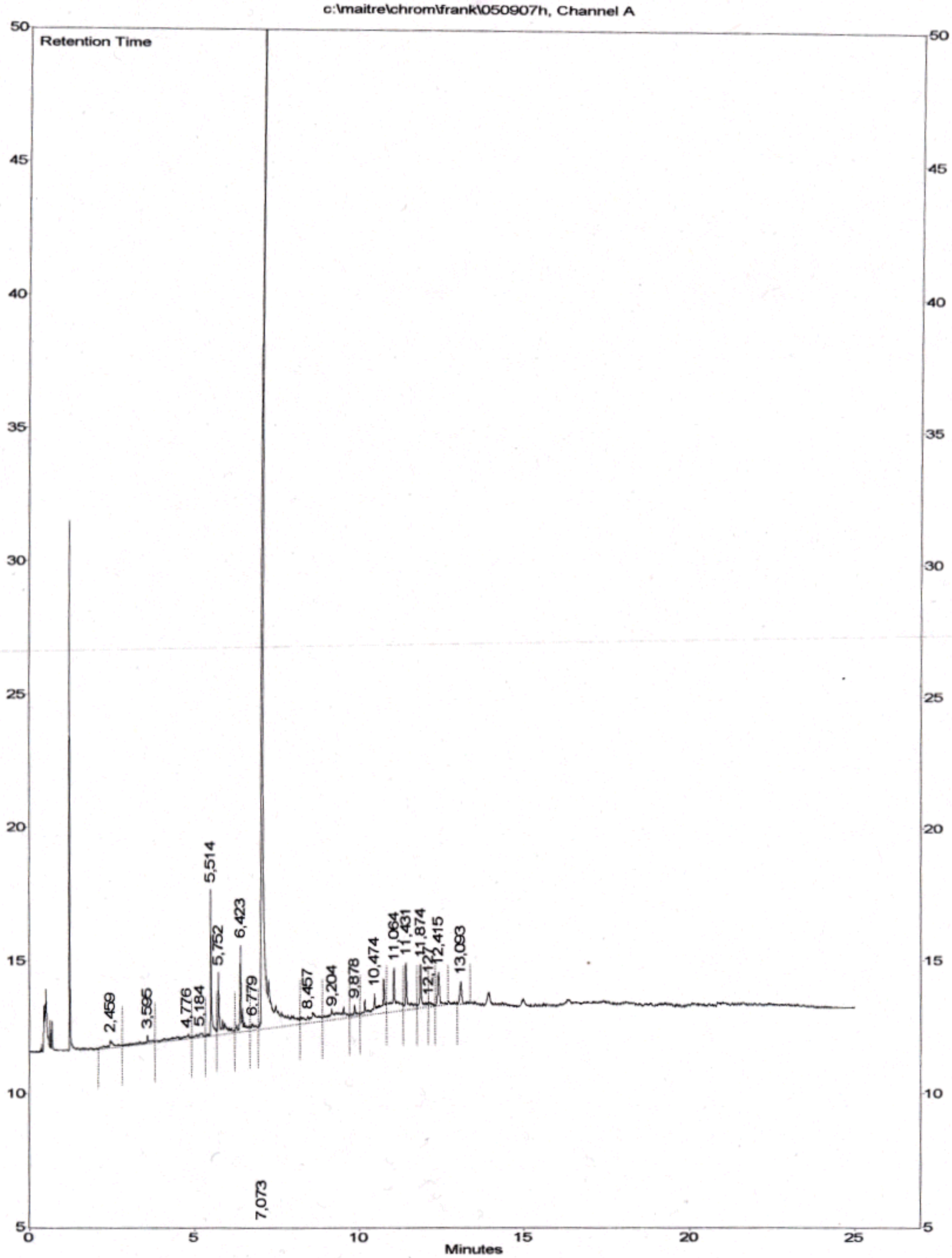
SPME C2 6.46 mmol/L



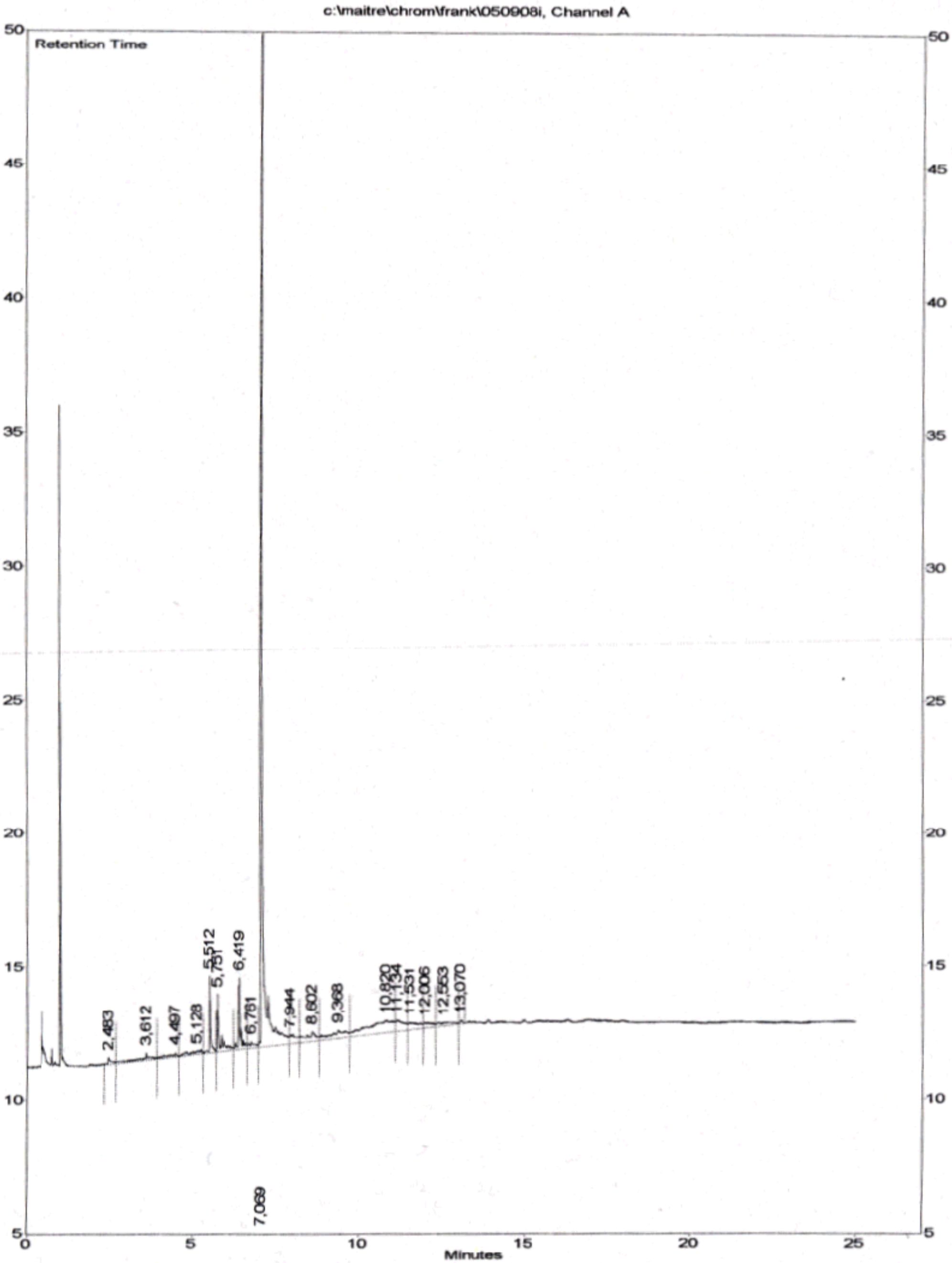
SPME C3 5,80 mmol/L



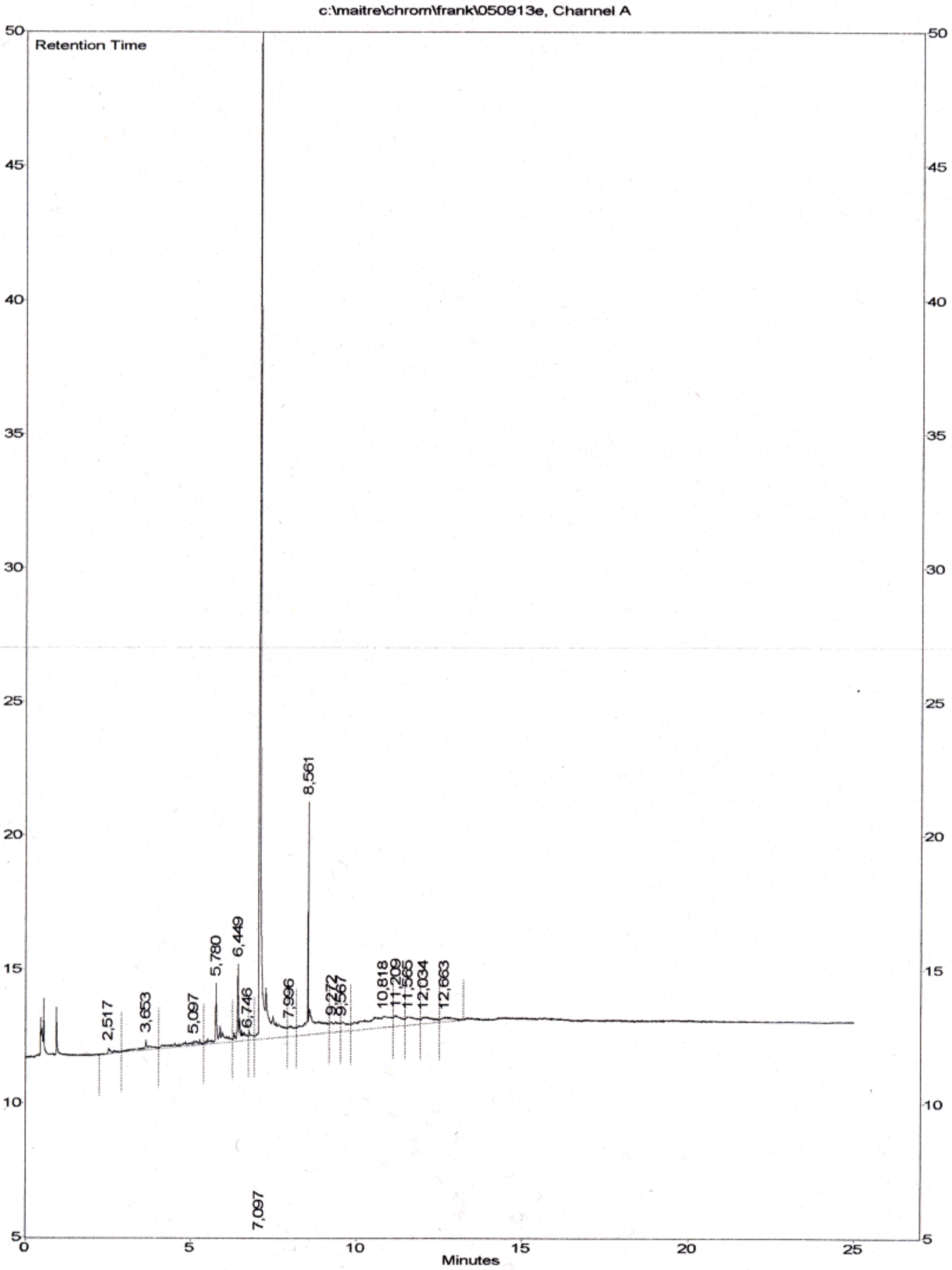
SPME D1 3.36 mmol/L

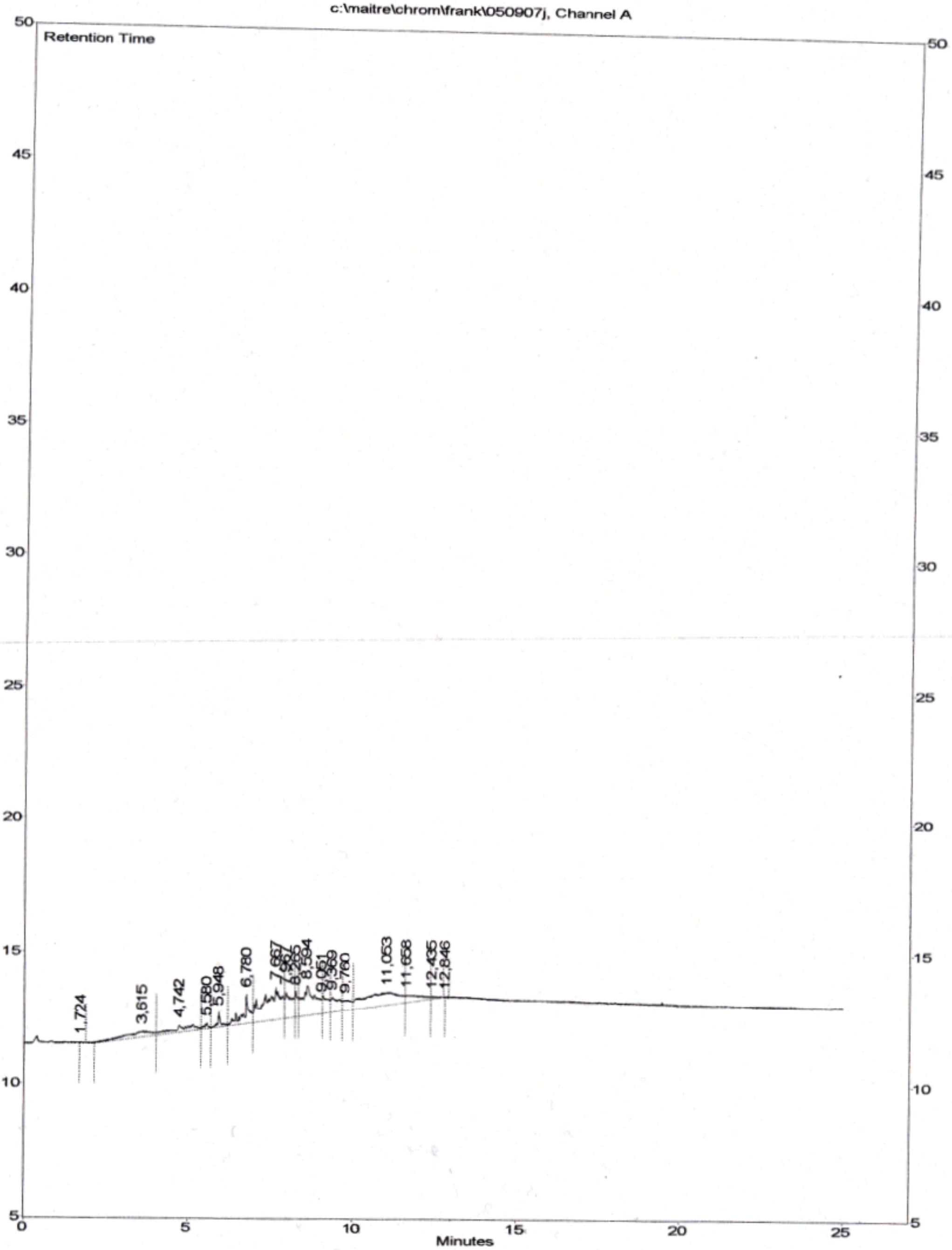


SPME D2 2,67 mmol/L



SPME D3 3.26 mmol/L

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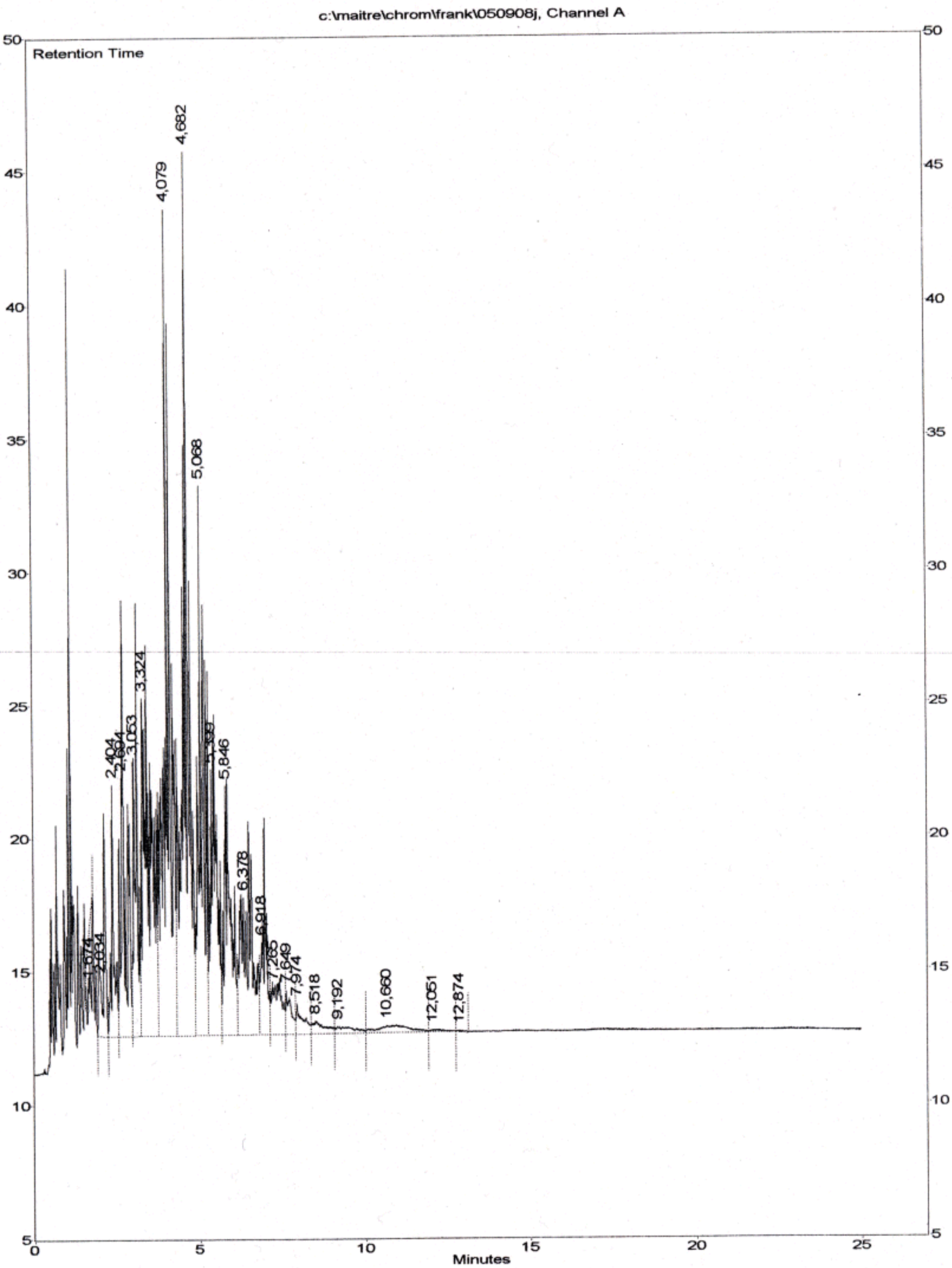
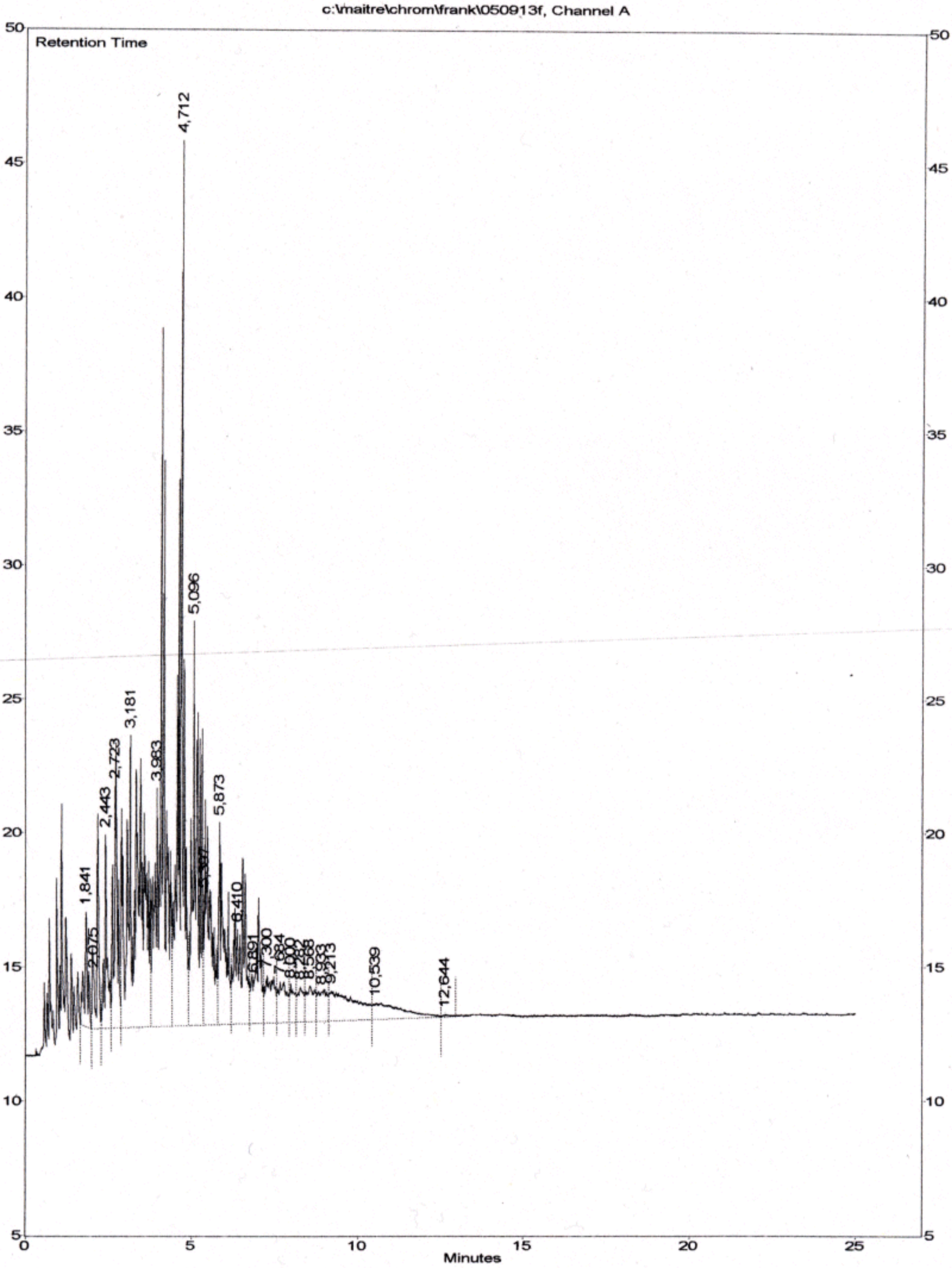
Retention Time
PA
Minutes
0
5
10
15
20
25
1,724
3,615
4,742
5,580
5,948
6,780
7,667
8,594
9,051
9,369
9,760
11,053
11,658
12,435
12,846

SPME E2 24.3 mmol/L



SPME E3 21.26 nmol/L



Appendix 7. Report and Chromatograms EXXONMOBIL

Memorandum

To T. F. Parkerton

Re 05MRL 183

Final Report - 0536490
"Aqueous BPH Analysis"

From D. J. Letinski

cc Archives
G. W. Trimmer
QAU

Date November 17, 2005

This memo serves as the final report for Study 0536490 and describes the test methods and results of the Bioavailable Petroleum Hydrocarbon (BPH) analysis of the water samples described below.

Approval Signatures

_____ _____
G. W. Trimmer, B.A. Date
Laboratory Coordinator

_____ _____
D.J. Letinski, M.S. Date
Study Director

Test Substances: MRD-05-364 (Effluent A)
MRD-05-365 (Effluent B)
MRD-05-366 (Effluent C)
MRD-05-367 (Effluent D)
MRD-05-368 (Effluent E)

Summary of Test Procedure:

The test substances can be generically described as effluent samples and were refrigerated upon receipt but then analyzed "as received". Sample aliquots of ca. 20 mL were placed in septum sealed glass vials with no headspace and placed on a LEAP Technologies CTC Analytics Combi PAL autosampler configured for automated SPME injections. A 30u PDMS (0.132uL) SPME fiber (Supelco) was equilibrated with each sample for 100 minutes with rapid agitation (250 rpm) and no headspace. A single fiber was used for all automated sample analyses.

The SPME fiber and liquid hydrocarbon standards were analyzed on a Perkin-Elmer Autosystem XL gas chromatograph with a flame ionization detector and 15 m x 0.53 mm id capillary column with 1.5 μm Rtx-1 stationary phase (Restek). A series of monoaromatic hydrocarbon standards was analyzed and the response factor of 2, 3 dimethylnaphthalene was used for quantification. The three levels of 2, 3 dimethylnaphthalene standards corresponded to 0.126, 0.629 and 1.89 nanomoles.

Additional BPH analyses were performed on effluent samples "A" (MRD-05-364) "C" MRD-05-366) and "E" (MRD-05-368) using a manual BPH technique. In this procedure, ca. 140 mL samples were placed in bottles each containing a small glass stir bar. The bottles were sealed with septum caps and no headspace. A 100u PDMS (0.612uL) was exposed to each sample and the samples mixed by rapid stirring for approximately 24 hours. The 100u fibers were then manually injected using the same GC-FID conditions used to analyze the automated samples described above.

The FID attenuation setting was -2 for all analyses. However, analysis of the 100u PDMS fiber for Effluent "C" was repeated using a FID setting of -1 since the single peak that comprised this sample was off-scale at the earlier attenuation as the response exceeded the detector range. At the attenuation -1 setting, only the two higher 2, 3 dimethylnaphthalene calibration levels were used for quantification.

Results

BPH results are listed in Table 1 and the reporting units are micromoles (umol) as 2, 3 dimethylnaphthalene/millilter (mL) PDMS. Effluents "A" (MRD-05-364) "B" (MRD-05-365) and "D" (MRD-05-367) yielded the lowest BPH concentrations. Effluents "A" and "B" yielded non-detectable concentrations using the 30u PDMS fiber while the "A" sample had a mean BPH concentration of 0.791 umol/mL PDMS using a100u PDMS fiber. The mean BPH concentration for effluent "D" was 3.58 umol/mL PDMS using the 30u fiber. Figure 1 presents overlaid GC-FID chromatograms of a BPH analytical standard and effluents "A", "B" and "D" using the 30u PDMS fiber. The 2, 3 dimethylnaphthalene standard elutes at approximately 6.0 minutes. For these three samples, effluent "D" had a detectable peak eluting at approximately 7.5 minutes.

Effluent "C" had a mean BPH of 7.51 umol/mL PDMS using the 30u PDMS fiber and 9.61 using the 100u fiber. Figure 2 presents overlaid chromatograms of effluent "C" using both the 30 and 100u fibers at the same (-2) attenuation. This sample is comprised primarily of a single chromatographic component eluting at approximately 7.5 minutes. This appears to be the same retention time as the single peak detected for effluent "D" though approximately twice the magnitude. This peak also elutes just after the retention time of the standard reference compound 9-methylanathracene (a single methylated three ring PAH).

Effluent "E" had a mean BPH of 23.2 umol/mL PDMS using the 30u PDMS fiber and 35.6 using the 100u fiber. Figure 3 presents overlaid chromatograms of effluent "E" comparing the 30 and 100u fiber analysis. The chromatographic profile indicates a

complex mixture eluting between approximately three and eight minutes. The significantly higher BPH concentration measured using the 100u fiber may be attributable to the greater sensitivity of this fiber detecting more very low concentration components than the 30 u fiber. It does not necessarily indicate that the automated technique with the 30u fiber did not reach equilibrium with that sample.

Table 1. BPH Results

EMBSI Sample ID	Sample ID	BPH Method	PDMS Film Thickness	FID Attenuation	rep	BPH Conc [umol as 2,3-DiMeNphthln/mL PDMS]	mean
MRD-05-364	Effluent "A"	auto	30u	-2	1	ND	
					2	ND	ND
		manual	100u	-2	1	0.551	
					2	1.03	0.791
MRD-05-365	Effluent "B"	auto	30u	-2	1	ND	
					2	ND	ND
MRD-05-366	Effluent "C"	auto	30u	-2	1	7.41	
					2	7.60	7.51
		manual	100u	-2	1	*9.47*	
					2	*12.2*	
					3	*11.0*	*10.9*
		manual	100u	-1	1	8.97	
					2	11.1	
					3	8.77	9.61
MRD-05-367	Effluent "D"	auto	30u	-2	1	3.58	
					2	3.58	3.58
MRD-05-368	Effluent "E"	auto	30u	-2	1	23.5	
					2	22.9	23.2
		manual	100u	-2	1	30.6	
					2	30.0	
					3	46.1	35.6

ND - not detected. Detection limits ~ 1 umol as 2,3-DiMeNphthln/mL PDMS for 30 u PDMS

~ 0.2 umol as 2,3-DiMeNphthln/mL PDMS for 100 u PDMS

results in italics - off scale, exceeded detector range

Figure 1.
GC-FID Chromatograms
Hydrocarbon Standard and SPME Injections
of Effluents "A", "B" and "D"

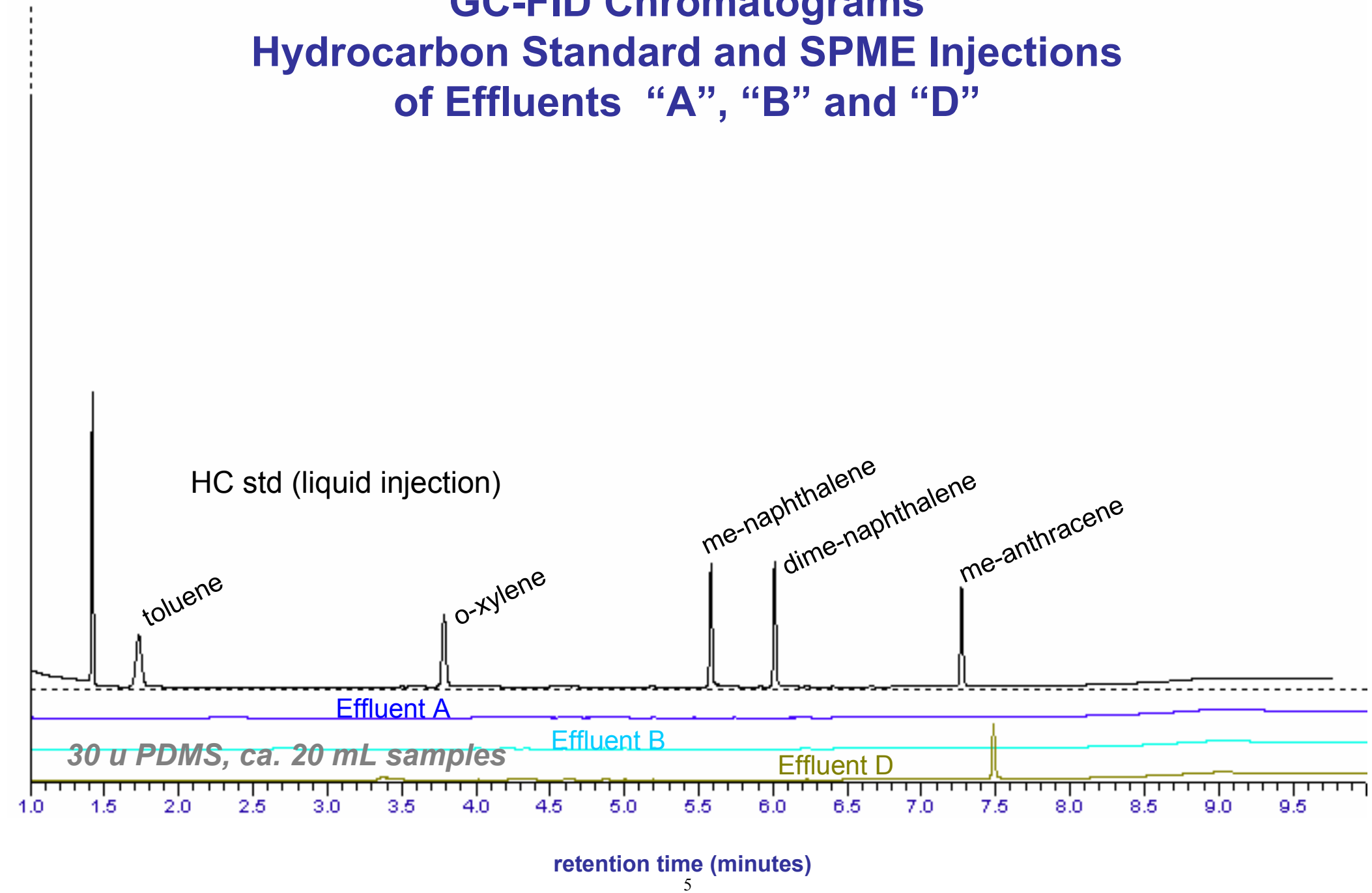


Figure 2.
GC-FID Chromatograms
SPME Injections of Effluent "C"

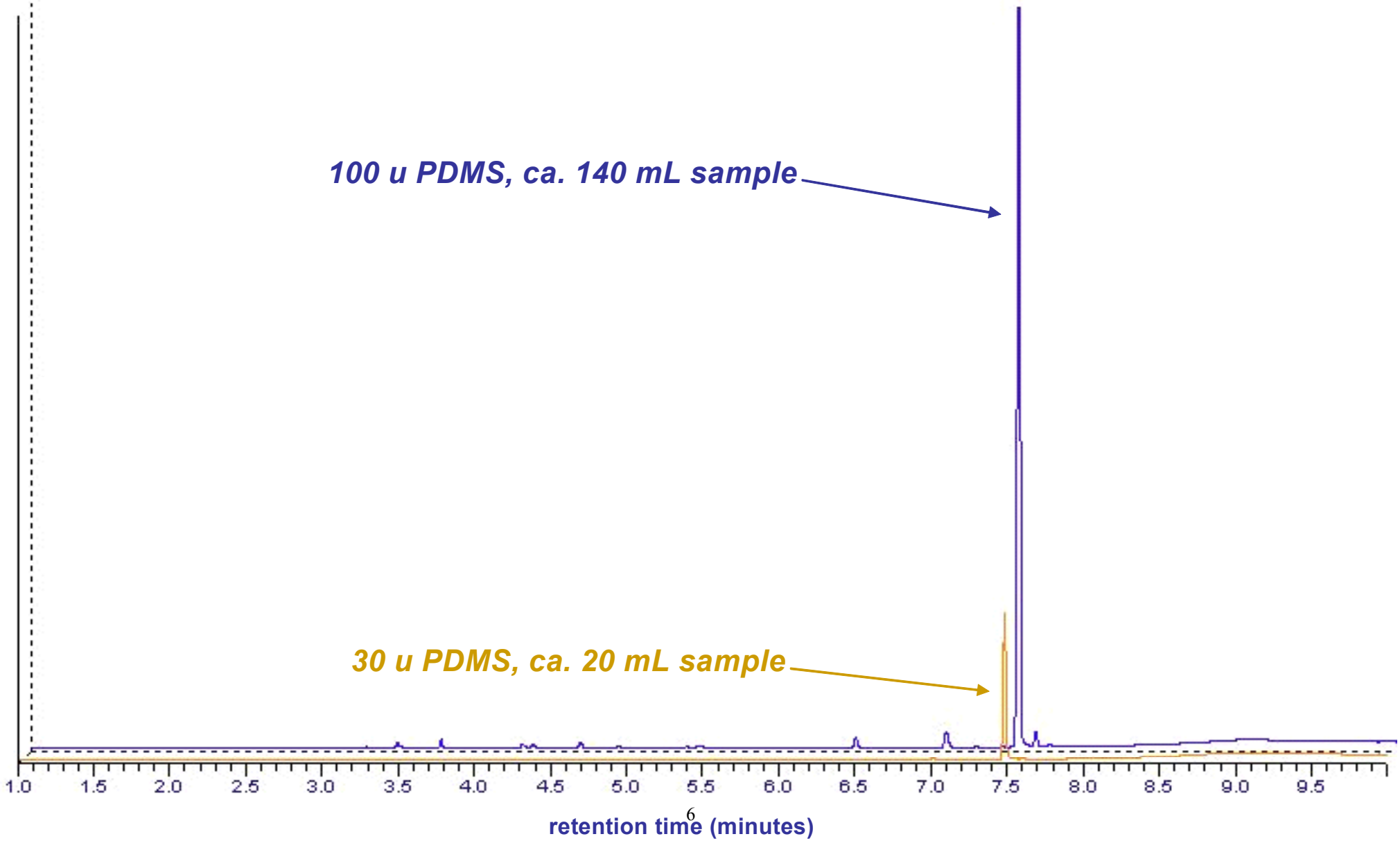
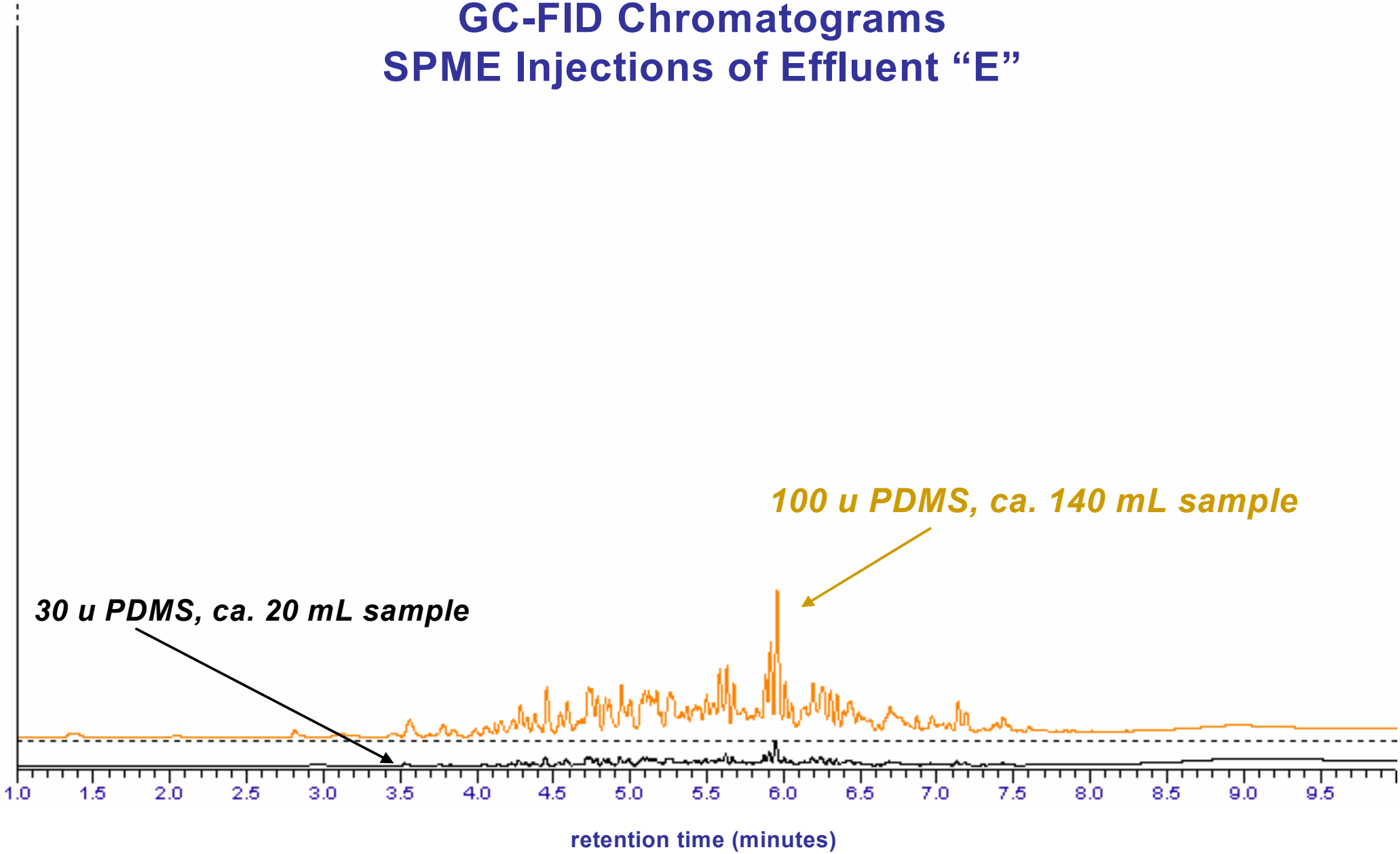


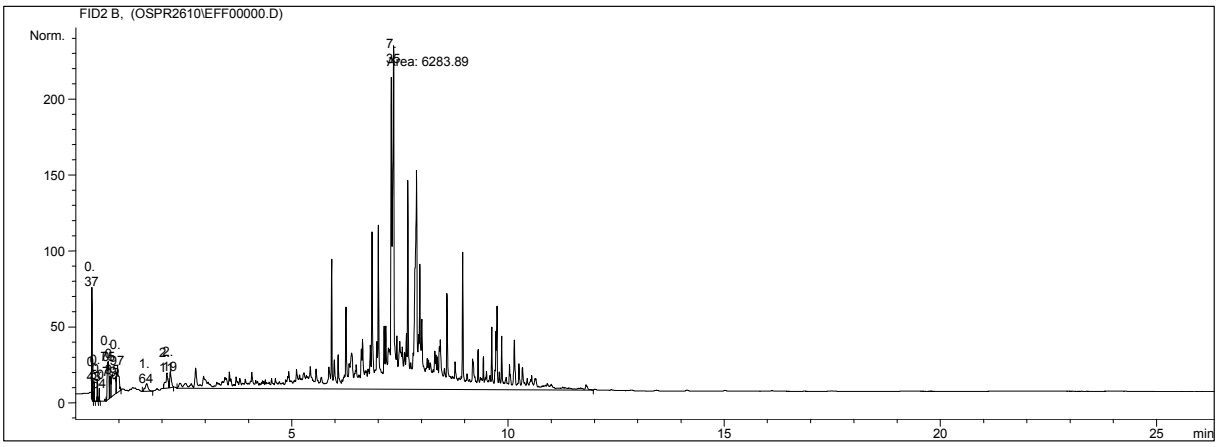
Figure 3.
GC-FID Chromatograms
SPME Injections of Effluent "E"



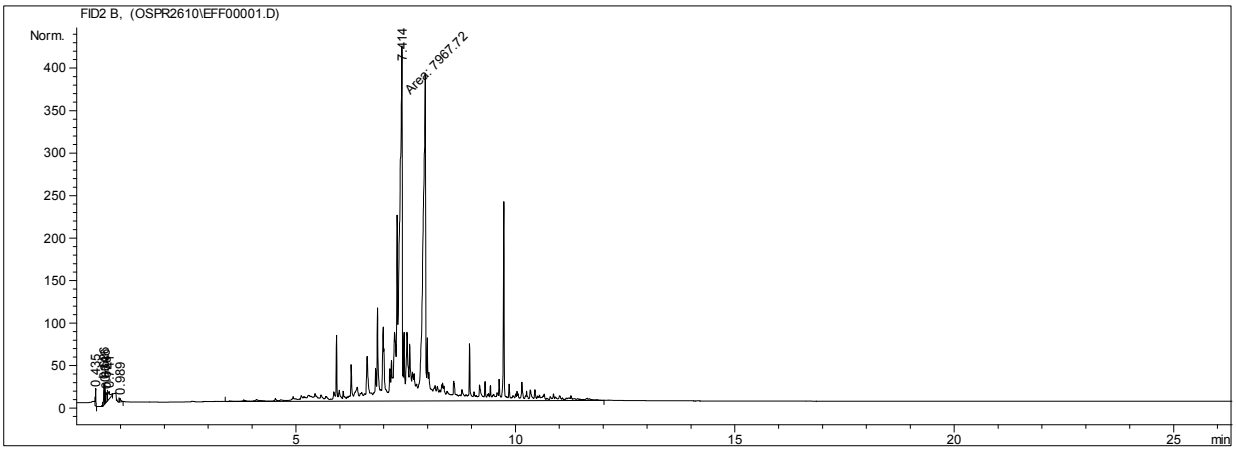
Appendix 8. Report and Chromatograms RIVO

RIVO data - SPME

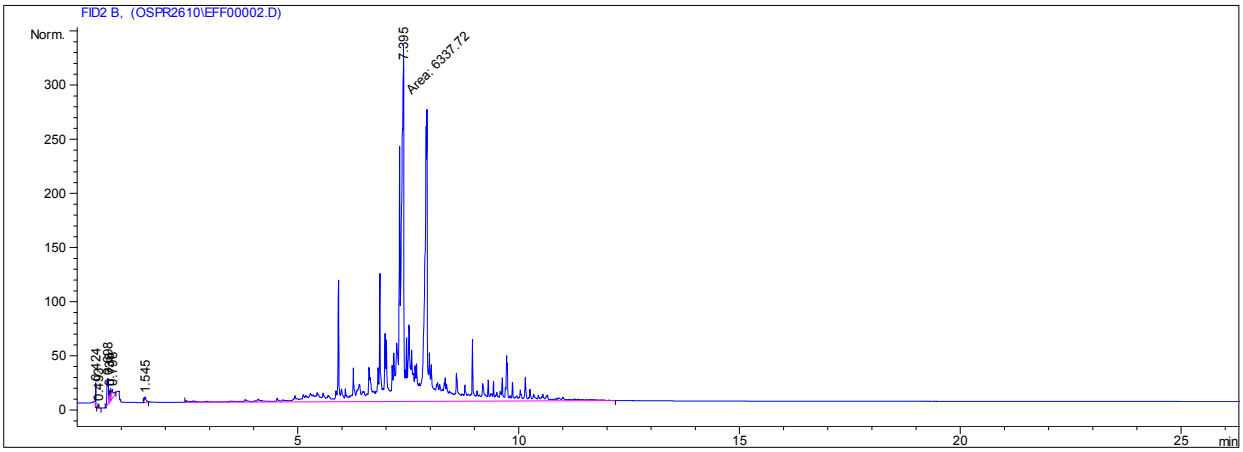
A1



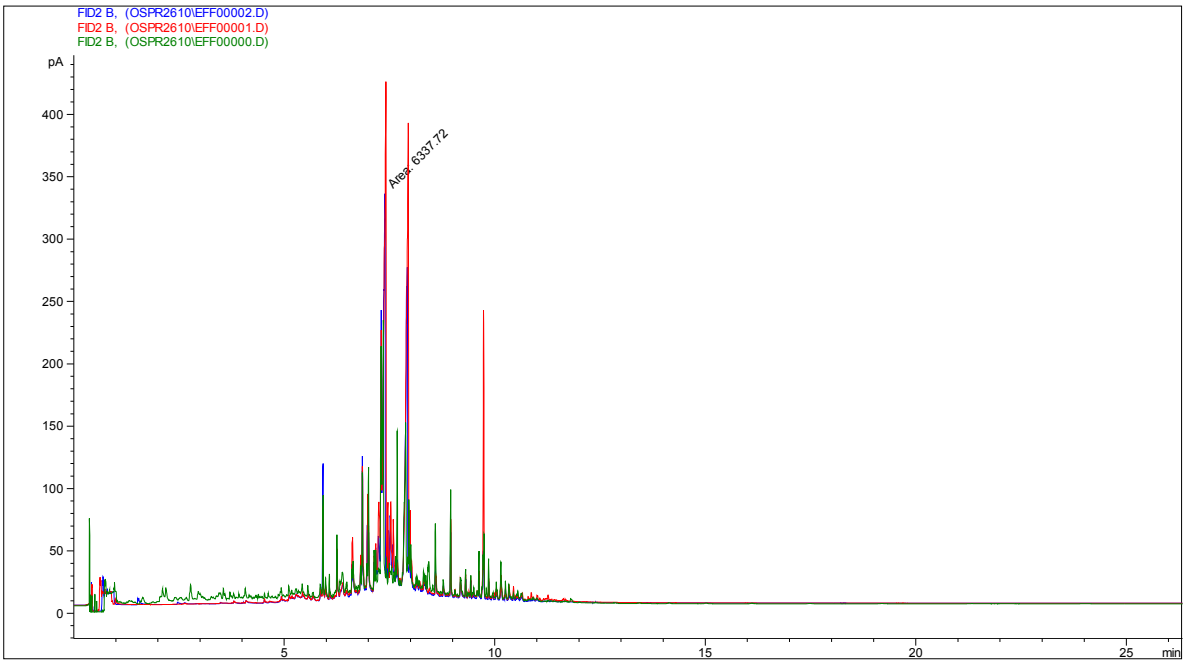
A2



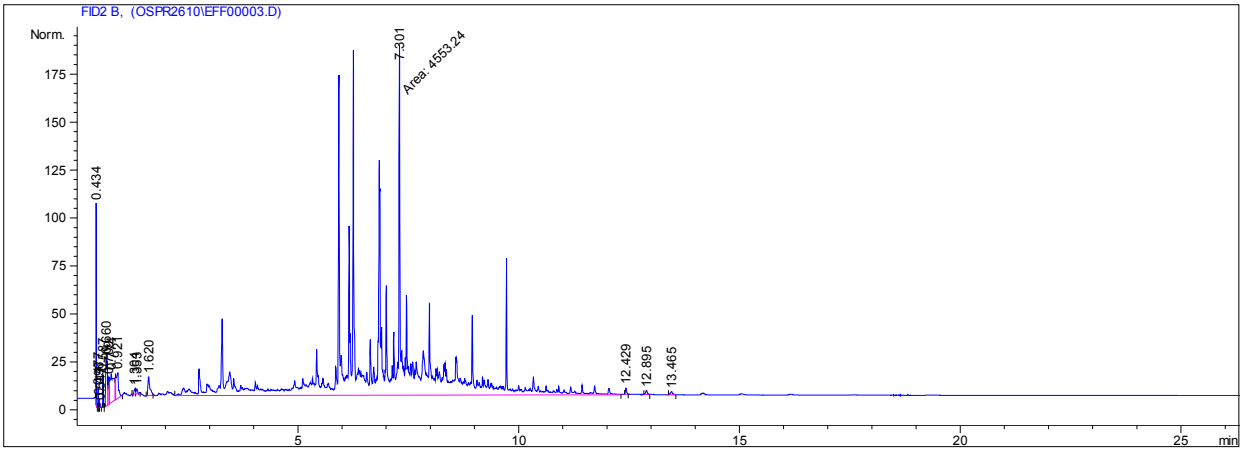
A3



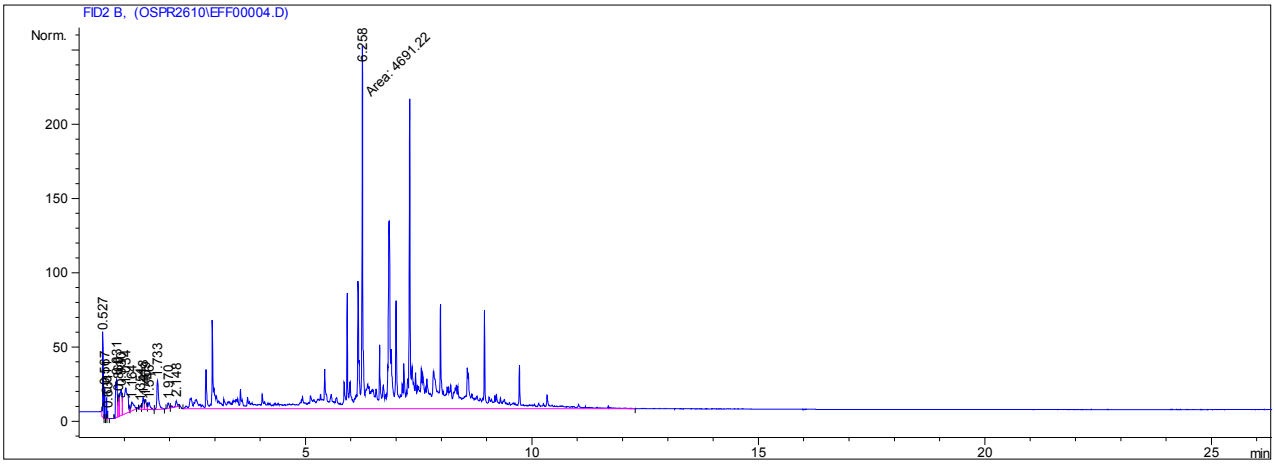
Overlay A1 t/m A3



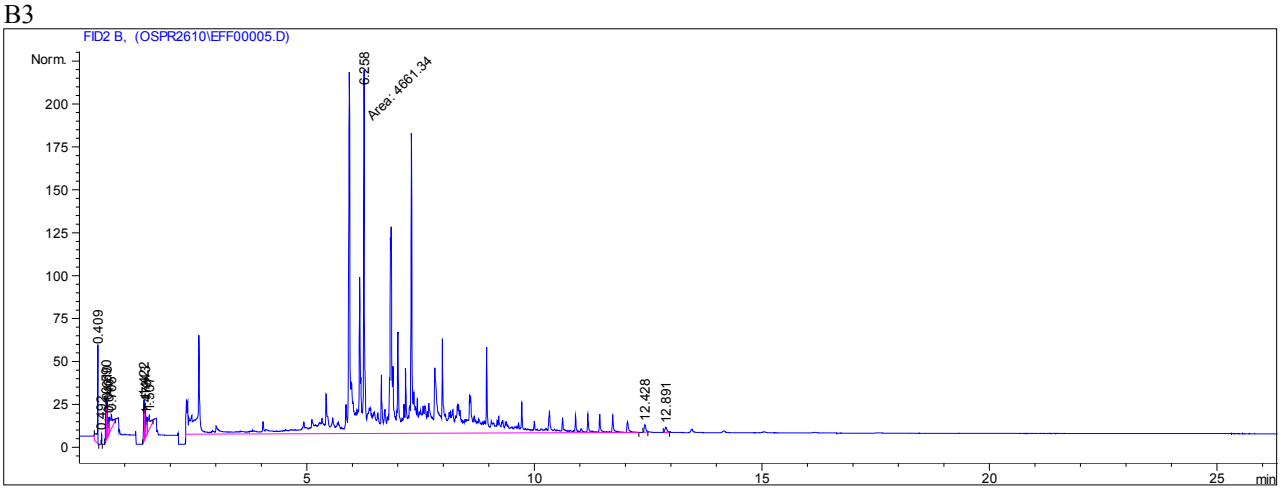
B1



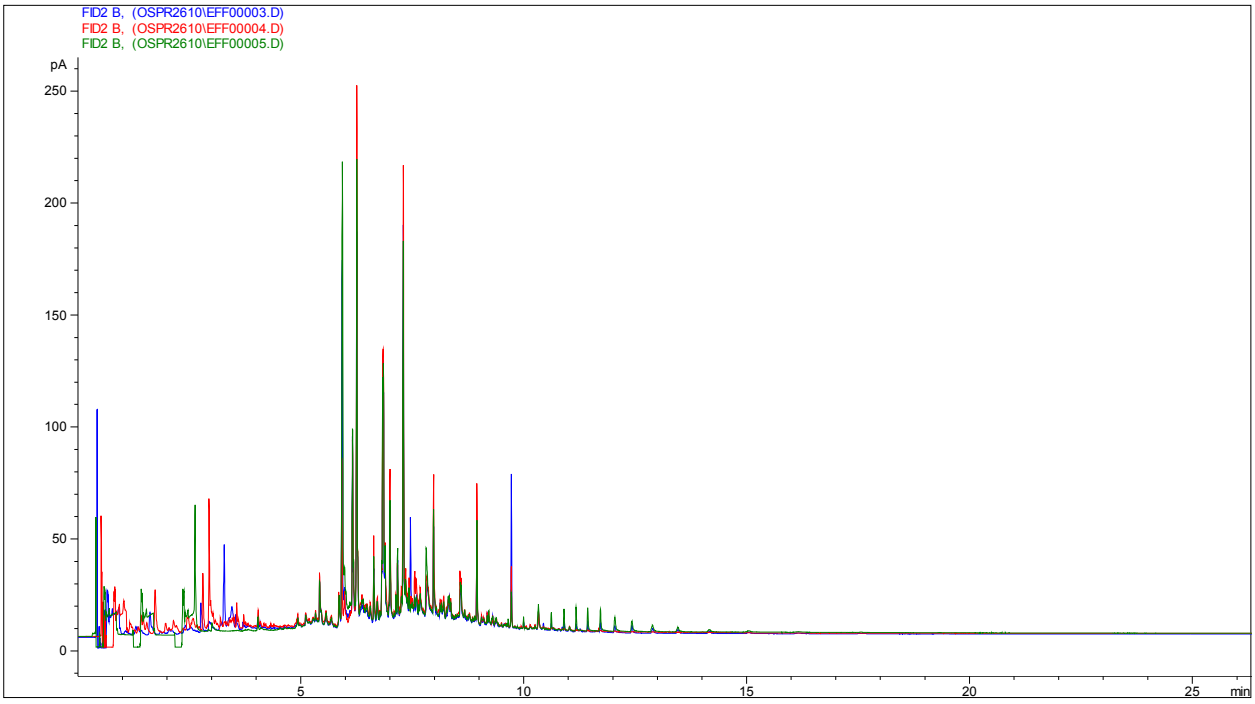
B2



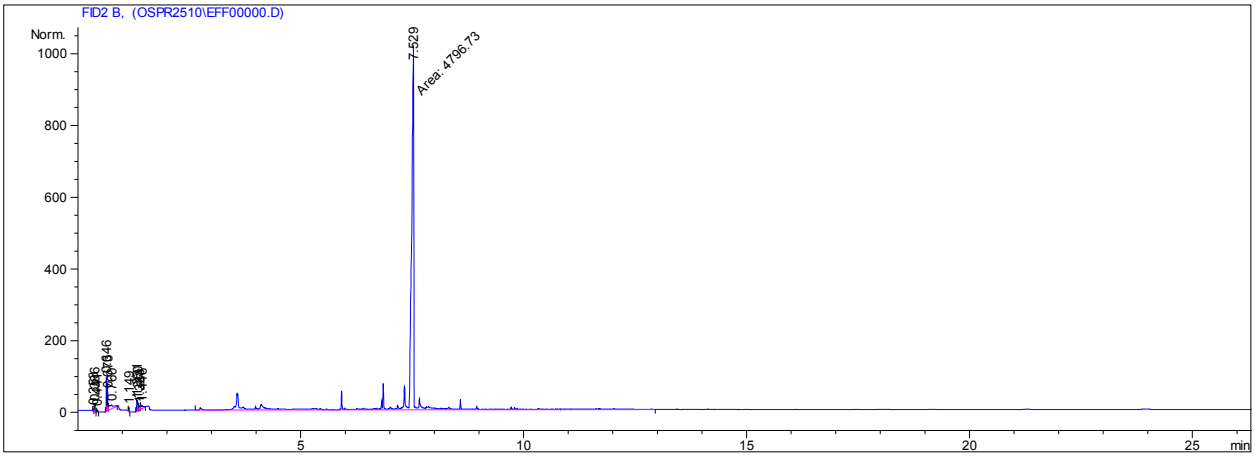
B3


FID2 B, (OSPR2610\EFF00005.D)
Norm.
6.258
Area: 4661.34
0.409
12.428
12.891
min

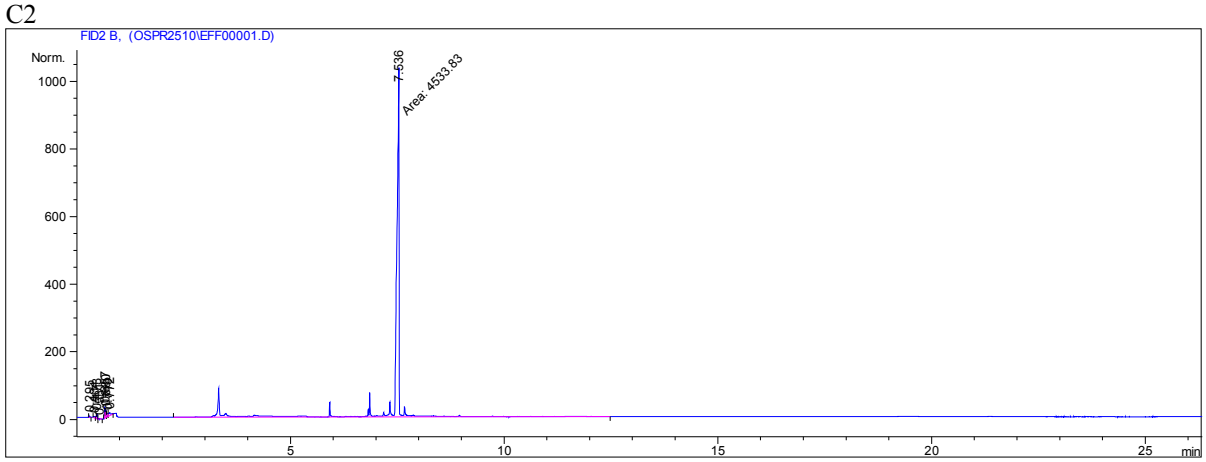
B1 t/m B3


FID2 B, (OSPR2610\EFF00003.D)
FID2 B, (OSPR2610\EFF00004.D)
FID2 B, (OSPR2610\EFF00005.D)
pA
min

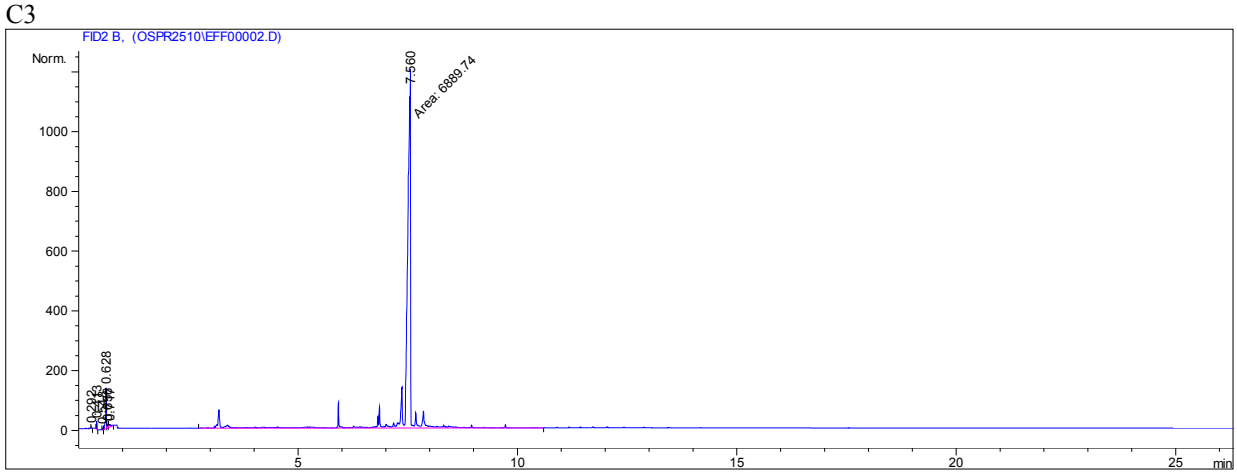
C1


FID2 B, (OSPR2510\EFF00000.D)
Norm.
7.529
Area: 4796.73
min

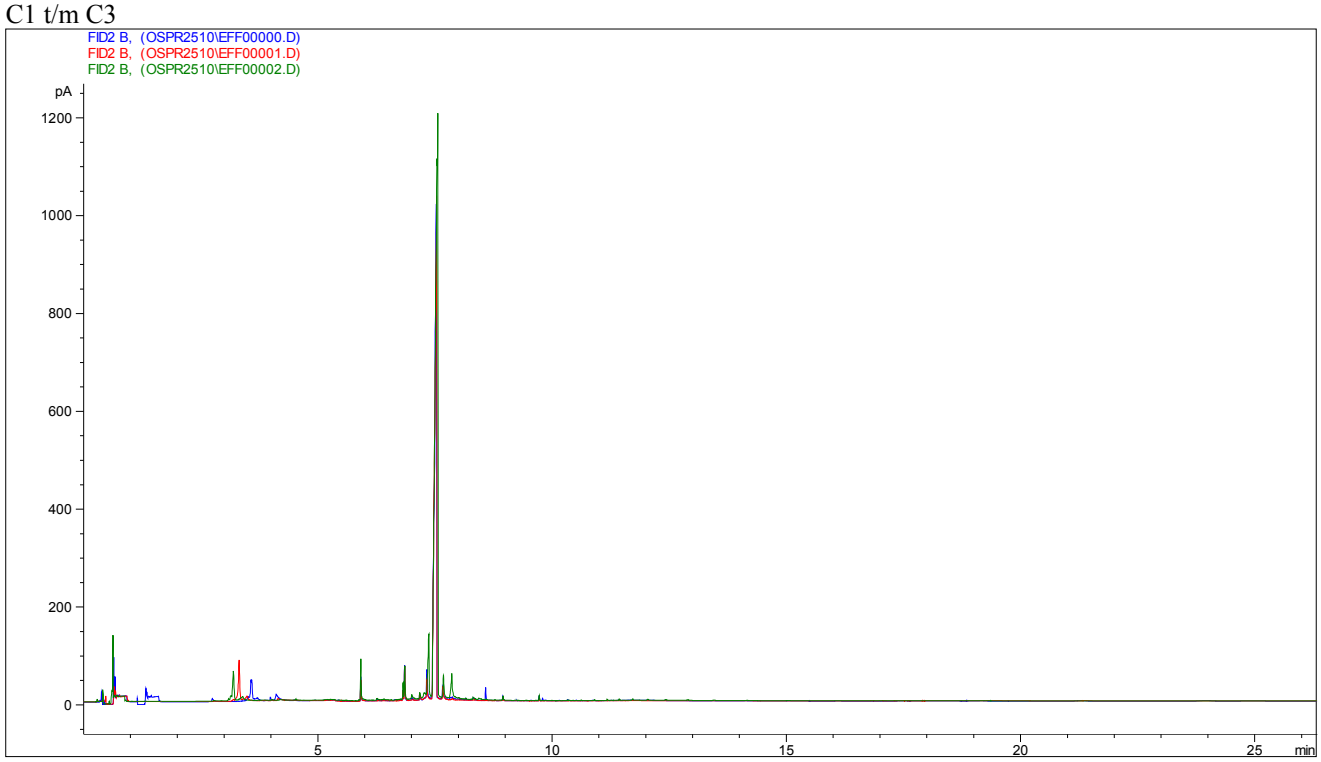
C2


FID2 B, (OSPR2510\EFF00001.D)
Norm.
7.536
Area: 4533.83
min

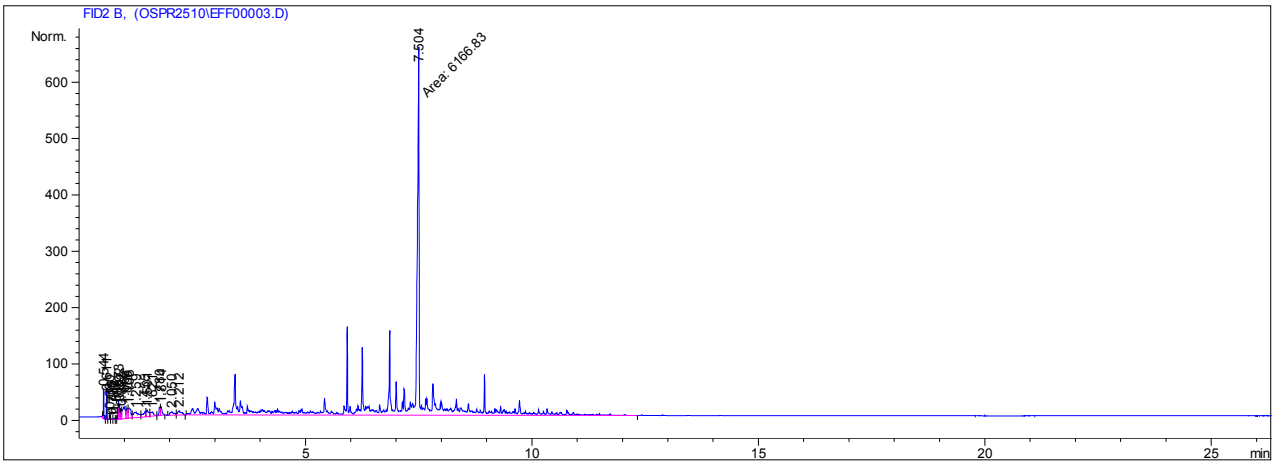
C3


FID2 B, (OSPR2510\EFF00002.D)
Norm.
7.560
Area: 6889.74
min

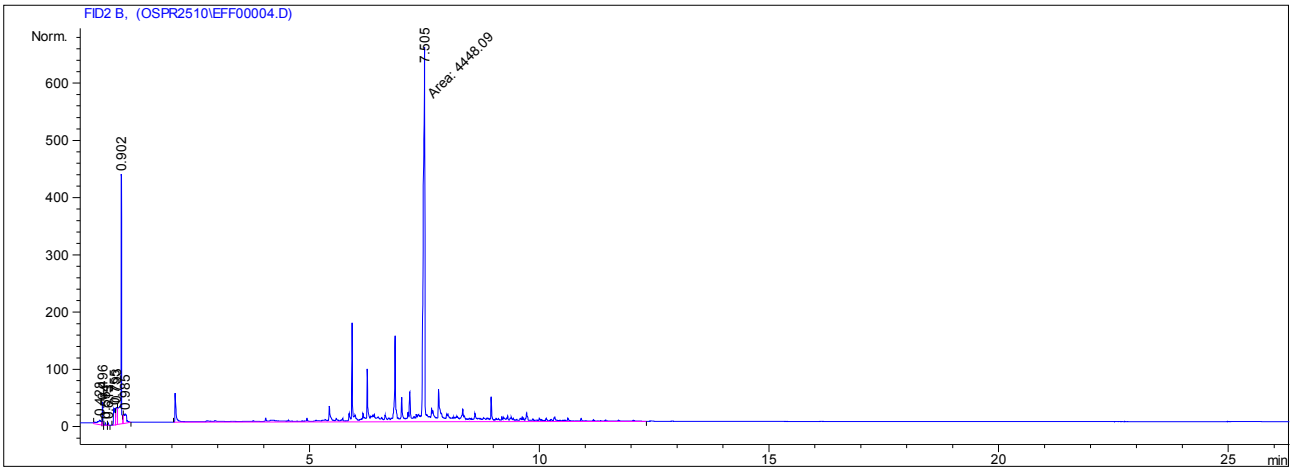
C1 t/m C3


FID2 B, (OSPR2510\EFF00000.D)
FID2 B, (OSPR2510\EFF00001.D)
FID2 B, (OSPR2510\EFF00002.D)
pA
min

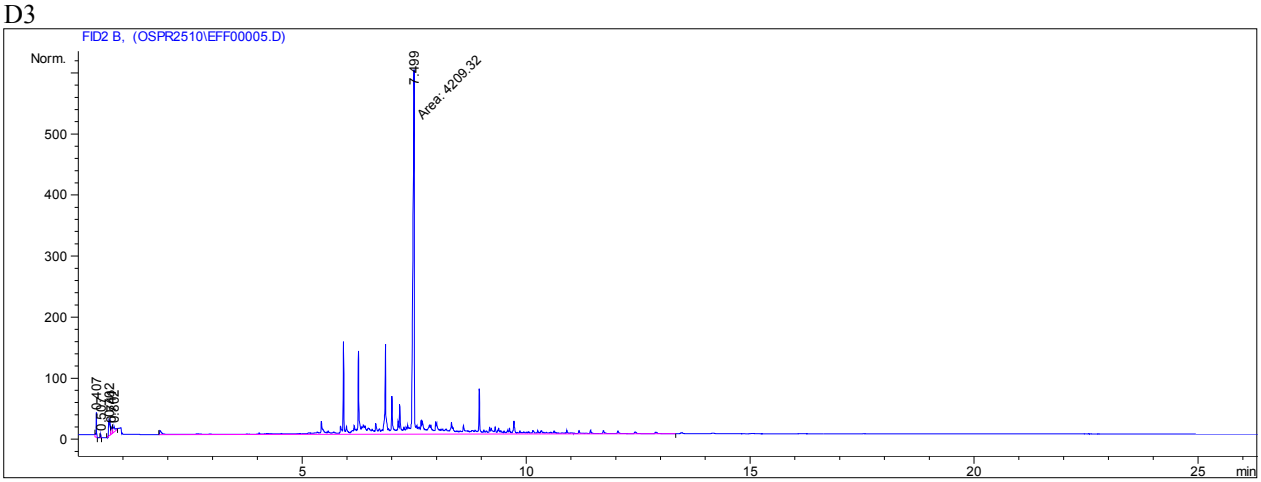
D1


FID2 B, (OSPR2510\EFF00003.D)
Norm.
7.504
Area: 6166.83
min

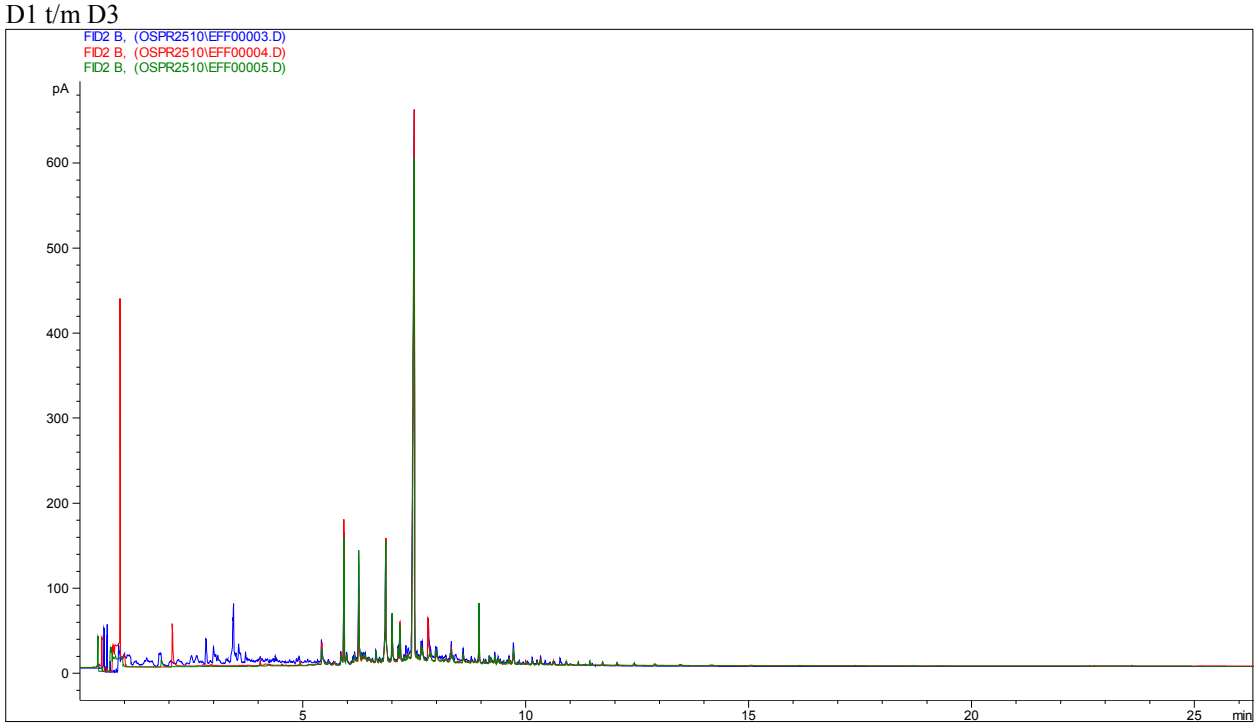
D2


FID2 B, (OSPR2510\EFF00004.D)
Norm.
7.505
Area: 4448.09
0.902
min

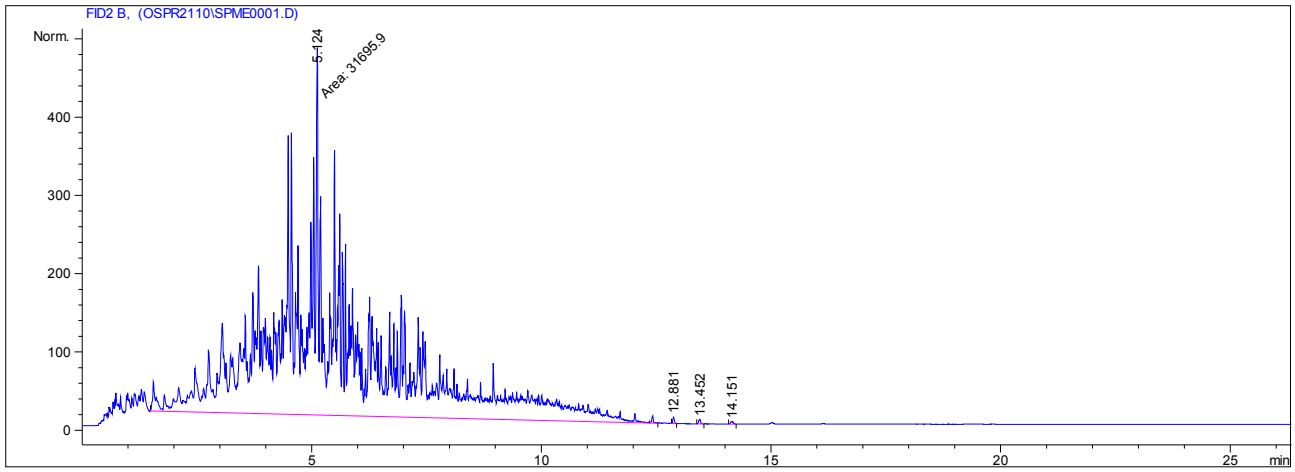
D3

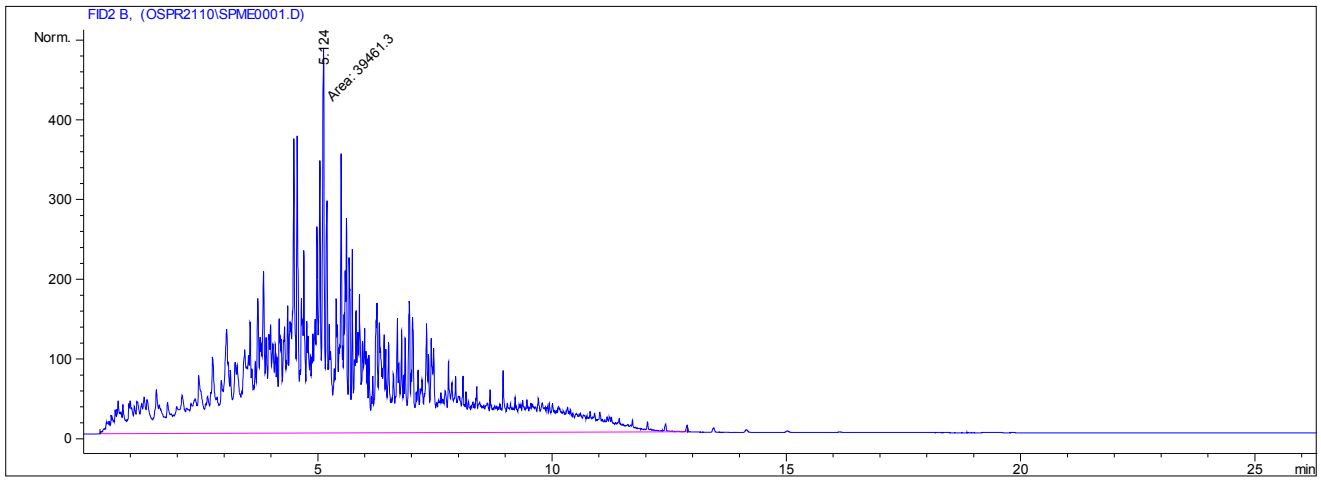

FID2 B, (OSPR2510\EFF00005.D)
Norm.
7.499
Area: 4209.32
min

D1 t/m D3

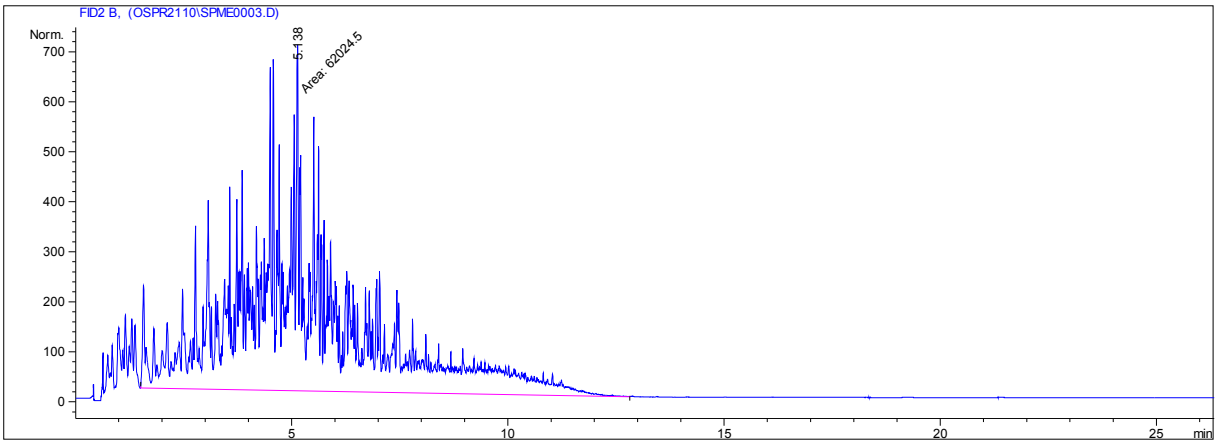

FID2 B, (OSPR2510\EFF00003.D)
FID2 B, (OSPR2510\EFF00004.D)
FID2 B, (OSPR2510\EFF00005.D)
pA
600
500
400
300
200
100
0
5
10
15
20
25
min

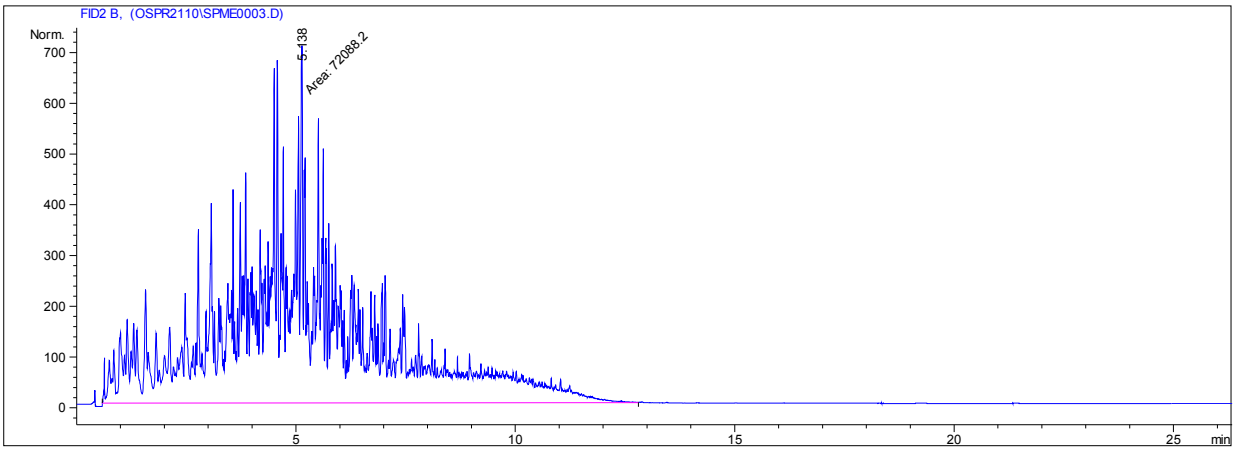
E1


FID2 B, (OSPR2110\SPME0001.D)
Norm.
5.124
Area: 31695.9
12.881
13.452
14.151
400
300
200
100
0
5
10
15
20
25
min

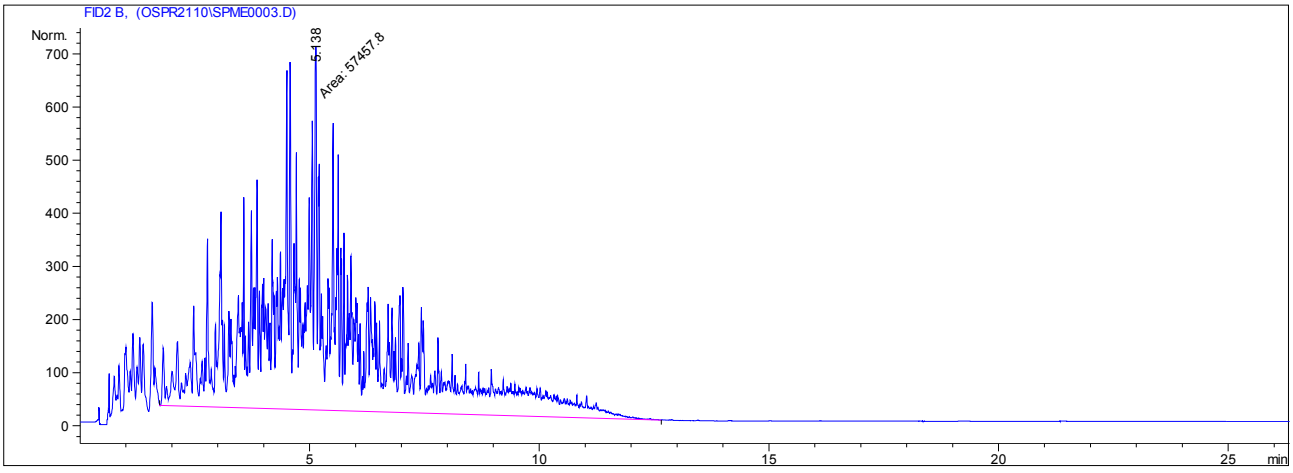

FID2 B, (OSPR2110\SPME0001.D)
Norm.
5.124
Area: 39461.3
400
300
200
100
0
5
10
15
20
25
min

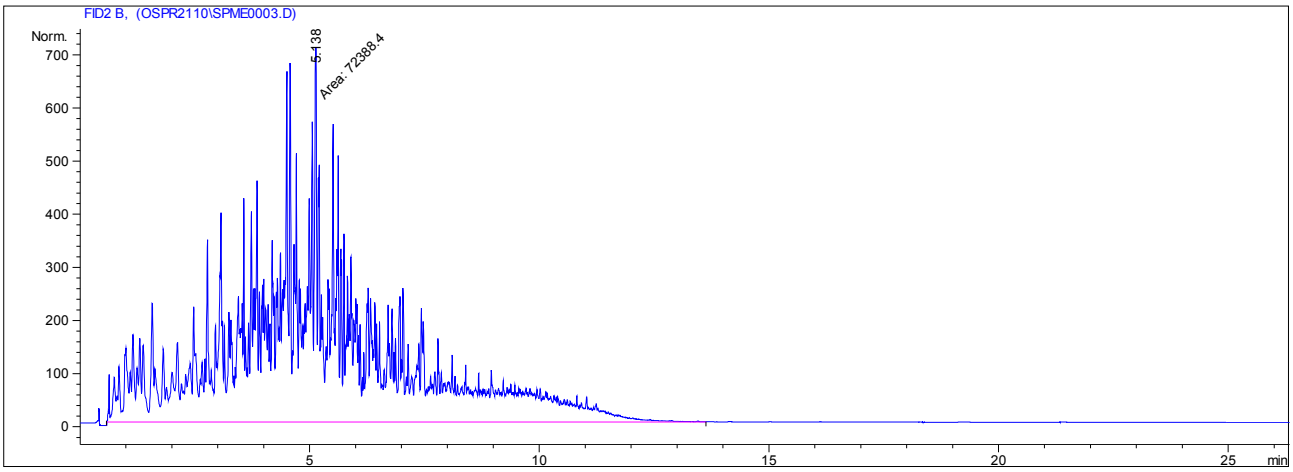
E2


FID2 B, (OSPR2110\SPME0003.D)
Norm.
700
600
500
400
300
200
100
0
5.138
Area: 62024.5
5
10
15
20
25
min

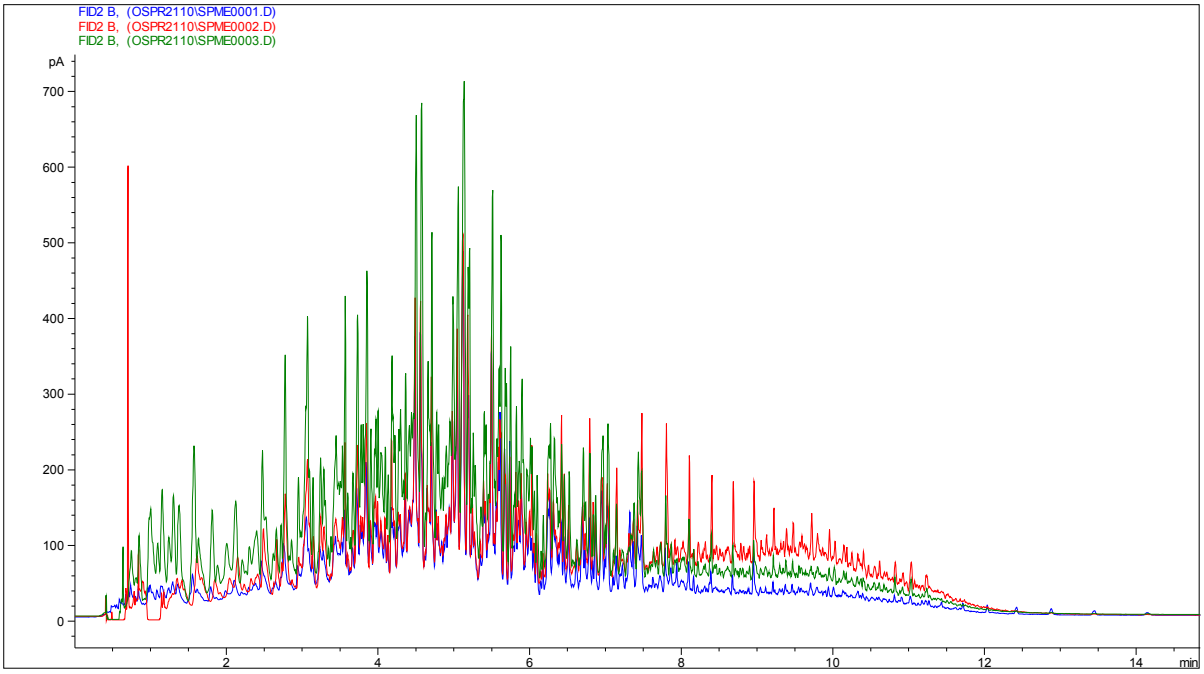

FID2 B, (OSPR2110\SPME0003.D)
Norm.
700
600
500
400
300
200
100
0
5.138
Area: 72088.2
5
10
15
20
25
min

E3


FID2 B, (OSPR2110\SPME0003.D)
Norm.
700
600
500
400
300
200
100
0
5.138
Area: 57457.8
5
10
15
20
25
min

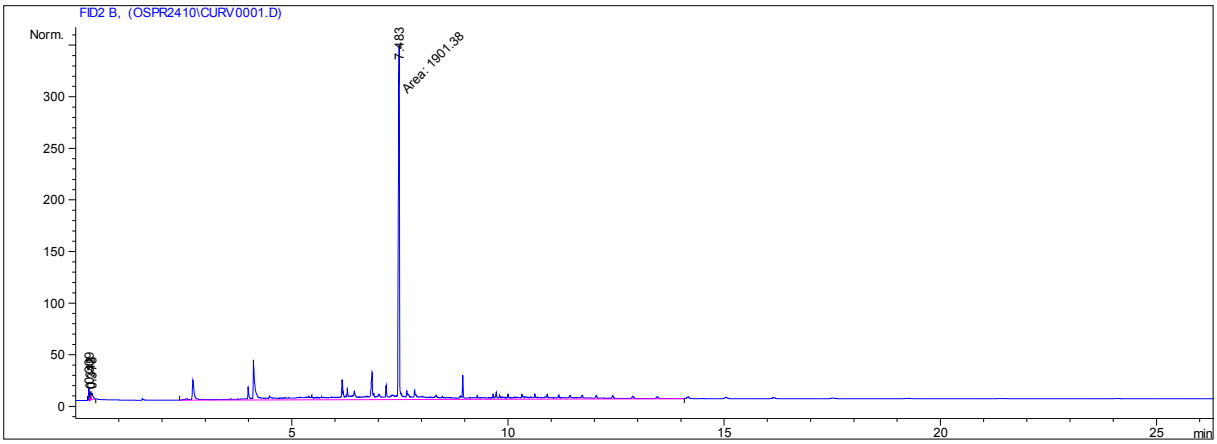


E1 t/m E3

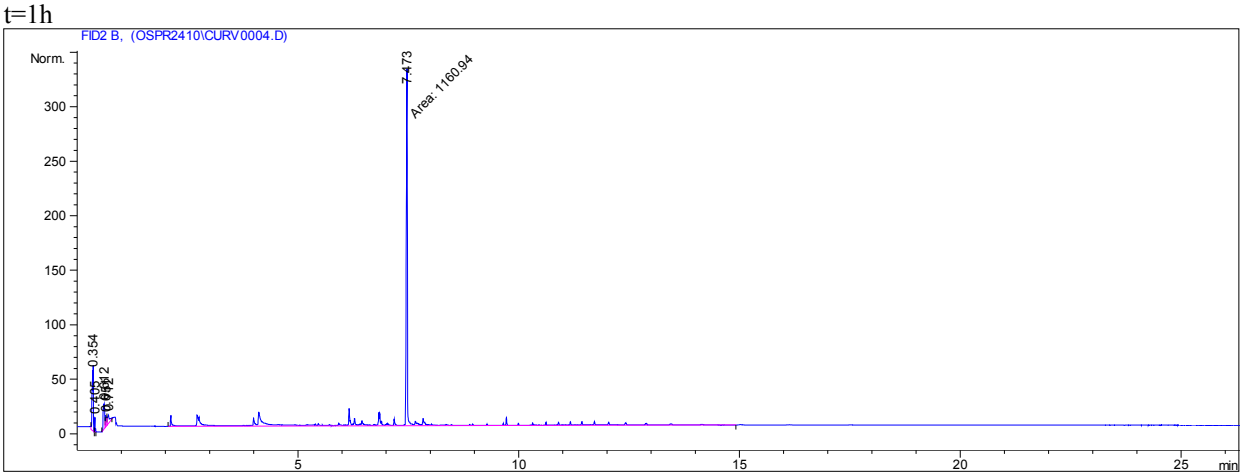


RIVO DATA – UPTAKE CURVE

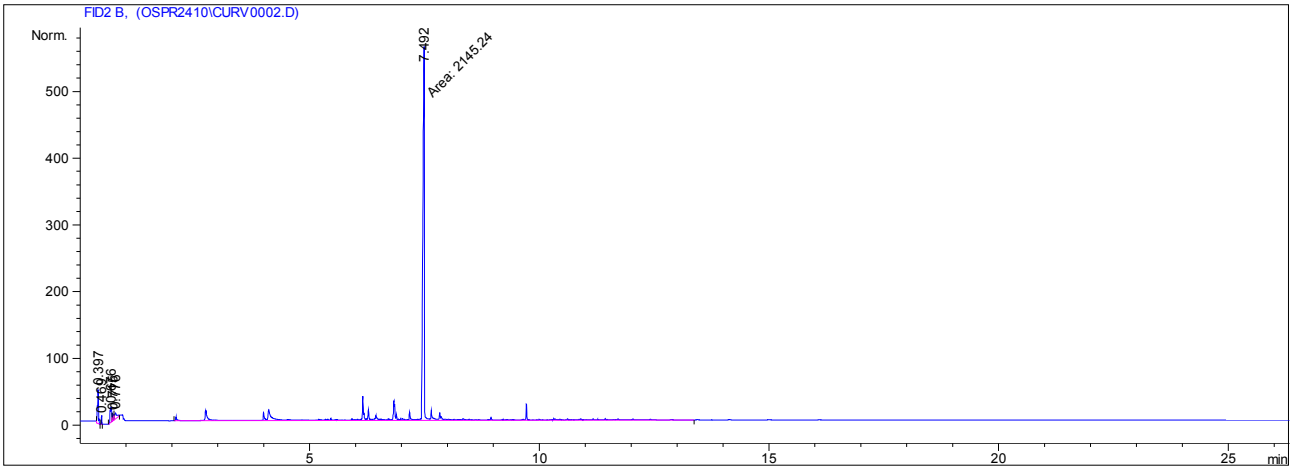
t=0.5h



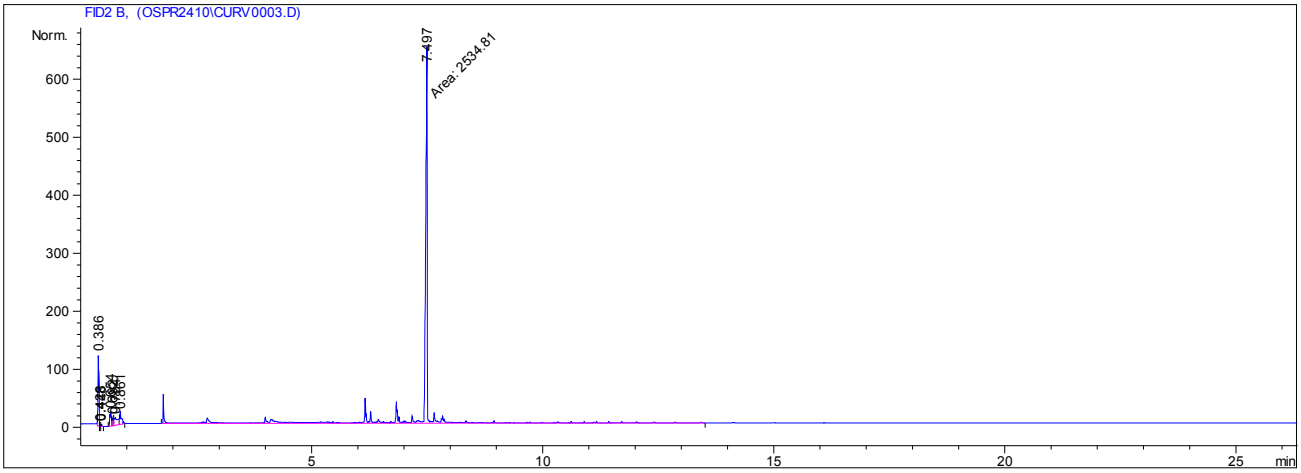
t=1h


FID2 B, (OSPR2410\CURV0004.D)
Norm.
7.473
Area: 1160.94
0.354
min

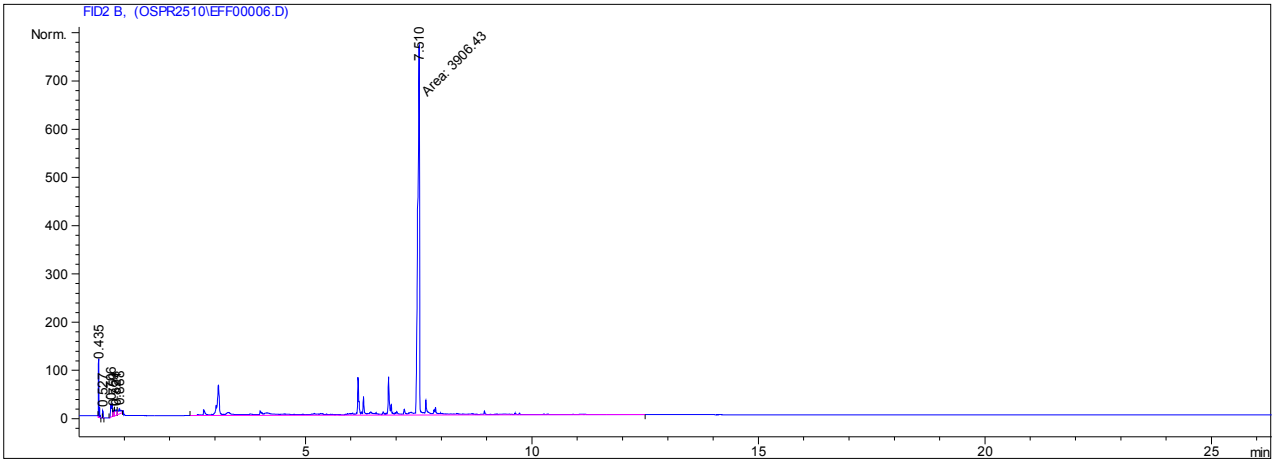
t=2h


FID2 B, (OSPR2410\CURV0002.D)
Norm.
7.492
Area: 2145.24
0.397
min

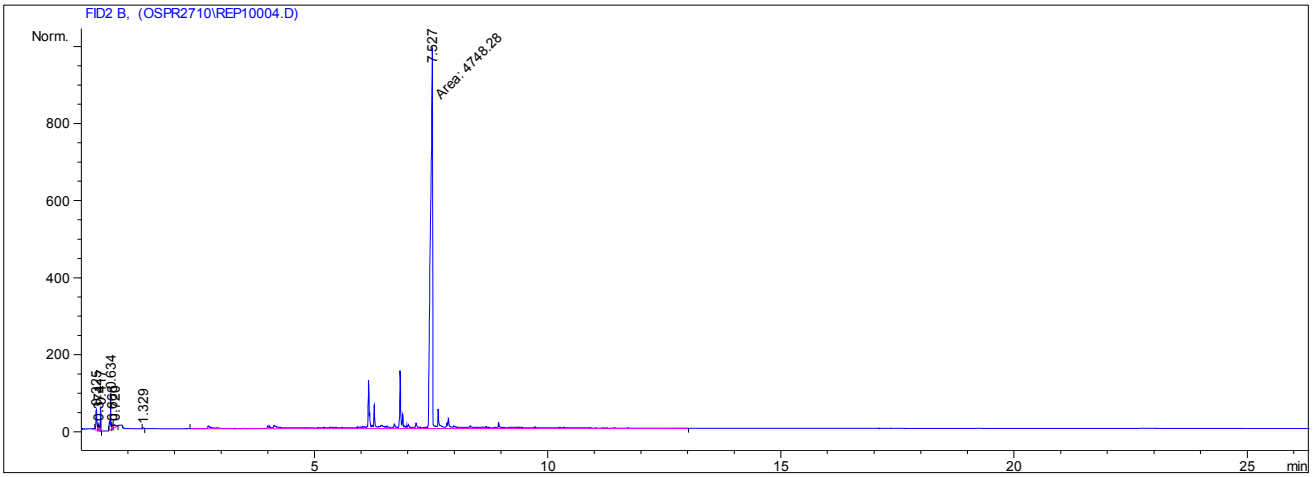
t=4h


FID2 B, (OSPR2410\CURV0003.D)
Norm.
7.497
Area: 2534.81
0.386
min

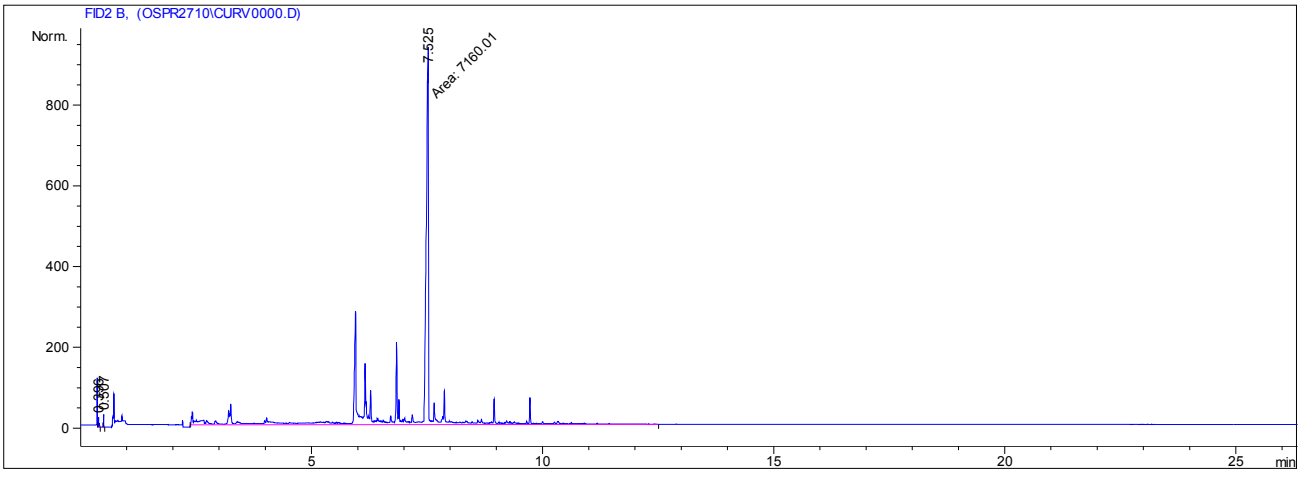
t=8h



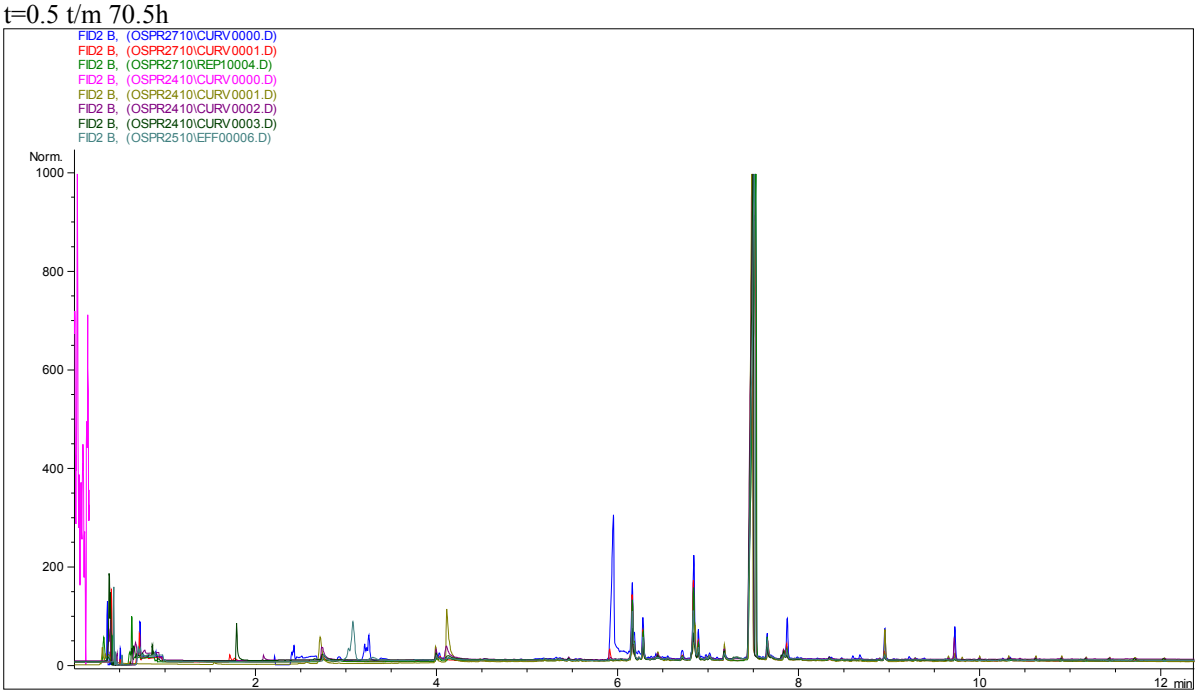
t=24h



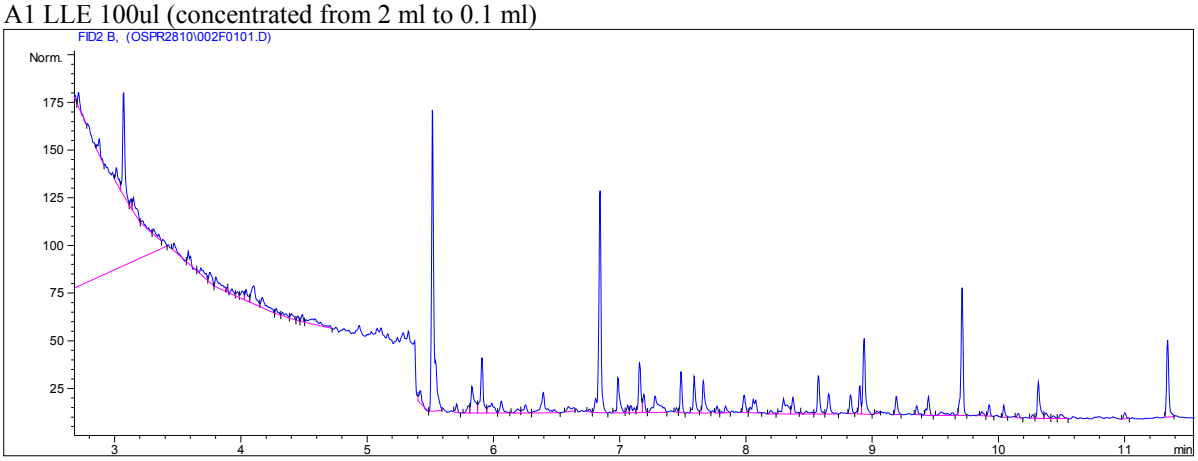
t=48h



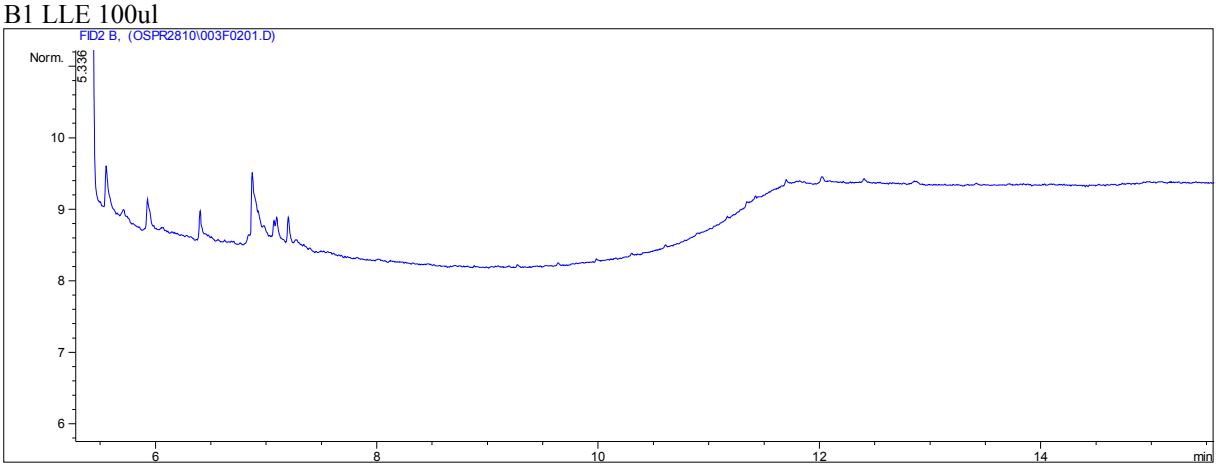
t=0.5 t/m 70.5h



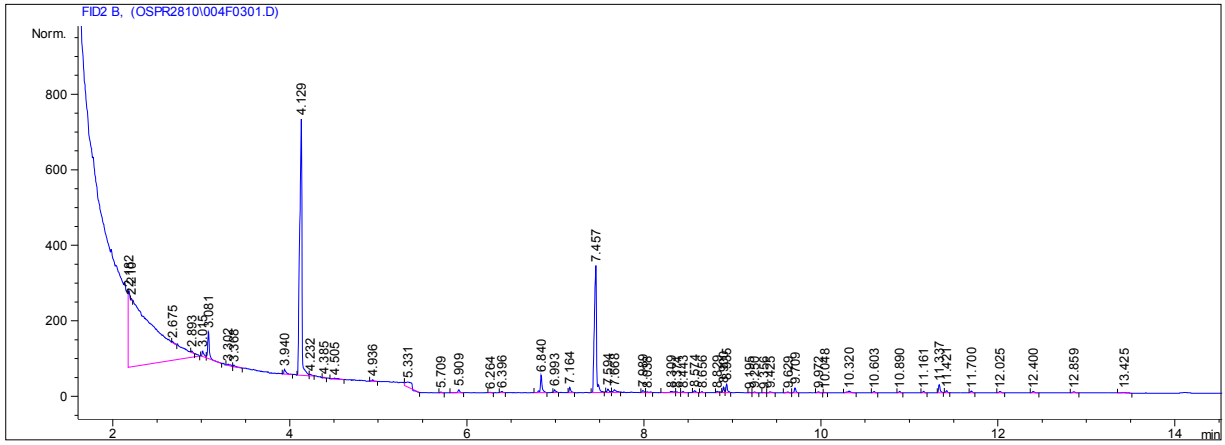
A1 LLE 100ul (concentrated from 2 ml to 0.1 ml)



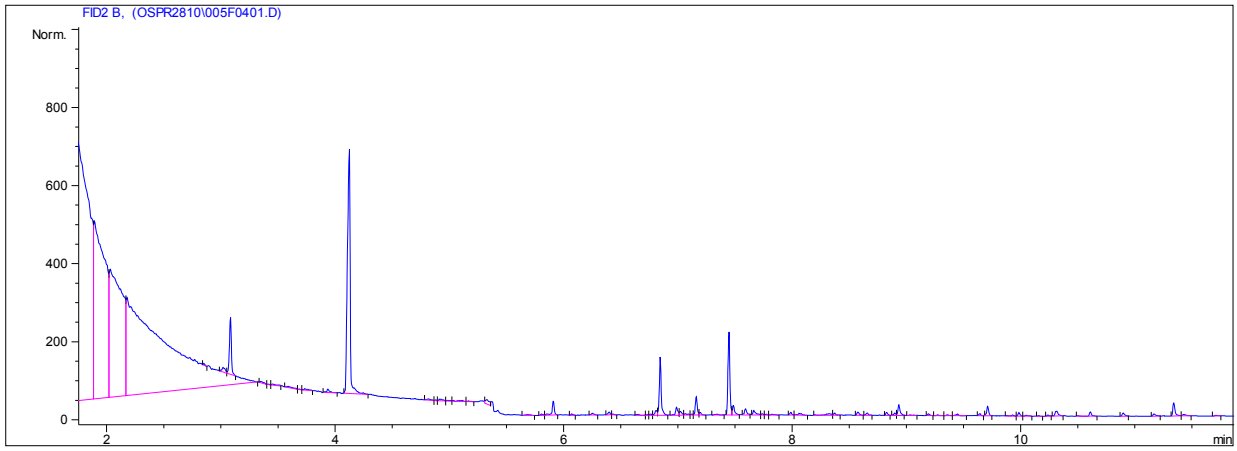
B1 LLE 100ul



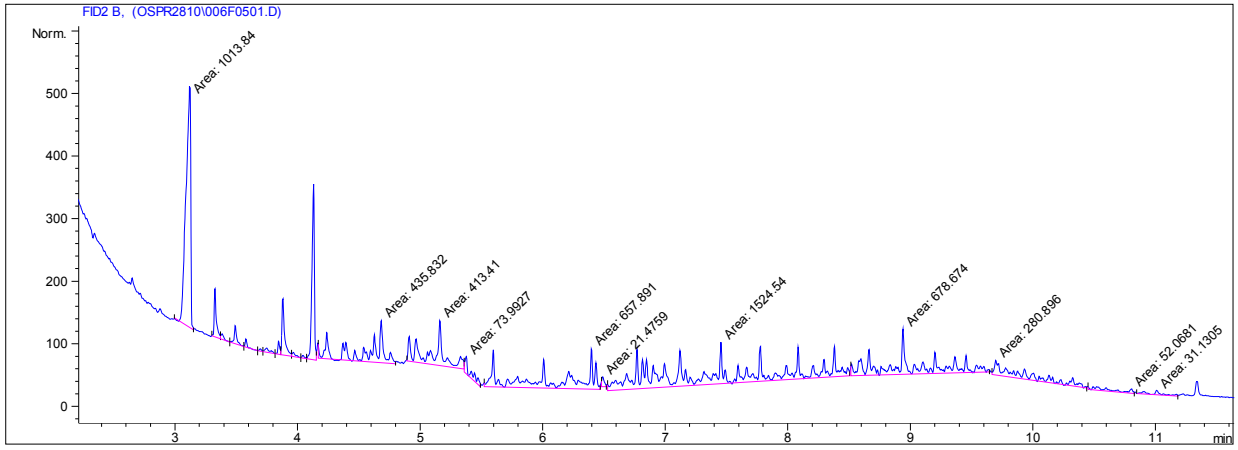
C1 LLE 100ul



D1 LLE 100ul



E1 LLE 100ul



Chromatogrammen vergelijking met meerdere fibers in 1 fles