Analysis of fatty acids in fatty acid-based herbicides by gas chromatography

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Detecting the exposure to fatty acid-based herbicides in the environment

Herbicide products are widely used as weed-controlling agents by both professionals and individuals. The formulas of some of those products contain chemicals that may have harmful impacts on the environment, which incentivized governments and health authorities to ban some of those herbicides. Researchers and producers were also encouraged to synthesize and examine weed-controlling agents that are greener and more environmentally sustainable. Some products of the modern generation of herbicides depend mainly on fatty acids and natural oils to achieve their mode of action as weed-controlling agents. Nonanoic acid, also known by its trivial name as pelargonic acid, is widely used as the main ingredient in the so-called bio-herbicides. However, the friendly environmental impact of those bio-herbicides is yet to be investigated. Research and studies that could achieve that goal, implicitly, shall include analytical methods to extract, detect and quantify those products in environmental matrices.

The aim of this work was to identify the fatty acid composition in a commercial fatty acid-based herbicide product and to detect it in environmental matrices. Soils collected from different locations were spiked with different amounts of the herbicide in order to mimic the exposure to the herbicide in nature. The extractions of FA´s from the soil were performed on a supercritical fluid extraction system using extraction solvent consisting of supercritical carbon dioxide and methanol. Methanol was used as a co-solvent to tune the polarity of the extraction solvent in order to create solubility conditions that are optimal for the extraction of the FA´s from the soils. Qualitative and quantitative analysis's were performed on gas chromatography systems equipped with flame ionization detectors or mass spectrometry detectors when higher degree of selectivity was needed. The free fatty acids used to construct the models as well as lipids in the extractions were analyzed in their fatty acid ester form after derivatization under acidic conditions. The qualitative analysis was performed by constructing retention index models for the fatty acid methyl esters to detect and identify the corresponding fatty acids in the herbicide and the soils. The quantitative analysis was performed by constructing internal calibration models for the fatty acid methyl esters.

The fatty acids of the herbicide in question could be identified using GC-FID and GC-MS. The herbicide was found to contain octanoic acid, pelargonic acid, 2-methyl octanoic acid, and decanoic acid, with pelargonic acid and 2-methyl octanoic acid having the highest

abundance, respectively. The constructed internal calibration models showed acceptable R^2 values that ranged between 0.992 and 0.996. The performance of the analysis showed good precision where repeatabilities for the peak areas and retention times with RSD% values ranged from 1.32 to 7.69 and from 0.011 to 0.783, respectively. Validation of the extraction process of FA´s from the spiked soils was estimated quantitatively in terms of recovery percentage for the concentrations of pelargonic acid. Recoveries of pelargonic acid in the spiked soils were below the acceptable criterion in five spiked samples with values: 8%, 22%, 62%, 66 %, and one sample with 129%, while three spiked samples were within the acceptable range, two samples with 79% and one sample with 100%. The low obtained recoveries may indicate that a better extraction method can be considered in future work in order to achieve optimal extraction conditions.

Abstract

Introduction:

Fatty acids-based herbicides are considered to be a green alternative to synthesized weed control products. However, the environmentally sustainable effect of those herbicides needs more investigation to be confirmed, which raises the need to develop analytical methods to detect the exposure to those herbicides.

Aim:

The aim of this work was to develop a chemical analysis method that enables identification of short-chain fatty acids (SCFA´s) and medium-chain fatty acids (MCFA´s) in a commercial fatty acid-based herbicide product, and to set up extraction and gas chromatography (GC) methods that can be used to detect the exposure to the product in environmental matrices.

Methods:

Fatty acids (FA´s) were extracted from the herbicide by liquid-liquid extraction (LLE) using n-heptane as organic solvent. The FA´s obtained from the extraction were derivatized under acidic conditions into fatty acid methyl esters (FAME´s) to be analysed on gas chromatography-flame ionization detector (GC-FID) system and gas chromatography-mass spectrometry (GC-MS) system. A standard solution of known FAME´s was used to build up a retention index (RI) model in order to identify FAME´s in the samples. Soil samples were collected and spiked with different volumes of the herbicide. FA´s were extracted from the soil samples on a supercritical fluid extraction (SFE) system using extraction solvent consisting of 90% supercritical carbon dioxide (SC-CO_2) and 10% methanol (MeOH) (v/v). The extractions were then derivatized under acidic conditions and the obtained FAME´s were analysed by GC-FID and GC-MS systems.

Results:

The herbicide product was found to contain octanoic acid, pelargonic acid, decanoic acid, and 2-methyoctanoic acid. R^2 values of the calibration curves ranged between 0.992 and 0.996. Repeatabilities for the peak areas and retention times had RSD% values varied between 1.32 - 7.69 and 0.011-0.783, respectively. Recoveries of pelargonic acid in the spiked soils were estimated to vary between 8% and 128%.

Conclusion:

The composition of herbicide content consists of medium chain FA´s. FA´s of the herbicide could be detected in soil exposed to the herbicide by GC-FID and GC-MS, where MS showed higher selectivity to detect FA´s. FA´s can be extracted from soil by SFE systems using extraction solvent consists of SC-CO_2 and MeOH, however more work could be required to investigate the optimum parameters of the extraction method. Derivatization step to prepare FAME´s from FA´s together with the extraction process directly affect the characterizations and the overall certainty of an analytical method.

Keywords:

Fatty acids | Gas chromatography | Green herbicides | Retention index | Supercritical carbon dioxide extraction

Acknowledgement

I would like to show my gratitude to the entire analytical chemistry group for all the fruitful cooperation, the great help and for providing a very healthy working atmosphere during the project work. I would like to thank my supervisors Peter Spégel, Margareta Sandahl, and my examiner Charlotta Turner, Centre of Analysis and Synthesis, Lund University, for their guidance and useful feedback during the project work and in report writing. Lastly, I would like to pay special thanks to Thamani Freedom, Fiona Nermark, Mynte Norberg and Bassadi Ncube for the great help in the labs and also with operating the instruments during the project time.

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1 List of abbreviations

t´R(n+1)	Adjusted retention time of the alkane eluting after the analyte.
t´R(n)	Adjusted retention time of the alkane eluting before the analyte.
t´R(X)	Adjusted retention time of the analytes
ANOVA	Analysis of variance
a.u.	Arbitrary area units
R^2	Coefficient of determination
C_{FA}	Concentration of fatty acid
C_{FAME}	Concentration of fatty acid methyl ester
C_{IS}	Concentration of internal standard
D	Derivatization factor
DF	Dilution factor
SANCO	Directorate General Health and Consumers; European Commission
EMA	European Medicines Agency
FAME´s	Fatty acid methyl esters
FA´s	Fatty acids
FID	Flame ionization detector
GC	Gas chromatography
IS	Internal standard
ICH	International Council for Harmonization
LLE	Liquid-liquid extraction

MS	Mass spectrometer
MCFA´s	Medium chain fatty acids
n	Number of carbon atoms in the alkane
C	- Number of carbon atoms in the fatty acid
	- Number of carbon atoms in the fatty acid methyl ester, excluding the methyl carbon
R	Recovery
RSD	Relative standard deviation
RI	Retention index
RF	Response factor
tR	Retention time
SCFA´s	Short chain fatty acids
SC-CO_2	Supercritical carbon dioxide
SFE	Supercritical fluid extraction
FDA	United States Food and Drug Administration

2 Introduction

Controlling weeds has always been an important demand in the farming industry, not only for agricultural purposes but also for other application fields such as gardens, landscapes, and forests. Although the weed does not affect the crops by direct action, it still can act on reducing the nutrition supply for the crops by competing for the nutrition resources in the soil and by blocking the exposure to the sun. In agriculture, controlling weeds may have a big impact on the production of the crops, in both quality and quantity. Thus, herbicide production and their applications are important key players in the process to cope with the global demand for increased crop production as one of the most important resources of food supply.[1]

Most weed control products on the market are chemically synthesized products with a broad variety of chemical reagents. This makes the treatment circle of the herbicidal applications may end with potentially harmful consequences on the environment, people's health, and other non-target organisms, where chemical residues from the herbicides may remain for years in the water and the soil. During the past decades, governments started to take actions to control the use of weed killers. In Sweden for instance, the Swedish Chemicals Agency "KEMI" decided to set new rules and restrictions on the use of weed killers. The agency established new classification of the chemicals used in weed controlling products dividing them into three groups where two of the three groups can only be used by professionals, and not allowed to be used in home gardens or public parks anymore. The rules, which came into act on October 2021, regulate the use of products that contain glyphosphate, pyrethrin, flupyradifurone, and acetamiprid.[2, 3]

Developing more environmentally sustainable herbicides could be a parallel process that goes along with limiting the use of less sustainable herbicides. Such sustainable herbicides, also known as bio-herbicides, are based and derived from organic acids or oils that can naturally occur in the environment. Herbicides that are based on pelargonic acid (Figure 1) as an active agent are quite commonly used in private gardens as well as in farming lands forvariety of agriculture applications.[1]

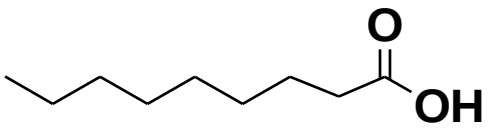


Figure 1: Chemical structure of Pelargonic acid (C9 FA).

In general, the herbicidal action of FA´s on plant´s tissues happens mainly due to stripping of the cuticular waxes, which leads to desiccation of the foliage.[4,5] FA´s mode of action may also be due to their ability to enhance the rate of formation of reactive oxygen species (ROS) and lipid-membrane peroxidation.[6] In a study to evaluate the pre-mentioned desiccant activity of nine FA´s in the range of 2 to 10 carbons, Coleman and Penner concluded that caprylic acid (eight carbons) and pelargonic acid (nine carbons) were found to be the most effective.[7] Moreover, the efficiency of FA´s herbicidal action was found to be related to its capacity to penetrate thylakoid and plasma membranes. An explanation suggests that the desiccant activity of the FA´s is due to a compromise between their intermediate levels of affinity for lipid bilayers and the mismatch in the alkyl chain length between the acid carbon chain and the hydrophobic tails of the membrane phospholipids, which causes bilayer destabilization.[8] Pelargonic acid, as a MCFA´s, was found to have the optimal combination between both factors.[5]

The reported high efficiency of pelargonic acid as weed killer, alongside with the fact that pelargonic acid is a naturally occurring fatty acid, could be a good reason to consider pelargonic acid-based herbicides as environmentally sustainable herbicides. The claim to an environmental sustainable activity of pelargonic acid products may also arise from the pieces of evidence that show low toxicity towards non-target organisms, such as birds, fish, and other species.[1] However, this environmentally sustainable effect is still doubtful and yet to be completely confirmed. For example, a study to investigate the effect of the exposure to pelargonic acid on zebra fish reported a decrease in circulating hormone levels in both male and female fishes.[6] Those doubts could also be supported with some disadvantages that arise from the composition of the herbicide products, where it may contain some other compounds that may be formed as byproducts during the production process.[7,8] In addition to that, pelargonic acid-based products are available in a broad variety of different concentrations, which may make it difficult to be tracked.[1-3] Furthermore, lack of data on relevant toxicological effects on the environment when using those bio-herbicides, especially in the case of commercial use by individuals.[1] Such doubts about the green characterizations of FA´s-based herbicide products consequently increased the demands for developing analytical chemistry methods that can provide more information about the exposure to FA´s-based herbicides.

GC is a very common and effective technique that has been used for decades to detect FA´s in sample matrices.[9] Moreover, using gases as mobile phases may make GC a very good choice as more sustainable and greener analytical application.[9] GC systems equipped with FID and MS detectors are commonly used to detect and quantify FA´s.[10,11] Both techniques can provide sensitive and robust analytical methods with high level of precision, trueness and accuracy; GC-MS, however, is considered to be more superior to GC-FID due to the higher selectivity that MS detector can provide.[12] GC requires FA´s to be derivatized to the corresponding esters become volatile enough to be eluted from a GC column without thermal decomposition.[11-13] FA´s can also be analyzed in the free form with no need for derivatization by using specialized columns which minimize the adsorption of the active carboxyl groups on the stationary phase.[14]

Using the retention indices as analytical characterization parameter is a very robust approach to design analytical GC methods to identify FAME´s. Using the same column, RI of a given analyte can relate the retention of the analyte to that of an alkane in homologous alkane series ; providing high independency from the chromatographic systems. The RI of the alkane can be calculated from Equation (1), then RI of a given analyte can be calculated using Kovat's equations. Equation (2) can be used in case of isothermal analysis while Equation (3) is used if the analysis was performed using a gradient program.[15,16]

$$RI = 100\ n \quad (1)$$

$$RI = 100\ n + 100[\log t´R(X) - \log t´R(n)] / [\log t´R\ (n+1) - \log t´R(n)] \quad (2)$$

$$RI = 100\ n + 100\ [\ (t´R(X) - t´R\ (n)/\ (t´R(n+1) - t´R(n)] \quad (3)$$

Where:

RI: retention index.

n: carbon number of the alkane eluting before the analyte.

t´R(X): adjusted retention time of the analyte.

t´R(n): adjusted retention time of the alkane eluting before the analyte.

t´R(n+1): adjusted retention time of the alkane eluting after the analyte.

3 Materials and Methods

3.1 Chemicals

3.1.1 Chemicals and Reagents

Pentane and tetradecane solvents were obtained from Fluka Chemie GmbH (Buchs, Switzerland. N-hexadecane solvent was obtained from BDH Laboratory Supplies (Poole, England). N-undecane solvent was obtained from E. Merck (Darmstadt, Germany). N-heptane was obtained from VWR Chemicals (Leuven, Belgium). Dodecane solvent was obtained from Merck Schuchardt OHG (Hohenbrunn, Germany). Octane, nonane, decane, hexanoic acid, nonanoic acid, tetradecanoic acid, hexadecanoic acid, heptadecanoic acid and homologous alkane solution (n: 8-20) were obtained from Sigma Aldrich (Steinheim, Germany). Supelco 37 Component FAME´s Mixture solution, octanoic acid, decanoic acid, dodecanoic acid, methyl decanoate, and methyl dodecanoate were obtained from Supelco analytical (Bellefonte, USA). 3 M methanolic HCl anhydrous solution (GC derivatization grade and LiChropur^{TM} quality) was obtained from Merck KGaA (Darmstadt, Germany).

3.1.2 Standard solutions

A standard solution of each FA: hexanoic acid (C6), octanoic acid (C8), nonanoic acid (C9), decanoic acid (C10), dodecanoic acid (C12), tetradecanoic acid (C14), hexadecanoic acid (C16) and heptadecanoic acid (C17) was prepared by weighing the FA in a glass test tube then it was derivatized into FAME using the method described in 3.3.1; see Appendix 8.1, Table 9 for gravimetric data. A standard solution of FA´s mixture was prepared by mixing FA´s (C: 6, 8, 9, 10, 12, 14, 16, and 17) in a glass test tube; the FA´s mixture was then derivatized into FAME´s using the method described in 3.3.1 (see Appendix 8.1, Table 10).

3.1.3 Internal standard solution

0.087 g of dodecanoic acid (C12) was dissolved in 40 mL of n-heptane to be used as an internal standard (IS).

3.1.4 Calibration solutions

A stock solution of FA´s (C: 6, 8, 9, 10, 14, 16, and 17) was prepared by dissolving 0.05 g of each FA in 20 mL n-heptane. Calibration solutions were prepared by the means of a serial

dilution to get final FA´s concentrations of 1.5, 5, 15, 25, 60, 100, 250,600, 1000, 1700, 2100 µg/mL. 1 mL of dodecanoic acid solution as IS was added to each calibration solution and the solvent was dried under a nitrogen stream. The solution was then derivatized into FAME´s using the method described in 3.3.1. Also, see Appendixes 8.4.1 and 8.4.2 for further explanations about preparing the calibration solutions.

3.1.5 Preparation of Alkane series

20 µL of: (pentane, n-octane, n-nonane, n-decane, n-undecane, n-dodecane, tetradecane, and n- hexadecane) were mixed with 10 mL n-heptane in 25 mL glass test tube and the test tube was closed with a plastic cap closure.

3.2 Instrumentation (see further explanations, Appendix 8.4.3)

3.2.1 Analysis of fatty acids in the herbicide by gas chromatography-flame ionization detector

The analysis was performed using an Agilent GC-FID instrument, model 6890N (from Agilent Technologies, Inc., Wilmington, DE, USA), equipped with HP-5 column (30 m × 320 µm inner diameter × 0.25 micron film thickness). The GC was operated in split mode with a split ratio of 100:1, using nitrogen as a carrier gas at flow rate of 1 ml/min. The injection temperature was set to 250 °C and the injection volume was 0.2-0.5 µL. The oven temperature program was set to: initial temperature: 70 °C; rate 1: 18 °C/min to 200 °C, hold time: 1 minute; rate 2: 20 °C/min to 300 °C, hold time: 5 minutes. The detector temperature was set to 260 °C, airflow rate: 400 mL/min, hydrogen fuel flow rate: 40 mL/min and makeup gas flow rate: 40 mL/min.

3.2.2 Analysis of fatty acids in blank soil and spiked soil by gas chromatography-flame ionization detector

The analysis was performed using an Agilent GC-FID system, model 7890B (from Agilent Technologies, Inc., Wilmington, DE, USA), equipped with DB-5 column (30 m × 0.25 mm inner diameter × 0.25 µm film thickness). The GC analysis was operated on splitless mode, using nitrogen as a carrier gas at flow rate of 1 mL/min. The injection temperature was set to 250 °C and injection volume was 1 µL. The oven temperature program was set to: initial temperature: 70 °C; rate 1: 5 °C/min to 140 °C, hold time: 1 minute; rate 2: 20 °C/min to 300 °C, hold time: 5 minutes. The detector temperature was set to 310 °C, airflow rate: 400 mL/min, hydrogen fuel flowrate: 40 mL/min and makeup gas flow rate: 40 mL/min.

3.2.3 Analysis of fatty acids by gas chromatography-mass spectrometry

The analysis was performed using an Agilent GC-MS instrument model 6890N and Agilent GC-MS instrument model 7890A (from Agilent Technologies, Inc., Wilmington, DE, USA), both instruments were equipped with DB-5ms column (30 m × 0.25 mm inner diameter × 0.25 µm film thickness) and Agilent 5973N mass detector, with the electron ionization (EI) source set to 70 eV, in full scan mode. The GC analysis was operated on splitless mode, using helium as a carrier gas at flow rate of 1 mL/min. The injection temperature and the oven temperature program were set at the same parameters used in *3.2.2*.

3.3 Methods

3.3.1 Derivatization of fatty acids

All lipids and FA´s in this work were derivatized into FAME´s by the following method[17]:

1 mL of 3 M methanolic HCl was mixed with the oil in a glass test tube. The test tube was closed with a Teflon-lined lid, shaken for 2 minutes and vortexed for 10 seconds. The tube was then submerged in a water bath at 60 °C for 2 hours. The test tube was removed from the water bath and allowed to cool down in room temperature. 1 mL water was added and the mixture was shaken for 4 minutes. Then 1.5 mL of heptane was added and the mixture was allowed to phase-separate. The organic layer was then collected and the solvent was evaporated under a gentle stream of nitrogen. The obtained extracts containing the FAME´s were then dissolved in 1 mL n-heptane and diluted one more time before the GC analysis. The introduced dilution factor (DF) was then included in the final calculations for quantification of FAME´s in the samples according to Equation (6).

3.3.2 Evaluation of the derivatization method

0.025 g of dodecanoic acid was weighed in a glass test tube and then derivatized to FAME according to the method described in 3.3.1; the extracted FAME was then diluted in 10 mL n-heptane. A standard solution of methyl decanoate (2500 µg/mL) was prepared by dissolving 0.025 g of methyl decanoate in 10 mL n-heptane. The two solutions were analyzed on the GC-FID according to the method in *3.2.2*. An estimation of the derivatization step's efficiency was performed by comparing the arbitrary area units (a.u.) obtained from the injections of the two solutions.

3.3.3 Lipid extraction from the herbicide and derivatization into fatty acid methyl esters

10 mL of the herbicide were transferred to a 25 mL flask and the acidity of the solution was adjusted to pH 2.6 by adding 37% HCl dropwise under stirring. The solution was then transferred to a 25 mL glass test tube; 10 mL n-heptane was then added and the test tube was closed with a Teflon-lined lid. The mixture was mixed by shaking for 2 minutes and let to settle until two-phases could be clearly observed. The organic layer was collected and the solvent was evaporated to dryness under a nitrogen stream. The obtained extract was derivatized into FAME´s using the method described in *3.3.1.*

3.3.4 Soil Sampling and preparation of soil samples spiked with the herbicide

Soil samples were collected from 3 different locations. 2 soils were collected from Grönbo Hage and Rya åsar in Borås city, Västra Götaland County, Sweden; 1 soil was collected from Rövarekulan, Löberöd, Skåne County, Sweden. For each soil, a hole of 20 cm depth and 10 cm width was dug and 500 grams of soil were collected in polyethylene bags. The soil was transferred to the lab in an icebox and then kept in fridge at 5 $^{\circ}$C for 18 hours. Soil was dried in the oven at 50 $^{\circ}$C for 2 hours then sieved through 177-micron sieve and stored at – 18 $^{\circ}$C. Eight spiked soils were prepared where 15 grams of soil were spiked with a different volume of the herbicide: 0.25 mL, 0.5 mL, 1 mL, 2 mL, 3 mL, 4 mL, 5 mL and 6 mL. The soil were then mixed with constant stirring by spatula for 7 to 10 minutes; solvents were let to slow-evaporate overnight in a fume hood at room temperature. After drying, the soils were carefully mixed again with constant stirring using spatula. The soils were transferred to glass test tubes and were shaken for 5 minutes. The tubes were then closed with plastic cap closures and kept in the freezer at -4 °C until extraction, which took place in the same week.

3.3.5 Lipid extraction from soils and derivatization into fatty acid methyl esters

Blank and spiked soil samples were extracted on Waters SFE system model (ASFE, MV-10) from Waters Ges.m.b.H (Massachusetts, USA). 3 g soil were added into the vessel and mixed with 3 g of glass beads. The extraction method was performed using 90% CO_2 and 10% MeOH as co-solvent (v/v), with a flow rate 2 mL/min at 80 $^{\circ}$C, 1 minute static time and 15 minutes dynamic time, and using n-heptane as makeup solvent with a flow rate of 0.5 mL/min. The solvent was evaporated under a gentle stream of nitrogen and extracts were then stored at -18 $^{\circ}$C until derivatization. For derivatization, 1 mL of dodecanoic acid with added to the extract and derivatization was then performed using the method describe in 3.3.1.

3.3.6 Identification of fatty acids

RI of FAME´s in the standard solution was calculated according to Equation (3). The calculated RI of FAME´s were plotted against their retention times (tR), and a fitted model was obtained. The linear equation of the fitted function was then used to calculate RI of the unknown analytes in the herbicide from tR. The RI´s of the FAME´s in the mixture were also plotted against their carbon number (C), excluding the methyl carbon. The fitted equation obtained from this function was then used to predict C of each analyte in the herbicide sample using their calculated RI´s.

3.3.7 Quantification of fatty acids in spiked soil samples

When using IS to calibrate for FAME´s in a typical calibration, the response of the detector to each analyte is related to the response of the detector to the IS with a relative response factor (RF) according to Equation (4).[11,15]

$$RF = (C_{IS}/C_{FAME}) * (A_{FAME}/A_{IS}) \quad (4)$$

Where C_{IS} and A_{IS} are the concentration of IS and the peak area of IS respectively. C_{FAME} and A_{FAME} are the concentration of the FAME and the peak area of the FAME, respectively. Calibration curves obtained from plotting the response ratio (A_{FAME}/ A_{IS}) against the concentration ratio of (C_{FAME}/C_{IS}) for each FAME, where the RF can be presented in the slope of the linear function of the calibration curve, Equation (5).

$$\frac{A_{FAME}}{A_{IS}} = k * \frac{C_{FAME}}{C_{IS}} \pm m \quad (5)$$

Where *k* and *m* are the slope and the y- intercept of the calibration curve, respectively. However, the calibration curves in this work were constructed using calibration solutions prepared from the derivatization of FA´s, not by using pre-synthesized standards FAME´s, where the concentration of the FA´s prior to derivatization was used instead of the concentration of the FAME. This approach was chosen so that the relationship obtained from the calibration curves would include a correction factor for the derivatization yield; see further explanations, Appendix 8.4.4.

Concentrations of FAME´s obtained from Equation (5) was then used to determine the FA content in the spiked soil samples ($C_{FAspiked}$) according to Equation (6), and reported as mg FA per gram soil.

$$C_{FAspiked} = \frac{1mL * DF * C_{FAME}}{3\ g} \quad (6)$$

Where $C_{FASpiked}$ is the concentration of the FA in the spike sample presented in mg per g soil, 1 mL is the volume of the solvent used to dissolve FAME´s, DF is the dilution factor used to prepare the sample for the GC analysis, and 3 g is the mass of the soil used to extract the FA.

3.3.8 Evaluation of the method performance

The linearity of the models was validated by the determination coefficient (R^2) of the regression equation obtained from the calibration curve of each FA in the calibration solution.[18,19]

The trueness of the method was evaluated by calculating recovery (R); i.e. relative bias due to analyte loss, according to Equation (7) and reported as percentage (%).[19]

$$R\ (\%) = \frac{m_{extracted} - m_{blank}}{m_{\ spiked}} * 100 \quad (7)$$

Where $m_{extracted}$, m_{spiked} , m_{blank} are the mass of the analyte quantified in the spiked soil sample, the mass of the analyte spiked in the soil, and the mass of the analyte quantified in the blank soil (non-spiked), respectively; and $m_{blank} = 0$ if the analyte was not detected in the blank soil. The recovery was evaluated according to the criteria of the Directorate General Health and Consumers; European Commission (SANCO), considering that acceptable recovery is when $70 \leq R \leq 120$.[19]

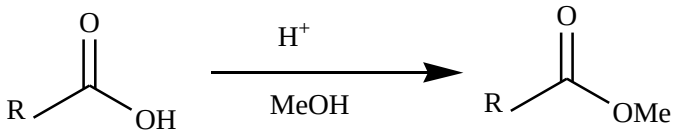
Precision of the performance of the GC analysis was validated for the concentrations of the herbicide's FA´s in three soil samples where each soil was spiked with a different concentration of the herbicide: 0.033, 0.20, and 0.33 mL/ g soil. The validation was done by performing an intra-day (within-run) repeatability assay and an inter-day (between-run) repeatability assay. For the intra-day repeatability assay, the test was performed by analyzing three different aliquots for each of the three concentrations on the GC-FID, at three different times during the same day. Due to lack of time, inter-day repeatability assay was performed only one time for each sample, where one aliquot of each of the three samples was analyzed

on the GC-FID two days later. Repeatabilities for the peak areas of FAME´s of the herbicide's FA´s detected in the soil samples were also tested in samples spiked with the three different concentrations of the herbicide: 0.033, 0.20, and 0.33 mL/ g soil, where 3 aliquots for each of the three concentrations were analyzed 3 times on the GC-FID during the same day. In addition, repeatability tests were performed for tR of the corresponding FAME´s of the herbicide's FA detected in the soil samples. The precision tests were reported as %RSD according to the formula: %RSD = (standard deviation/mean) X100.[20,21] The results were evaluated according to the criteria of U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) which states that acceptable RSD values should be within 15%.[19-21]

4. Results and discussions

4.1 Evaluation of the derivatization step

Scheme 1: Acidic methylation of fatty acid into fatty acid methyl ester



To test for the performance of the derivatization step (scheme 1), the chromatograms obtained from individual injection of standard solutions prepared from pre-synthesized methyl decanoate and pre-synthesized methyl dodecanoate was compared with FAME´s solutions prepared from the derivatization of decanoic acid and dodecanoic acid (Figures 2-5). Chromatograms showed identical retention times (6.5 minutes for methyl decanoate and 7.8 minutes for methyl dodecanoate), showing a piece of evidence that the derivatization step had occurred successfully.

Figure 2: Chromatogram of FAME derivatized from decanoic acid, tR: 6.512.

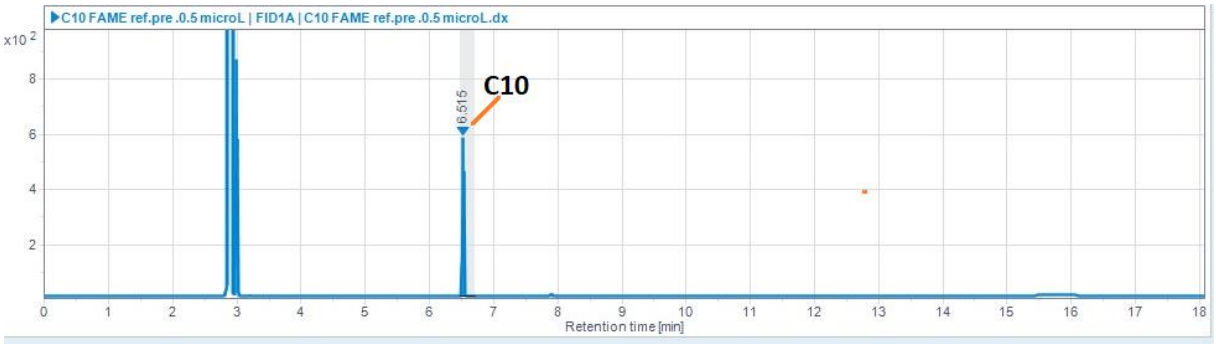


Figure 3: Chromatogram of pre-synthesized methyl decanoate, tR: 6.515.

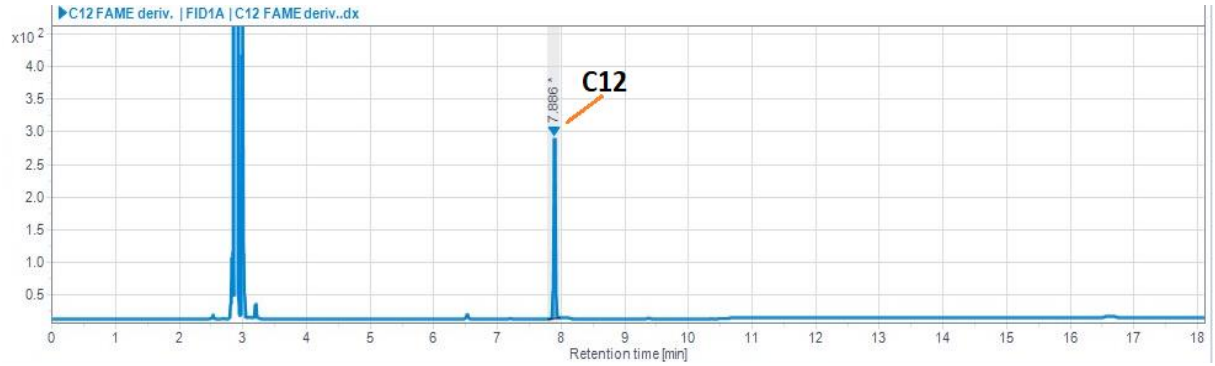


Figure 4: Chromatogram of FAME derivatized from dodecanoic acid, tR: 7.886.

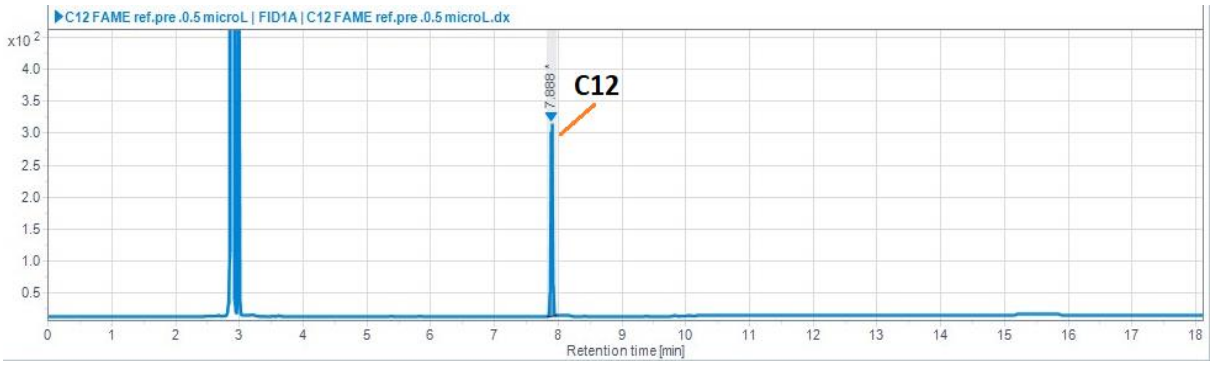


Figure 5: Chromatogram of pre-synthesized methyl dodecanoate, tR: 7.888.

The efficiency of the derivatization step was evaluated for dodecanoic acid according to the method mentioned in 3.3.2. Figure 6 shows the peak areas obtained from the injection of the FAME derivatized from dodecanoic acid and the injection of methyl dodecanoate, which were found to be 32492 a.u. and 32511 a.u; respectively, with peak ratio 99.94%. This relatively quantified yield percentage could indicate good efficiency of the derivatization method.

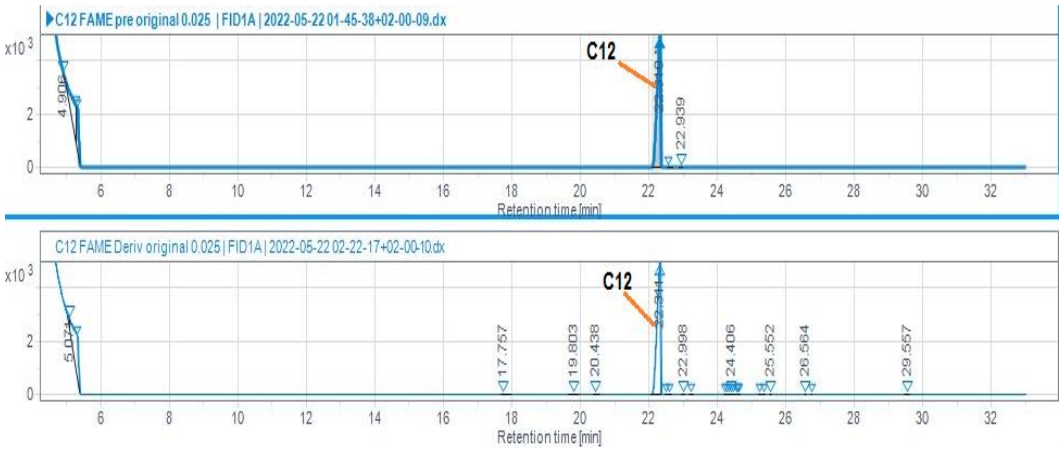


Figure 6: Chromatograms of methyl dodecanoate (top), and FAME derivatized from dodecanoic acid (bottom).

The reactivity of derivatization step was also tested to be working successfully for the rest of the FA´s (C: 8, 9, 14, 16, and 17), see Figures 17-20 in Appendix 8.2 for chromatograms. The efficiency was not evaluated for each individual FA. However, the evaluated high yield for dodecanoic acid (99.94%) may indicate good efficiency for the rest of the FA´s since they were derivatized by using the same method and conditions. Table 9 in Appendix 8.1 presents gravimetric data from derivatizations of each FA individually. Derivatizations gave yields that varied between 22% and 94% (w/w). It was found that the yield was higher for longer chain FAME´s (95% for C>10). The yield obtained from C: 10, 9, and 8, was 89%, 88% and 83 %; respectively, while C6 FAME had the lowest yield with 22%. The low yield of shorter chain FAME´s can be due to their high volatility, which could be a reason to loss of yield during evaporating the solvents.

4.2 Identification of unknown fatty acids in the herbicide

The FAME´s in the herbicide sample could be identified by comparing tR with chromatograms obtained from individual injection of each FAME, the standard FAME´s mixture and Supelco 37 component FAME´s mixture (see Appendix 8.2; Figures 17-21).

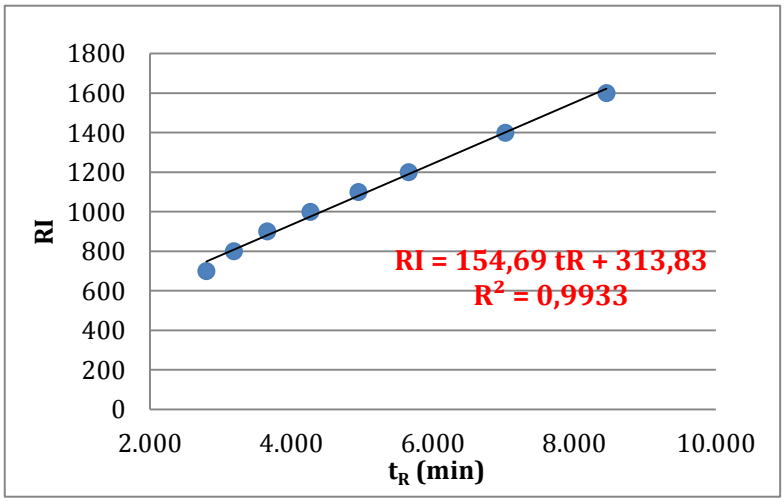


Figure 7: Plot of retention index (RI) of alkanes (n: 7, 8, 9, 10, 11, 12, 14, and 16) against their retention time (tR).

RI´s of alkanes n: 7, 8, 9, 10, 11, 12, 14, and 16 calculated from Equation (1) were plotted against tR, (Figure 7). The equation of the obtained linear function was used to calculate tR for alkanes: C13, C15, and C17. RI of each FAME in the standard solution was calculated using Equation (3). However, the tR of the FAME´s were used instead of t`R in order to

simplify handling the data. This approach was chosen after comparing the RI´s the pre-synthesized C10 and C12 FAME´s, calculated by using the fitted mode, with their RI´s in the literature and the database of National Institute of Standards and Technology (NIST).[22,23] The calculated RI of each FAME in the standard solution were also compared to its RI in the data bases, Table 1. The comparisons showed minor shifts in RI´s values indicating that using tR instead of t`R would not affect the results in a misleading way. The peaks of FAME´s in the herbicide were also confirmed by their RI´s on column DB-5 which was used in the quantification analysis, (Table 1).

Table 1: Retention indices of FAME´s in the standard solution, and FAME´s identified in samples extracted from the herbicide. The results are from GC-FID for column Hp-5 which was used to identify FAME´s and for column DB-5 which was used in quantification. C is the carbon number of the FAME excluding the methyl carbon. C8* stands for carbon number in methyl, 2-mehyloctanoate, excluding the methyl ester and the branched γ-methyl.

C	Calculated RI of FAMEs mixture	Calculated RI of FAME´s from herbicide HP-5 column	Calculated RI of FAME´s from herbicide on DB-5 column	RI from NIST Database
6	919	-	-	924
8	1123	1118	1128	1128
9	1222	1223	1248	1227
10	1313	1329	1343	1328
12	1521	_	_	1527
14	1753	_	_	1726
16	1957	_	_	1926
8*	_	1159	1171	1154

The calculated RI´s of the FAME´s were plotted against their tR and the relationship was fitted using a linear function, (Figure 8). The fit gave a good description of the data points with R^2 = 0.999. A linear function was chosen for this fit over a second or a third degree polynomial function since the range of FA´s content in the herbicide was expected to vary between SCFA´s and MCFA´s; using a polynomial function in this case could lead to over-fitting the data and hence less accurate results. The equation obtained from the linear function was then used to calculate the RI of unknown FAME´s in the sample directly from tR.

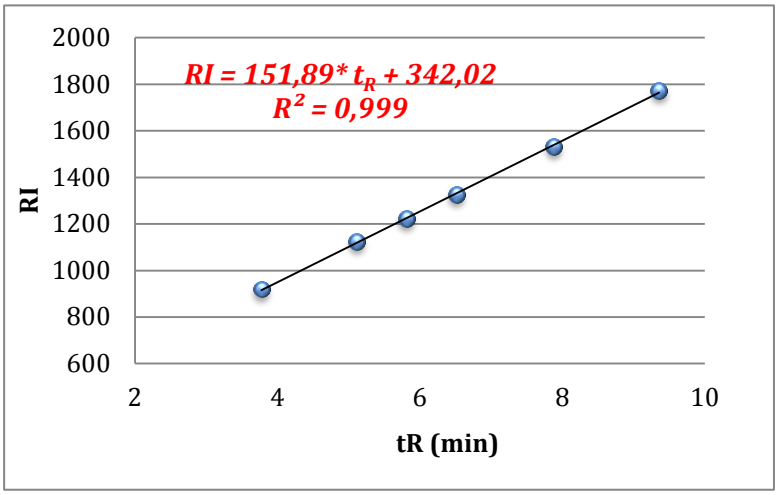


Figure 8: Plot of retention indices (RI) of FAME´s (C: 6, 8, 9, 10, 12, and 14) against their retention time (tR).

The validity of the previous fit could be confirmed by the data obtained after plotting the RI´s of the FAME´s against C, excluding the methyl carbon. Figure 9 shows that a linear relationship was also obtained with R^2= 0.999; RI´s of FAME´s with higher carbon chain; e.g. C≥14, were still in the linear range. By comparing the RI´s of the analytes in the herbicide that were obtained from the last two fits with the databases, it could be confirmed that samples prepared from the herbicide contained C: 8, 9, and 10 FAME´s. The peaks of C: 8, 9, and 10 FAME´s in the herbicide samples, Figure (10), could also be identified by comparing tR with the chromatogram of the standard FAME´s mixtures.

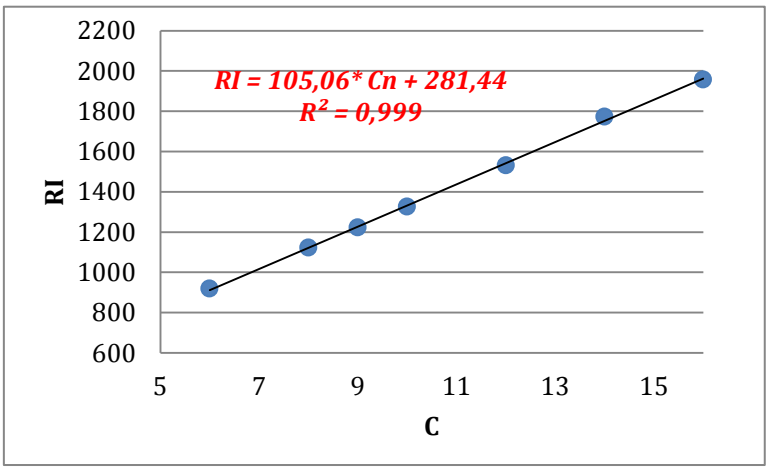


Figure 9: Plot of retention indices (RI) of FAME´s (C: 6, 8, 9, 10, 12, 14 and 16) against their carbon number (C), excluding the methyl carbon.

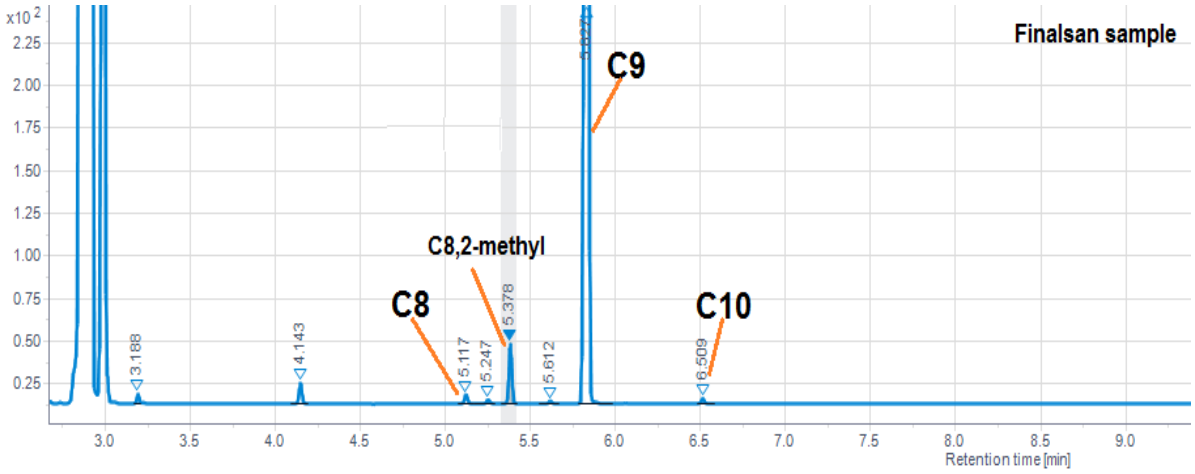


Figure 10: Chromatogram of FAME´s from the herbicide, column HP-5. Analytes identified are: Methyl octanoate (C8), methyl pelargonate (C9), methyl decanoate (C10) and methyl, 2-methyloctanoate (C8, 2-methyl).

The analyte eluted at tR= 5.378, (Figure 10), was found to have the second largest peak area in the sample after C9 FAME. The calculated RI of the analyte using the RI fitted model was C=8.5 and the anlayte could not be identified by GC-FID. When the herbicide sample was analyzed on the GC-MS, the mass spectrum of the analyte showed molecular ion of m/z=172. The mass spectrum showed also a fragment ion of m/z= 88 which could be formed as a result of a Mclafferty rearrangement (Figure 11).[24] The identification of the analyte was confirmed to be methyl, 2-methyloctanoate, when compared with spectra database in NIST library (Figure 12). The peaks of FAME´s in the herbicide were also identified by comparing the mass spectra with NIST library, (Figures 13-16).

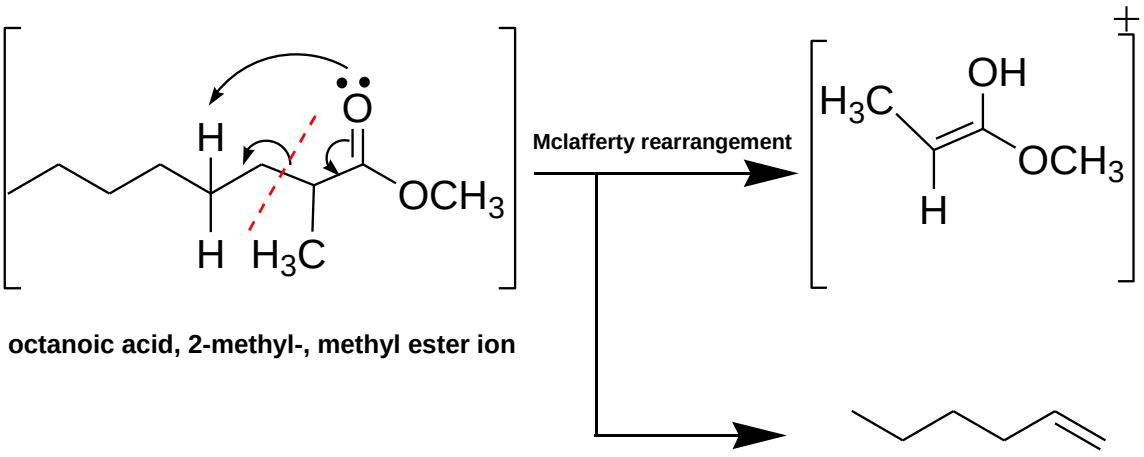


Figure 11: Formation of the Mclafferty ion in the fragmentation of methyl,2-methyl octanoate.

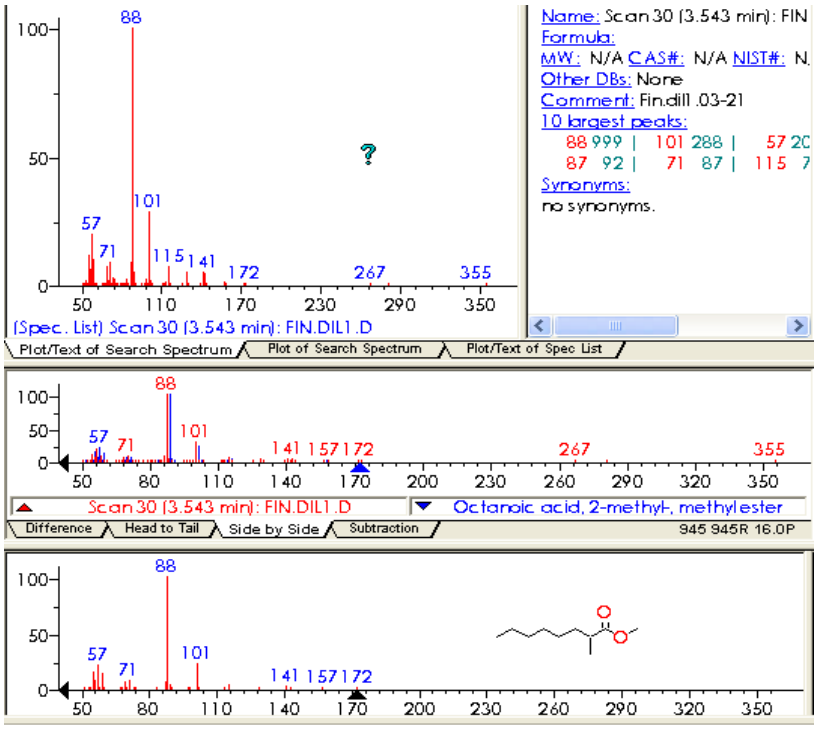


Figure 12: Mass spectra of methyl, 2-methyl octanoate. Spectrum of the analyte (top), spectrum from NIST library (bottom), comparison between the two spectra (middle).

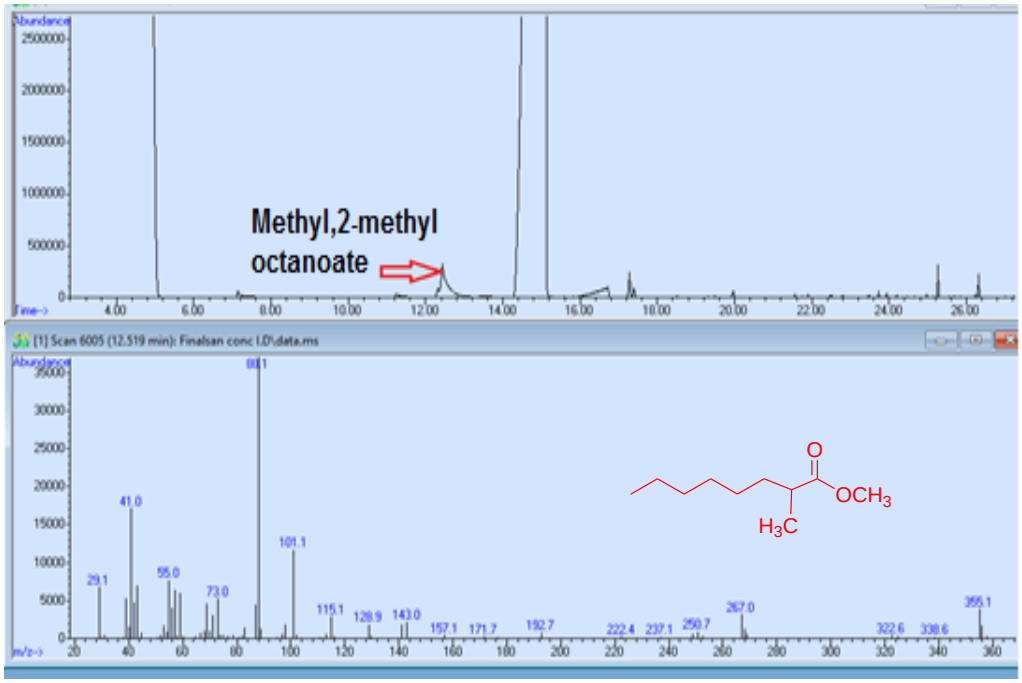


Figure 13: Chromatogram and mass spectrum of methyl,2-methyl octanoate in the herbicide sample.

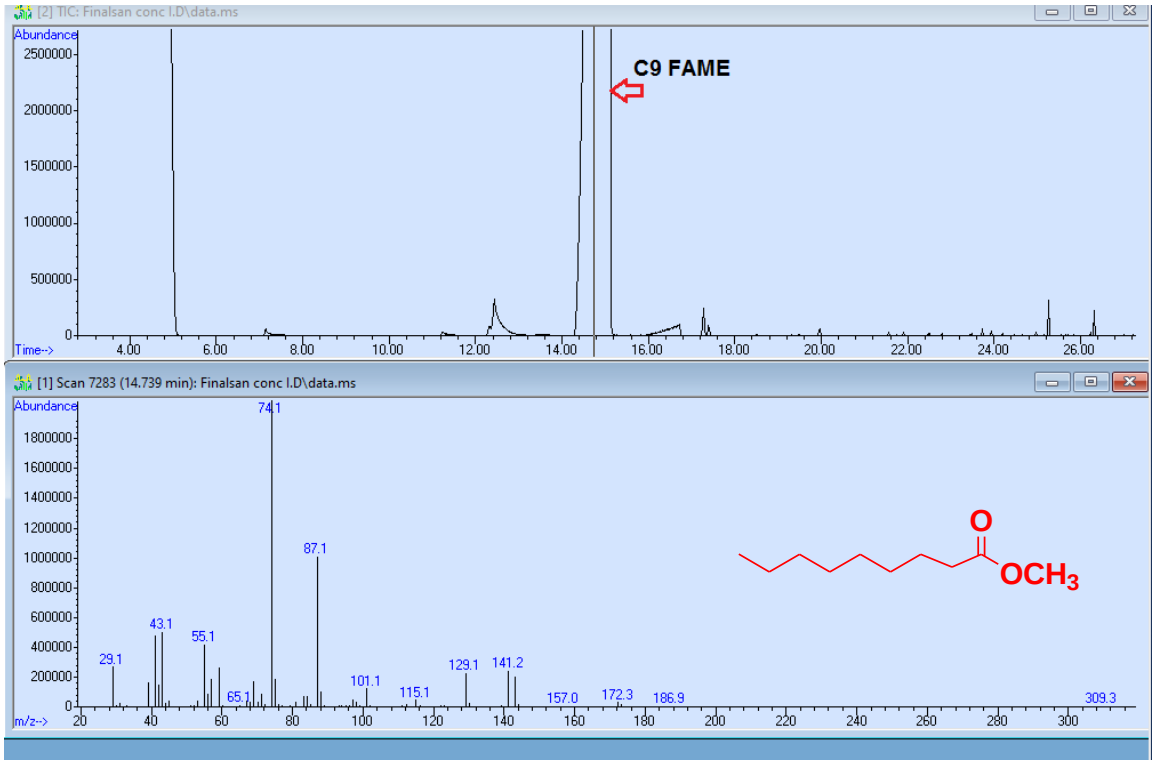


Figure 14: Chromatogram and mass spectrum of methyl pelargonate, (C9) FAME, in the herbicide sample.

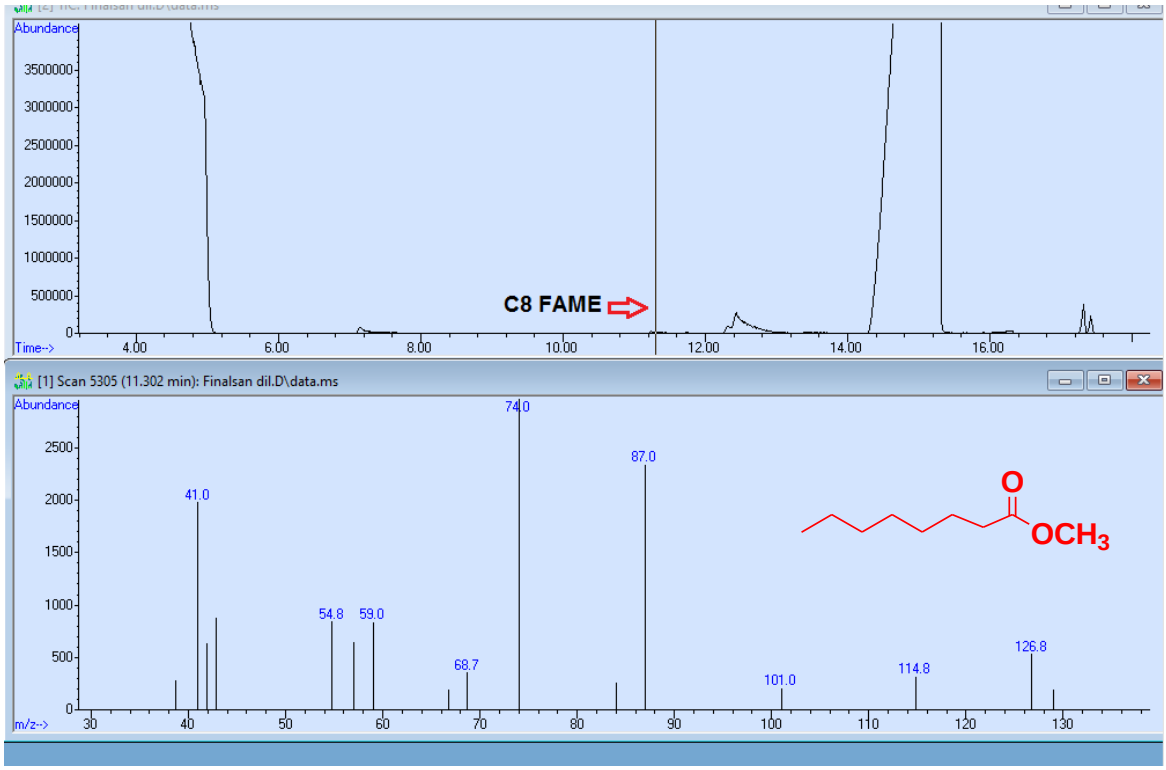


Figure 15: Chromatogram and mass spectrum of methyl octanoate, (C8) FAME, in the herbicide sample.

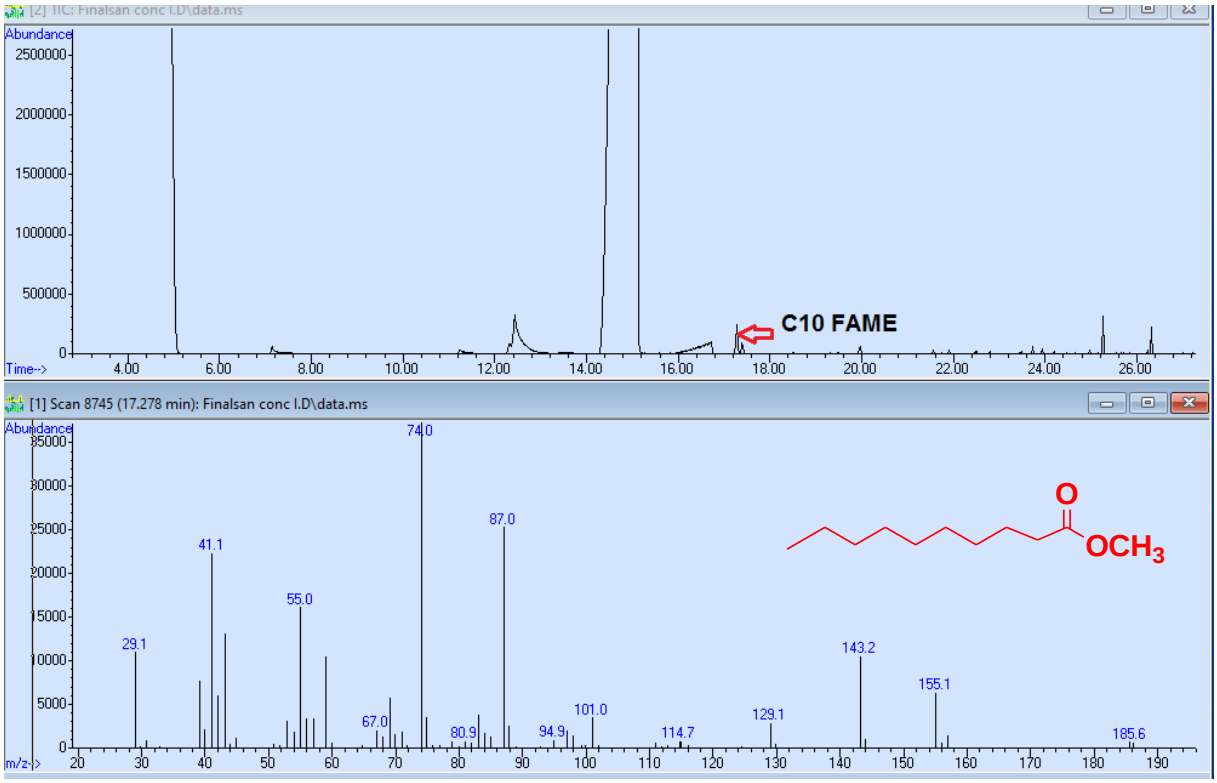


Figure 16: Chromatogram and mass spectrum of methyl decanoate, (C10) FAME, in the herbicide sample.

4.3 Supercritical fluid extraction of blank and spiked soil samples

The soils were collected from nature reserve locations mentioned in 3.3.4. Choosing nature conservation areas was in order to minimize contamination in the samples and to avoid soils that might have been pretreated with herbicides or chemical products that contain any of the FA´s in question in this study. Extraction of blank and spiked soil with the herbicide was performed on SFE system using a mixture of SC-CO_2 and MeOH as extraction solvent in an attempt to make the analysis as a green as possible. The volume of extraction solvent consisted of 90% SC-CO_2 and 10% MeOH as co-solvent; extractions were performed according to the method mentioned in 3.3.5 which was adapted from reference [25]; see (Table 11, Appendix 8.1) for the gravimetric data of extracts and derivatization yields. Using 10% MeOH as co-solvent was thought to be a good approach since it would increase the polarity, hence the solubility of the extraction solvent, enhancing the extraction of the MCFA´s of the herbicide. One more reason for making extraction conditions more suitable

for SCFA´s was to get as good information as possible about SCFA´s content in the soil to decide which FA to exclude when choosing a suitable IS.

FA´s of the herbicide; i.e. octanoic acid, pelargonic acid, 2-methyl octanoic acid, and decanoic acid, were not detected in the blank soil collected of Rya åsar and Grönbo. This may indicate that MCFA´s of the herbicide, detected in the spiked samples from these two soils, are originated from the herbicide. In contrary to that, pelargonic acid was detected in the blank soil collected from Rövarekulan with an estimated content of 0.455 mg/ g soil, which may indicate that the access of pelargonic acid content detected in the spiked soil could be originated from the spiking process. Moreover, neither of the other FA´s content of the herbicide was detected in the blank soil from Rövarekulan, which again raises the hypothesis that those detected FA´s in the soil originated from the herbicide.

A complete evaluation of the SFE extraction process by estimating the recovery for all the FA´s of the herbicide in the soil samples could not be performed. This was due to that the herbicide was in liquid form and its FA´s content could not be extracted by using the SFE system. Recoveries in the spiked samples were estimated only for pelargonic acid since it was the only FA with known concentration, (see further explanations, Appendix 8.4.5). The concentration of pelargonic acid used as a reference was the concentration reported in the product label and in the safety data sheet of the herbicide (28 mg/mL).[26] However, the recovery assay of pelargonic acid might be used as an indicator to predict recoveries of the other FA´s of the herbicide as well. This hypothesis could be supported by the close convergence in physical and chemical characteristics between pelargonic acid and the other FA´s in question, particularly, the hydrophobic characteristics of the analytes. Where LogP values of octanoic acid as the least hydrophobic FA in the herbicide and decanoic acid as the most hydrophobic one are 3.050 and 4.040, respectively, while pelargonic acid lies in between with LogP =3.420.[27]

Recoveries; i.e. relative bias due to analyte loss, of pelargonic acid were evaluated in samples spiked with the eight different concentrations of the herbicide, Table 2. The resulted showed a large variation range in the recoveries of pelargonic with R% varied between 8.2% and 128.6%, where the R% of 5 samples lied outside the acceptable range; i.e. 70>R %> 105. Recoveries of pelargonic acid in four samples were estimated to be below the acceptable range with R% values: 8, 22, 62 and 66. R% of pelargonic acid in Rövarekulan soil spiked with 0.4 mL herbicide/g soil was only 79%, although pelargonic acid was detected in the

blank soil (0.455 mg/ g soil), which may rise the hypothesis of pelargonic acid loss in the spiked soils. In contrast, Grönbo soil spiked with 0.017 mL herbicide/g soil was found to have enhancement in the recovery of pelargonic acid with 129 R%, although pelargonic acid was not detected in the blank soil.

Table 2: Pelargonic acid content in the spiked soil samples reported as mass in (mg/g soil), recovery reported as R in (%). *(T)* stands for triplicate injections; the content is reported as the mean value in (mg/g soil). (*) refers to that pelargonic acid was detected in the blank soil collected from Rövarekulan (0.455 mg/ g soil), and it was included in the recovery calculation according to Equation 7.

Volume of herbicide Spiked (mL/g soil)	**Mass of Pelargonic acid (mg/ g soil)**	**Recovery of Pelargonic acid (%)**
0.017	0.600	129
0.033^{T} (triblicate injection)	0.937 ± 0.002	100 ± 0.2
0.067^{T}	0.411	22
0.13	0.306	8
0.20^{T} (triblicate injection)	3.710 ± 0.011	66 ± 0.2
0.27	5.920	79
0.33^{T} (triblicate injection)	5.760 ± 0.019	62 ± 0.2
0.4	9.166*	79*

In SFE, recovery is a quantitative estimation for the extraction process, including extraction of the analyte from the matrix and then carrying it to the collecting point. This process can be influenced by number of factors such as volume and composition of the extraction solvent,

time, pressure, instrumentation, and most importantly breaking the bonds between the analyte and the matrix and subsequently move the analytes out of the matrix.[28] Performing the extractions by using the same solvent volume, time, and pressure decrease the influence of those parameters as reasons for the large variation range that was noticed in the recoveries of pelargonic acid where R% ranged between 8% and 129 %. Moreover, using an automated SFE system minimizes the hypothesis that systematic errors could be resulted from instrumentation. Therefore, breaking the bonds between the analyte and the soil, and then move it out of the soil particles into the extraction solvent could be the step that had the biggest impact to produce such low and diverse recoveries. This step is not only dependent on the solubility of the FA´s in the extraction solvent but also on moving the analytes out of the matrix i.e. diffusivity of the FA´s from the soil particles into the extraction solvent, and on the overall composition of the matrix.

Solubility of SC-CO_2 as an extraction solvent depends on the overall polarity of the solvent, which is regulated by the amount of co-solvent. The poor extraction recoveries may indicate that using 10% MeOH as co-solvent was not a satisfactory approach where it might have increased the polarity to a higher level than optimal conditions required for the extraction of SCFA´s and MCFA´s. In addition to that, the influence of the matrix composition might also have affected the solubility of the extraction solvent in terms of the degree of moisture in the soil. Variations in the amount of water that might retain in the soil after drying could lead to variations in the overall polarity of the extraction medium in the vessel, hence variations in the solubility power of the extraction solvent. Moreover, GC-FID analysis of the blank soils showed that the main FA´s content in the soils is quite hydrophobic long chain FA´s (Appendix 8.2, Figures 26, 30, 32). The great hydrophobic characteristics of the soils could be a reason for lowering the overall yield of the extracted FA´s since the impact of increasing the polarity of the extraction solvent could be higher on lowering the extraction yield of more hydrophobic analytes; i.e. longer chain FA´s.

One more parameter that could play a role in controlling the extraction process is the particle size of the soil. Under a given extraction time, the SFE extraction rate is a function of mass transfer kinetics which is controlled by either the convective mass transfer between the soil material surface and the fluid phase or the diffusion of the analyte in the soil particle, or both. In addition to that, the particle size of the matrix determines the amount of difficult–to–access and easy to access solute in the solid phase; i.e. amount of extracted and non-extracted FA´s from the soil.[29] Using 177-micron sieve could have led to that all particles of diameter smaller than 177-micron would pass through the sieve, which might lead to different extents of

diversity in the particle sizes from one sample to another, and subsequently lead to differences in the extraction rates.

In summary, the obtained inadequate and inconsistent recovery for pelargonic acid, together with the pre-discussed errors that affected the extraction process, limited the ability of estimating the variations within the extraction process. Such limitation decreased the trueness and the overall reliability of the method, since the extraction process is the key step in sample preparation that gives the most significant variation in an analytical performance.[30]

4.4 Evaluation of the analysis performance

A comparison between the calibration curves obtained from the internal calibration and calibration curves obtained from external calibration that both plots had quite close R^2 values with a little advantage in the interest of the internal standard calibration method for FA´s with carbon chain length: C≤10 (Figures 37,38, and 39, Appendix 8.3). Moreover, the peak areas of methyl decanoate showed constant response with RSD = 3.9%. However, the poor linearity of the models makes the performance of methyl decanoate, as a good IS to quantify the FA´s under investigation to be questionable, since linearity plays the most important role to evaluate the performance of a given IS.[31]

The linearity of the calibration curves increased for the longer chain FAME´s; this could be due to the lower volatility, which minimizes the loss in the derivatization yield even at lower concentrations. The calibration curves of FAME´s obtained from plotting the peak area ratios against the concentration ratios showed curves with had R^2 >0.99 at the concentration range 1.5- 600µg/mL, (Table 3). Although R^2 >0.99 may indicate acceptable linearity, it was found that better linearity could be achieved in similar studies with $R^2 \geq 0.999$.[32,33] Calibration curves of C8 and C9 were obtained using only 3 and 4 data points, respectively, due to lack of data points in the working range for C8 and a deviated point in the case of C9, (see Appendix 8.3, Figures 37-42 for calibration curves). The low linearity of the obtained calibration curves, especially for the shorter FA´s C8 and C9, could be explained by the fact that the calibration curves were constructed using FAME´s prepared by the derivatization of the FA´s and not by using pre-synthesized standards FAME´s. Although the derivatization step showed high efficiency of 99% yield in case of dodecanoic acid as shown in section 4.1, the preparation steps still might be subjected to errors during sample preparation. Since visual evaluation was not sufficient to prove the linearity of the curves, the fitted models were tested statistically by regression analysis. Using Microsoft Excel and Minitab software, Analysis of Variance

(ANOVA) output for the models showed significant statistical linear relationships (*p-value of* $F < 0.05$).[34]

Table 3: The table presents the internal standard calibration parameters of the FAME´s in terms of the regression equation and linearity of the calibration curve. The linearity is reported as the coefficient of determination (R^2). C is the number of carbon atoms in the FAME, excluding the methyl carbon.

FAME C	Regression Equation	Coefficient of determination
C8	y = 0,4293x - 0,013	0.996
C9	y = 0.8471x - 0.0105	0.992
C10	y = 1.2734x + 0.0007	0.993
C14	y = 1.5094x + 0.0094	0.995
C16	y = 1,4251x + 0,0173	0.996
C17	y = 1.4456x + 0.0092	0.994

Methyl hexanoate (C6) could not be detected below 1700 µg/mL, while C8 could be detected at 25µg/mL. FAME´s with longer carbon chains (C: 9, 10, 14, 16, and 17) could be detected at 1.5 µg/mL. The slope of the calibration curves also increased as the carbon chain gets longer, which indicated better calibration sensitivity. These differences in calibration sensitivity can be explained by the mechanism of the FID detector where the response is not only dependent on the concentration but also on the mass of the analytes.[35]

Table 4 presents the results obtained from repeatability tests for the concentrations of FA´s as described in 3.3.8. Results of the intra-day assay showed RSD % values varied from 0.18 to 3.54 while the inter-day assay showed %RSD values ranged between 0.01 and 2.46. The obtained results were within the acceptable range (RSD < 15%), which indicates satisfactory repeatability of the analysis process and also a good performance of the GC-FID instrument. Even though, the assay could not be used to validate the precession of the whole method since such validation requires precision measurements for concentrations of FA´s in all the replicate extractions obtained from the SFE process.

Table 4: Table shows results of repeatability tests for concentrations of FA´s from the analysis of the spiked soil samples. The tests were performed as described in 3.3.8 and repeatabilities are reported as RSD in (%).

Assay	Volume of herbicide spiked (mL/g soil)	RSD of Octanoic Acid (%)	RSD of 2-methyl octanoic acid (%)	RSD of Pelargonic acid (%)	RSD of Decanoic acid (%)
Intra-day (n=3)	0.03	0.35	0.54	0.18	1.16
	0.20	1.14	0.46	0.28	0.66
	0.33	3.54	0.53	0.33	1.60
Inter-day (n=4)	0.03	0.26	0.29	0.15	0.01
	0.20	0.78	0.34	0.22	0.43
	0.33	2.46	0.05	0.27	0.23

In addition to that, the obtained results from the repeatabilities for peak areas and tR of the FAME´s showed solid evidences for a precise performance of the instrument, Tables 5 and 6. The peak areas showed good repeatabilities where the instrument gave constant response to the four FAME´s in question with RSD% values ranged between 1.32 and 7.69, while the repeatabilities of tR showed RSD% values ranged between 0.011 and 0. 0.783.

Table 5: Repeatabilities of peak areas of FAME´s in spiked soil samples are reported as RSD in (%), (n = 3). All the reported results were collected from injections that were performed during the same day.

Volume of herbicide spiked (mL/g soil)	RSD for peak areas of Methyl octanoate (%)	RSD for peak areas of Methyl, 2-methyloctanoate (%)	RSD for peak areas of Methyl pelargonate (%)	RSD for peak areas of Methyl decanoate (%)
0.033	2.48	1.32	2.01	2.47
0.200	4.55	2.20	2.55	3.23
0.333	7.69	2.41	2.35	2.47

Table 6: Repeatability of the tR of FAME´s in the spiked soil samples. tR are reported as mean values in minutes and repeatabilities are reported as RSD in %, (n =9). The results are collected from injections that were performed during the same day.

FAME	Retention time (min)	RSD (%)
Methyl octanoate	12.040 ± 0.006	0.051
Methyl, 2-methyoctanoate	13.152 ± 0.008	0.064
Methyl pelargonate	15.103 ± 0.118	0.783
Methyl decanoate	17.788 ± 0.002	0.011
Methyl dodecanoate (IS)	22.161 ± 0.032	0.145

4.5 Quantitative analysis of the spiked soil samples

Due to pre-mentioned outcomes from the previous two sections, the performed quantitative analysis could not be considered reliable enough to obtain accurate results but rather a scanning approach to get a rough estimation of the FA´s content in the spiked samples and what to be considered in future work. Moreover, it is not guaranteed that the molecular environment of the spiked analyte would be the same as the native analyte since the molecular interactions experienced by the analyte may differ between the two environments. This difference would be expected to affect the behavior of the analyte along the different processes in a given analysis, especially sample preparation and extraction, which would eventually affect the quantification results. Quantification results of the FA´s content in the spiked soil samples are presented in Table 7.

Table 7: Summary of the herbicide is FA´s content in the spiked soil samples. Concentrations are calculated according to Equation (6) and reported as mass in (mg /g soil). ND stands for not detected. The asterisk symbol (*) refers to that signal-to-noise ratio was lower than 10. The (^{T}) symbol refers to triplicate injections of 3 aliquots of the same sample during the same day, reported as mean value in mg.

Volume of herbicide spiked (mL/g soil)	Octanoic Acid (mg /g soil)	2 Methyl, Octanoic Acid (mg /g soil)	Pelargonic Acid (mg /g soil)	Decanoic Acid (mg /g soil)
0.017	0.0271	0.0429	0.6001	0.0043*
0.033^{T}	0.0288 ± 0.0001	0.0589 ± 0.0003	0.9373 ± 0.0017	0.0065* ± 0.00003
0.067	ND	ND	0.4109	0.0072
0.130	ND	ND	0.3062	0.0091
0.200^{T}	0.0581 ± 0.0007	0.1715 ± 0.0008	3.7097 ± 0.0105	0.0168 ± 0.0001
0.276	ND	ND	5.9199	0.0262
0.330^{T}	0.0714 ± 0.0025	0.2630 ± 0.0014	5.7604 ± 0.0192	0.0295 ± 0.0004
0.400	0.0928	0.3881	9.1661	0.0412

Pelargonic acid content in the spiked samples varied between 0.600 mg and 9.166 mg per gram soil. Blank soil collected from Rövarekulan was found to contain pelargonic acid with content 0.445 mg per gram soil, which represented 4.98% of the total pelargonic acid content that was quantified in the spiked soil.

The calibration curve of C8 FAME was used for quantification of methyl, 2-methyl octanoate. Although methyl, 2-methyl octanoate has the same molecular weight as C9 FAME (172 g/mol), the calibration curve of C9 FAME was not used for quantification. This approach was chosen due to the big difference in concentration between methyl, 2-methyl octanoate and C9 FAME in the samples which might lead to misleading results, since the response of the FID detector is concentration dependent.[35] The poor recovery observed for pelargonic acid could be expected to have even bigger impact on the analytes of lower concentrations, which might be the reason that octanoic acid and 2-methyl octanoic acid were not detected in some of the

samples. 2-methyl octanoic acid showed the second highest abundance among the herbicide FA´s content in the spiked samples where it ranged between 0.0429 and 0.3881 mg/g soil. Octanoic acid was found to have the lowest concentration in the samples with concentrations ranged between 0.0271 and 0.0928 mg/g soil. Decanoic acid had the lowest concentration that was less than 0.0412 mg/g soil in all soils.

Table 8: Table presents the calculated ratios of each fatty acid per total content of the herbicide's fatty acids detected in the spiked soil samples. Ratios are reported in (%) and were only calculated for samples where all fatty acids could be detected.

Volume of herbicide Spiked (ml/g soil)	Octanoic Acid	2 Methyl Octanoic Acid	Pelargonic Acid	Decanoic Acid
0.017	3.98	6.31	89.08	0.63
0.033	2.79	5.71	90.87	0.63
0.20	1.47	4.34	93.77	0.42
0.333	1.17	4.29	94.06	0.48
0.400	0.96	4.01	94.61	0.43

It was also observed that the ratios of the herbicide's FA´s content did not have the same values in the samples extracted from the spiked soil, Table 8. The individual ratio of octanoic acid, 2methyl octanoic acid, and decanoic acid per the total content of the herbicide's FA´s were found to increase with lowering the amount of the herbicide spiked in the soil from 0.4 to 0.017 mL herbicide/g soil. Octanoic acid ratio increased from 1% to 4%; the ratio of 2-methyl octanoic acid increased from 4% to 6%; decanoic acid ratio increased from 0.43% to 0.63 %. On the other hand, pelargonic acid ratio increased with increasing the amount of herbicide spike from 89% to 95%. This dominance of pelargonic acid in samples spiked with larger amount of the herbicide may have arisen from higher molecular interaction of pelargonic acid with the extraction solvent due to more abundance, as the analyte with the highest abundance in the herbicide, and not necessarily due to better chemical properties; i.e. hydrophobicity. However, pelargonic acid and 2-methyl octanoic acid had the highest ratios in all samples, making them the most reliable analytes to detect the herbicide in sample matrices.

5 Conclusion

In conclusion, GC-FID/MS showed good efficacy in detecting FA´s in the fatty acid-based herbicide and in soils that were exposed to the herbicide, by separating and detecting the corresponding FAME´s derivatives. GC-MS system showed higher selectivity to identify FAME´s than GC-FID. The FA-based herbicide product used in the study was found to contain octanoic acid, pelargonic acid, decanoic acid, and 2-methyl octanoic acid; pelargonic acid and 2-methyl octanoic acid could be the most reliable analytes to detect exposure to the product in sample matrices. Although the low recovery levels, a certain conclusion cannot be drawn regarding using SFE with extraction solvent consist of 90% SC-CO2 and 10% MeOH (v/v) to extract FA´s from soil that was exposed to the herbicide; this area of the work may need more research. The derivatization of FA´s to FAME´s beside the extraction process, with the inclusive sample preparation steps, directly affects the reliability of the method of FA´s analysis. Low efficacy of these processes limits the method´s characterizations; e.g. sensitivity, precision, trueness, and accuracy; hence, the overall certainty of the method. Thus, the use of methods and models constructed by these techniques is conditional on the high efficacy and to be used with caution if the efficacy is questionable.

6 Future aspects

Working on developing a method to use SFE to extract FA´s from samples spiked with FA-based herbicides. The effect of co-solvent as well as temperature, pressure, extraction time on the extraction yield can be investigated. An attempt to investigate the impact of decreasing the polarity of solvent on the extraction yield was done by extracting a blank sample from Grönbo soil by the method in [36]; using 100% SC-CO_2 with pressure of 350 bar under 45 minutes dynamic time. The method resulted triplicate extractions of 0.024, 0.020, and 0.023 (g extract/ g soil), which is about 4 to 5 times as much as the extract obtained from using 10% MeOH. However, the FA´s content in theses extracts were not quantified due to lack of time. More research can also be done on other sample matrices spiked with lower concentrations than those used in this study for a better optimization to the goal of trace analysis. The identified FA´s in the herbicide can be subjected to studies with biological purposes that aim to investigate the environmental impact and track the toxicological effects of the identified FA´s on both targeted and non-targeted organisms.

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8 Appendix

8.1 Gravimetric data

Table 9: Table presents the gravimetric yield from derivatization of each FA individually. The step was performed to confirm that methylation of FA´s was successful, and to identify the retention time of each FAME individually. The last raw presents the result from the derivatization of the lipid extracted from the herbicide by LLE. FAME´s resulted from this step was used only for the identification of the FAME´s by their tR. Data reported as mass in (g), yield is reported as (w/w) percentage in (%).

	Mass of FA (g)	**Mass of the FAME´s yield (g)**	**Yield percentage, (w/w), (g FAME per g oil) (%)**
Hexanoic acid (C6)	0.051	0.011	22.20
Octanoic acid (C8)	0.046	0.038	83.08
Pelargonic acid (C 9)	0.046	0.040	88.02
Decanoic acid (C10)	0.037	0.033	88.65
Dodecanoic acid (C12)	0.041	0.040	97.57
Tetradecanoic acid (C14)	0.042	0.040	96.63
Hexadecanoic acid (C16)	0.039	0.038	96.67
Heptadecanoic acid (C17)	0.039	0.037	95.34
Herbicide sample	0.260	0.143	55.00

Table 10: Gravimetric data of FA´s in the standard solution mixture that was derivatized to FAME´s mixture; the obtained FAME´s mixture was used to construct the retention indices models. Masses of the FA´s are reported in (g).

FA	**Mass of FA (g)**
Hexanoic acid (C: 6)	0.067
Octanoic acid (C: 8)	0.064
Pelargonic acid (C: 9)	0.057
Decanoic acid (C:10)	0.050
Dodecanoic acid (C:12)	0.041
Tetradecanoic acid (C:14)	0.028

Hexadecanoic acid (C: 16)	0.025

Table 11: Gravimetric data of the extractions and the derivatization yield from blank soils and soils spiked with the herbicide. The extractions were performed on Waters SFE system model (ASFE, MV-10), using extraction solvent consists of 10% MeOH and 90% and 90% SC-CO_2 (v/v), according to the method described in 3.3.5. The masses of the extracts and derivatization yields are reported in (g); the yield percentages are reported as (weight/weight) percentage in (%).

Soil	Volume of spiked herbicide (mL/g soil)	Mass of the extract (g)	Mass of derivatization yield (g)	Yield Percentage (w/w) (%)
Grönbo Blank		0.0050	0.0020	39.92
Grönbo	0.017	0.0041	0.0031	75.61
Grönbo	0.033	0.0071	0.0060	84.51
Grönbo	0.067	0.0303	0.0057	18.81
Grönbo	0.130	0.0350	0.0230	65.71
Grönbo	0.276	0.0269	0.0190	70.63
Grönbo	0.333	0.0242	0.0246	101.65
Rya åsar Blank		0.0044	0.0040	90.91
Rya åsar	0.200	0.0172	0.0178	103.49
Rövarekulan Blank		0.0090	0.0041	45.56
Rövarekulan	0.400	0.0410	0.0339	82.68

8.2 **Additional results regarding the qualitative analysis**

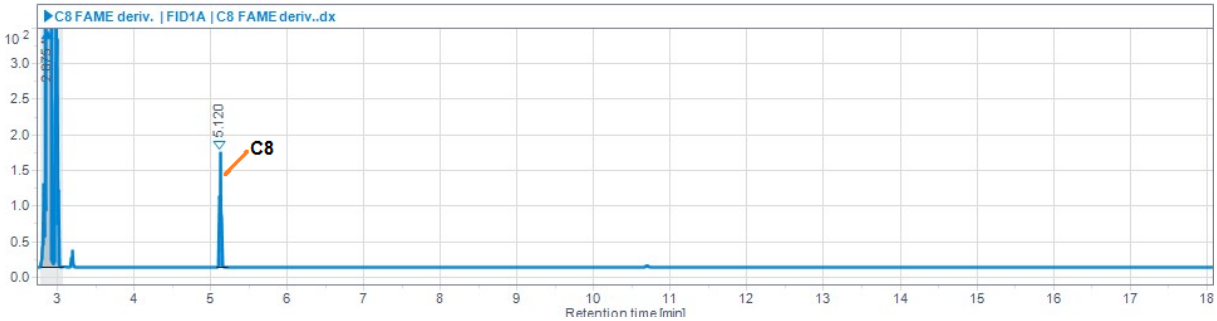

Figure 17: Chromatogram of FAME derivatized from octanoic acid; HP-5 column.

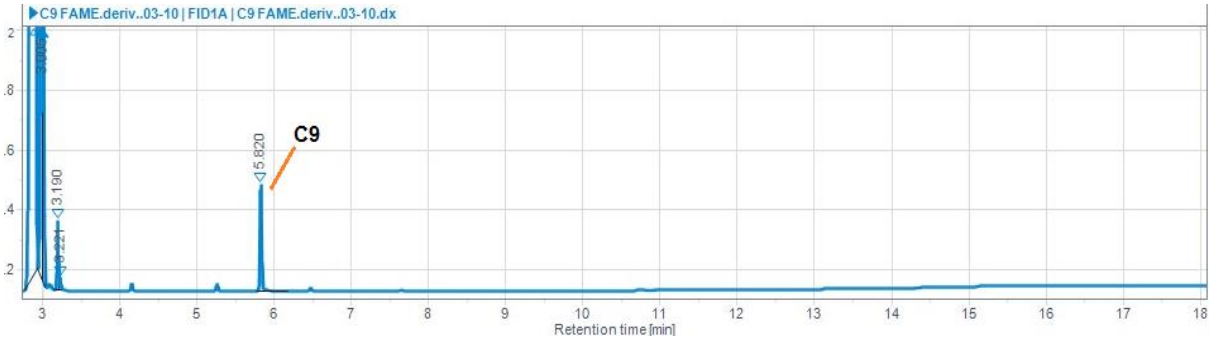

Figure 18: Chromatogram of FAME derivatized from pelargonic acid; HP-5 column.

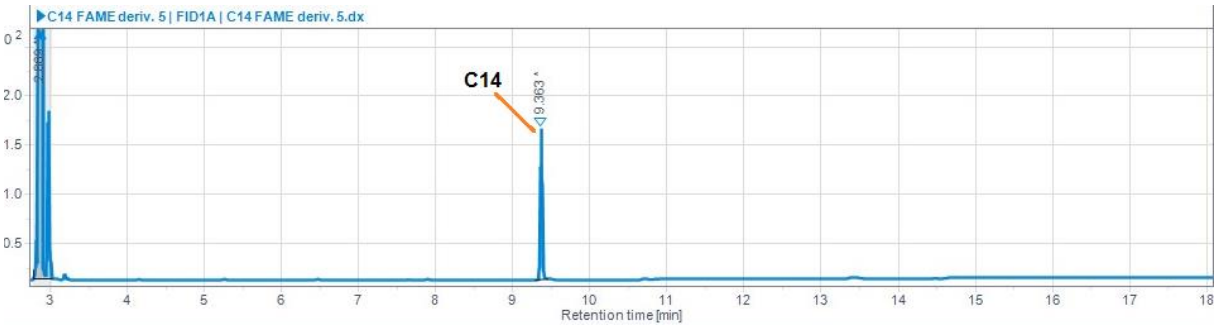

Figure 19: Chromatogram of FAME derivatized from tetradecanoic acid; HP-5 column.

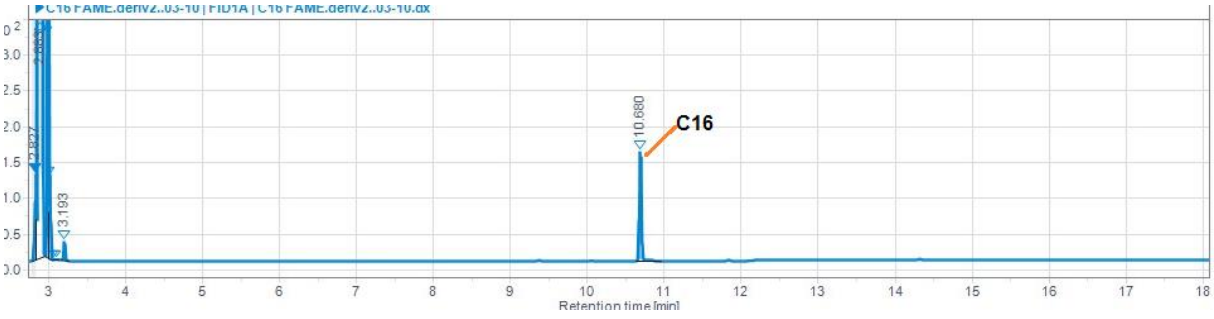

Figure 20: Chromatogram of FAME derivatized from hexadecanoic acid; HP-5 column.

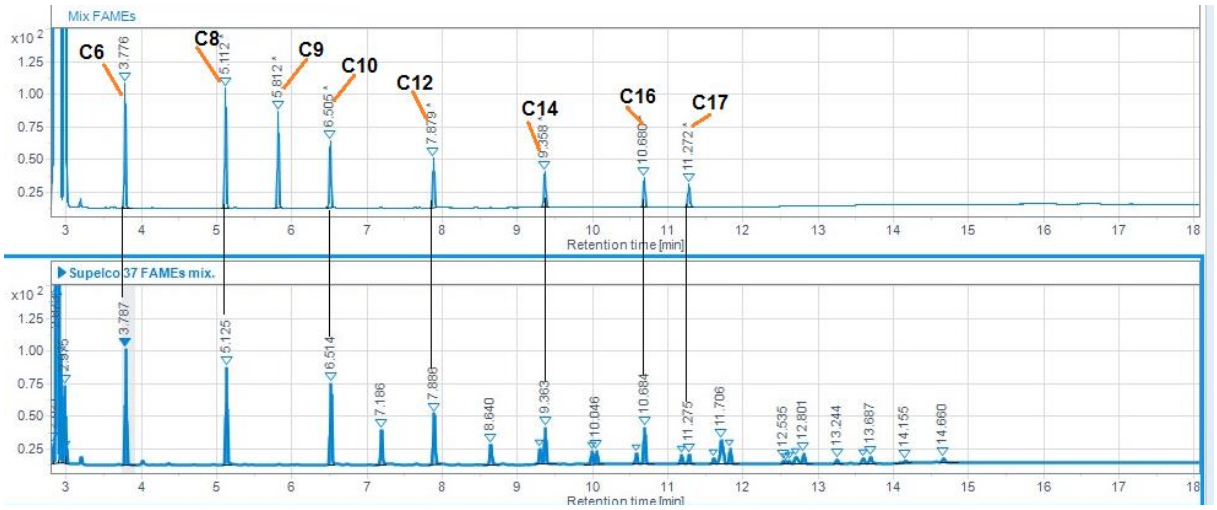

Figure 21: Chromatogram of FAME´s mixture C:6,8,9,10,12,14,16 and 17 (above), and supleco 37 component FAME´s mixture (below); HP-5 column.

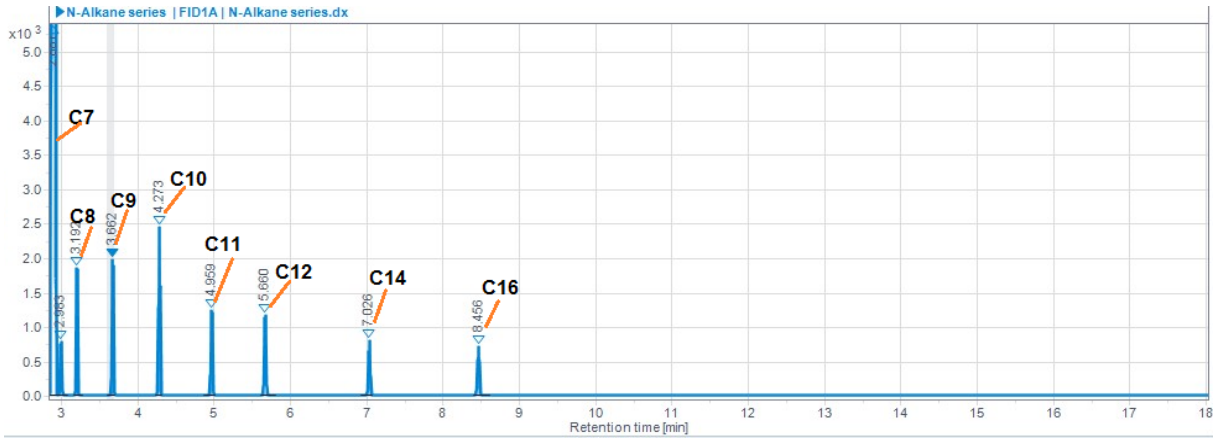

Figure 22: Chromatogram of alkane series (n: 7,8,9,10,11,12,14,16), HP-5 column.

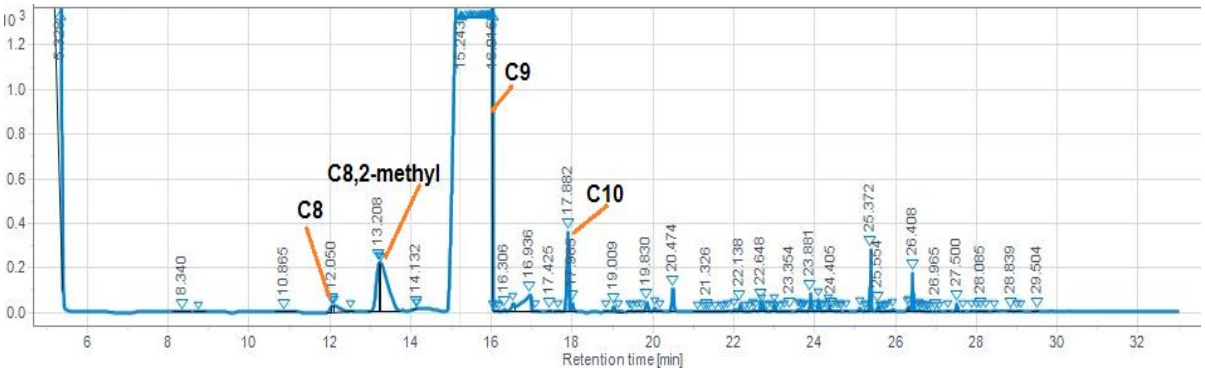

Figure 23: Chromatogram of FAME´s from the herbicide sample, DB-5 column.

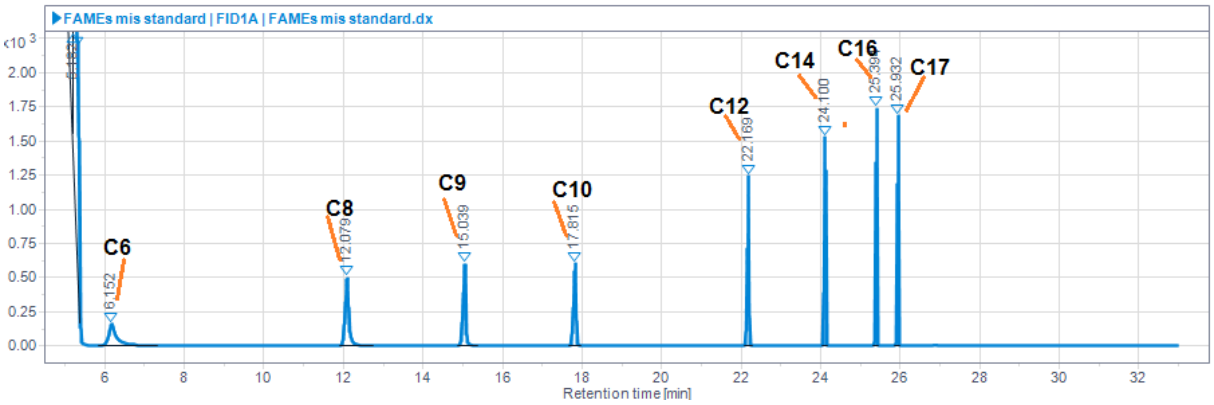

Figure 24: Chromatogram of FAME´s standard mixture (C:6,8,9,10,12,14,16, and 17), DB-5 column.

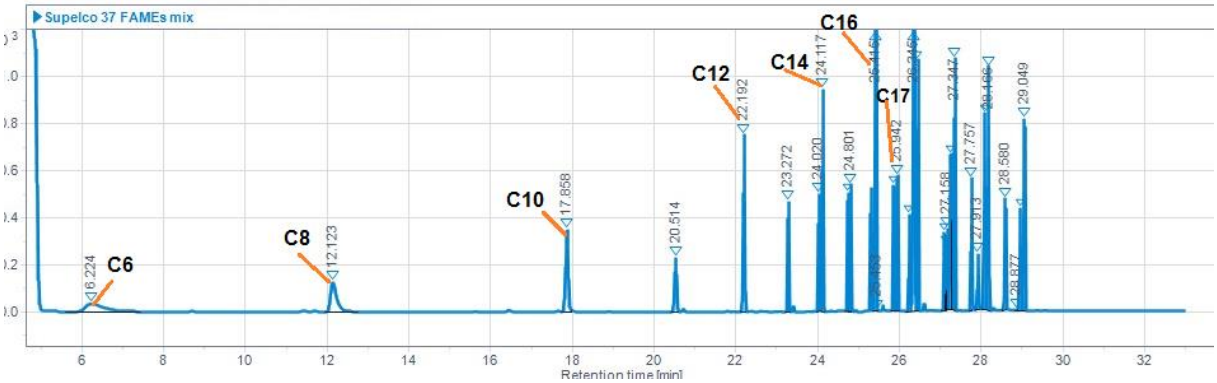

Figure 25: Chromatogram of FAME´s of Supelco 37 components mixture, DB-5 column.

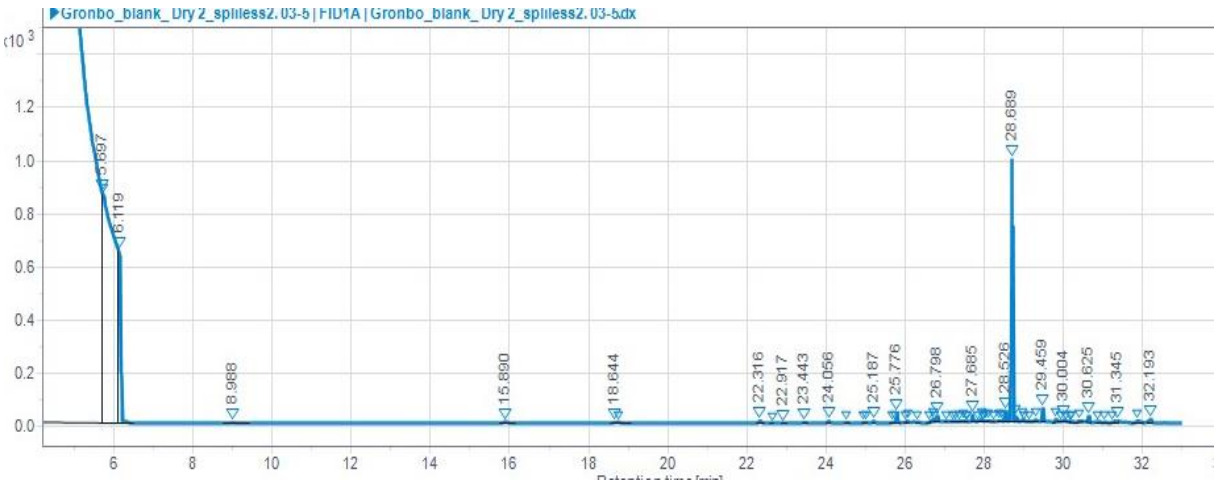

Figure 26: Chromatogram of FAME´s of blank sample from Grönbo soil, DB-5 column.

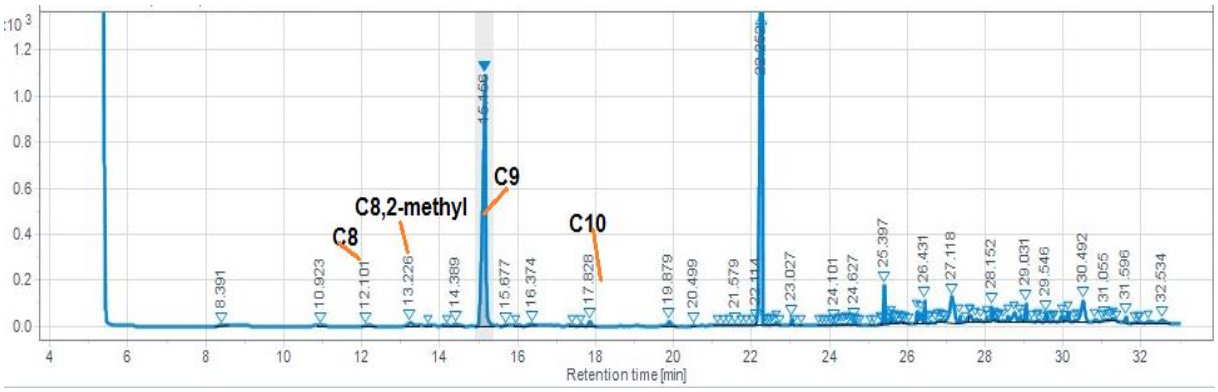

Figure 27: Chromatogram of FAME´s of Grönbo soil spiked with 0.017 mL/g soil, DB-5 column.

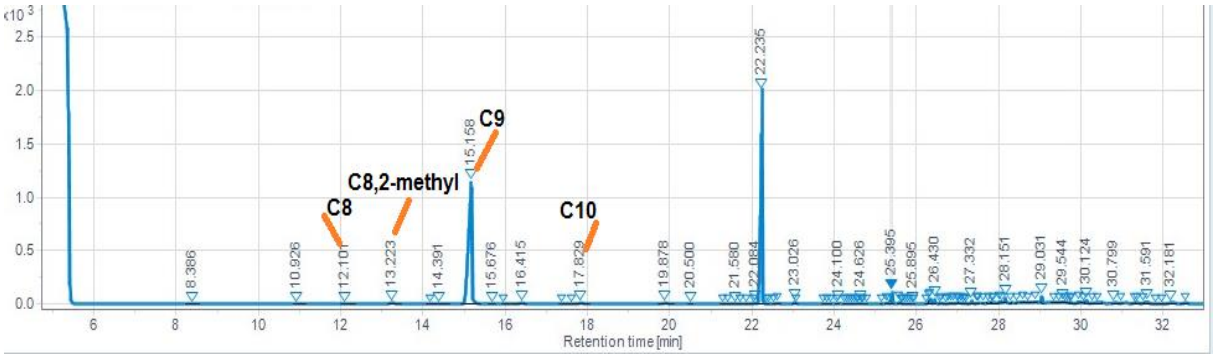

Figure 28: Chromatogram of FAME´s of Grönbo soil spiked with 0.033 mL/g soil, DB-5 column.

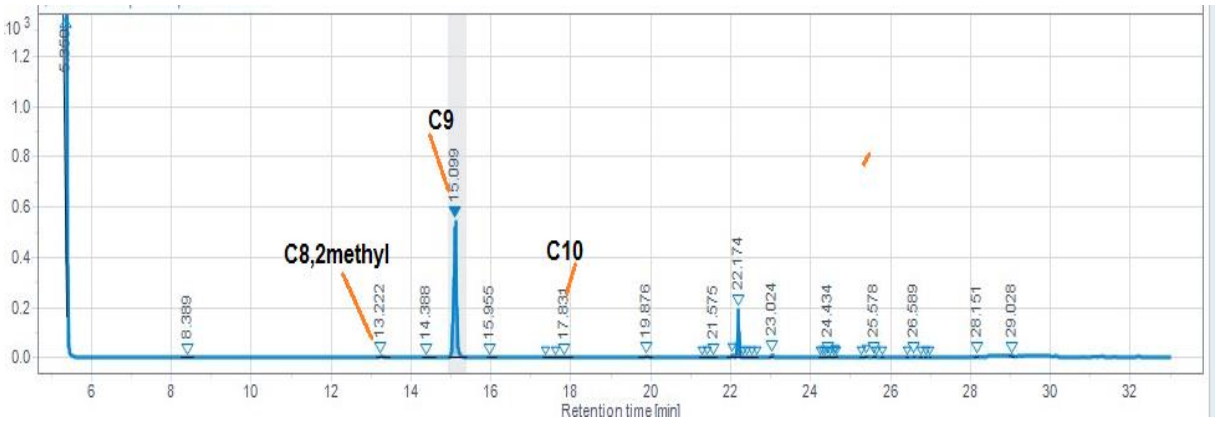

Figure 29: Chromatogram of FAME´s of Grönbo soil spiked with 0.33 mL/g soil, DB-5 column.

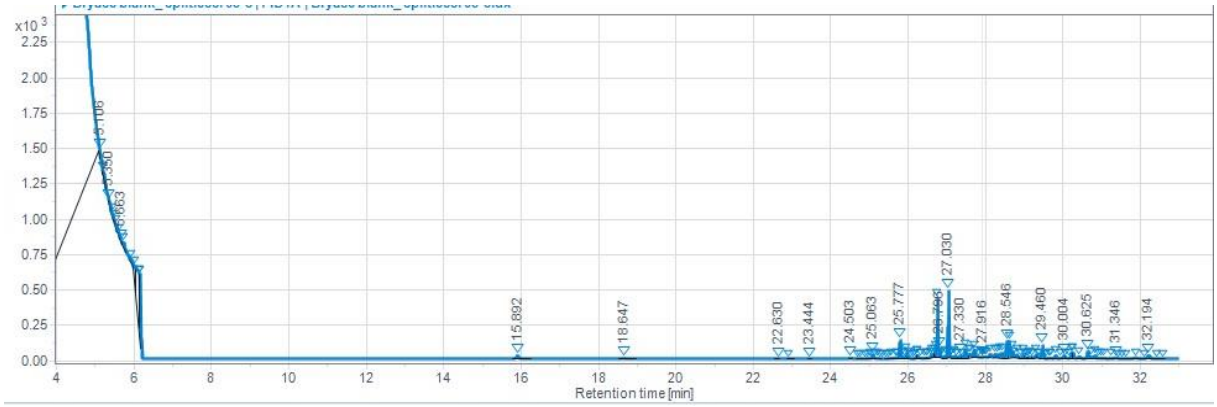

Figure 30: Chromatogram of FAME´s of blank sample form Rya åsar soil, DB-5 column.

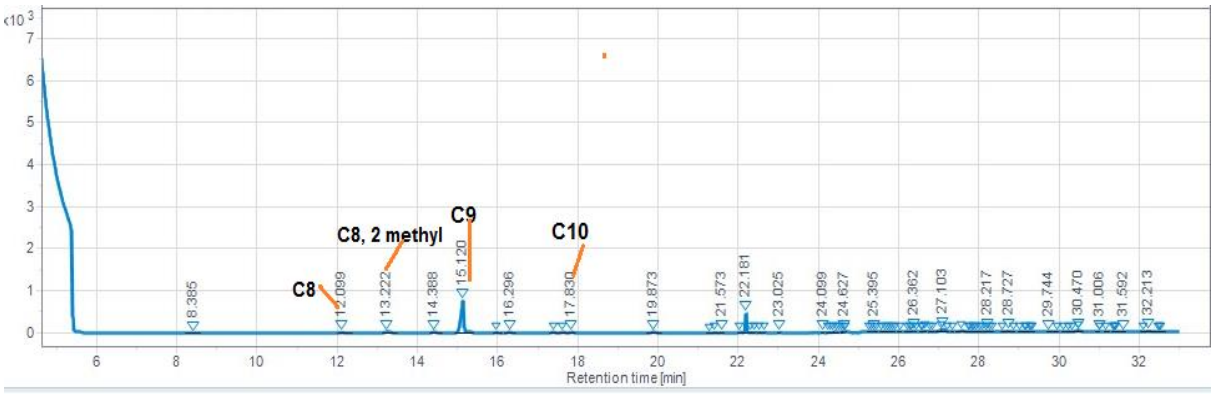

Figure 31: Chromatogram of FAME´s of Rya åsar soil spiked with 0.2 ml/g soil, DB-5 column.

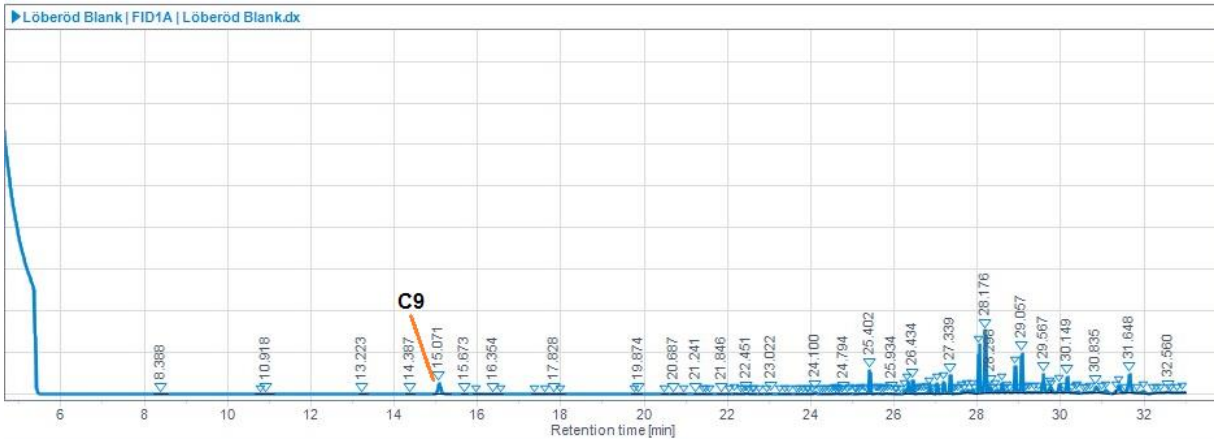

Figure 32: Chromatogram of FAME´s of balnk sample from Rövarekulan soil, DB-5 column. Pelargonic acid was detected in the soil and labeled by its corresponding FAME (C9).

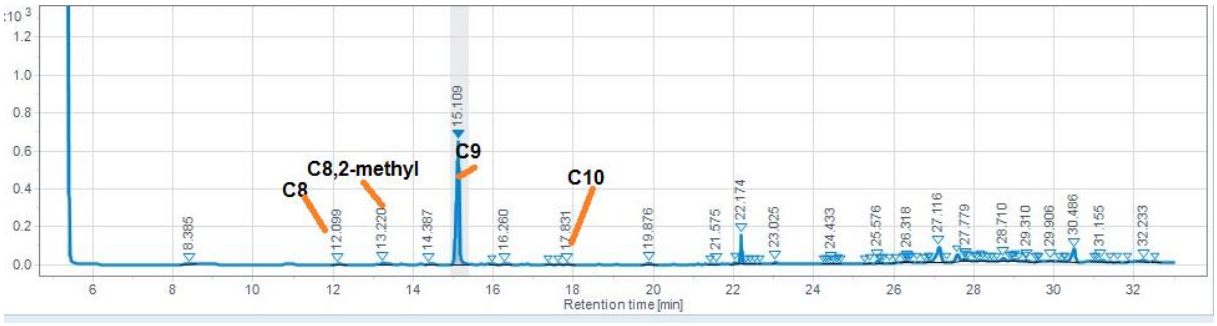

Figure 33: Chromatogram of FAME´s of Rövarekulan soil sapmle spiked with 0.4 ml/g soil, DB-5 column.

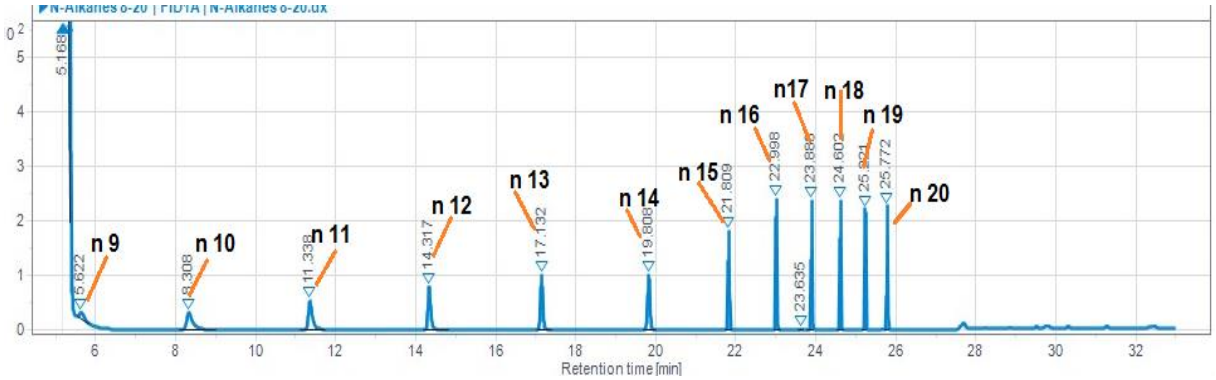

Figure 34: Chromatogram of alkane series (n: 8-20), DB-5 column.

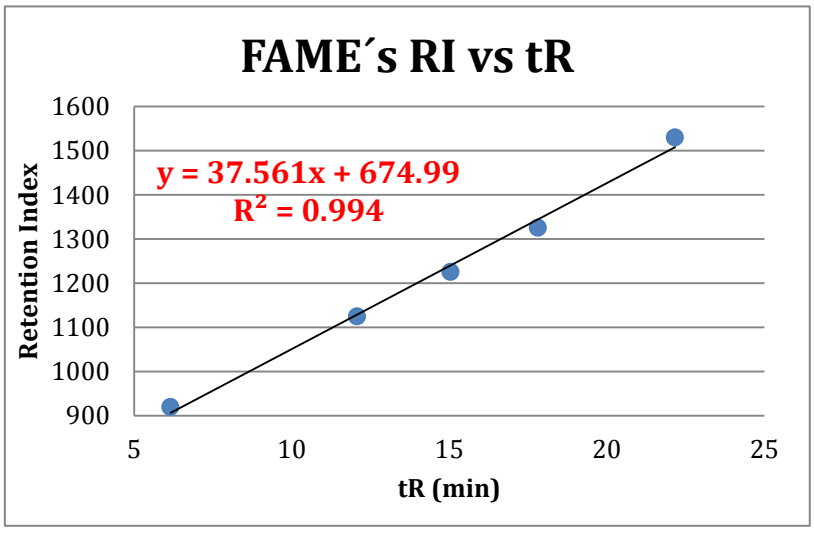

Figure 35: Plot of retention indices (RI) of the FAME´s in the standards solution mixture plotted against their retention times (tR), Column DB-5

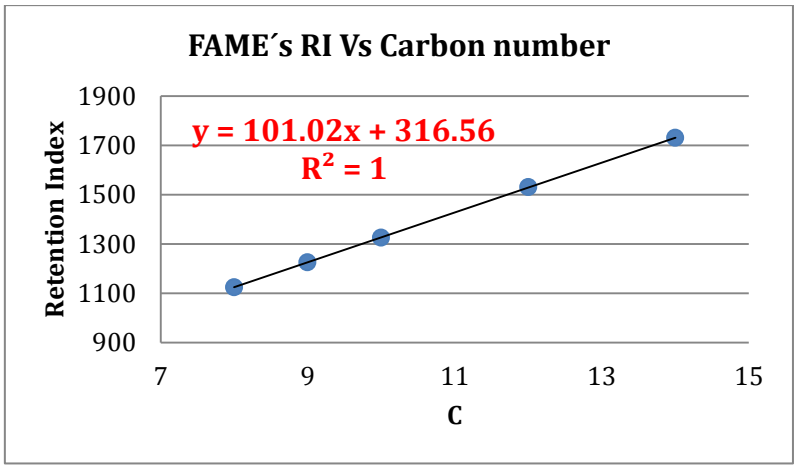

Figure 36: Plot of retention indices (RI) of the FAME´s in the standards solutions plotted against their carbon numbers (C); excluding the carbon of the methyl group, Column DB-5

8.3 Additional results regarding quantitative analysis and the analysis performance

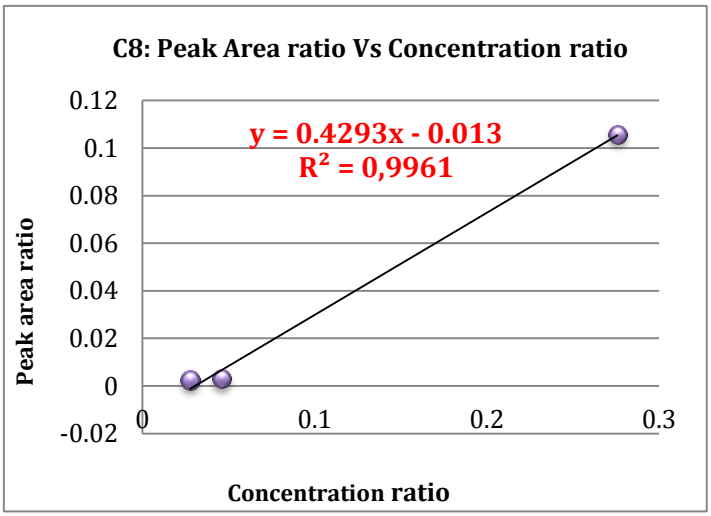

Figure 37 (a): Interal calibration curve of C8 FAME, peak area ratio (A_{FAME}/A_{IS}) against concentration ratio (C_{FA}/C_{IS}).The lowest data point on the peak ratio axis ($A_{FAME}/A_{IS)} \neq 0$.

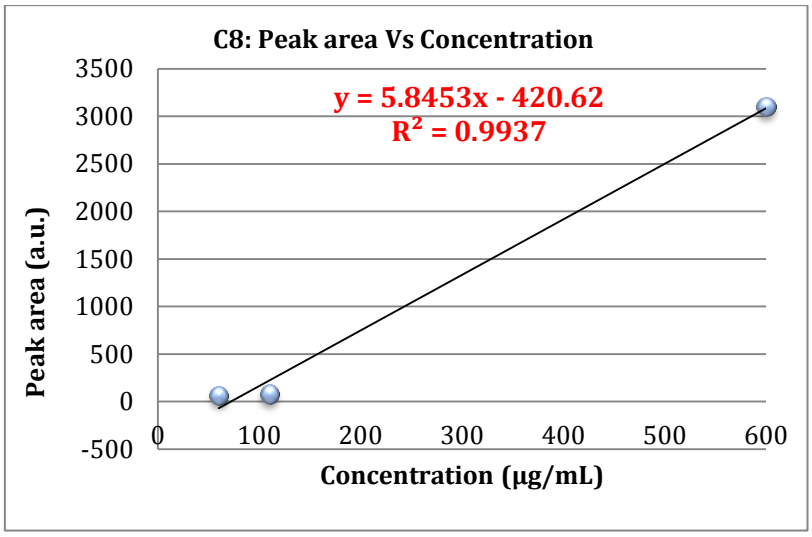

Figure 37 (b): External calibration curve of C8 FAME, peak area in (a.u.) against concentration in (µg/mL).The lowest data point on the peak area axis (Peak area) ≠ 0.

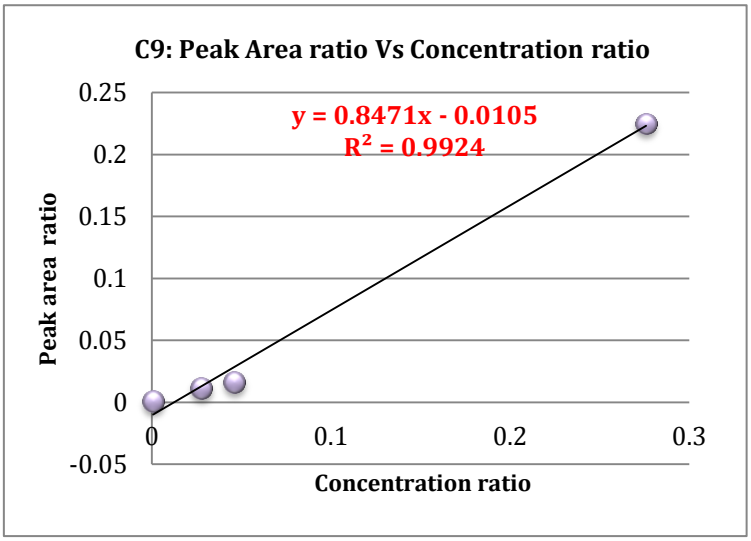

Figure 38 (a) : Internal calibration curve of C9 FAME, peak area ratio (A_{FAME}/A_{IS}) against concentration ratio (C_{FA}/C_{IS}).The lowest data point on the peak ratio axis ($A_{FAME}/A_{IS)} \neq 0$.

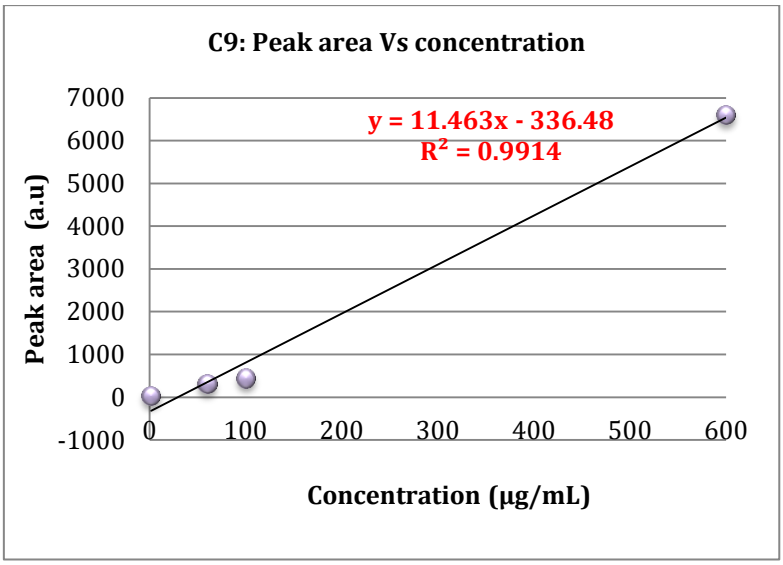

Figure 38 (b): External calibration curve of C9 FAME, peak area in (a.u.) against concentration in (µg/mL).The lowest data point on the peak area axis (Peak area) ≠ 0.

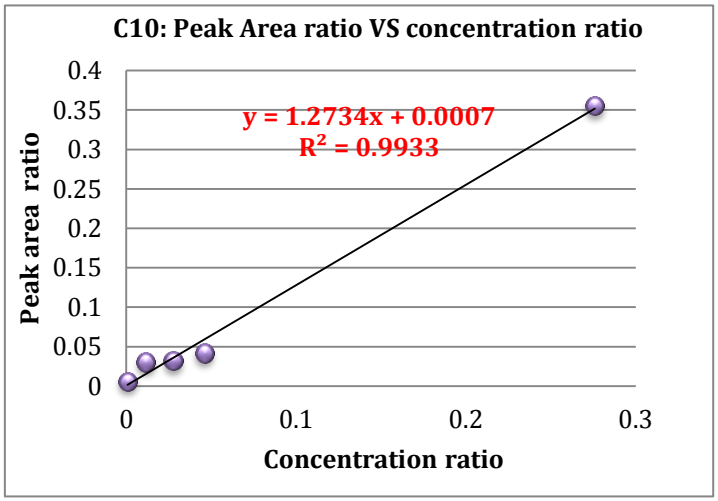

Figure 39 (a): Internal calibration curve of C10 FAME, peak area ratio (A_{FAME}/A_{IS}) against concentration ratio (C_{FA}/C_{IS}).The lowest data point on the peak ratio axis (A_{FAME}/A_{IS}) ≠ 0.

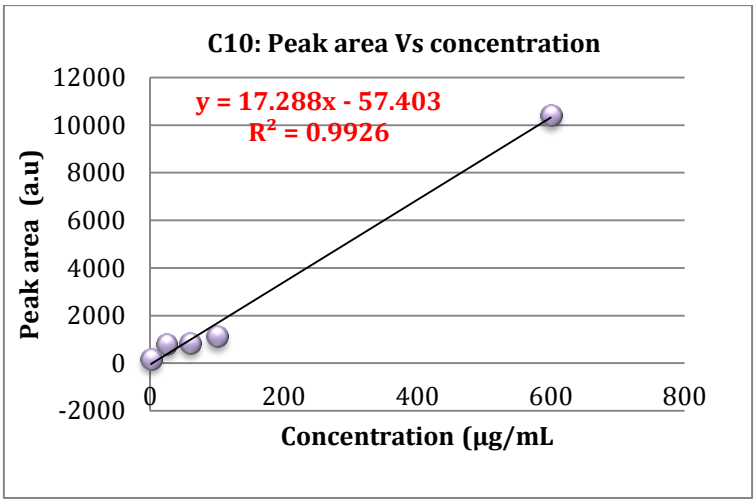

Figure 39 (b): External calibration curve of C10 FAME, peak area in (a.u.) against concentration in (µg/mL).The lowest data point on the peak area axis (Peak area) ≠ 0.

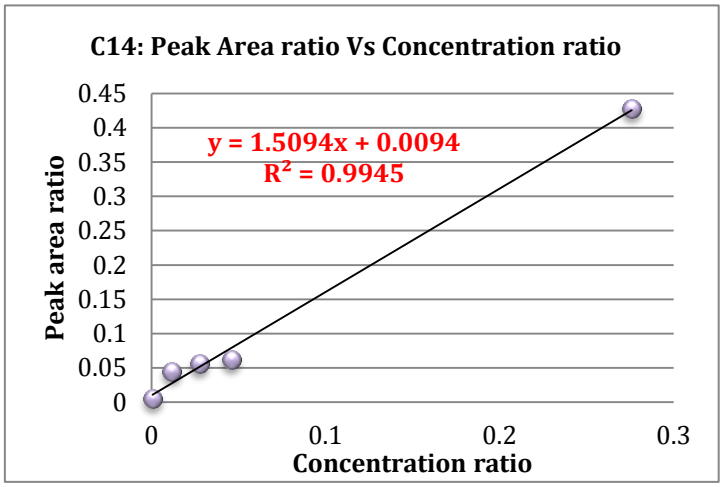

Figure 40: Internal calibration of C14 FAME, peak area ratio (A_{FAME}/A_{IS}) against concentration ratio (C_{FA}/C_{IS}).The lowest data point on the peak ratio axis ($A_{FAME}/A_{IS)} \neq 0$.

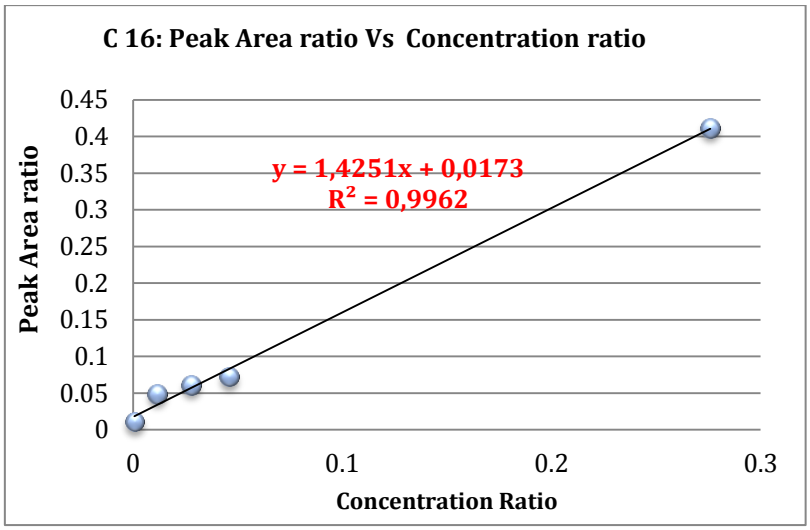

Figure 41: Internal calibration of C16 FAME, peak area ratio (A_{FAME}/A_{IS}) against concentration ratio (C_{FA}/C_{IS}).The lowest data point on the peak ratio axis ($A_{FAME}/A_{IS)} \neq 0$.

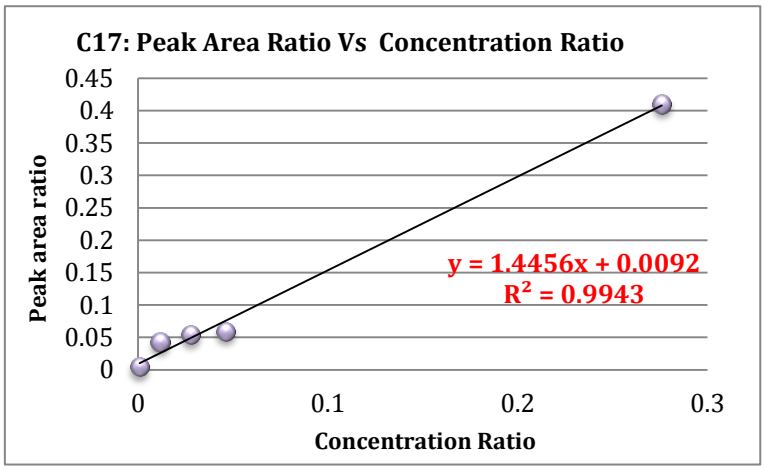

Figure 42: Internal calibration of C17 FAME, peak area ratio (A_{FAME}/A_{IS}) against concentration ratio (C_{FA}/C_{IS}).The lowest data point on the peak ratio axis ($A_{FAME}/A_{IS)} \neq 0$.

8.4 Further explanations

8.4.1 Reason for preparing calibration solutions with wide range

Calibration curves were constructed using a wide range of concentrations due to the big difference in concentration of FA´s in the herbicide. This big difference was observed from the ratios of the peak areas of the FAME´s during the qualitative analysis. For example, the peak areas of methyl pelargonate and methyl, 2-methyloctanoate had a ratio of 49:1. In

addition to that, the quantification process and part of the extraction process were carried out in parallel; i.e. it was not clear what concentration would be expected in the soil samples. Due to those reasons, a wide range of concentrations was prepared to cover the expected working range for analytes with high concentration, considering that 0–150% of the expected analyte concentration should be covered when constructing the calibration curve.[37]

8.4.2 Procedures of introducing the internal standard to the samples

For The preparation of the FAME´s calibration solutions, the FA´s were first mixed with 1 mL of the IS solution and the solvents were evaporated under gentle stream of nitrogen, then dissolved again in 1 mL methanolic HCl for derivatization. The extracted FAME´s obtained from the derivatization step were dried again under gentle stream of nitrogen and then dissolved in 1 mL n-heptane for GC analysis. The procedures were performed in that order due to two reasons. First, in order to avoid formation two phases in the glass tube during the derivatization. Second, to make sure that FA´s before the derivatization and the FAME´s obtained from the derivatization are dissolved in equal volumes; i.e. they have the same concentration. Having the same volume along the sample preparation steps is criterial to get the benefit of using the FA´s concentrations prior derivatization to construct calibration curves and the included correction factor for the derivatization step could be correct. The same procedures were performed to prepare the FAME´s from the spiked samples for GC analysis.

8.4.3 Reasons for using different instruments during the analysis

Due to a technical problem with Agilent GC-MS instrument model 6890N, the analysis was carried on using Agilent GC-MS model 7890A. This was not expected to affect the reliability of the analysis since both instruments are equipped with the same column DB-5 column, hence the same selectivity and analyte-stationary phase interaction is also expected; mass detectors in both instruments were set to the same parameters. In addition, due to lack of time by the end of the project period, it was decided to perform the quantification analysis on Agilent GC-FID instrument model 7890B instead of Agilent GC-FID system model 6890 N, taking advantage of auto-injector of the first. The peaks of all analytes in question were identified by comparing tR with the standards solutions and confirmed by RI´s models on both column.

8.4.4 Reasons for using concentration of fatty acid prior to derivatization to construct the calibration curves

The analytes of interest in this work, i.e. FA's, needed to be derivatized to their corresponding FAME´s in order to be quantified by the instrumentation used in the work; i.e. GC systems equipped with Hp-5 and DB-5 columns, (5%-Phenyl)-methylpolysiloxane nonpolar stationary phase). In addition, a known concentration is required to construct a calibration curve; however, the only known concentration is the concentration of FA´s prior to derivatization. Although the derivatization method used in the work was estimated to be 99% efficient for decanoic acid and has been efficiently used in previous works, the yield percentage can still be varying. This variation is controlled by a derivatization factor (D) which may range between 0 and 1, Equation (8).

$$[FAME] = [FA] * D \qquad (8)$$

So, when using calibration solutions prepared from known concatenations of FA´s; the constructed calibration curves would include a correction for the derivatization yield where the concentration of the FAME in Equation (5) is nothing but the concertation of FA multiplied by the derivatization factor.

8.4.5 Quantifying FA´s content in the herbicide to be used as references for recovery in SFE

Two attempts were performed to quantify octanoic acid, methyl, 2- methyloctanoic acid, and decanoic acid in the herbicide extracted by LLE (Figures 43,44), in order to use the obtained concentrations as references to calculate their recoveries (bias) in the SFE extraction. However, the recovery levels of pelargonic acid obtained from the two attempts were much lower than 70%. This low recovery minimized the trueness, hence the accuracy, of the quantification; thus the obtained concentrations could not be used as references to estimate the recovery of the three FA´s in SFE.

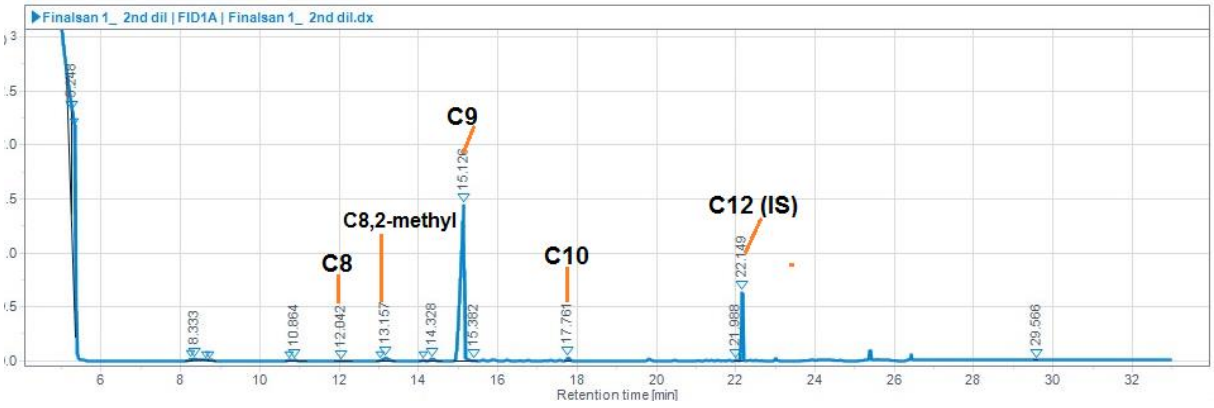

Figure 43: Chromatogram of FAME´s of the herbicide form the first attempt to quantify the FA´s content in the herbicide. Methyl octanoate (C8), methyl, 2-methyl octanoate (C8,2-methyl), methyl pelargonate (C9), methyl decanoate (C10), methyl dodecanoate (C12) as internal standard.

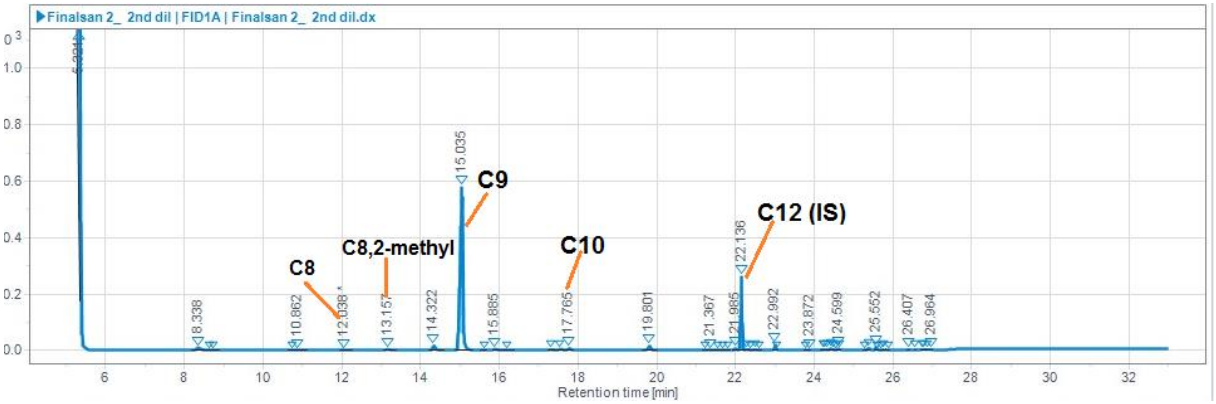

Figure 44: Chromatogram of FAME´s of the herbicide form the second attempt to quantify the FA´s content in the herbicide. Methyl octanoate (C8), methyl, 2-methyl octanoate (C8,2-methyl), methyl pelargonate (C9), methyl decanoate (C10), methyl dodecanoate (C12) as internal standard.