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DIPLOMARBEIT

Titel der Diplomarbeit

Contribution to the analysis and quality control of Peru Balsam

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Contents

1. Introduction

During the last decade a comeback of nature orientated medicine could easily be witnessed. Numerous studies in this field are trying to ascertain the efficacy of plants used in traditional medicine.
The plant kingdom provides a multitude of flavours and fragrances which have found their way into everyday life. The most important sources of flavour and fragrance materials worldwide are **essential oils**: the volatile aromatic oily liquids obtained from odoriferous plant parts -- whether flowers, fruits, leaves, roots, or wood. They are applied as flavouring for foods, soft drinks and confectionaries, in pharmaceutical products, and as fragrances in perfumes, cosmetics, and household and industrial products. [1]
Recent advances in physico-chemical separation and analysis techniques have greatly extended the knowledge about the chemical composition of essential oils. [1]

This study forms part of the program courses of the pharmaceutical studies to obtain pharmacian diploma at the University of Vienna / Universität Wien. It was performed in the Faculty of Pharmacy of the University of Barcelona / Universitat de Barcelona, following a Socrates/Erasmus, European student exchange programme and was carried out during the summer semester 2007.

1.2. Objectives of the research

The bark and wood of tropical trees often contain big amounts of non-volatile constituents next to their volatile compounds which form the essential oil. These non-volatile compounds are known as resins and their solution in the essential oil is called oleoresin. Oleoresins can be received by e.g. cutting the bark or provoking other injuries. What you obtain are often sticky and glutinous liquids. Resins are not or just very little soluble in water but good soluble in lipophilic solvents. [4]

The following studies were carried out with the oleoresin obtained from *Myroxylon balsamum*. This oleoresin, Peru balsam, finds its origin in El Salvador.

The objectives of the research were:

1. To extract and determine the essential oil composition of Peru balsam by applying two different extraction methods, apart from the distillation as described in the Pharmacopoeia, and to compare the results among each other. Furthermore, the obtained results were then compared with those obtained by applying the distillation method.
2. To make an attempt in designing a new HPLC method that serves to detect and quantify the main constituents of the Peru balsam, which could be useful for its quality control and could be considered for its possible inclusion in the Pharmacopoeia.

Extracting methods applied were: SFE-CO_2 (Supercritical Fluid Extraction with CO_2) and SPME (Solid Phase Micro Extraction).

Qualitative analyses were made by GC-MS (Gas Chromatography coupled to Mass Spectrometry), the results being compared with the data in the Wiley 6.1 Library, and by GC-FID, calculating retention indices based on fatty acid methyl ester retention times and on n-paraffin hydrocarbons retention times (Kovats indices).

Quantitative analyses were based upon GC-peak areas, obtained by GC-FID.

All gas chromatography experiments were done with polar and non-polar stationary phases.

HPLC peaks could be quantified by adding an internal standard.

2. Peru balsam – general information

Peru balsam is obtained from the tree *Myroxylon balsamum* (L.) Harms var. *pereirae* (Royle) Harms
Order: Fabales
Subfamily: Faboideae
Family: Fabaceae
Genus: Myroxylon

Botanical synonyms:

- *Myrosperum erythroxylum* (Allem)
- *Myrosperum peruiferum* (De Candolle)
- *Myrosperum secundum* (Klotzsch)
- *Myroxylon pedicelatum* (Lamarck)
- *Myroxylon pereirae* (Klotzsch)
- *Myroxylon peruiferum* (Linneo f.)

Definition and characters of *Balsamum peruvianum* as described in the European Pharmacopoeia (2005):

Definition:
Peru balsam is the balsam obtained from the scorched and wounded trunk of *Myroxylon balsamum* (L.) Harms var. *pereirae* (Royle) Harms. It contains not less than 45,0% m/m and not more than 70,0% m/m of esters, mainly benzyl benzoate and benzyl cinnamate. [2]

Characters:
A dark brown, viscous liquid which is transparent and yellowish-brown when viewed in a thin layer; the liquid is not sticky, it is non-drying and does not form threads; practically insoluble in water, freely soluble in ethanol, not miscible with fatty oils, except for castor oil. [2]

The Balsamum peruvianum has its origin in San Salvador (Central America) and can further be found in Honduras, Guatemala, Cuba, Mexico, Costa Rica and Panama but not in Peru. The drug first was imported from Spain through Peruvian ports. That's where the drug's name comes from.

Crude Peru balsam is a dark brown, thick liquid with an aromatic smell of cinnamon and vanilla, and a bitter taste. To remove it from the tree, the bark is alternately scorched and beaten. The balsam in the bark is obtained by boiling. Following removal of strips of bark from the tree, the exposed wood also secretes balsam. The material is absorbed into rags wrapped around the tree, which are then boiled in water. The balsam sinks to the bottom and is then collected. [6]
Peru balsam is an oleoresin which contains small amounts of free benzoic and cinnamic acids and about 50-70% of cinnamein. [4]
This **cinnamein** fraction extracted out of the alkaline solution with ether contains about **25 – 40% benzyl benzoate** and **10 – 25% benzyl cinnamate.** [4]
Further constituents are **alpha- and beta- nerolidol** (3 – 5%), small amounts of **vanillin** as well as methyl esters of **cinnamic acid** and **benzoic acid.** [4]

The essential oil is obtained by hydro distillation of the balsam using a Clevenger type apparatus as described in the European Pharmacopoeia (2005). [5]

2.1. The Fabaceae family

As circumscribed by the APG (Angiosperm Phylogeny Group) system, the Fabaceae is a large family, Fabaceae *sensu lato.* The International Code of Botanical Nomenclature allows the use of Fabaceae *s.l.* and Leguminosae as equivalent botanical names at the family rank. The APG-system uses the name Fabaceae. [7]

However, the family Fabaceae can also be circumscribed differently as Fabaceae *sensu stricto,* for example in the Cronquist system. In such classifications the subfamilies Mimosideae and Caesalpinioideae are raised to the rank families, Mimosaceae and Caesalpiniaceae. The remaining group has the equivalent botanical names of Fabaceae and Papilionaceae (but not Leguminosae). The APG treats this

group at the rank of subfamily, with the name Faboideae (it is equivalent with the name Leguminoseae being Papilionoideae). [7]

The Leguminoseae *sensu lato* is the third largest family of flowering plants (after Asteraceae and Orchidaceae) with 650 genera and over 18,000 species. These are commonly called legumes and the family contains some of our most valuable food crops, such as beans, peas, peanuts, soybeans, and lentils. Other members of the family are important sources of animal food or green manure. Some genera such as *Acacia* and *Mimosa* are ornamental trees and shrubs. Still other members of the family have medicinal or insecticidal properties, or yield important substances like gum Arabic, tannins, dyes, or resins. [7]

The leaves are stipulate, nearly always alternate, and range from pinnately or palmately compound to simple. The petiole base is commonly enlarged into a pulvinus. The flowers are slightly to strongly perigynous, zygomorphic, and commonly in racemes, spikes, or heads. The perianth commonly consists of a calyx and corolla of 5 segments each. The petals are overlapping (imbricate) in bud with the posterior petal (called the banner or flag) outermost in position. The petals are free except for variable connection of the two lowermost ones called the keel petals. The lateral petals are often called the wings. The androecium most commonly consists of 10 stamens in two groups (i.e., they are diadelphous with 9 stamens in one bundle and the 10^{th} stamen more or less distinct). The pistil is simple, comprising a single style and stigma, and a superior ovary with one locule containing 2 to many marginal ovules. The fruit is usually a legume. A significant characteristic of legumes is that they host bacteria in their roots, within structures called root nodules. These bacteria known as rhizobia have the ability to take nitrogen gas (N_2) out of the air and convert it to a form of nitrogen that is usable to the host plant (NO_3^- or NH_3). This process is called nitrogen fixation. The legume, acting as a host; and rhizobia, acting as a provider of usable nitrate, from a symbiotic relationship. [7]

2.2. Overview of the botanical features of *Myroxylon balsamum*

Figure 1: *Myroxylon balsamum* *[19]*

Root, stem and leaves (figure 1): M. balsamum is a tree up to 26m in height with a spreading crown which starts to branch at about 13 to 19m. The bark is smooth, yellowish grey or brown with numerous lenticels. The leaves are usually odd-pinnate and have 4 to 7 obovate, acuminate, coreacious, short-petioled leaflets. The upper surface of the leaves is dark green and the lower surface pale green. [6]

Flower and fruit (figure 1): The androgynous flowers are on approximately 12cm long pedicels and simple richly blossomed racemes. The calyx is inferior, broadly tubular or oblong-campanulate, dark green and has short, rough hairs. The 5 petals are white and stemmed. The standard petal is almost circular. The stamens are bright red. The ovary is on a long stem and has 1 valve. The fruit is a one-seeded indehiscent, winged pod with brown-red reniform and distinctly curved seeds. [6]

2.3. Historical references

The medicinal history of Peru balsam began with the Indians of Central and South America who used to apply the drug to stop bleeding and promote wound healing by stimulating the granulation effects of the skin. Peru balsam was also known to have diuretic effects and was also used to expel worms.

Peru balsam has mild antiseptic properties and is said to promote the growth of skin cells [10].

The balsam has been used in dentistry in the treatment of dry socket (post extraction alveolitis) and as a component of dental impression material. It formerly was used widely as a treatment for scabies, and it has been used in suppositories to treat haemorrhoids [6].
Peru balsam has been used for the treatment of topical wounds and infections, and as a flavouring agent in the food industry [10].
Today, Peru balsam forms part of various pharmaceutical preparations and perfumes. It may only be applied externally and not internally because of its possible allergic secondary effects.

2.4. Earlier research about Peru balsam

Peru balsam is a contact allergen and contact dermatitis occurs frequently with this product [8].
It may cause dermatitis in individuals who have sensitivity to benzoin resinoids. The main contact sensitizers in Peru balsam have been identified and include cinnamates, benzoates, and terpenoids. Additional patch testing is recommended in Peru balsam sensitive individuals to trace how sensitization is acquired [9].

Studies have evaluated the use of Peru balsam as a treatment of wounds [11].

Peru balsam has been used topically as 5% to 20% formulations for wounds and burns; there are no recent well-controlled clinical studies to support appropriate dosing.

PART A – COMPOSITION OF THE VOLATILE FRACTION OF PERU BALSAM BY DIFFERENT EXTRACTION METHODS

3. Essential oils

Essential oils have many functions for people. Most obvious is their role in fragrance materials, but they are equally important as flavouring materials and in medicine. [1]

Medicine and Pharmacy understand essential oils as volatile, strong smelling mixtures of substances that are products of the secondary metabolism of plants. They differ entirely, chemically and physically, from fixed oils that are constituted of fatty acids. This definition overlaps partly with the definition given by the European Pharmacopoeia:
Odorous products, usually of complex composition, obtained from a botanically defined herbal raw material by steam distillation, dry distillation or by a suitable mechanical process without heating. Essential oils are usually separated from the aqueous phase by a physical process that does not significantly affect their composition. [2]

Ecological conditions influence many aspects of plant growth and may as well influence the composition of an essential oil. Also the physical process by which essential oils are obtained may influence the chemical composition of an essential oil. Water distillation, steam distillation, hydro diffusion, expression and solvent extraction produce different essential oils from the same plant material, because not all components are extracted equally well by each process during which individual components may undergo chemical changes. Such differences are generally minor, but are important for the quality of the essential oil. [1]

Volatile compounds are to be found in most plant species, but just the ones that contain more than 0,01% (up to 10%) are interesting. The families that contain biggest amounts of essential oils are Apiaceae, Asteraceae, Cupressaceae, Lamiaceae, Myrtaceae, Pinaceae, Poaceae, Piperaceae, Rutaceae, Zingiberaceae and Verbenaceae. [3]

Essential oils are complex mixtures of sometimes up to hundreds of chemical compounds. Most of these compounds can be grouped into a few major classes, but there are also many components of essential oils that bear little resemblance to these classes. [1]

One primary has to distinguish between terpenoid and none-terpenoid structures. The group built out of *none-terpenes* consists of aliphatic compounds, benzene derivates and sulphur compounds. Further, benzene derivates can either show C_6 - C_1 construction like benzoic acid derivates as vanillin, or C_6 - C_3 construction like phenylpropanoids as for example anethol, eugenol and cinnamic aldehyde.

Terpenes can be classified as mono-terpenes, sesqui-terpenes and diterpenes. The latter group only occurs in very small amounts. All types of terpenes can show aliphatic, aromatic or cyclic structures. Cyclic structures vary from monocyclic to bi- or even tricyclic. Further, terpenes can be oxygenated through alcohol, aldehydes or ketons as structural residues. Similarly they can occur simply as hydrocarbons. Terpenes and terpene derivates constitute a widely represented group of substances. Although they show wide structural diversity, they share a common characteristic: they are built from 2 (monoterpenes), 3 (sesquiterpenes) or more isoprene (C_5H_8) units.

Monoterpenes conform to the molecular $C_{10}H_{16}$. Examples include alpha-terpinene, gamma-terpinene, limonene, alpha-phellandrene, beta-phellandrene, alpha-pinene, beta-pinene.

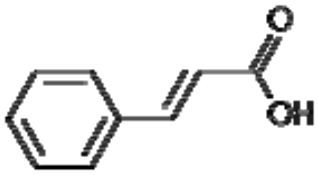
Sesquiterpenes are compounds generated from 3 isoprene units and conforming to the formula: $C_{15}H_{24}$. As there are so many, it is impossible to generalize about their molecular structure. Farnesene is perhaps the most simple, acyclic example; many sesquiterpenes are bicyclic, having two C_6-rings or a C_6 and a C_5 ring.

Aliphatic compounds are acyclic organic compounds. The chain of C-atoms in these compounds may be straight or branched and some of the bonds between C-atoms may be unsaturated.

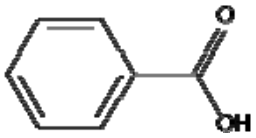
Benzene derivates, also known as benzenoids, are compounds containing a characteristic benzene ring. Esters of aromatic alcohols and aliphatic acids are of interest in flavours and fragrances because of their characteristic odour properties. Benzyl acetate is the main component of jasmine oil and gardenia oil, phenylethyl acetate is an aroma compound found in several essential oils and in many fruits,

benzyl benzoate is a major component of Peru balsam and is a commonly used fixative and modifier of heavy blossom fragrances in perfumery. [1]

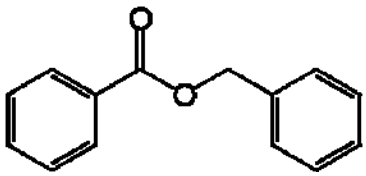
Here, some structures of the most important volatile constituents of the Peru balsam are shown:



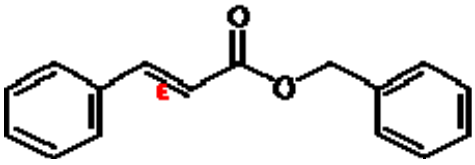
Cinnamic acid



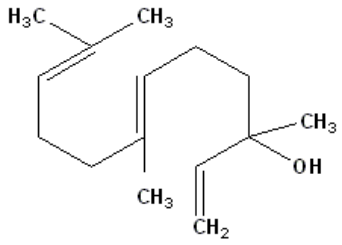
Benzoic acid



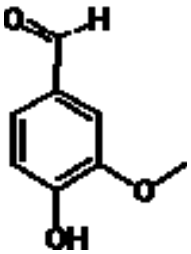
Benzyl benzoate



Benzyl cinnamate



Nerolidol



Vanillin

Pharmacological effects of essential oils are numerous. Many of them have antimicrobial properties. Camphor, rosemary and mustard oil irritate skin locally; eucalyptol and menthol are good expectorants while thyme is spasmolytically active. Cinnamon and carnation oil are able to drive out pest. Nutmeg and wormwood oils are toxicologically important. But most of them are used as food flavours, spices and in mouth care, as well as in cosmetics, especially in perfume industry. [3]

Pharmacopoeia tests the identity and purity of essential oils. Physically they are tested on relative density, angle of refraction, optical activity and solidification point. Additionally they are tested on colour, scent, taste, solubility and impurities with water, fixed oils, heavy metals, esters and halogens. [3]

4. Extraction

The operating principle of any sample preparation method is to partition analytes between the sample matrix and an extracting phase [13].

4.1. Solid Phase Micro Extraction / SPME

4.1.1. General Introduction

The concept of using an adsorbent material to extract trace organic compounds from an aqueous sample was developed in the 1980s, and its application has been extensively reviewed. Sorbents are now used to extract organic compounds from various matrices including water, air, and even soil. A sorbent with a strong affinity towards organic compounds will retain and concentrate those compounds from a very diluted aqueous or gaseous sample. Many sorbents are specifically suited for the extraction of different groups of organic compounds with various degrees of selectivity. One widely used sorbent technique is solid phase extraction (SPE). Application of sorbing material permanently attached to a fiber addresses the limitations of SPE and allows reuse of the same extraction phase. The resulting technique is called SPME [13].

Simplicity and convenience of operation make SPME a superior alternative to more established techniques in a number of applications.
SPME allows rapid extraction and transfer to analytical instruments [13].

The SPME process has two steps: partitioning of analytes between the coating and the sample matrix, followed by desorption of concentrated extracts into an analytical instrument. In the first step, the coated fiber is exposed to the sample or its headspace, which causes the target analytes to partition from the sample matrix into the coating. The fiber bearing concentrated analytes is then transferred to an instrument for desorption, whereupon separation and quantitation of extracts can take place [13].
Extraction was carried out with a Supelco *micro syringe*:
Fiber holding support: 57330-U
Fiber: 57348-U, with coating consisting of bivinylbenzene / Carboxen on polidimethylsiloxane on a Stable Flex fiber.

The metal rod, which serves as the piston in a micro syringe, is replaced with stainless steel microtubing having an inside diameter slightly larger than the outside diameter of the fused silica rod. Typically, the first 5mm of the coating is removed from a 2 cm long fiber which is then inserted into the micro tubing. High temperature epoxy glue is used to permanently mount the fiber. Sample injection is then very much like standard syringe injection. Movement of the plunger allowed exposure of the fiber during extraction and desorption and its protection in the needle during storage and penetration of the septa. [13]
Expansion of air caused by temperature increase allows removal of desorbed analytes from the extracting phase. [13]
The transport of analytes from the matrix into the coating begins as soon as the coated fiber has been placed in contact with the sample. Typically, SPME extraction is considered to be complete when the analytes concentration has reached distribution equilibrium between the sample matrix and the fiber coating. In practice, this means that once equilibrium is reached, the extracted amount is constant within the limits of experimental error and it is independent of further increase of extraction time. Of course the extraction can be interrupted and the fiber analyzed prior to equilibrium. To obtain reproducible data, however, constant convection conditions and careful timing of the extraction are necessary. [13]

Extraction modes

Three basic types of extractions can be performed using SPME:

1. direct extraction,
2. headspace configuration, and
3. membrane protection approach. [13]

In the *direct extraction mode*, the coated fiber is inserted directly into the sample and the analytes are transported directly from the sample matrix to the extracting phase. [13]

In the *headspace mode*, the analytes need to be transported through the barrier of air before they can reach the coating. This modification serves primarily to protect the fiber coating from damage by high molecular weight and other non-volatile interferences present in the sample matrix. Amounts of analytes extracted into the

coating from the same vial at equilibrium using direct and headspace sampling are identical as long as sample and gaseous headspace volumes are the same. This is caused by the fact that the equilibrium concentration is independent of fiber location in the sample/headspace system. If the above condition is not satisfied, a significant sensitivity difference between the direct and headspace approaches exists only for very volatile analytes. [13]
Temperature has a significant effect on the kinetics of the process by determining the vapour pressure of the analytes. In fact, the equilibration times for volatiles are shorter in the headspace SPME mode than for direct extraction under similar agitation conditions. [13]

The main purpose of the *membrane barrier* is to protect the fiber against damage, similar to the use of headspace SPME when very dirty samples are analyzed. However, membrane protection is advantageous for determination of analytes having volatility too low for the headspace approach. In addition, a membrane made from appropriate material can add a certain degree of selectivity to the extraction process. The kinetics of membrane extractions are substantially slower than for direct extraction, though, because the analytes need to diffuse through the membrane before they can reach the coating. Use of thin membranes and increased extraction temperatures will result in faster extraction times. [13]

The efficiency of the extraction process is dependent on the *distribution constant.* This is a characteristic parameter that describes properties of a coating and its selectivity toward the analytes versus other matrix components. Specific coatings can be developed for a range of applications. Coating volume determines method sensitivity as well, but thicker coatings result in longer extraction times. [13]

Because of its solvent-free nature, SPME can be interfaced conveniently to analytical instruments of various types. Only extracted analytes are introduced into the instrument, since the extracting phase is non-volatile. The sensitivity of determinations using the SPME technique is very high, facilitating trace analysis. Although in most cases the entire amount of analytes is not extracted from the sample, all material that is extracted is transferred to the analytical instrument,

resulting in good performance. Also, the solvent-free process results in narrow bands reaching the instrument, giving taller, narrower peaks and better quantitation.
The analytical instrument used most frequently with SPME has been the *gas chromatograph*. The split should be turned off during SPME injection. Under these conditions, the desorption of analytes from the fiber is very rapid, not only because the coatings are thin but because the high injector temperatures produce a dramatic decrease in the coating/gas distribution constant and an increase in the diffusion coefficients. [13]

Applications:

A lot of research has been done on samples of environmental origin: air, water, sludge, and soils. The majority of applications have been developed for aqueous matrices.
A unique feature of SPME for application to analysis of food and drugs is its ability, in some cases, to extract substances from products without opening the package. For example, flavours present in wine can be checked before sale or purchase by introducing a fiber into the headspace of the wine through the cork of the bottle. A similar process can be applied to on-line product monitoring of each individual item to ensure the best possible quality.
Food and pharmaceutical products are frequently contaminated by volatile organic solvents, which are used in manufacturing and processing of these goods. Considering the frequent complexity of their matrices, the analytical procedures can be quite involved. Static headspace is frequently used for this purpose, but SPME has been found to be a good alternative for analysis of both contaminated pharmaceuticals and foods because of its superior sensitivity, especially for less volatile compounds.
Another interesting application of SPME to analysis of food, is to quantify caffeine in soft drinks, for example tea and cola [13].

4.1.2. SPME- extraction conditions

To extract the most volatile constituents out of the Peru balsam by SPME corresponding to the headspace method, 0,4g of the sample were weighed into a vial, sealed and equilibrated for 30 min in a water bath. Water temperature was ranged from 35°C to 50°C and 60°C. After 30 min of equilibration, the syringe was injected into the vial and the fiber was exposed into the headspace. This exposition of the fiber lasted for 30min and once for 60 min.
In summary, four extraction conditions combining different extraction times and temperatures were used:

1. 30min at 35°C
2. 30min at 50°C
3. 30min at 60°C
4. 60min at 50°C

Each extraction was performed four times in order to analyze the volatiles extracted by GC-FID and GC-MS in polar and non-polar columns. Identification and quantification of the peaks obtained were carried out as described in chapter 5.
The results were combined in excel-tables and are shown in chapter 6.

The injections that were carried out are listed here, also showing the corresponding file-name and can be looked through in chapter 9.1 and 9.3.:

- SPME3035:
  Injected in GC-FID with polar column under the file-name “SP3035P2”
  Injected in GC-MS with polar column under the file-name “SPME3035”
  Injected split less in GC-FID with non-polar column under the file-name “SP3035AP”
  Injected split less in GC-MS with non-polar column under the file-name “SP3035MS”
- SPME3050:
  Injected in GC-FID with polar column under the file-name “SPM3050P”
  Injected in GC-MS with polar column under the file-name “SPME3050”
  Injected split less in GC-FID with non-polar column under the file-name “SP3050AP”

- SPME6050:
  Injected split less in GC-FID with non-polar column under the file-name "SP6050AP"
  Injected split less in GC-MS with non-polar column under the file-name "SP6050MS"
- SPME3060:
  Injected in GC-FID with polar column under the file-name "SPM3060P"
  Injected in GC-MS with polar column under the file-name "JOAN"
  Injected in split less in GC-FID with non-polar column under the file-name "SP3060AP"
  Injected split less in GC-MS with non-polar column under the name "SP3060MS"

4.2. Super-critical Fluid Extraction / SFE – CO_2

4.2.1. General Introduction

The technology of supercritical fluid extraction (SFE) offers one the opportunity to efficiently and economically improve recovery, increase reproducibility, decrease the use of halogenated solvents, and provide cleaner extracts to the measurement instrument [15].
Supercritical fluid extraction is the process by which a supercritical fluid removes sample components (analytes) and extracts them from a sample matrix (everything else). The supercritical fluid moves the analytes to the trap where the fluid evaporates, leaving the sample components behind. The sample is then reconstituted with rinse solvent and the rinse solvent containing sample fractions is washed to the output vials. [14]
The solvent properties of a supercritical fluid depend on its polarity and density. When the fluid density changes, the sample components that will be extracted from the sample also change [14].
CO_2 is the most widely used fluid in SFE.

The key steps in SFE are

1. contacting the supercritical fluid with the sample in order to dissolve some part of the total extractables from the sample,
2. moving the solution of supercritical fluid plus extracted components away from the rest of the sample, and
3. precipitating the extracted components in a collecting region by removing the supercritical fluid. [14]

The Hewlett-Packard 7680T is a supercritical fluid extractor that performs sample preparation of solid, semisolid, and liquid samples by exploiting the properties of supercritical carbon dioxide. [14]

Components of the HP 7680T:

The extraction module

The extraction module is where the extraction steps and rinse sub steps of supercritical fluid extraction take place. The thimble containing the sample is placed in the thimble carousel which carries it into the extraction chamber where the components are then extracted. The components are collected on the trap and rinsed into vials in the fraction output ray. The rinse solvent bottles sit in the bin on top of the module. [14]

The thimbles and vials

The sample is placed in a container called a thimble to perform an extraction using the HP 7680T. The thimble is placed in the thimble carousel. When the sequence is started, the carousel moves the thimble into the extraction chamber and the extraction method is started. The extracted fractions are washed to small sampler vials in the fraction output tray. [14]

The extraction fluid

CO_2 removes components from the sample matrix and deposits them on the trap. [14]

The trap

The sample components are collected on the trap. The supercritical CO_2 with the sample components enters the trap through the nozzle where it is depressurized. The components are collected on the trap while CO_2 exits the trap to the vent. During a rinse step, the extracted components are dissolved in the rinse solvent and moved from the trap through the exit line either to an automatic sampler vial or waste. [14]

An extraction method consists of all extraction steps and associated rinse sub steps. Extraction takes place inside the extraction module and occurs in the following three stages:

1. The supercritical CO_2 removes sample components from the sample.
2. The supercritical fluid solution containing the extracted components is pumped to the trap where the supercritical CO_2 evaporates, leaving the extracted components behind.
3. Rinse solvent dissolves the extracted components from the trap and transports them to a vial or to waste. [14]

One of the very attractive physicochemical properties of supercritical fluids is their gas-like pressure-volume behaviour: they expand with decreases in pressure (increases in volume). Expanding the fluid to ambient pressure removes the extraction fluid from the mixture as a gas, leaving the extracted components precipitating out of the expanding gas-stream. The combination of fluid pump plus flow restrictor provides a means to provide

1. high pressures on the upstream side of the restrictor,
2. expansion to ambient pressures on the downstream side of the restrictor, and
3. movement of the fluid stream. [14]

A fluid becomes supercritical when it is held at temperatures sufficiently high (above its critical temperature) so that intermolecular attractive forces are insufficient for condensation into a liquid. No matter how high the pressure, a fluid above the critical temperature will not condense into a liquid. The number of molecules per unit volume

can be essentially that of liquids, but the tendency to expand as the volume increases is gas-like.
Supercritical fluids dissolve like liquids and handle like gases [14].

The significant properties of Super critical Fluids that relate to extraction processes are:

1. solvating power is directly related to density (which near the critical point is pressure and temperature dependent),
2. relatively high diffusivity and low viscosity, and
3. minimal surface tension [15].

The physical stage of a substance of fixed composition can be described by a phase diagram. There are three lines describing the sublimation, melting, and boiling processes. These lines also define the regions corresponding to the gas, liquid, and solid states [15].

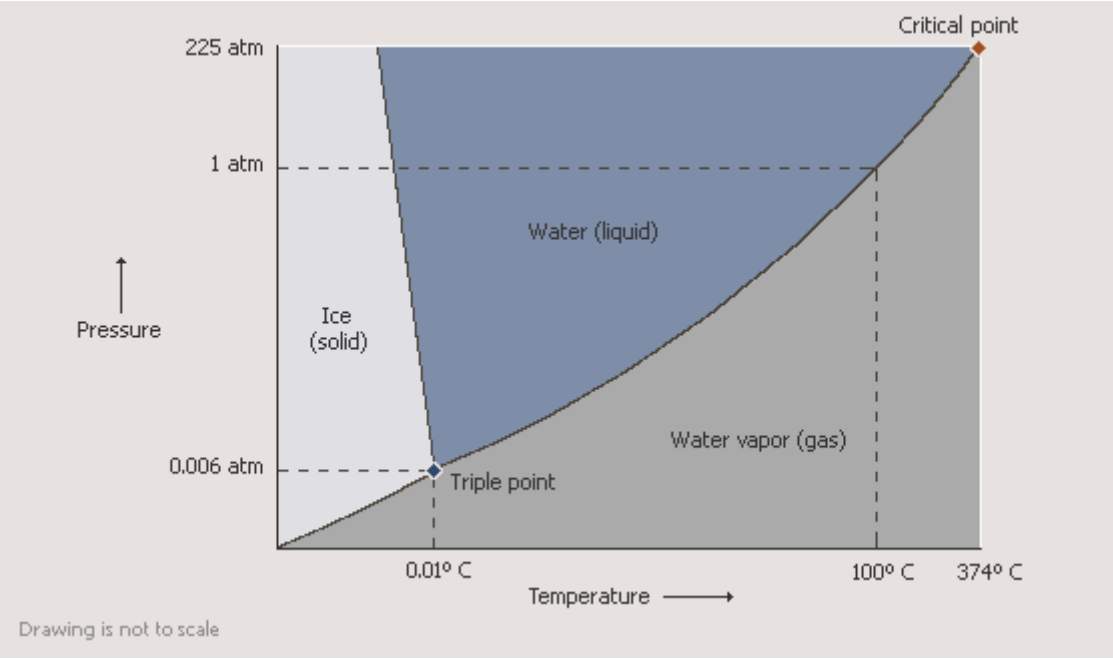


Figure 2: phase diagram

The vapor pressure (boiling) starts at the triple point and ends at the critical point. At this point we can define a SF as any substance that is above its critical point. At this point we can define a SF as any substance that is above its critical temperature and critical pressure. *The critical temperature* is therefore the highest temperature at

which a gas can be converted to a liquid by an increase in pressure. *The critical pressure* is the highest pressure at which a liquid can be converted to a traditional gas by an increase in the liquid temperature. In the so-called critical region, there is only one phase and it possesses some of the properties of both a gas and liquid. Supercritical and liquid CO_2 (sub critical) con both be used as solvents.
The solvating power of the supercritical fluid is highly dependent on its temperature and pressure. At low pressure the solvent power of CO_2 surprisingly decreases with rising temperature; whereas at high pressures it increases in a straightforward fashion. Density decreases dramatically with an increase in temperature at low pressure; whereas at higher pressure, changes in temperature have much less effect on density. Thus density, not pressure, to a first approximation is proportional to the solvent power of the SF.

- Solvent power of a supercritical fluid increases with density at a given temperature.
- Solvent power of a supercritical fluid increases with temperature at a given density. [15]

The properties of gas-like diffusivity and viscosity, coupled with liquid-like density, combined with the pressure-dependent solvating power SFs have provided the impetus for applying SF technology to analytical separation problems. Finally, the low (essentially zero) value of surface tension of SFs allows *better penetration* into the sample matrix relative to liquid solvents.
In summary, solvating strength in the supercritical region is a direct function of density-which in turn is dependent on system pressure (at constant temperature) or temperature (at constant pressure). [15]

High - purity CO_2 serves as the fluid of choice for all extractions. Replacement choices for pure CO_2 will, in over 90% of the cases, employ CO_2 with an organic modifier added (to increase the fluid polarity) rather then an alternate fluid such as propane or sulphur hexafluoride.
The advantages of using CO_2-based fluids are:

1. it is non-flammable
2. it is non-toxic
3. it is less expensive than reagent grade liquid solvents

4. it is available in a high state of purity, and
5. it can be vented to the atmosphere or recycled without harm. [15]

Since 1989, American proponents of SFE have consistently stressed its potential in the environmental market. More recently, SFE has been evaluated in such areas as pharmaceutical analysis, pesticide residue analysis, and “truth in labelling” of foods, polymer additive screening, and municipal drinking water assessment [15].

SFE-CO_2 – extraction conditions

Two extractions of the volatile constituents of *Myroxylon balsamum* were performed by SFE-CO_2.

A) The first one was a three step process increasing CO_2 density and recovering the volatiles extracted in each step separately in three vials, as indicated below.

extraction-conditions for	step1	step2	step3
density (g/ml)	0,35	0,45	0,55
pressure (bar)	91	111	131
temperature (C)	55	55	55
extraction solvent	CO_2	CO_2	CO_2
flow rate CO_2 (ml/min)	1	1	1
balance time (min)	0,5	0,5	0,5
extraction time (min)	7	7	7
sample volume (mg)	250	250	250

recovery-conditions equally for	step1/2/3
a. extraction trap conditions	
restrictor temperature (C)	45
trap temperature (C)	-5
special trap	TENAX
compensation volume (ml)	1,0
b. fraction obtention	

solvent	Hexanol
volume (ml)	0,5
restrictor temperature (C)	10
trap temperature (C)	-5

Vials number 1 to 3 were obtained, one for each step. The sample-composition of each vial was then analyzed by injection in GC-MS and GC-FID instruments with polar and non polar columns as described in chapter 5. Thus the peaks were identified qualitative and quantitative. The results are shown in the tables added to chapter 6 and the chromatograms are shown in chapter 9.2 and 9.4.

B) The second extraction was carried out in a single step using a single CO_2 density for a longer time. The same conditions used above were applied, with the following changes undertaken:

extraction-conditions for	*vial4*
density (g/ml)	0,55
extraction time (min)	12
sample volume (mg)	500

The extract was recovered in vial number 4, and was analyzed by GC-MS and GC-FID with both column types, polar and non polar.

All together 4 vials were obtained and injected in the analytical GC instruments:

- vial1: 1μl injected in GC-FID apolar under the file-name “PB4FID”
  1μl injected in GC-FID polar under the file-name “PB4FID2”
  1μl injected in GC-MS apolar under the file-name “PB4MS2”
  1μl injected in GC-MS polar under the file-name “PB4”

- vial2: 1μl injected in GC-FID apolar under the file-name “PB2FID”

1μl injected in GC-FID polar under the file-name “PB2FID2”

1μl injected in GC-MS apolar under the file-name “PB2MS2”

1μl injected in GC-MS polar under the file-name “PB2”

- vial3: 1μl injected in GC-FID apolar under the file-name “PB3FID”

1μl injected in GC-FID polar under the file-name “PB3FID2”

1μl injected in GC-MS apolar under the file-name “PB3MS2”

1μl injected in GC-MS polar under the file-name “PB3”

- vial4: 1μl injected in GC-FID apolar under the file-name “PB6FID”

1μl injected in GC-FID polar under the file-name “PB6FID2”

1μl injected in GC-MS apolar under the file-name “PB6MS4”

1μl injected in GC-MS polar under the file-name “PB6”

- further injections:

0,1μl alkanes injected in GC-FID apolar under the file-name “ALKANOMS”

0,1μl methylic esters injected in GC-FID apolar under the file-name “ESTEREMS”

0,1 μl alkanes injected in GC-FID polar under the file-name “ALKSC”

0,1 μl methylic esters injected in GC-FID polar under the file-name “ESTSC”

1μl of vial4 and 0,1μl alkanes injected in GC-FID apolar under the file-name “ALKPB6MS”

1μl of vial4 and 0,1 μl methylic esters injected in GC-FID apolar under the file-name “EMEPB6MS”

1μl of vial4 and 0,1 μl alkanes injected in GC-FID polar under the file-name “ALKPB6SC”

1μl of vial4 and 0,1 μl methylic esters injected in GC-FID polar under the file-name “EMEPB6SC”

5. Principles of Gas Chromatographic analysis

5.1. Introduction

In a very short time gas chromatography, GC, has become the premier technique for separation and analysis of volatile compounds. It has been used to analyze gases, liquids, and solids - the latter usually dissolved in volatile solvents. Both organic and inorganic materials can be analyzed and molecular weight can range from 2 to over 1000 Daltons. Gas chromatographs are the most widely used analytical instruments in the world. [18]

Gas Chromatography is a separation method in which the components of a sample partition between two phases: one of them is a stationary bed with a large surface area and the other one is a gas which percolates through the stationary bed. The sample is vaporized and carried by the mobile gas phase (the carrier gas) through the column. Samples partition into the stationary liquid phase, based on their solubility at the given temperature. The components of the sample (called solutes or analytes) separate from one another, based on their relative vapour pressures and affinities for the stationary bed. This type of chromatographic process is called elution. [18]

The use of a gas for the mobile phase requires that the system be contained and leak-free, and this is accomplished with a glass or metal tube referred to as the column. [18]

The tendency of a given component to be attracted to the stationary phase is expressed in chemical terms as an equilibrium constant called the *distribution constant*, K_c, sometimes also called the partition coefficient. In chromatography, the greater the value of the constant, the greater the attraction to the stationary phase. $K_c = (A)_S/(A)_M$ ….The distribution constant is defined as the concentration of the solute A in the stationary phase divided by its concentration in the mobile phase. This constant is a true thermodynamic value which is temperature dependent; it expresses the relative tendency of a solute to distribute itself between the two phases.

Advantages of Gas Chromatography:

- Efficient, providing high resolution
- Sensitive, easily detecting ppm and often ppb
- Non-destructive, making possible on-line coupling; e.g., to mass spectrometer (MS)

- Highly accurate quantitative analysis, typical RSDs of 1-5%
- Requires small samples, typically μl
- Reliable and relatively simple

Because GC is excellent for quantitative analysis, it has found wide use for many different applications. Sensitive, quantitative detectors provide fast, accurate analyses, and at a relatively low cost. [18]
In GC as well as in distillation techniques, temperature is a major variable, but gas chromatographic separations are also dependent upon the chemical nature (polarity) of the stationary phase. This additional variable makes GC more powerful. [18]

Disadvantages of gas Chromatography

- Limited to volatile samples
- Not suitable for thermally labile samples
- Fairly difficult for large, preparative samples
- Requires spectroscopy, usually mass spectroscopy, for confirmation of peak identity.

In summary: for the separation of volatile materials, GC is usually the method of choice due to its speed, high resolution capability, and ease of use. [18]

The basic parts of a simple gas chromatograph are carrier gas, flow controller, injector, column, detector, and data system.
Today, the most popular columns are made of fused silica and are open tubes with capillary dimensions. The stationary liquid phase is coated on the inside surface of the capillary wall. [18]

Figure 3: Chromatography column [19]

In summary a Gas Chromatograph functions as follows. An inert carrier gas (like helium) flows continuously from a large gas cylinder through the injection port, the column and the detector. The flow rate of the carrier gas is carefully controlled to ensure reproducible retention times and to minimize detector drift and noise. The sample is injected (usually with a micro syringe) into the heated injection port where it is vaporized and carried into the column, typically a capillary column 15m, 30m or 60m long, coated on the inside with a thin (0.2μm) film of high boiling liquid (the stationary phase). The sample partitions between the mobile and stationary phases, and is separated into individual components based on relative solubility in the liquid phase and the relative vapour pressures. [18]

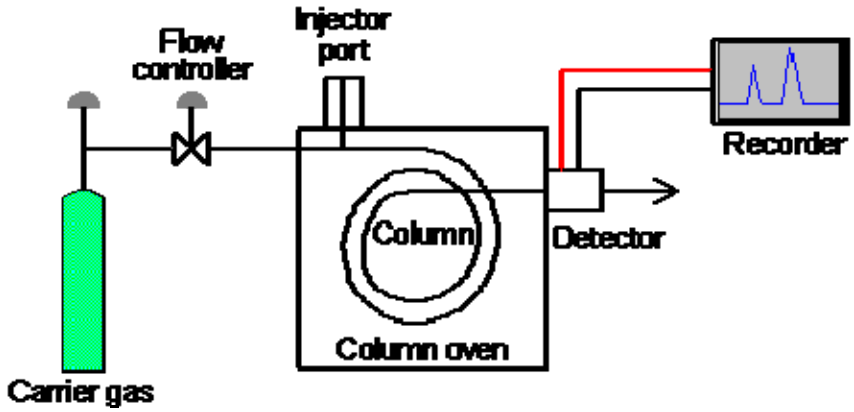


Figure 4: Gas Chromatograph [19]

In sample injection one has to distinguish between split less injection and injection with split. Split less means, that the whole amount of injected sample will be applied on the column. Whereas split-mode means, that just a small amount of the injected liquid reaches the column. The most part of the sample is evaporated. [18]

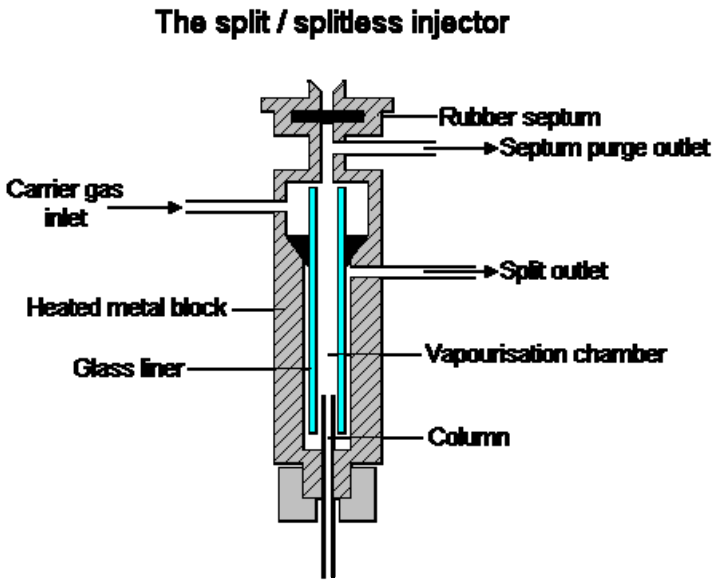


Figure 5: Split/ Split less injector [19]

After the column, the carrier gas and sample pass through a detector. This device measures the quantity of the sample, and generates an electrical signal. This signal goes to a data system/integrator which generates a chromatogram (the written record of analysis). [18]

The main purpose of the carrier gas is to carry the samples through the column. It is the mobile phase and it is inert and does not interact chemically with the sample. A secondary purpose is to provide a suitable matrix for the detector to measure the sample components. [18]

It is important that the carrier gas be of high purity because impurities such as oxygen and water can chemically attack the liquid phase in the column and destroy it. The injection port should be hot enough to vaporize the sample rapidly so that no loss in efficiency results from the injection technique. On the other hand, the injection port temperature must be low enough so that thermal decomposition or chemical rearrangement is avoided. [18]

A detector senses the effluents from the column and provides a record of a chromatography in the form of a chromatogram. The detector signals are proportionate to the quantity of each solute/analyte making possible quantitative analysis. [18]

5.2. GC-FID

The most common detector is the Flame Ionization Detector, FID. It has the desirable characteristics of high sensitivity, linearity, and detectivity and yet is relatively simple and inexpensive. The FID is not very selective and detects all organic compounds. The magnitude of this signal (peak height or peak area) is proportional to the amount of analyte and is the basis for quantitative analysis. [18]

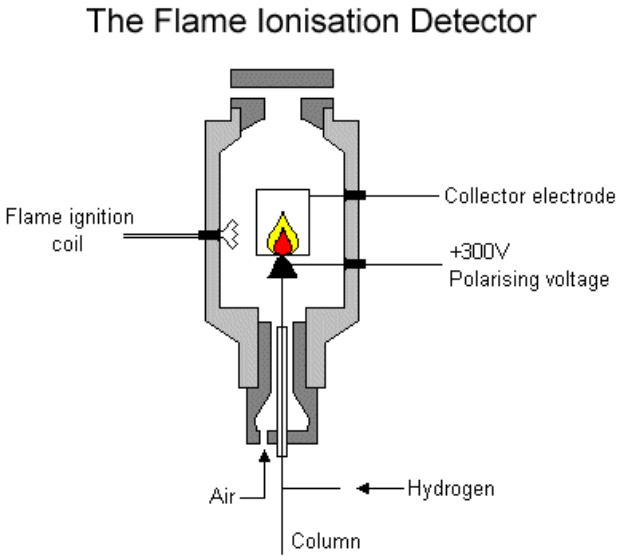


Figure 6: Flame Ionisation Detector [19]

The FID is an example of the ionization detectors invented specially for GC.
The column effluent is burned in a small oxy-hydrogen flame producing some ions in the process. These ions are collected and form a small current that becomes the signal. When no sample is being burned, there should be little ionization, the small current arising from impurities in the hydrogen and air supplies. [18]
Thus, the FID is a specific property-type detector with characteristic high sensitivity. The FID responds to all organic compounds that burn in the oxy-hydrogen flame. The signal is approximately proportional to the carbon content, giving rise to the so-called *equal per carbon* rule. [18]
For efficient operation, the gases (hydrogen and air) must be pure and free of organic material that would increase the background ionization. Their flow rates need to be optimized. For open tubular columns that have flows around 1ml/min, make-up gas is added to the carrier gas to bring the total up to about 30ml/min.
Compounds not containing organic carbon do not burn and are not detected.[18]

5.3. GC-MS

Gas chromatography-mass spectrometry occupies a special place among the analytical techniques involved in the investigations of plant volatiles as it combines a maximum of information with the use of minimum material. [17]

Although GC provides a very good separation of compounds, it cannot be used alone for identification of peaks. On the other hand, mass spectroscopy (MS) is one of the most sensitive detection techniques. In addition, it is easily coupled to a GC system, providing a good combination for detection. The only problem that occurs is that MS just works under vacuum conditions, and thus the carrier gas must be evacuated before the sample is subjected to MS. [17]

Mass spectroscopy is based on the bombardment of organic molecules with electrons and converting them to highly energetic positively charged ions which can break up into smaller ions. The ions are deflected by the magnetic field onto the collecting detector, which is combined with an amplifier and a data editing system. Results are presented in mass spectra that show a relative abundance and a mass to charge ratio (m/z) of the ions. With the help of computers and digital libraries (through the matching of obtained data with existing data in the libraries), most of the compounds can easily and rapidly be identified [17].

Figure 7 : Gas chromatograph 6890 connected to mass spectrometer HP 5971A [19]

5.4.Analytical conditions

For *qualitative and quantitative analysis* a Hewlett Packard 6890 Gas Chromatograph was used. This instrument was equipped with a FID detector and connected to a computer. Data processing was achieved with the ChemStation software.

The following analytical conditions were adapted to the GC instruments:

- Fused silica capillary columns :
  - **Supelcowax™ 10** (60m long x 0,25mm internal diameter x 0,25µm film thickness), polar column
  - **Methyl silicone** (60m long x 0,25mm internal diameter x 0,25µm film thickness), non-polar column
- Carrier gas: Helium
- Flow rate: 1 ml/min
- Split ratio: 45.8 : 1 (45.8 ml/min) in column Supelcowax
- Split less in column Methyl silicone; in both columns at the GC-MS instrument
- Oven temperature: shown below
- Initial temperature: 60 °C
- Final temperature: 240 °C
- Heating rate:

Oven temp	°C / min	Next °C	Hold min	Run time
Initial		60	2	2
Ramp 1	3.00	150	0	32
Ramp 2	5.00	240	30	80
Ramp2`	5.00	240	50	100

Ramp 2` conditions were only adopted to the polar column during the SFE- injections into the GC-FID instrument. The enlargement of the runtime helped to resolve the last doubts of peak identification and served therefore to complete the qualitative analysis.

- Detector temperature: 270 °C
- Injector temperature: 220 °C

For *qualitative analysis* injection of the samples into a Hewlett Packard Gas Chromatograph 6890 equipped with the Mass Spectrometer 5971A, was also used. The capillary columns were connected directly to the detector.

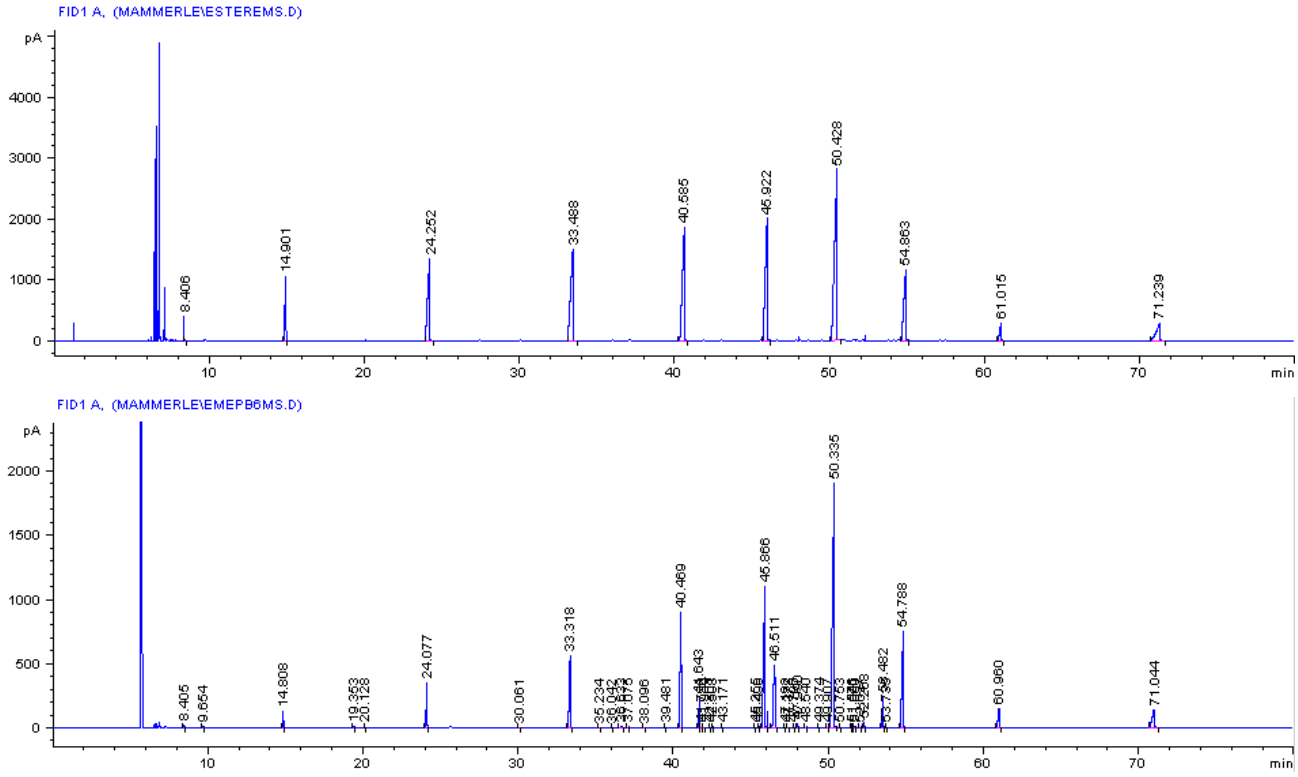
The analytical conditions were the same as listed above.
Mass spectra were obtained between m/z=30 and m/z=300, using an ionization energy of 70eV.
Again, the instrument was connected to a computer equipped with ChemStation software for data processing.

5.5. Identification of essential oil constituents

Identification, on the one hand, was achieved by calculating Retention indices for every peak in both stationary phases and, on the other hand, by comparison of the mass spectra with the data stored in digital library WILEY6.
Retention indices were achieved by injection of methyl esters of fatty acids and n-alkanes, also known as Kovat`s indices, in both column types.
Calculations were carried out corresponding to the formula given below:

$$\mathbf{I_R(A) = RI_{(n)} + 100 \times [(Rt_{(A)} - Rt_{(n)}) / (Rt_{(n+1)} - Rt_{(n)})]}$$

$I_R(A)$...Retention index of substance=peak A
$RI_{(n)}$...Retention index of reference (arbitrary given as 100); always to chose the peak in front of the substance A
$Rt_{(A)}$...Retention time of peak A
$Rt_{(n)}$...Retention time of the reference peak in front of peak A
$Rt_{(n+1)}$...Retention time of the reference peak after peak A



The first graphic above shows the chromatogram of n-alkanes obtained after a single injection into a non-polar column. Whereas the second graphic shows the chromatogram of a mixed injection of n-alkanes with sample also obtained after injection into a non-polar column.

5.6. Quantification of essential oil constituents

The amount of one peak is represented in % of the total sample. The peak areas were achieved without using correction factor. Again, each peak was quantified in polar and non-polar column.

6. Results and Discussion

Aim of the study was to identify and quantify the peaks in the sample by applying different extraction modes. The results achieved are presented in the tables below and additionally the results obtained by SPME and SFE were compared with the results achieved by distillation, which was carried out during an earlier study.
Identification of compounds was made by comparing the mass spectra obtained with data in Wiley 6.1 Library, and by calculating the retention indices. If the retention indices of some compounds corresponded with the retention indices in the literature, or if the mass spectra showed enough similarities with the mass spectra in the library, this was confirmed by writing an “X” in the tables. An “XX” means the spectrum and the index correspond, while “X0” means that the spectrum corresponds but the indices show greater differences. Some components in the tables below could only be identified by their MS because the according peaks were too small and therefore retention indices could not be calculated.

Presented below is a legend to provide better understanding of the following tables:
n° SC…..number of the peak in polar column
n° MS…..number of peak in non-polar column
name…..name of the extracted substance
masses/sc…..confirmation of the mass spectra obtained by polar column with the Wiley6 library
masses/ms…..confirmation of the mass spectra obtained by non-polar column with the Wiley6 library
RI-Est sc/exp…..Retention index calculated with fatty acid methyl esters in polar column/experimental
RI-Est sc/teoric…..Retention index calculated with fatty acid methyl esters in polar column/theoretical
RI-Est ms/exp..…Retention index calculated with fatty acid methyl esters in non-polar column/experimental
RI-Est ms/teoric…..Retention index calculated with fatty acid methyl esters in non-polar column/theoretical
conf RI/sc…..confirmation of the experimental Retention index with the theoretical Retention index / polar column
conf RI/ms…confirmation of the experimental Ret index with the theoretical Retention index/non-polar column
% ms…..quantification of the peak in % (“t” stands for traces and values smaller than 0,05%)
Rt(FID)sc….Retention time of the peak obtained by FID in polar column
Rt(MS)sc…..Retention time of the peak obtained by MS in polar column
Rt(FID)ms…..Retention time of the peak obtained by FID in non-polar column
Rt(MS)ms…..Retention time of the peak obtained by MS in non-polar column
RI-Alk sc/exp…..Retention index calculated with n-alkanes (Kovat`s index) in polar column/experimental
RI-Alk sc/teoric…..Retention index calculated with n-alkanes in polar column/theoretical
Conf RI-Alk sc…..confirmation of the experimental Retention index with the theoretical Retention index calculated with n-alkanes / polar column
RI-Alk ms/exp….Retention index calculated with n-alkanes (Kovat`s index) in non-polar column/experimental
RI-Alk ms/teoric….Retention index calculated with n-alkanes in non-polar column/theoretical
Conf RI-Alk ms….. confirmation of the experimental Retention index with the theoretical Retention index calculated with n-alkanes / non-polar column

6.1. SPME

SPME 3035

nº SC	nº MS	name	masses	masses	RI-Est sc	RI-Est sc	RI-Est ms	RI-Est ms	conf RI	conf RI	%ms	Rt (FID)sc	Rt (MS)sc	Rt (FID)ms	Rt (MS)ms	RI-Alk sc	RI-Alk sc	conf RI-Alk sc	RI-Alk ms	RI-Alk ms	conf RI-Alk ms
			sc	ms	exp	teoric	exp	teoric	sc	ms						exp	teoric		exp	teoric	
2	6	**alpha-pinene**	x	x	119	117-119	258	209-210	xx	ox	**3,0**	13,65	2,71	20,35	22,06	1026	1030	x	1028	930	ox
5	3	**2-beta-pinene**	x	x		159-164	234	223-224		x	**0,7**		3,7	18,081	19,55		1118		980	970	x
8	4	**alpha-phellandrene**	x	x		193-194	245	235-242		x	**0,5**		4,76	19,2	20,79		1171		1005	997	x
9	11	**alpha-terpinene**	x	x		200	287	242-245		ox	**0,4**		5,07	23,033	24,97		1187		1084	1010	ox
10		**limonene**	x		x	208-209			xx		**t**		5,51				1206				
11	6	**beta phellandrene + 1,8-cineol**	x	x	210	210	258	257	xx	xx	**3,0**	19,85	5,74	20,348	22,06	1209	1215	x	1028	997	ox
12		**cis-ocimene**	x			214-219					**t**		6,41				1235				
13	8	**gamma-terpinene**	x	x	223	226-228	271	264-276	x	xx	**1,8**	20,95	6,75	21,609	23,39	1236	1249	x	1054	1046	x
14	1	**styrene**	x	x	232		185				**16,4**	21,69	6,98	13,95	15,09	1254			888		
17	5	**p-cymene**	x	x		238-240	252	250		xx	**t**		7,36				1275				
18		**6-methyl-5-heptene-2-one**	x			267-288					**t**		9,48								
19	12	**allo-ocimene**	x	x			305	306		xx	**1,3**		10,70	24,806	26,88		1396		1121	1117	x
21		**acetic acid**	x								**t**		13,26								
22	24	**alpha copaene**	x	x		345-348	439	431-435		x	**0,5**		14,88	36,29	39,22		1487		1381	1369	x
23		**formic acid**	x								**t**		15,36								
24	2	**benzaldehyde**	x	x	377	361	214	213	x	x	**15,7**	33,57	16,05	16,275	17,56	1552			940		
25	15	**terpinen-4-ol**	x	x		405	329	326-327		xx	**0,7**		19,21	27,00	29,35		1597		1167	1158	x
26	10	**methylbenzoat**	x	x		405					**t**		19,76								
27		**Acetophenona**	x		441						**t**	37,99	20,84								
28	14	**Ethylbenzoate**	x	x	446		320				**1,9**	38,30	21,49	26,14	28,37	1691			1150		
29	27	**trans-beta-farnesene**	x	x	455	431	472	469	ox	x	**0,8**	38,91	21,84	38,615	41,58	1711	1666	ox	1448	1447	xx
30		**benzylformate**	x								**t**		22,20								
31	16	**alpha-terpineol**	x	x		442-444	335	329-332		x	**0,2**		22,89	27,486	29,86		1692		1177	1169	x
32	9	**guaiacol**	x	x	538	504	278		ox		**0,7**	43,64	28,57	22,20	24,05	1887			1067		
33	5	**benzylalcohol**	x	x	544	516	252	258	ox	x	**29,0**	43,93	29,35	19,779	21,24	1898			1017		
34	37	**nerolidol**	x	x	612	613-615	522	526	xx	x	**7,6**	47,22	35,15	41,80	45,00	2045	2037	x	1553	1545	x
35	14	**benzoic acid**	x	x	918		320				**t**	62,86	43,46								
36	43	**benzylbenzoate**	x	x		870	613	603-613		xx	**2,5**		47,17	46,52	50,20				1737		
15	7	**trans beta ocimene**	x	x			265	258-261		x	**7,9**			21,01	22,71				1043	1035	x
	9	**eugenol**		x				415			**t**				37,2					1324	
	20	**beta caryophyllene**		x				451-453			**t**				37,46					1408	
x	21	**vanillin**		x				417			**t**				38,22						
	23	**cycloisosativene**		x			436				**t**			36,05	39,01				1376		x
	25	**geranyl acetone**		x							**t**				40,96					1425	
	26	**ethylcinnamate + unknown**		x							**t**				41,28						
	31	**alpha farnesene**		x			494	489-495		xx	**0,2**			40,18	43,25				1497	1495	xx
	35	**cis alpha bisabolene**		x				513							44,52						
		total % identified									**94,8**										

According to the relatively low temperature the table shows, that foremost monoterpenes were extracted. This is reasonable because monoterpenes are more volatile than sesquiterpenes and other non-terpenic structures present in the Peru balsam. The constituents with the highest percentage like benzyl alcohol, styrene, benzaldehyde, trans beta cymene and nerolidol, a sesquiterpene, were therefore extracted the most.

SPME6050

nº SC	nº MS	name	masses sc	masses ms	RI-Est sc exp	RI-Est sc teoric	RI-Est ms exp	RI-Est ms teoric	conf RI sc	conf RI ms	%ms	Rt (FID)sc	Rt (MS)sc	Rt (FID)ms	Rt (MS)ms	RI-Alk sc exp	RI-Alk sc teoric	conf RI-Alk sc	RI-Alk ms exp	RI-Alk ms teoric	conf RI-Alk ms
1		**alpha-pinene**	x		119	119			x		**t**	13,67	2,75			1026	1030	x			
4		**beta-pinene**	x			159-164					**t**		3,60				1118				
5	4	**limonene**	x	x		208	257	250-258		xx	**0,3**		5,41	20,25	22,04		1206		1027		
6		**cis-beta-ocimene**	x			214-219					**t**		6,32				1235				
7		**gamma-terpinene**	x			226-228					**t**		6,58				1249				
8	5	**trans-beta-ocimene**	x	x	232	227	264	258-261	x	x	**1,1**	21,71	6,82	20,89	22,73	1255	1253	xx	1039	1035	x
9	1	**styrene**	x	x	240		183				**2,3**	22,38	6,97	13,79	15,02	1272			886		
10		**acetic acid**	x								**t**		13,18								
11	18	**cycloisosativene**	x	x							**t**		14,41								
12	19	**alpha copaene**	x	x		345-354	438	431-435		x	**0,5**		14,98	36,18	39,23		1487		1379	1369	x
15	2	**benzaldehyde**	x	x	377	361	213	213	x	xx	**3,3**	33,58	16,07	16,14	17,56	1552			936		
16		**terpinen-4-ol**	x			405					**t**		19,26								
17	8	**methylbenzoate**	x	x	408	405	281	284	x	x	**0,3**	35,95	19,77	24,48	22,47	1618			1073		
18		**acetophenone**	x		424						**t**	36,95	20,85			1649					
19	9	**ethylbenzoate**	x	x	441		319				**0,8**	37,99	21,56	26,03	28,38	1681			1147		
20	23	**trans-beta-farnesene**	x	x	445	431	471	469	x	x	**1,9**	38,32	22,07	38,54	41,63	1691	1666	ox	1447	1447	x
21	6	**benzyl-formate**	x	x			270				**0,3**		22,23	21,46	23,37				1052		
22		**alpha-terpineol**	x			433-434					**t**		22,97				1692				
23	27	**alpha murolene**	x	x		460		485-520			**t**		23,81		43,44		1718			1486	
24	28	**beta-bisabolene**	x	x		464	496	495-498		x	**0,3**		24,00	40,31	43,57		1723		1500	1495	x
25		**alpha-farnesene (Z/E)**	x								**t**		24,24				1746				
	25	**alpha curcumenen**		x			478	480		xx	**0,1**			39,04	42,68				1462	1466	x
	25a	**alpha amorphene**		x			484	475-484		xx	**0,3**			39,42	42,68				1474		
26	26	**alpha-farnesene (E,E)**	x	x	455	460	493	489-495	x	x	**1,5**	38,92	25,10	40,10	43,28	1711	1746	ox	1494	1495	x
27	7	**guaiacol**	x	x	538	504	277		ox		**0,3**	43,65	28,57	22,09	24,05	1887			1064		
28	3	**benzylalcohol**	x	x	544	516	251	258	ox	x	**11**	43,94	29,32	19,69	21,25	1899			1015		
29		**benzylalcohol**	x		544	516			ox		**t**	43,94	29,45			1899					
30		**unknown**			588						**t**	46,12	33,20			1994					
31		**unknown**			599						**t**	46,71	34,07			2021					
32	31	**nerolidol**	x	x	612	613	526	526	xx	xx	**36**	47,23	35,75	41,98	45,22	2046	2037	x	1559	1545	x
33	22	**ethyl cinnamate**	x	x			466				**0,5**		37,22	38,14	41,26				1435		
34	14	**eugenol**	x	x		624-667	411	415		x	**0,2**		37,99	34,29	37,2		2159		1331	1324	x
36	11	**benzoic acid**	x	x			344				**7,1**		43,48	28,30	30,33				1194		
38	16	**vanillin**	x	x			427	417		x	**1,5**			35,42	38,33						
39	37	**benzylbenzoate**	x	x	918	870	618	603-613	ox	x	**28**	62,89	47,42	46,74	50,46	>2300			1747		
41	20	**cinnamic acid**	x	x		979	453	480		ox	**0,4**		50,88	37,34	40,00				1407		
	13	**unknown**					401				**0,1**			33,59	36,5				1313		
	15	**beta caryophyllene**		x				451-453			**t**				37,47					1408	
	17	**unknown**					406				**0,4**			35,79	38,79				1369		
	21	**geranyl acetone**		x			463				**0,2**			37,95	40,99				1429	1425	x
	24	**unknown**									**t**				42,1						
	30	**cis alpha bisabolene**		x			503	513		x	**0,3**			40,77	44,54				1517		
	33	**unknown**					538				**0,4**			42,62	45,97				1582		
	36	**trans farnesol**		x			596	586-599		xx	**0,1**			45,69	49,33				1701	1696	x
		total % identified									**98,5**										

This table shows, that with increasing temperature and extraction time more less volatile constituents can be extracted, like sesquiterpenes and other structures which do not correspond to the family of terpenes. Therefore a very high percentage of nerolidol was extracted with 36% in comparison to SPME3035 with only 7,6%. Other constituents that are clearly extracted in bigger amounts than the others are benzyl benzoate and benzyl alcohol. The rest of the constituents only show small percentages.
This method extracted slightly more constituents than the other two methods, which have a shorter extraction time.

SPME3060

n° SC	n° MS	name	masses sc	masses ms	RI-Est sc exp	RI-Est sc teoric	RI-Est ms exp	RI-Est ms teoric	conf RI-Est sc	conf RI-Est ms	%ms	Rt (FID)sc	Rt (MS)sc	Rt (FID)ms	Rt (MS)ms	RI-Alk sc exp	RI-Alk sc teoric	conf RI-Alk sc	RI-Alk ms exp	RI-Alk ms teoric	conf RI-Alk ms
	1	**styrene**		x			184				**1,8**			13,87	15,00				887		
1		**alpha-pinene**	x		122	117-119			x		**t**	13,88	2,75			1034	1030	x			
3	5	**trans-beta-ocimene**	x	x	235	227	264	258-261	x	x	**0,8**	21,93	6,99	20,90	22,72	1260	1253	x	1040	1035	x
4		**acetic acid**	x								**t**		13,28								
5	18	**alpha copaene**	x	x	360	345-354	438	431-435	x	x	**0,4**	32,21	14,91	36,18	39,22	1516	1487	ox	1379	1369	x
6	2	**benzaldehyde**	x	x	379	361	213	213	ox	xx	**2,4**	33,77	16,02	16,19	17,56	1558			938		
7		**terpinen-4-ol**	x			405					**t**		19,19				1597				
8	8	**methylbenzoate**	x	x		405	281	284		x	**0,2**		19,72	22,48	24,57				1073		
9		**Acetophenone**	x		437						**t**	37,81	20,8			1675					
10	9	**ethylbenzoate**	x	x			319				**0,6**		21,49	26,03	28,37				1147		
11	22	**trans-beta-farnesene**	x	x	448	431	471	469	ox	xx	**1,8**	38,46	22,00	38,53	41,63	1695	1666	ox	1446	1447	xx
12	6	**benzyl formate**	x	x			270				**0,3**		22,18	21,48	23,32				1052		
13	26	**alpha murolene**	x	x		460		485-520			**t**		23,73		43,43		1718			1486	
14	27	**beta-bisabolene**	x	x		464	496	495-498		xx	**0,4**		23,92	40,3	43,56		1723		1500	1495	x
15		**alpha-farnesene (Z,E)**	x			460					**t**		24,16								
16	25	**alpha-farnesene(E,E)**	x	x	473	471	493	489-495	x	xx	**1,4**	40,03	25,01	40,094	43,27	1750	1746	x	1494	1495	xx
17	7	**guaiacol**	x	x		504	277				**0,2**		28,53	22,09	24,05				1065		
18	3	**benzylalcohol**	x	x	546	516	251	258	ox	x	**8,8**	44,02	29,35	19,67	21,24	1902			1015		
	4	**limonene**		x			257	250-258		xx	**0,2**			20,27	22,04				1027		
19		**unknown**			589						**t**	46,18	33,17			1996					
20		**unknown**			601						**t**	46,75	34,01			2023					
21	32	**nerolidol**	x	x	613	613-615	526	526	x	xx	**38**	47,29	35,74	41,99	45,25	2049	2037	x	1559	1545	x
23	21	**ethyl cinnamate**	x	x			466				**0,4**		37,19	38,14	41,25				1435		
24	13	**eugenol**	x	x	671	650-674	401	415	xx	x	**0,1**	49,79	37,95	33,59	37,19	2172	2159	x	1313	1324	x
25	12	**vinyl-methoxy-phenol**	x	x							**t**		38,65								
26	11	**benzoic acid**	x	x	918		343				**6,0**	62,81	43,34	28,22	30,5				1193		
28	15	**vanillin**	x	x			x	417		x	**t**		46,05	~35,5	38,35						
29	38	**benzylbenzoate**	x	x		870	619	603-613		x	**31,1**		47,34	46,76	50,51				1748		
31	19	**cinnamic acid**	x	x		979	455	480		ox	**0,8**		50,89	37,42	40,13				1413		
32	39	**benzyl cinnamate**	x	x							**t**		57,67		58,94						
	20	**geranyl acetone**		x			463				**0,2**			37,95	40,98				1428	1425	x
	36	**farnesol, trans**		x			585	586-599		xx	**0,1**			45,14	48,75				1679	1696	ox
	12	**unknown**					388				**0,4**			32,41	35,3				1285		
	16	**unknown**					432				**0,3**			35,79	38,79				1369		
	20	**geranyl acetone**		x			463				**0,2**			37,95	40,98				1429	1425	x
	28	**cis gamma bisabolene**		x							**t**				43,83						
	29	**beta sesquiphellandren**		x			503	501-505		xx	**0,7**			40,76	44,05				1517	1510	x
	30	**trans gamma bisabolene**		x							**t**				44,28						
	31	**cis alpha bisabolene**		x				513			**t**				44,52						
	34	**unknown**					538				**t**			42,625	45,97				1582		
	35	**unknown**									**t**			43,27	46,69						
	37	**trans farnesol**		x			596	586-599		xx	**0,1**			45,69	49,32				1701	1696	x
		total % identified									97,8										

Because of the high temperature again mainly sesquiterpenes and other less volatile components were extracted. Nerolidol was extracted the most with 38%. Followed by benzyl benzoate with 31,1%. All the other constituents that were identified only show relative percentages under 10%.

6.2.SFE

Step1/vial1/first extraction

nº SC	nº MS	name	masses sc	masses ms	RI-Est sc exp	RI-Est sc teoric	RI-Est ms exp	RI-Est ms teoric	conf RI-Est sc	conf RI-Est ms	%ms	Rt (FID)sc	Rt (MS)sc	Rt (FID)ms	Rt (MS)ms	RI-Alk sc exp	RI-Alk sc teoric	conf RI-Alk sc	RI-Alk ms exp	RI-Alk ms teoric	conf RI-Alk ms
1		**toluene**	x			121					**t**		2,89								
2		**benzyl alcohol**	x		541	516			ox		**t**	41,68	29,14								
	1	**beta farnesene**		x				469			**t**				41,6				1547	1447	ox
	2	**alpha curcumene**		x							**t**				42,54						
	3	**alpha-farnesene(E,E)**		x							**t**				43,29						
	4	**beta bisabolene**		x				495-498			**t**				43,57				1591	1495	ox
3	5	**nerolidol**	x	x	609	613-615	519	526	x	x	**8,4**	45,24	34,98	41,62	44,96	2043	2037	x	1547	1545	x
4		**benzoic acid**	x								**t**	50,04	43,64								
6	6	**benzyl benzoate**	x	x	916	870	611	603-613	ox	xx	**48,9**	59,38	46,87	46,4	50,23				1732		
7		**cinnamic acid**	x			979		480			**t**		50,96								
8		**benzyl cinnamate**	x		>1000		770				**t**	88,46	57,66	53,43	59,12				2060		
9		**unknown**	x								**t**		58,43								
		total % identified									**57,3**										

Step2/vial2/first extraction

n° SC	n° MS	name	masses	masses	RI-Est sc	RI-Est sc	RI-Est ms	RI-Est ms	conf RI-Est	conf RI-Est	%ms	Rt (FID)sc	Rt (MS)sc	Rt (FID)ms	Rt (MS)ms	RI-Alk sc	RI-Alk sc	conf RI-Alk sc	RI-Alk ms	RI-Alk ms	conf RI-Alk ms
			sc	ms	exp	teoric	exp	teoric	sc	ms						exp	teoric		exp	teoric	
2	1	**benzylalcohol**	x	x	541	516	256	258	ox	x	**0,3**	41,64	29,15	20,14	21,1				1024		
	2	**unknown**		x							**t**				34,37						
	3	**trans beta farnesene**		x			425	469		ox	**0,3**			35,24	41,65				1547	1447	ox
	4	**unknown**		x							**t**				41,99						
	5	**alpha curcumene**		x							**t**				42,59						
	6	**alpha farnesene Z/E**		x							**t**				42,81						
	7	**alpha farnesene E/E**		x			484	489-495		x	**0,2**			39,48	43,31				1475	1495	x
	8	**beta bisabolene**		x				495-498			**t**				43,61						
	9	**cis alpha bisabolene**		x							**t**				44,59						
3	10	**nerolidol**	x	x	611	613-615	520	526	x	x	**17,7**	45,23	35,05	41,64	45,03	2046	2037	x	1564	1545	ox
	11	**unknown**		x							**t**				45,38						
4		**benzoic acid**	x		695						**t**	48,94	43,62								
5	12	**benzylbenzoate**	x	x	916	870	612	603-613	ox	xx	**60,4**	59,42	47,25	46,45	50,34				1741		
6		**cinnamic acid**	x								**t**		50,98								
6b		**benzyl cinnamate**	x								**t**	71,4	~52								
7	13	**benzyl cinnamate**	x	x	>1000		768				**15,2**	88,59	57,74	53,44	59,04				2060		
		total % identified									**94,1**										

Step3/vial3/first extraction

n° MS	name	masses	masses	RI-Est sc	RI-Est sc	RI-Est ms	RI-Est ms	conf RI-Est	conf RI-Est	%ms	Rt (FID)sc	Rt (MS)sc	Rt (FID)ms	Rt (MS)ms	RI-Alk sc	RI-Alk sc	conf RI-Alk sc	RI-Alk ms	RI-Alk ms	conf RI-Alk ms
		sc	ms	exp	teoric	exp	teoric	sc	ms						exp	teoric		exp	teoric	
1	**benzyl alcohol**	x	x	518	516	x	258	xx	x	**t**	40,47	29,15	~19	~21						
2	**trans beta farnesene**		x			444	469		ox	**1,6**			36,62	41,64				1390	1447	ox
3	**alpha-farnesene (Z,E)**		x							**t**				43,31						
4	**alpha-farnesene(E,E)**		x			457	489-495		ox	**0,1**			37,55	43,61				1416	1495	ox
5	**beta bisabolene**		x							**t**				44,59						
7	**unknown**	x	x							**t**	44,02	32,96		45,38						
	unknown	x								**t**	44,63	33,8								
6	**nerolidol**	x	x	612	613-615	518	526	xx	x	**5,1**	45,28	35,11	41,55	45,05	2049	2037	x	1544	1545	xx
	benzoic acid	x		667						**t**	47,71	43,59								
	vanillin	x		671			417			**t**	47,89	46,05								
8	**benzyl benzoate**	x	x	918	870	611	603-613	ox	xx	**53,9**	59,55	47,34	46,42	50,42						
	unknown	x								**t**		48,1								
	cinnamic acid	x			979		480			**t**		50,97								
	benzyl cinnamate	x								**t**	71,43	52,17								
9	**benzyl cinnamate**	x	x							**37,0**	88,83	57,89	53,46	59,07						
	total % identified									**97,7**										

The number of components extracted and the total % identified increase with each step, even if just slightly. An increase in pressure hardly shows effects on the amount of the volatiles extracted. Whereas the percentages of benzyl benzoate and benzyl cinnamate rise with the pressure.

Vial4/second extraction

n° MS	name	masses sc	masses ms	RI-Est sc exp	RI-Est sc teoric	RI-Est ms exp	RI-Est ms teoric	conf RI-Est sc	conf RI-Est ms	%ms	Rt (FID)sc	Rt (MS)sc	Rt (FID)ms	Rt (MS)ms	RI-Alk sc exp	RI-Alk sc teoric	conf RI-Alk sc	RI-Alk ms exp	RI-Alk ms teoric	conf RI-Alk ms
4	**trans beta farnesene**	x	x		431	444	469		ox	**1,2**		21,7	36,64	41,7				1391	1447	ox
1	**benzyl alcohol**	x	x	541	516	247	258	ox	x	**0,2**	41,65	29,09	19,32	21,16						
2	**methyl benzoate**		x				284			**t**				24,52						
3	**methyl cinnamate**		x							**t**			~35	38,2						
5	**unknown**		x							**t**				42,03						
6	**alpha curcumene**		x				480			**t**				42,63					1466	
7	**alpha-farnesene (Z,E)**		x							**t**				42,85						
8	**alpha-farnesene(E,E)**		x			484	489-495		x	**0,1**			39,44	43,36				1474	1495	ox
9	**beta bisabolene**		x				495-498			**t**				43,66					1495	
10	**unknown**		x							**t**				44,09						
11	**cis alpha bisabolene**		x							**t**				44,63						
	unknown	x								**t**	43,99	32,92								
	unknown	x								**t**	44,61	33,72								
12	**nerolidol**	x	x	611	613-615	519	526	xx	x	**7,0**	45,22	35,07	41,59	45,14	2046	2037	x	1546	1545	xx
13	**unknown**		x			525				**0,1**			41,9	45,45				1557		
14	**unknown**		x			535				**0,1**			42,48	46,04				1577		
	benzoic acid	x		682						**t**	48,36	43,55			2196					
	vanillin	x		696						**t**	49,01	45,99			2229					
15	**benzyl benzoate**	x	x	916	870	612	603-613	ox	xx	**55,9**	59,41	47,29	46,46	50,56						
	cinnamic acid	x			979		480			**t**		50,92								
16	**benzyl cinnamate**	x	x							**34,4**	88,81	57,77	53,49	59,13						
	total % identified									**99,0**										

Here, the same pressure as in step3 of the first extraction was applied. Therefore the percentages of the extracted constituents are almost identical. Furthermore the same number of constituents was extracted. Again benzyl benzoate and benzyl cinnamate show the highest percentages, followed by nerolidol.

6.3. Comparison of the results with distillation

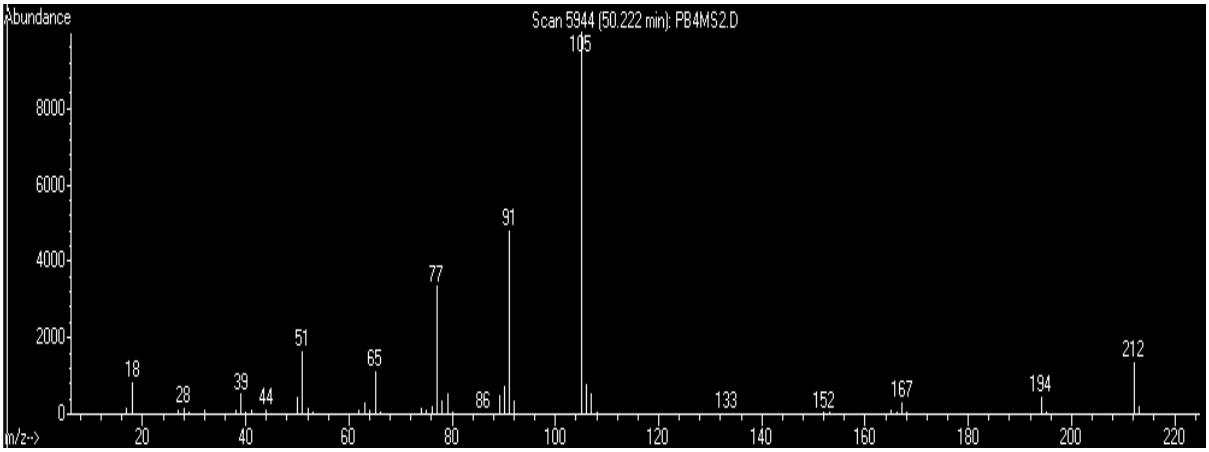
	%	%	%	%	%	%	%	%
constituents	SPME3035	SPME6050	SPME3060	SFEvial1	SFEvial2	SFEvial3	SFEvial4	Distillation
beta phellandrene+1,8-cineol	0,4	/	/	/	/	/	/	/
6-methyl-5-heptene-2-one	t	/	/	/	/	/	/	/
acetic acid	t	t	t	/	/	/	/	/
acetophenone	t	0,7	t	/	/	/	/	/
allo ocimene	1,3	/	/	/	/	/	/	/
alpha amorphene	/	0,3	/	/	/	/	/	/
alpha copaene	0,5	0,5	0,4	/	/	/	/	/
alpha curcumene	/	0,1	/	t	t	/	t	/
alpha farnesene (E,E)	0,2	1,5	1,4	t	0,2	0,1	0,1	0,2
alpha farnesene (Z,E)	/	t	t	/	t	t	t	/
alpha murolene	/	t	t	/	/	/	/	0,2
alpha phellandren	0,5	/	/	/	/	/	/	/
alpha pinene	3,0	t	t	/	/	/	/	/
alpha terpinene	0,4	/	/	/	/	/	/	/
alpha terpineol	0,2	t	/	/	/	/	/	0,2
aristolene	/	/	/	/	/	/	/	/
benzaldehyde	15,7	3,3	2,4	/	/	/	/	0,7
benzoic acid	t	7,1	6,0	t	t	t	t	0,1
benzyl alcohol	29,0	11	8,8	t	0,3	t	0,2	t
benzyl benzoate	2,5	27,9	31,1	48,9	60,4	53,9	55,9	44,6
benzyl cinnamate	/	/	t	t	15,2	37,0	34,4	1,3
benzyl formate	t	0,3	0,3	/	/	/	/	/
beta bisabolene	/	0,3	0,4	t	t	t	t	0,1
beta caryophyllene	t	t	/	/	/	/	/	/
beta farnesene	/	/	/	t	/	/	/	/
beta pinene	1,1	t	/	/	/	/	/	/
beta sesquiphellandren	/	/	0,7	/	/	/	/	/
cinnamic acid	/	0,4	0,8	t	t	t	t	0,1
cis alpha bisabolene	t	0,3	t	/	t	/	t	/
cis beta ocimene	t	t	/	/	/	/	/	/
cis gamma bisabolene	/	/	t	/	/	/	/	/
cycloisosativene	t	t	/	/	/	/	/	/
ethyl benzoate	1,9	0,8	0,6	/	/	/	/	1,1
ethyl cinnamate	t	0,5	0,4	/	/	/	/	0,4
ethylbenzene	/	/	/	/	/	/	/	/
eugenol	t	0,2	0,1	/	/	/	/	0,1
formic acid	t	/	/	/	/	/	/	/
gamma terpinene	1,8	t	/	/	/	/	/	/
geranyl acetone	t	0,2	0,4	/	/	/	/	0,1
guaiacol	0,7	0,3	0,2	/	/	/	/	/
limonene	t	0,3	0,2	/	/	/	/	/
methyl benzoate	t	0,3	0,2	/	/	/	t	0,2
methyl cinnamate	/	/	/	/	/	/	t	0,1
naphthalene	/	/	/	/	/	/	/	/
nerolidol	7,6	35,6	38,0	8,4	17,7	5,1	7,0	46,1
para cymene	t	/	/	/	/	/	/	/
patchoulene	/	/	/	/	/	/	/	/
styrene	16,4	2,3	1,8	/	/	/	/	0,1
terpinen-4-ol	0,7	t	t	/	/	/	/	0,3
trans beta farnesene	0,8	1,9	1,8	/	0,3	1,6	1,2	t
trans beta ocimene	7,9	1,1	0,8	/	/	/	/	/
trans caryophyllene	/	/	/	/	/	/	/	/
trans farnesol	/	0,1	0,2	/	/	/	/	/
trans gamma bisabolene	/	/	t	/	/	/	/	/
vanillin	t	1,5	t	/	/	t	t	/

The major constituents identified by *hydro distillation* are the sesquiterpene nerolidol with 46,1% and benzyl benzoate with 44,6%. 18 components of Peru balsam were identified all together. Next to the two components mentioned above, only 2 more achieved percentages slightly over the value of 1. Benzyl cinnamate and ethyl benzoate namely.

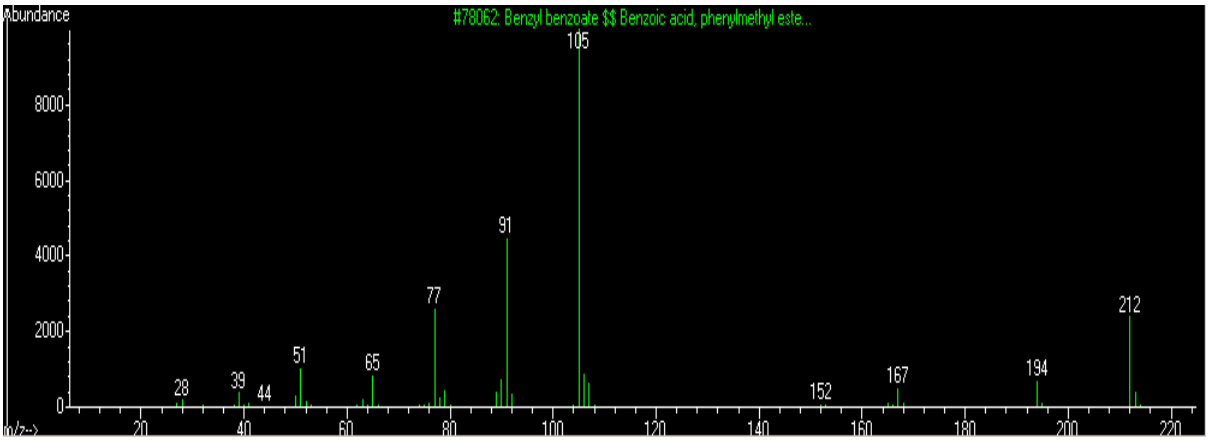
Major constituents in the volatile fraction extracted by *SPME* are nerolidol with 38%, benzyl benzoate with about 31%, as well as benzyl alcohol, benzaldehyde, benzoic acid and trans beta cymene with percentages around the value of 10.

*SFE´*s major constituents are formed by benzyl benzoate with around 60%, followed by benzyl cinnamate showing the highest value of 33% in vial4 and nerolidol with approximately 16% in vial2.

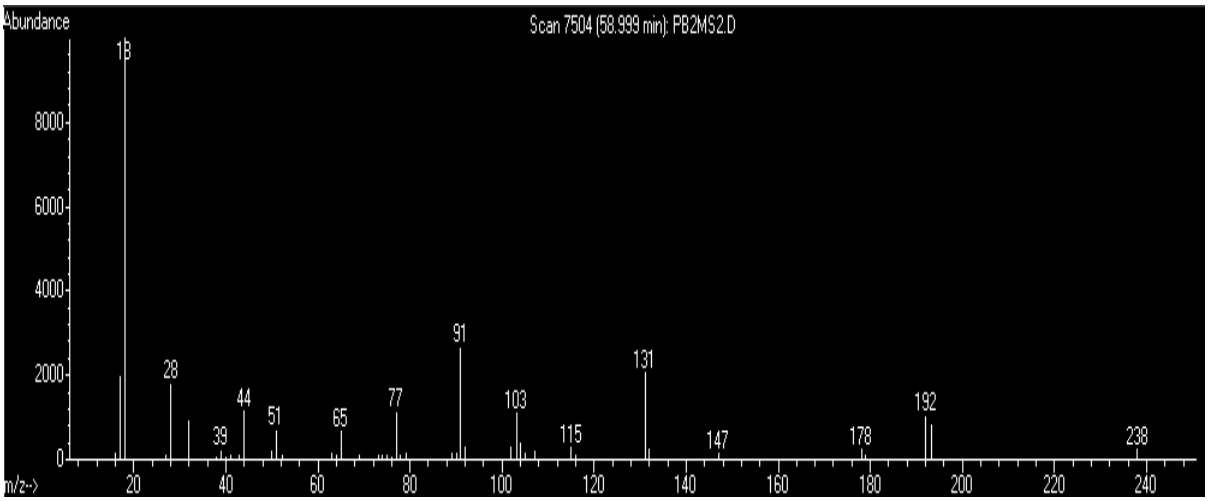
In summary the tables show an increased extraction of sesquiterpenes and other less volatile components when higher extraction temperatures are applied. The main constituents of the Peru balsam according to their relative percentages are benzyl benzoate, benzyl cinnamate, nerolidol, benzyl alcohol and benzaldehyde.
Further can be observed that with an increase of temperature the percentages of the monoterpenes are reduced to a minimum. Some even seem to have disappeared. That's because other components, like sesquiterpenes, increase in their percentages and therefore change the proportions.
It's evident that with SPME more constituents can be extracted than with SFE and distillation. Further research should be done to improve SPME extraction conditions.
The mass spectra of the main components as mentioned above are shown here:



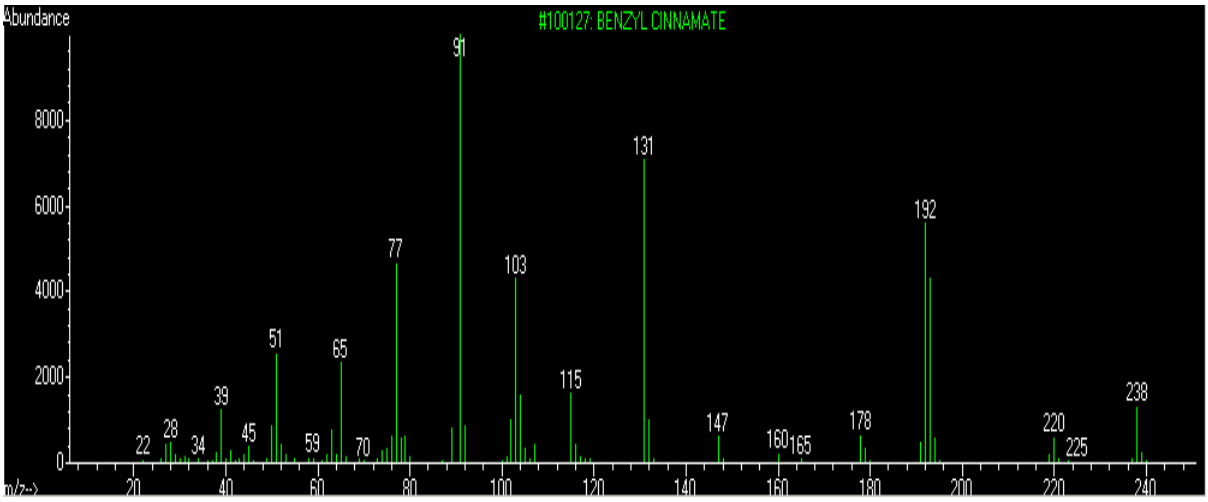
Benzyl benzoate out of the file „PB4MS2"



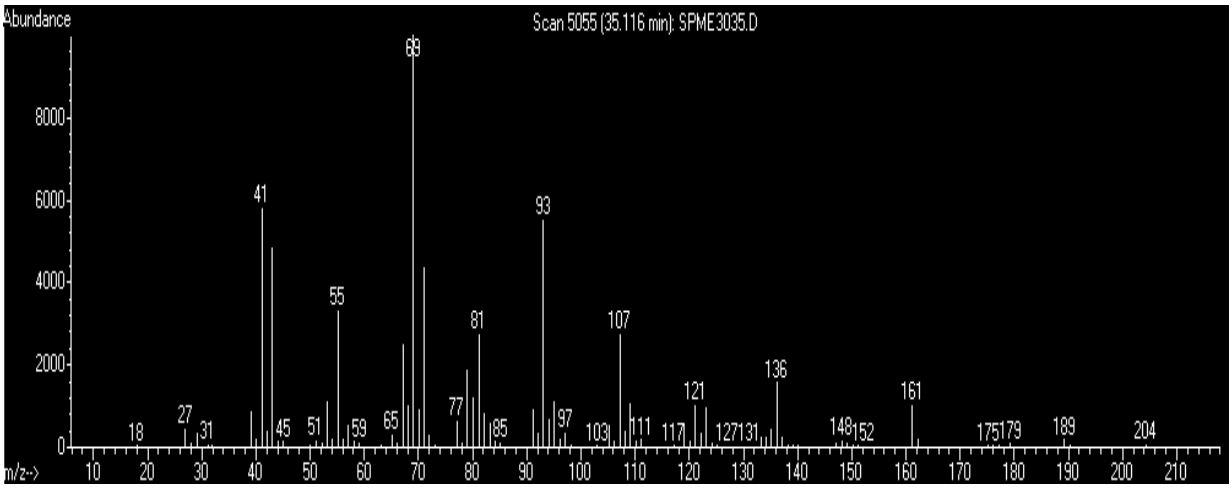
Benzyl benzoate out of the Wiley6 library



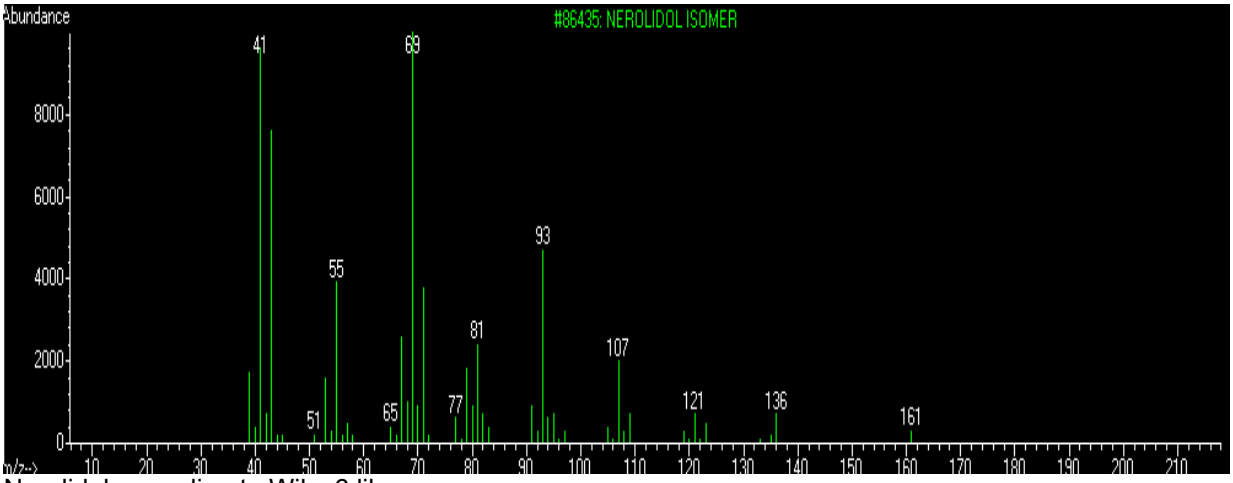
Benzyl cinnamate out of the file „PB2MS2“



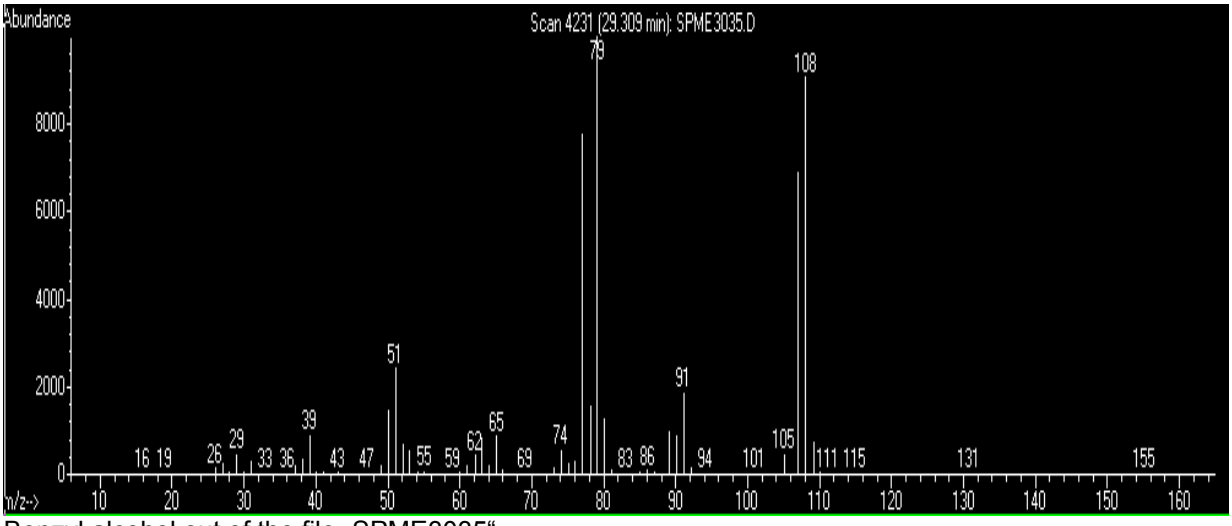
Benzyl cinnamate out of the Wiley6 library



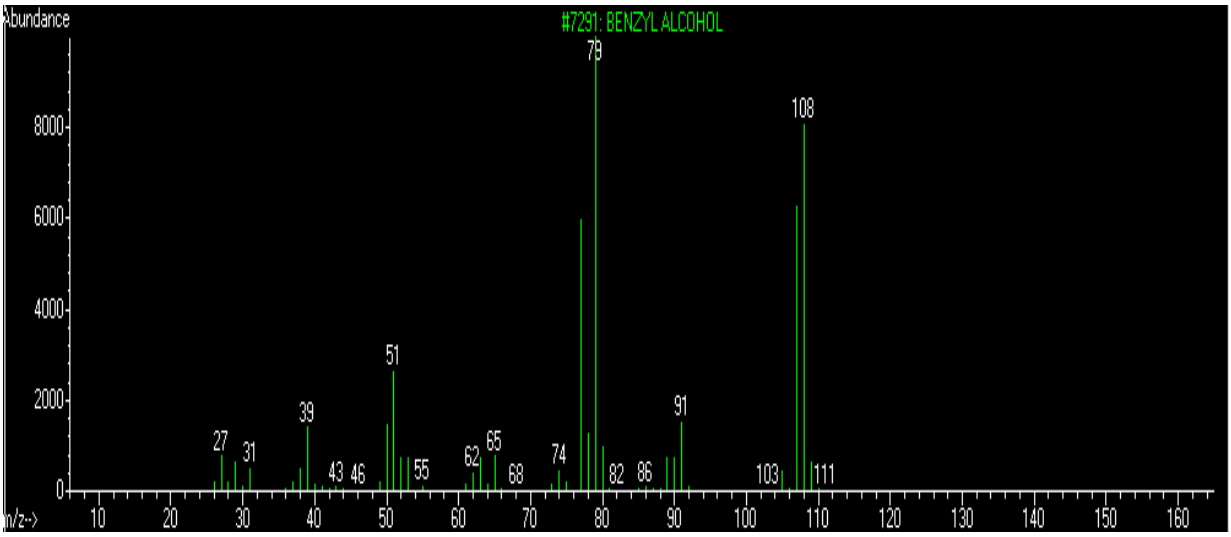
Nerolidol out of the file „SPME3035“



Nerolidol according to Wiley6 library



Benzyl alcohol out of the file „SPME3035“



Benzyl alcohol out of Wiley6 library

PART B – DESIGN OF AN HPLC METHOD FOR QUALITY CONTROL OF PERU BALSAM

7. High Performance Liquid Chromatography

7.1. General Introduction

Over the last 40 years chromatography has experienced a situation of continuous growth.

HPLC is best described as High Performance Liquid Chromatography since the essence of the technique is the highly resolutive separation which can be achieved by the use of uniform micro particulate chromatographic support and well-designed equipment. Other acronyms such as high pressure or high speed liquid chromatography are often used but do not reflect the essential features of the technique. [16]

The use of liquid chromatography for the analysis of biological materials has many advantages over the classical technique of gas chromatography, since the liquid mobile phase allows the separation and recovery of substances which are not readily volatilized. [16]

In addition, liquid chromatography is to be preferred for molecules which have high polarity, high molecular weight, and a number of ionic groups or thermal instability.

The theory of HPLC is not very different than that of gas liquid chromatography. The use of a liquid instead of a gaseous phase introduces several important constraints on the chromatographic system. Since liquids are more viscous (20 to 200 times) and exhibit lower diffusion rates (3000 to 30000 times) than gases, the separation column must be operated at higher pressures in HPLC than in GC. [16]

The analysis time is kept short in liquid chromatography by the use of small columns (typically 4mm by 30cm) and small particle sizes (<10ym) with a consequent improvement in equilibration rates and hence, analysis time. Another advantage with HPLC is that closed, reusable columns are used, thus hundreds of samples can be run through an individual column without repacking. [16]

7.2. Working conditions

The aim of the experiments that were carried out was to develop a method which can be applied to detect and quantify the most characteristically constituents of Peru balsam such as *benzoic acid*, *cinnamic acid*, *benzyl benzoate* and *benzyl cinnamate*. The method was meant to have a short runtime and the sum of the ester-components should correspond to the percentage demanded by the European Pharmacopoeia, which are a minimum of 45% and a maximum of 70%. The quantification was carried out by using an internal standard. Description of the calculation procedure and the corresponding results are presented below.

Development of the most suitable processing parameters required various tests. Parameters most likely to influence the outcome of the peaks in the chromatogram had to be adopted, improved and changed frequently, until the best mobile phase composition, detector wavelength, runtime and separation gradient were determined. Injections were carried out with the following samples:

- Solution of Peru balsam
- Reference solutions of: benzoic acid, cinnamic acid, benzyl benzoate and benzyl cinnamate
- Internal standard solutions.

The obtained chromatograms that served for the quantitative determination of the main constituents of the Peru balsam can be looked through in the appendix.

A Waters-HPLC instrument with following references was used for this study:

- Waters 1525
- Binary HPLC Pump
- Dual λ Absorbance Detector, Waters 2487
- Data processing through connection with computer with the software “Breeze”

The column is described as:

- Nucleosil 120, RP-C18 (Teknokroma, “Tracer”)
- Dimension: 25 x 0,45cm
- Particle dimension: 3μm

Designing a method, first of all, a mobile phase composition had to be chosen. According to literature mixtures of Acetonitrile : Water in different proportions varying from 70:30 to 60:40 and 50 : 50 were applied. As it turned out, it was necessary to

generate a gradient to achieve a better separation of the peaks, especially of the ester compounds. Examples for gradients that were tried are listed here:

- Acetonitrile : Water 70:30 → 100:0
- 25:75 → 75:25 → 100:0 → 75:25
- 5:95 → 75:25 → 100:0 → 75:25

Another variation of the mobile phase was tried out by adding small percentages of acids. These acids were meant to improve the appearance of the small peaks representing benzoic acid and cinnamic acid. 0,05% trichloracetic acid, 1% acetic acid, 0,1% acetic acid and 0,05% acetic acid were added to the mobile phase in different elution methods. None of these injections provided useful chromatograms. Thus, the decision was made not to add acid to the mobile phase.

Another very important parameter that had to be varied was the runtime. Different chromatograms from 20min up to 56 min were obtained and compared. It was necessary to find the shortest runtime possible still showing clearly separated peaks. For the first injections the wavelength λ=254 was applied. According to the absorption maximum of benzoic acid (λ =235nm) and cinnamic acid (λ =280nm), the dual detector was adapted to these two wavelengths.

Finally, the parameters which showed the best results and therefore were chosen to be applied for the analysis of the Peru balsam are shown here:

- λ = 235 / 280 nm
- Injected volume= 10μl
- Mobile phase= Acetonitrile : Water
- Gradient= 0min 5:95
  1min 5:95
  15min 90:10
  35min 90:10
  40min 5:95
- Run time= 40minutes
- Flow rate= 1ml/min

Further, to do the quantitative analysis an internal standard to be added to the Peru balsam solution had to be chosen. An internal standard enables to calculate the concentration of the constituents, represented as peaks, in the sample-solution. The most important properties of an internal standard have to be that the corresponding

peak does not interfere with the peaks to be calculated, that the peak-heights corresponds to the sample-peaks (not too high and not too small) and further, that the internal standard can be detected at the respective wavelengths, 235nm and 280nm.
Injections of Quercetin, p-coumaric acid and coumarin were undertaken. 1mg of each substance was dissolved separately in 10ml methanol. As it could be observed, coumarin showed the best properties to be used as internal standard.

The quantification with an internal standard required the following steps:

- preparation of the reference standard solution consisting of
  4,3mg benzoic acid
  6,85mg cinnamic acid
  100,57mg benzyl benzoate
  40,34mg benzyl cinnamate
  dissolved in 100ml MeOH
- preparation of an internal standard solution: 80,40mg Coumarin dissolved in 20ml MeOH
- preparation of several dilutions of the reference standard solution with the internal standard :
  Dilution1: 10ml reference standard solution + 1ml internal standard solution
  Dilution2: 5ml reference standard solution + 1ml internal standard solution
  Dilution3: 2,5ml reference standard solution + 1ml internal standard solution
  Dilution4: 1ml reference standard solution + 1ml internal standard solution
- Injection of the Dilutions: each dilution was injected three times
- Injection of the Sample solution consisting of 7,98mg Coumarin and 15,51mg Peru balsam dissolved in 20ml MeOH: injected three times
- Calculation of the response factor (“f”) for each substance and therefore each peak corresponding to every single dilution.
- Calculation of the percentages of the constituents of the Peru balsam.

For each substance (benzoic acid, cinnamic acid, benzyl benzoate, and benzyl cinnamate) the factor “f” was calculated three times for each dilution. These three factors were used to calculate the average value for each dilution. And out of these four different factors was again calculated the average value “f “. This result could

finally be used to calculate the percentage of the substance in the Peru balsam. These three results again served to calculate the average percentage, which represents the final result.
The chromatograms obtained by the injections are shown in the Appendix (9.5).

$$f = [A] / [C] \times A_{Coumarin} / A_{analyte}$$

f……response factor of the analyte (to the Coumarin in the respective dilution)
[A]……concentration of the analyte in the dilution
[C]……concentration of the Coumarin; has the same value in every dilution!
A……peak area

$$\% = A_{(Analyte)} \times (C_{Coumarin} / A_{Coumarin}) \times f \times V \times (100/g)$$

A……peak area
C……concentration in g/L
f……response factor
V……volume of sample (7,98mg Coumarin and 15,51 mg Peru balsam dissolved in 20ml MeOH) that was prepared in liter
g……gram of Peru balsam in the sample

7.3. Results

Here are given the results:
Benzoic acid concentration (235nm) = 1,56%
Cinnamic acid concentration (235nm) = 4,06%
Cinnamic acid concentration (280nm) = 4,89%
Benzyl benzoate concentration (235nm) = 30,84%
Benzyl cinnamate concentration (235nm) = 22,70%
Benzyl cinnamate concentration (280nm) = 23,83%
Sum of ester-constitutes (235nm) = 53,54%

The constituents cinnamic acid and benzyl cinnamate show higher results when calculated at their respective wavelength, which is 280nm. Nevertheless the differences are just minimal and therefore the question arises if it is necessary to carry out the analysis at both wavelengths.

The sum of the ester fractions, benzyl benzoate and benzyl cinnamate at 235nm, is 53,54% and corresponds therefore to the demand of the European Pharmacopoeia. These required aims were achieved: all peaks appear clearly separated from each other, the analysis time is as short as possible and the percentages of the ester-compounds are high enough.

The calculations in detail are shown here:

Sample: 7,98mg Coumarin + 15,51mg Perubalsam / 20ml MeOH
0,399 g/L coumarin
Coumarin /Standard: 0,201 g/L

Benzoic acid concentration (235nm)

4,3mg/100ml artificial sample

Dilution1 (10ml artificial sample + 1ml standard solution / 20ml MeOH)
0,0215 g/L Benz.ac.

f1/1=	0,25	**f1/2=**	0,27	**f1/3=**	0,28	**f1=**	**0,27**

Dilution2 (5ml artificial sample + 1ml standard solution / 20ml MeOH)
0,01075 g/L Benz.ac.

f2/1=	0,29	**f2/2=**	0,29	**f2/3=**	0,29	**f2=**	**0,29**

Dilution3 (2,5ml artificial sample + 1ml standard solution / 20ml MeOH)
0,005375 g/L Benz.ac.

f3/1=	0,27	**f3/2=**	0,32	**f3/3=**	0,29	**f3=**	**0,29**

Dilution4 (1ml artificial sample + 1ml standard solution / 20ml MeOH)
0,00215 g/L Benz.ac.

f4/1=	0,32	**f4/2=**	0,39	**f4/3=**	0,32	**f4=**	**0,35**

		f=	**0,30**
%1=	**1,63**		
%2=	**1,51**		**1,56 %**
%3=	**1,53**		

Cinnamic acid concentration (235nm)

6,85mg/100ml artificial sample

Dilution1 (10ml artificial sample + 1ml standard solution / 20ml MeOH)
0,03425 g/L cinn.ac.

f1/1=	0,67	**f1/2=**	0,51	**f1/3=**	0,42	**f1=**	**0,54**

Dilution2 (5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,017125 g/L cinn.ac.

f2/1=	0,64	**f2/2=**	0,60	**f2/3=**	0,46	**f2=**	**0,57**

Dilution3 (2,5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,0085625 g/L cinn.ac.

f3/1=	0,57	**f3/2=**	0,47	**f3/3=**	0,45	**f3=**	**0,50**

Dilution4 (1ml artificial sample + 1ml standard solution / 20ml MeOH)

0,003425 g/L cinn.ac.

f4/1=	0,55	**f4/2=**	0,51	**f4/3=**	0,51	**f4=**	**0,52**

		f=	**0,53**
%1=	**3,38**		
%2=	**3,81**		**4,06 %**
%3=	**4,99**		

Benzyl benzoate concentration (235nm)

100,57mg/100ml artificial sample

Dilution1 (10ml artificial sample + 1ml standard solution / 20ml MeOH)

0,50285 g/L benzyl benz.

f1/1=	0,32	**f1/2=**	0,32	**f1/3=**	0,32	**f1=**	**0,32**

Dilution2 (5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,251425 g/L benzyl benz.

f2/1=	0,31	**f2/2=**	0,30	**f2/3=**	0,30	**f2=**	**0,30**

Dilution3 (2,5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,1257125 g/L benzyl benz.

f3/1=	0,28	**f3/2=**	0,28	**f3/3=**	0,28	**f3=**	**0,28**

Dilution4 (1ml artificial sample + 1ml standard solution / 20ml MeOH)

0,050285 g/L benzyl benz.

f4/1=	0,30	**f4/2=**	0,30	**f4/3=**	0,30	**f4=**	**0,30**

		f=	**0,30**
%1=	**31,53**		
%2=	**30,30**		**30,84 %**
%3=	**30,70**		

Benzyl cinnamate concentration (235nm)

40,34mg/100ml artificial sample

Dilution1 (10ml artificial sample + 1ml standard solution / 20ml MeOH)

0,2017 g/L benzyl cinn.

f1/1=	1,49	**f1/2=**	1,44	**f1/3=**	1,47	**f1=**	**1,47**

Dilution2 (5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,10085 g/L benzyl cinn.

f2/1=	1,56	**f2/2=**	1,54	**f2/3=**	1,57	**f2=**	**1,56**

Dilution3 (2,5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,050425 g/L benzyl cinn.

f3/1=	1,47	**f3/2=**	1,49	**f3/3=**	1,49	**f3=**	**1,49**

Dilution4 (1ml artificial sample + 1ml standard solution / 20ml MeOH)

0,02017 g/L benzyl cinn.

f4/1=	1,64	**f4/2=**	1,55	**f4/3=**	1,58	**f4=**	**1,59**

		f=	**1,53**
%1=	**22,54**		
%2=	**23,36**		**22,70 %**
%3=	**22,20**		

Cinnamic acid concentration (280nm)

6,85mg/100ml artificial sample

Dilution1 (10ml artificial sample + 1ml standard solution / 20ml MeOH)

0,03425 g/L cinn.ac.

f1/1=	0,46	**f1/2=**	0,51	**f1/3=**	0,54	**f1=**	**0,51**

Dilution2 (5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,017125 g/L cinn.ac.

f2/1=	0,52	**f2/2=**	0,52	**f2/3=**	0,56	**f2=**	**0,53**

Dilution3 (2,5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,0085625 g/L cinn.ac.

f3/1=	0,48	**f3/2=**	0,54	**f3/3=**	0,54	**f3=**	**0,52**

Dilution4 (1ml artificial sample + 1ml standard solution / 20ml MeOH)

0,003425 g/L cinn.ac.

f4/1=	0,59	**f4/2=**	0,59	**f4/3=**	0,57	**f4=**	**0,58**

		f=	**0,54**
%1=	**5,17**		
%2=	**4,98**		**4,89 %**
%3=	**4,53**		

Benzyl cinnamate concentration (280nm)

40,34mg/100ml artificial sample

Dilution1 (10ml artificial sample + 1ml standard solution / 20ml MeOH)

0,2017 g/L benzyl cinn.

f1/1=	0,71	**f1/2=**	0,73	**f1/3=**	0,72	**f1=**	**0,72**

Dilution2 (5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,10085 g/L benzyl cinn.

f2/1=	0,73	**f2/2=**	0,72	**f2/3=**	0,71	**f2=**	**0,72**

Dilution3 (2,5ml artificial sample + 1ml standard solution / 20ml MeOH)

0,050425 g/L benzyl cinn.

f3/1=	0,65	**f3/2=**	0,69	**f3/3=**	0,68	**f3=**	**0,67**

Dilution4 (1ml artificial sample + 1ml standard solution / 20ml MeOH)

0,02017 g/L benzyl cinn.

f4/1=	0,70	**f4/2=**	0,71	**f4/3=**	0,70	**f4=**	**0,70**

		f=	**0,70**
%1=	**25,47**		
%2=	**23,41**		**23,83 %**
%3=	**22,60**		

7.4. Comparison with European Pharmacopoeia

The gravimetric determination of the ester-compounds, as described in the European Pharmacopoeia, was carried out not only to compare the results obtained by the chosen HPLC-method but also to show that the developed method is adequate and can be considered for its possible inclusion in the European Pharmacopoeia.

The definition given by the Pharmacopoeia for the Peru balsam says that:
"It contains not less than 45,0 per cent m/m and not more than 70,0 per cent m/m of esters, mainly benzyl benzoate and benzyl cinnamate." [2]

Assay given by the Pharmacopoeia to determine the ester-constituents of the Peru balsam:

To 2,50 g in a separating funnel add 7,5 ml of dilute sodium hydroxide solution R and 40 ml of peroxide-free ether R and shake vigorously for 10 min. Separate the lower layer and shake it with three quantities, each of 15 ml, of peroxide free ether R. Combine the ether layers, dry over 10 g of anhydrous sodium sulphate R and filter. Wash the sodium sulphate with two quantities, each of 10 ml, of peroxide-free ether R. Combine the ether layers and evaporate to dryness. Dry the residue (esters) at 100 °C to 105 °C for 30 min and weigh. [2]

This working-instruction was carried out three times. Each time the residue was dried to constant weight. Out of these three results was calculated the average and final percentage.

1.gravimetric valoration

weight of "round bottom flask":	74,24	g						
weight of Peru balsam:	2,52	g						
weight before drying the residue:			75,66	g	56,40	%		
weight after first drying-procedure:			75,66	g	56,27	%	**56,28**	**%**
weight after second drying-procedure:			75,66	g	56,27	%		

2.gravimetric valoration

weight of "round bottom flask":	74,25	g						
weight of Peru balsam:	2,51	g						
weight before drying the residue:			75,72	g	58,69	%		
weight after first drying-procedure:			75,65	g	56,09	%		
weight after second drying-procedure:			75,64	g	55,63	%	**55,62**	**%**
weight after third drying-procedure:			75,64	g	55,62	%		

3.gravimetric valoration

weight of "round bottom flask":	74,25	g						
weight of Peru balsam:	2,51	g						
weight before drying the residue:			75,75	g	60,09	%		
weight after first drying-procedure:			75,67	g	56,74	%		
weight after second drying-procedure:			75,66	g	56,34	%	**56,29**	**%**
weight after third drying-procedure:			75,66	g	56,24	%		

average value: **56,06 %**

The results obtained by HPLC-quantification (53,54%) and by the Pharmacopoeia-quantification (56,06%) differ only slightly. Thus the designed HPLC method can be considered as suitable.

8. Conclusion

Part A of this experimental work proves that SPME is a suitable method for the determination of the Peru Balsam's essential oil composition. Application of SPME led to identification and quantification of far more constituents than could be extracted by SFE-CO_2 or hydro distillation. Nerolidol and benzyl benzoate show the highest percentages in all these three extraction methods. Benzyl alcohol, benzaldehyde and benzoic acid as well show high percentages during extraction with SPME. One can resume that at low temperature foremost monoterpenes can be extracted and that with an increase in temperature and extraction time more less volatile constituents can be extracted and identified.

SFE especially served to detect benzyl cinnamate in higher amounts as well as the already mentioned main constituents. The higher the pressure and the longer the extraction time, the more constituents in higher amounts can be detected.

Both extraction methods SFE-CO_2 and SPME, but especially the latter one seem pretty promising and therefore further research should be done to improve extraction conditions.

The challenge of Part B was to make an attempt in designing a new HPLC method for appropriate quality control as demanded in the Pharmacopoeia. Various test injections had to be done until the best method parameters were found. By addition of an internal standard quantification of the main constituents, benzoic acid, cinnamic acid, benzyl benzoate and benzyl cinnamate could be achieved. Finally the gravimetric determination of the ester compounds, as described in the European Pharmacopoeia, was carried out not only to compare the results obtained by the developed HPLC-method but also to show that the method is adequate and can be considered for its possible inclusion in the European Pharmacopoeia.

9. References

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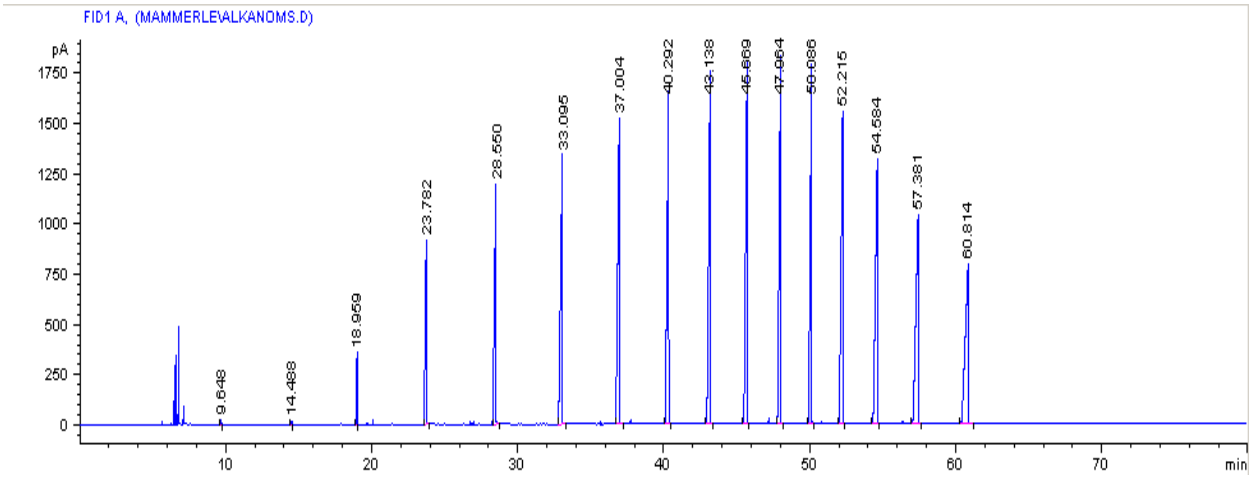
10. APPENDIX



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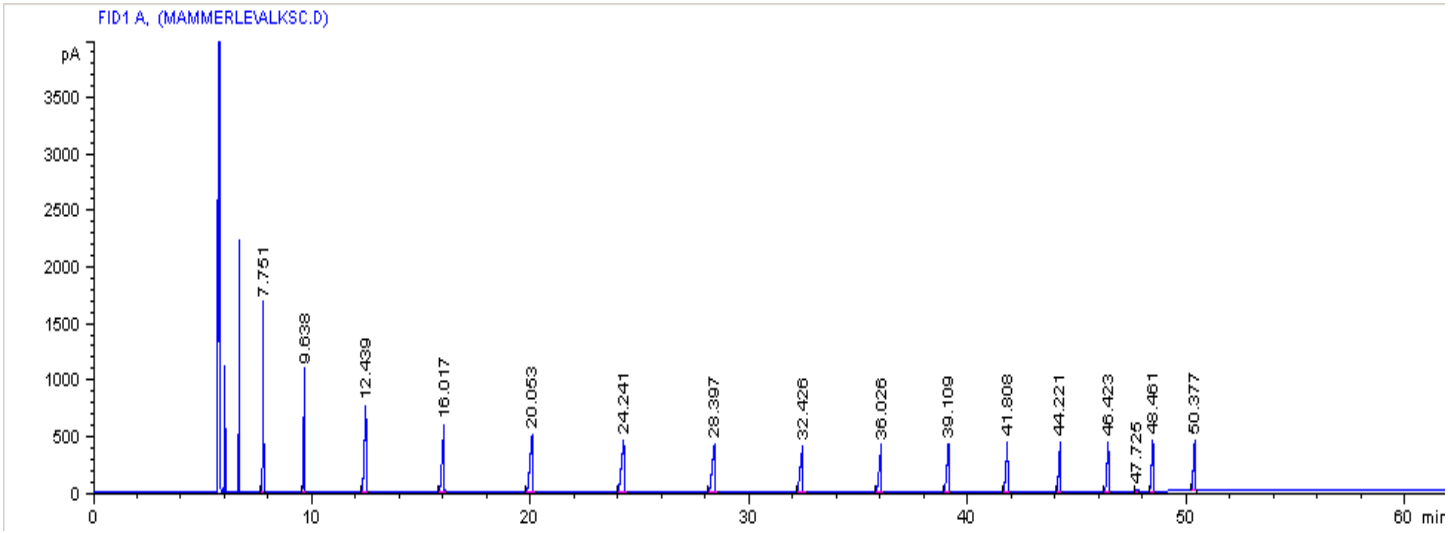

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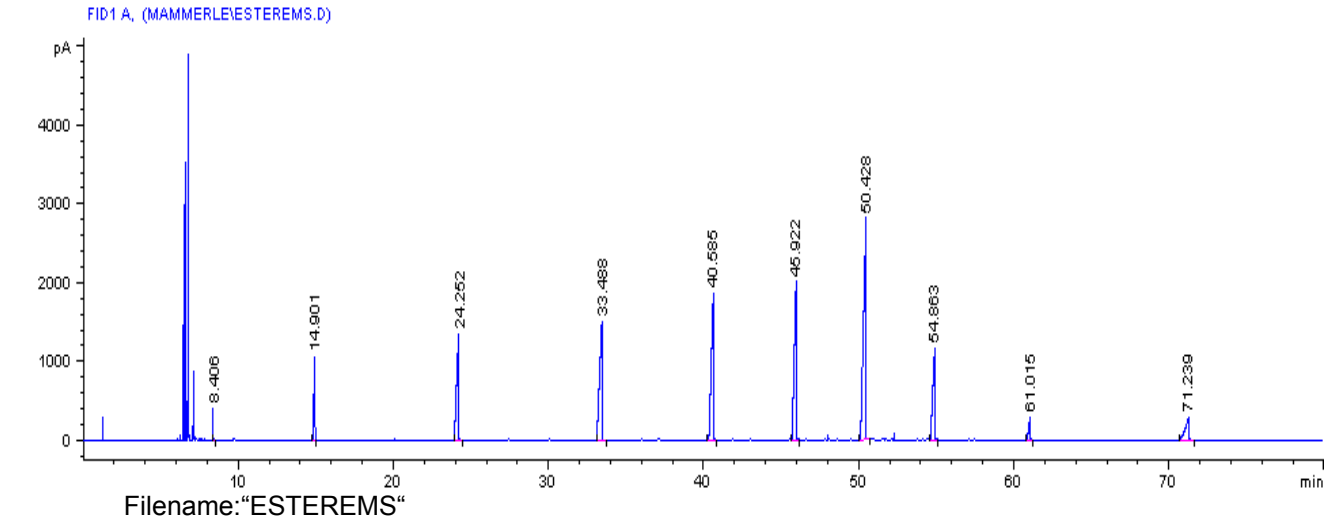

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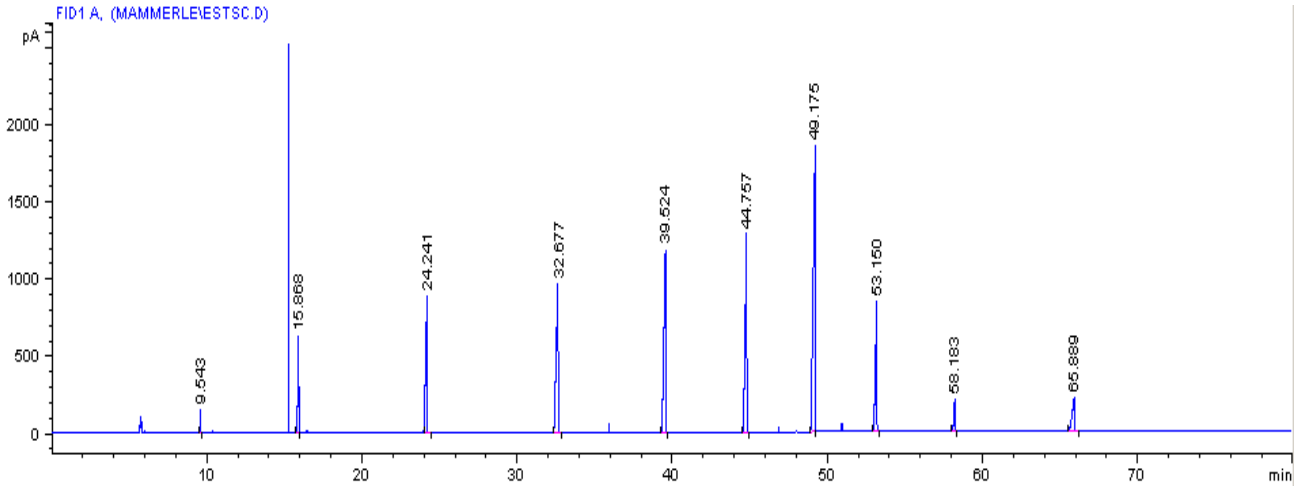

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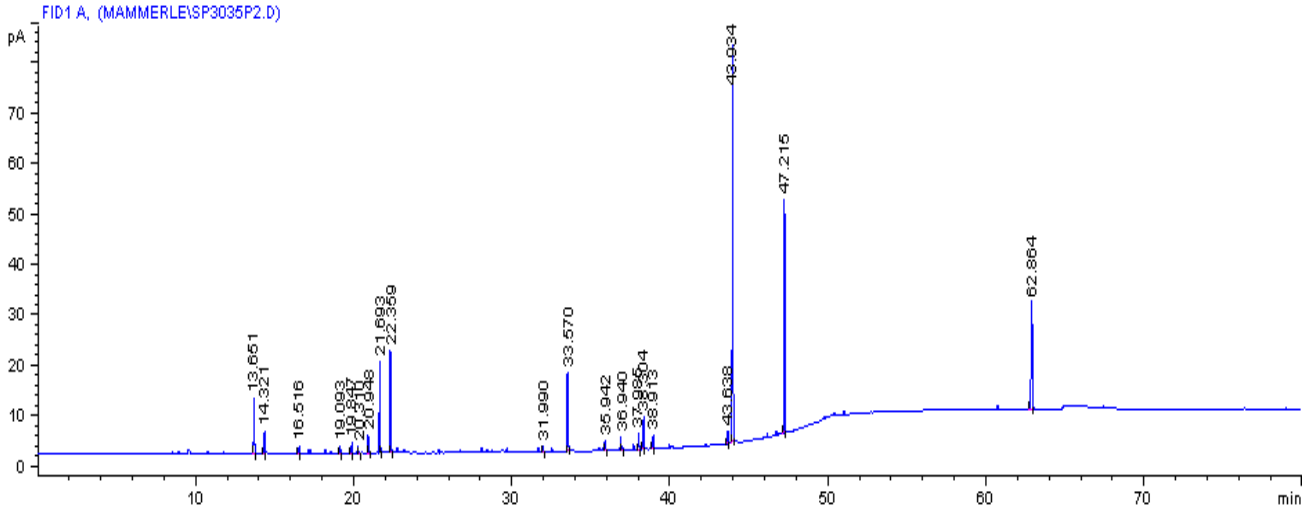

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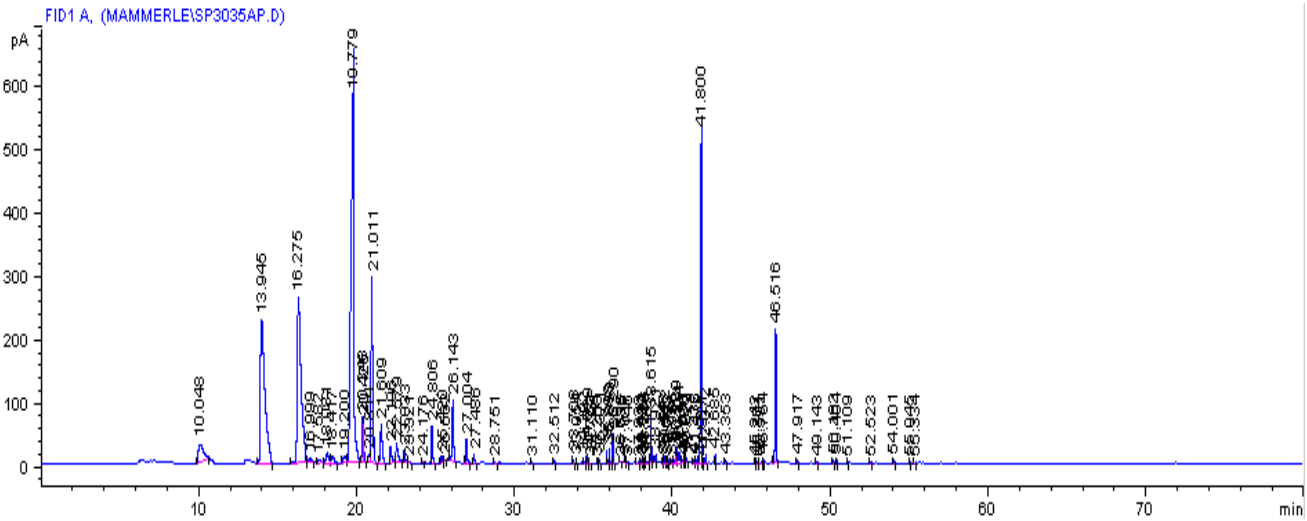

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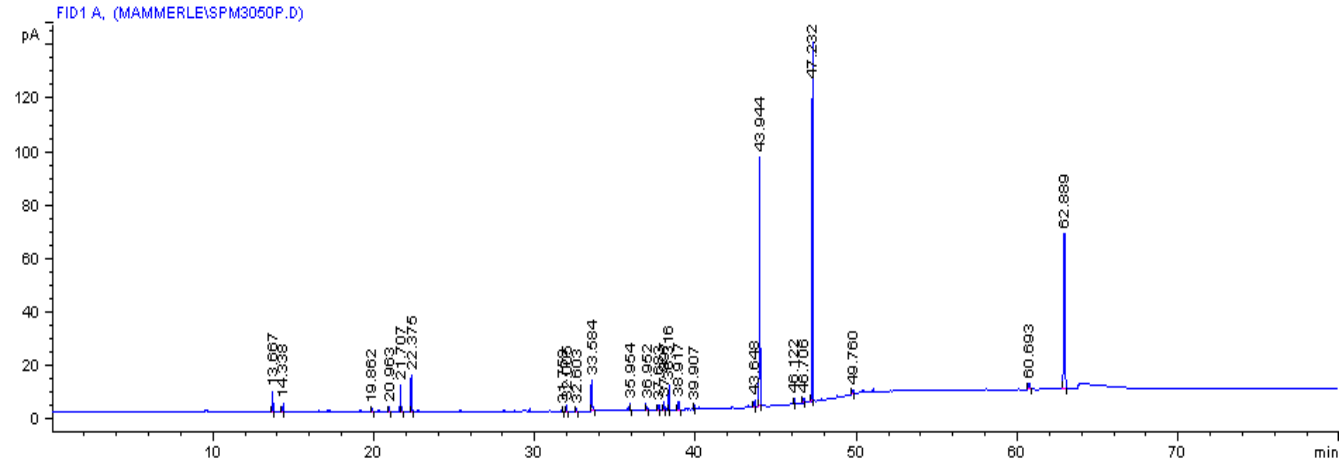

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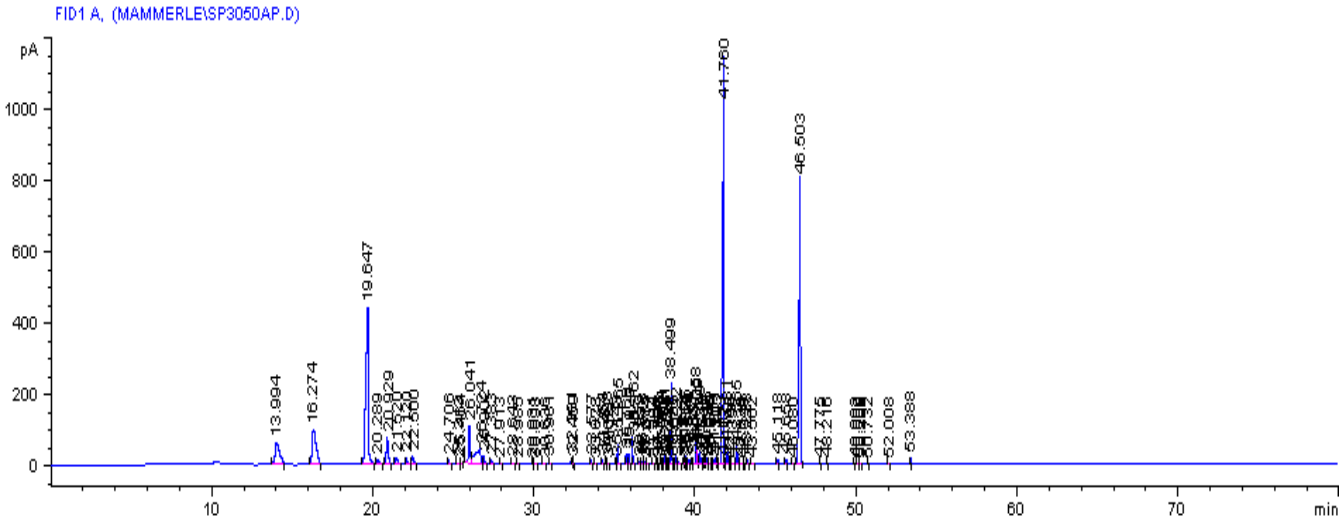

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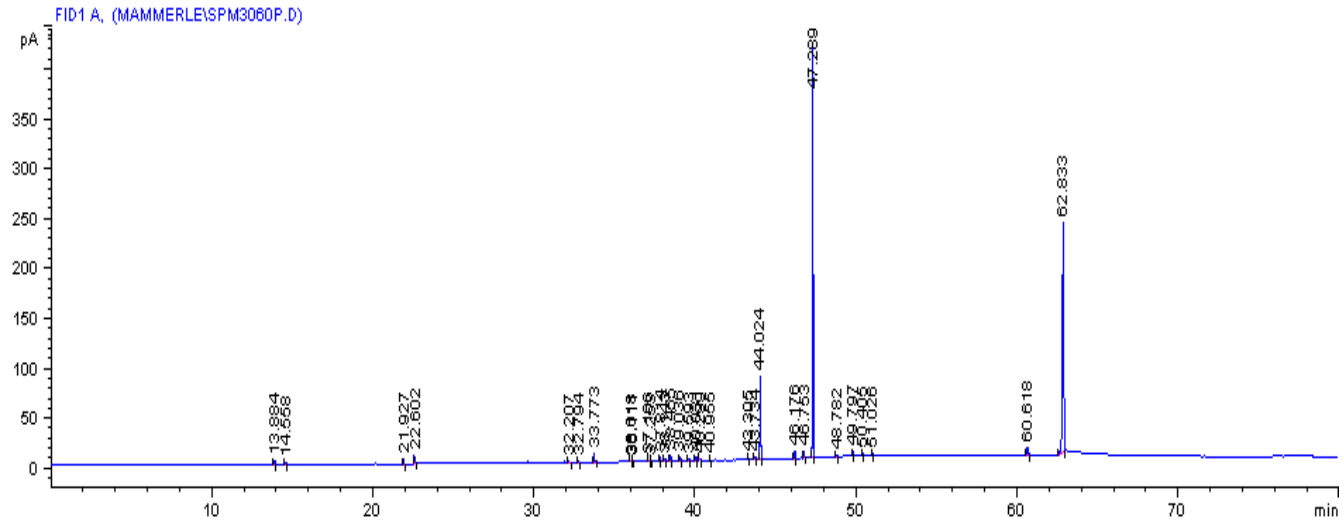

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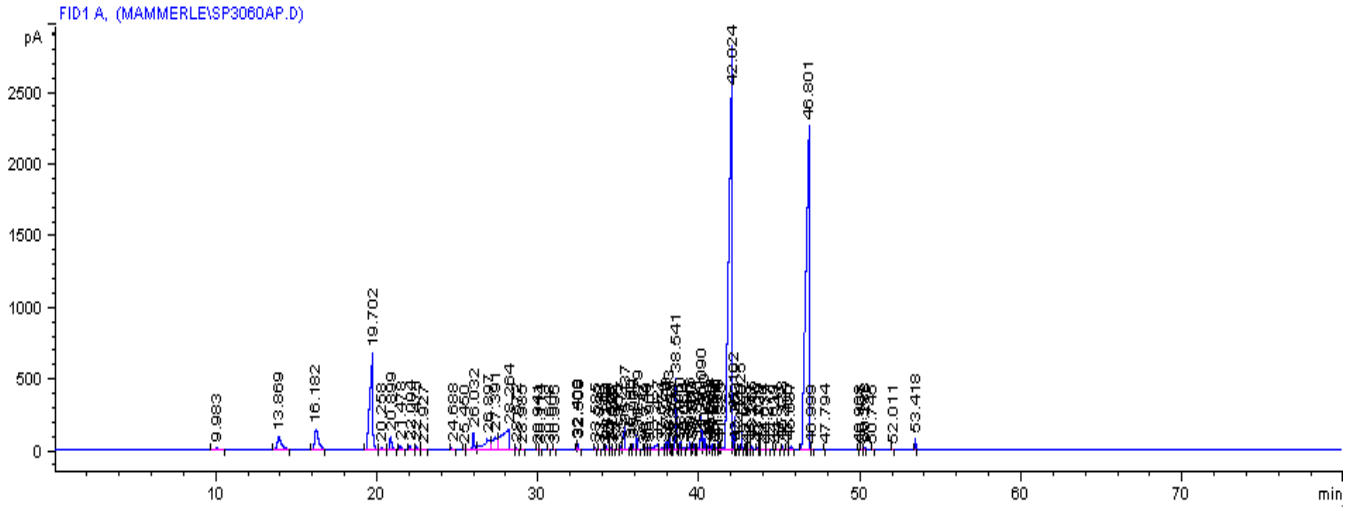

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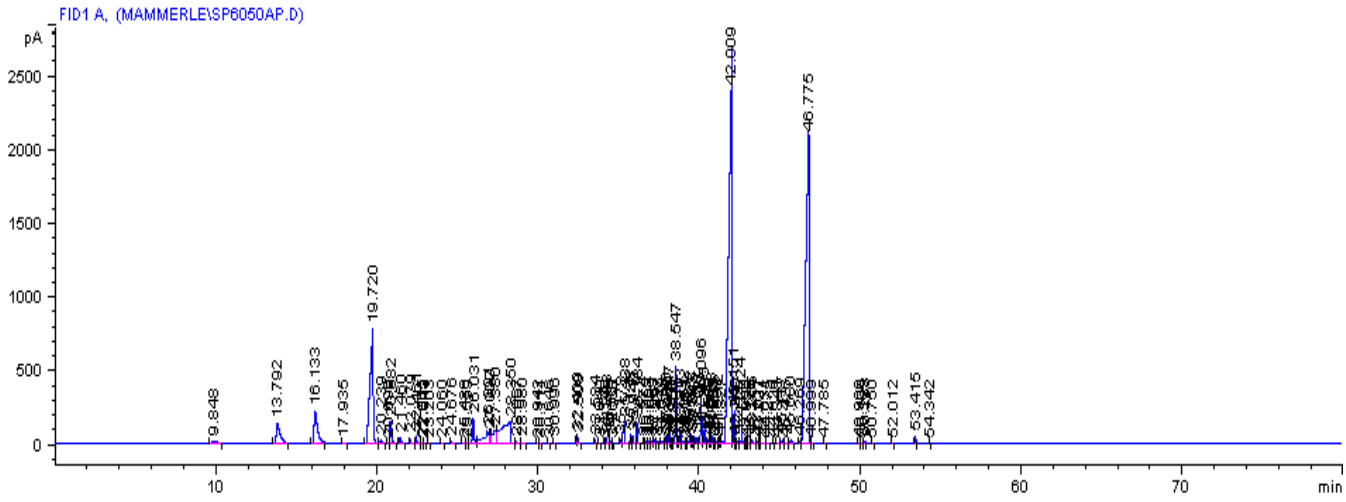

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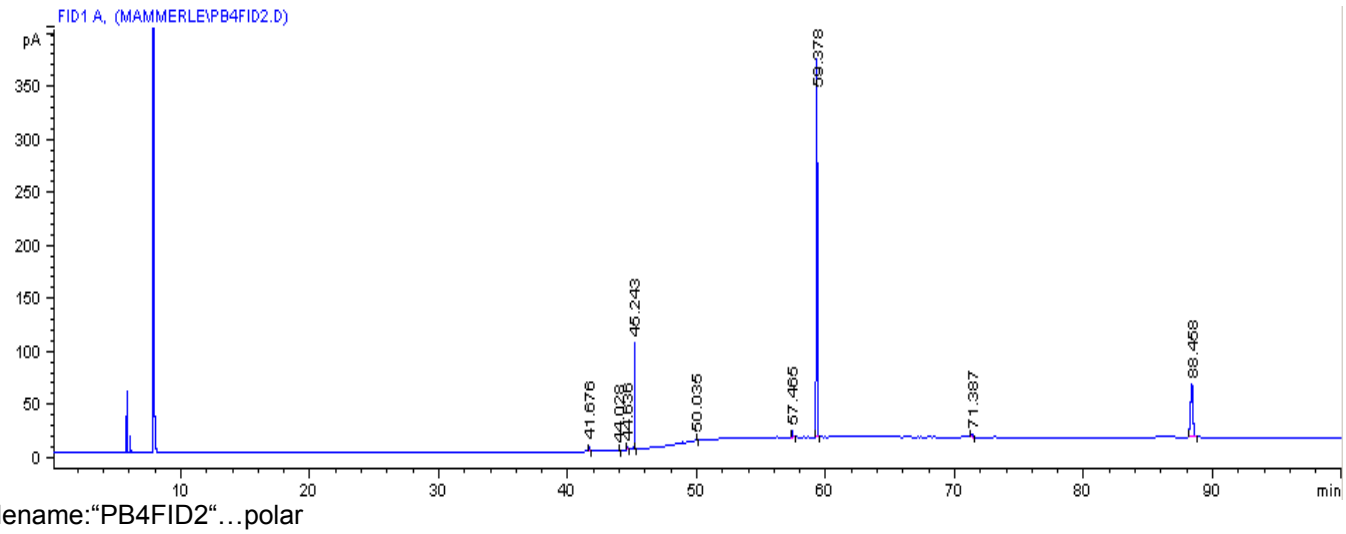

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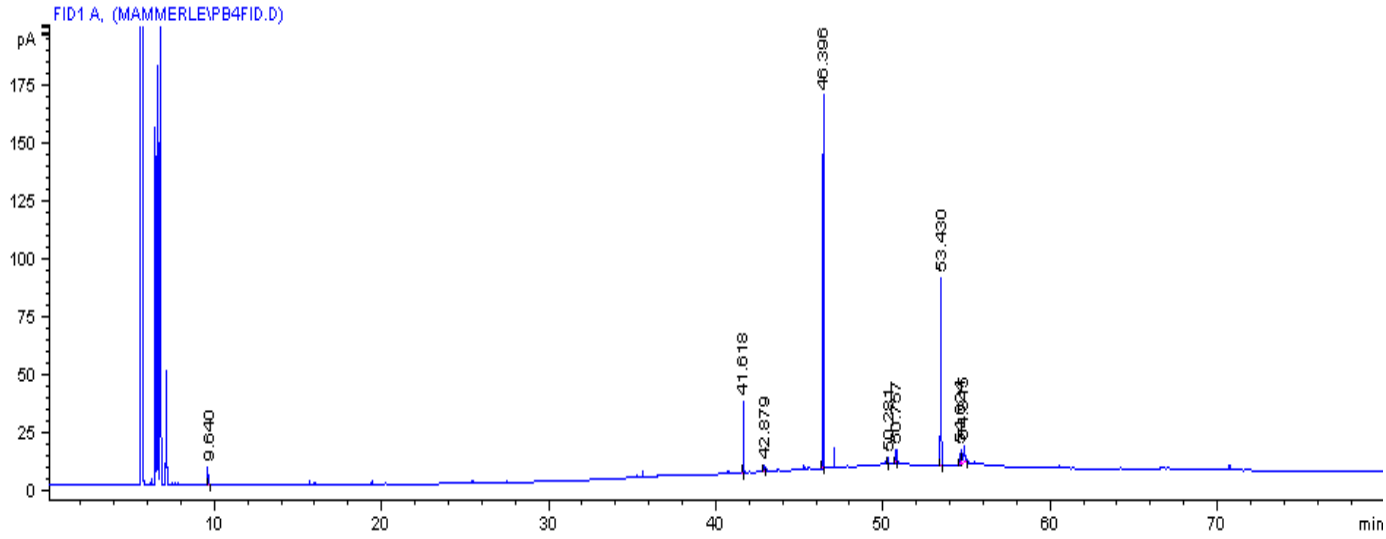

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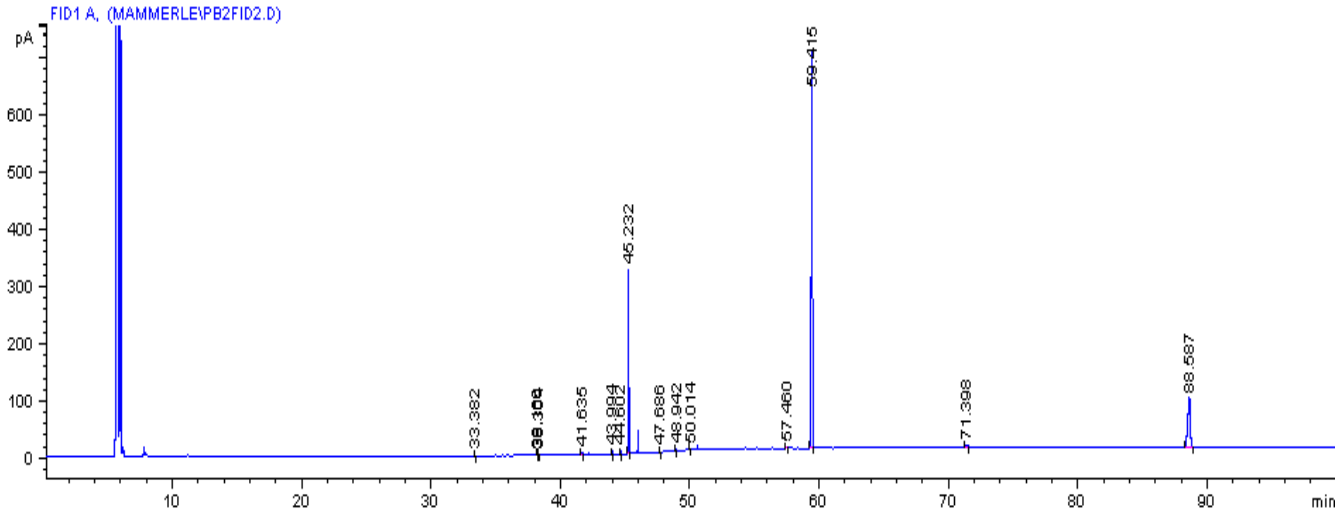

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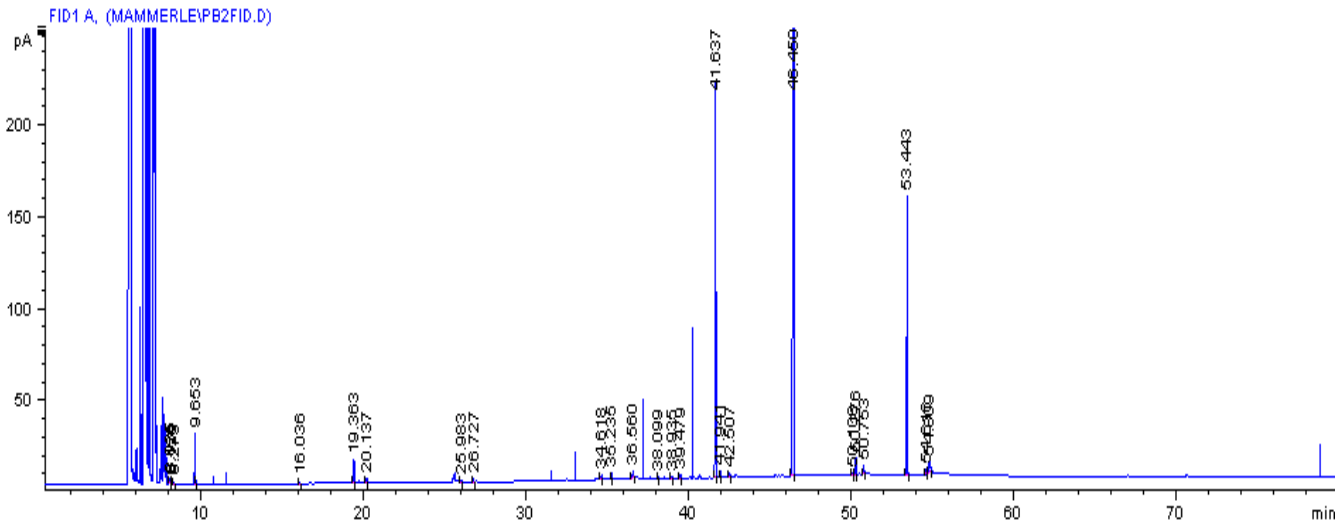

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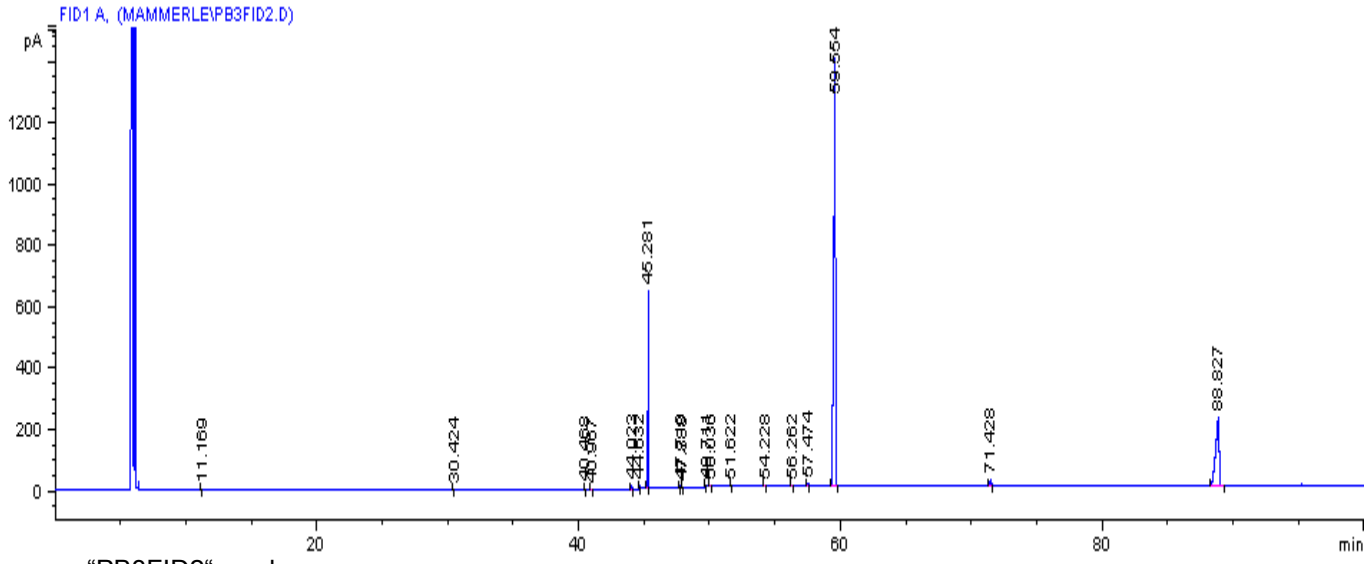

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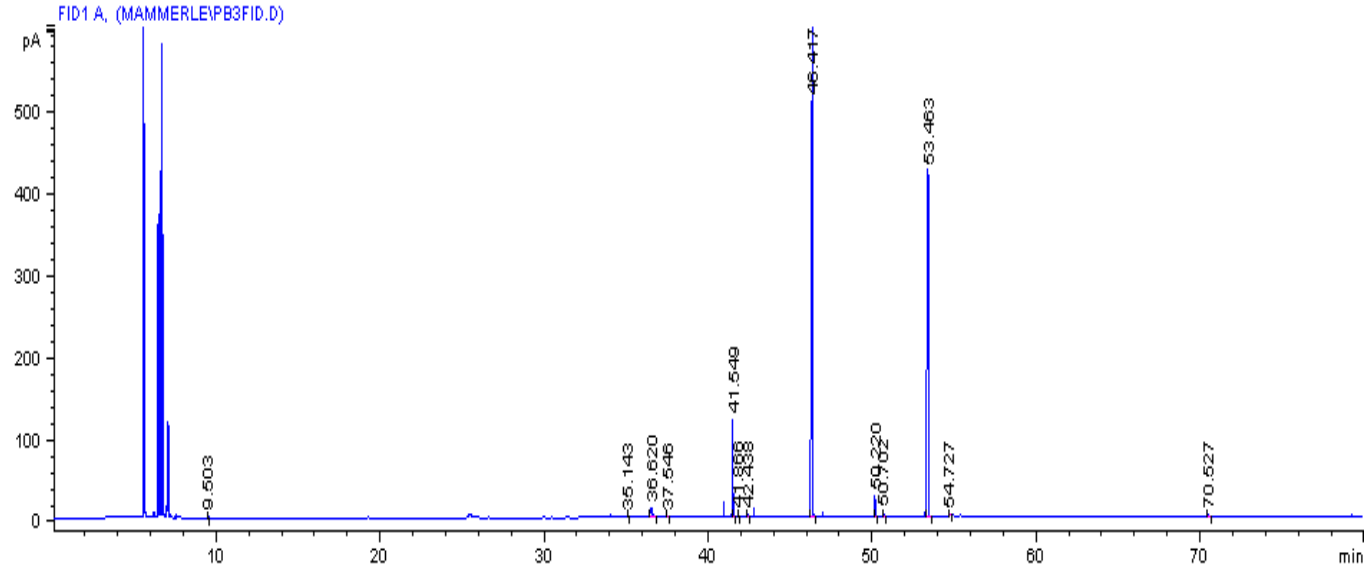

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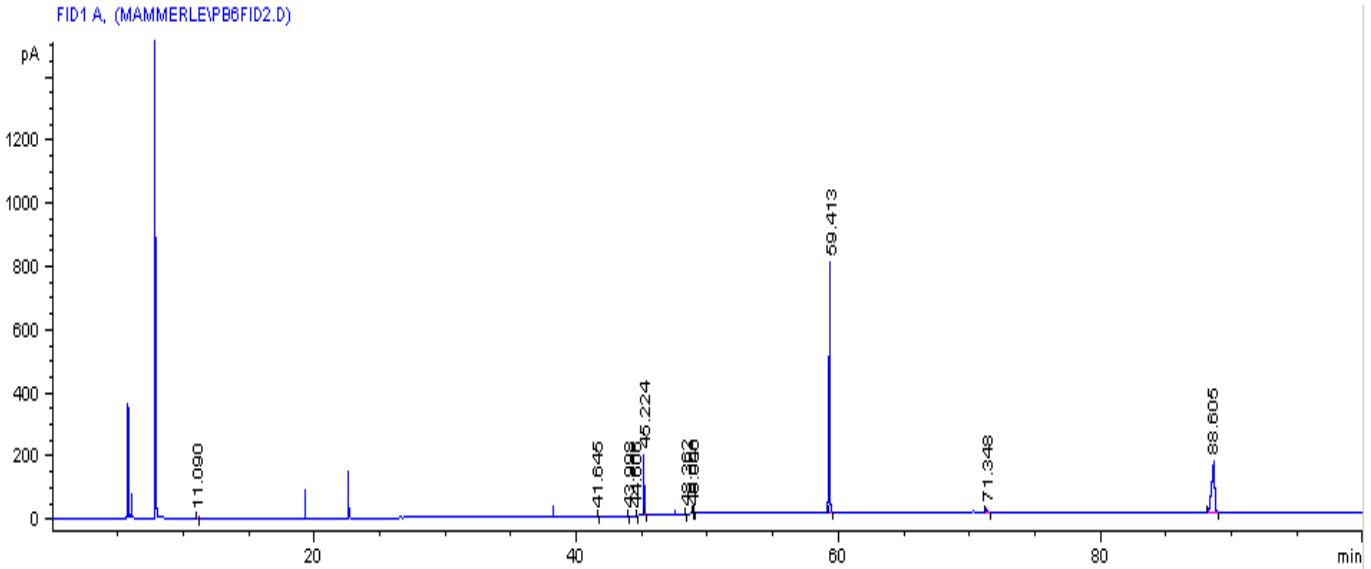

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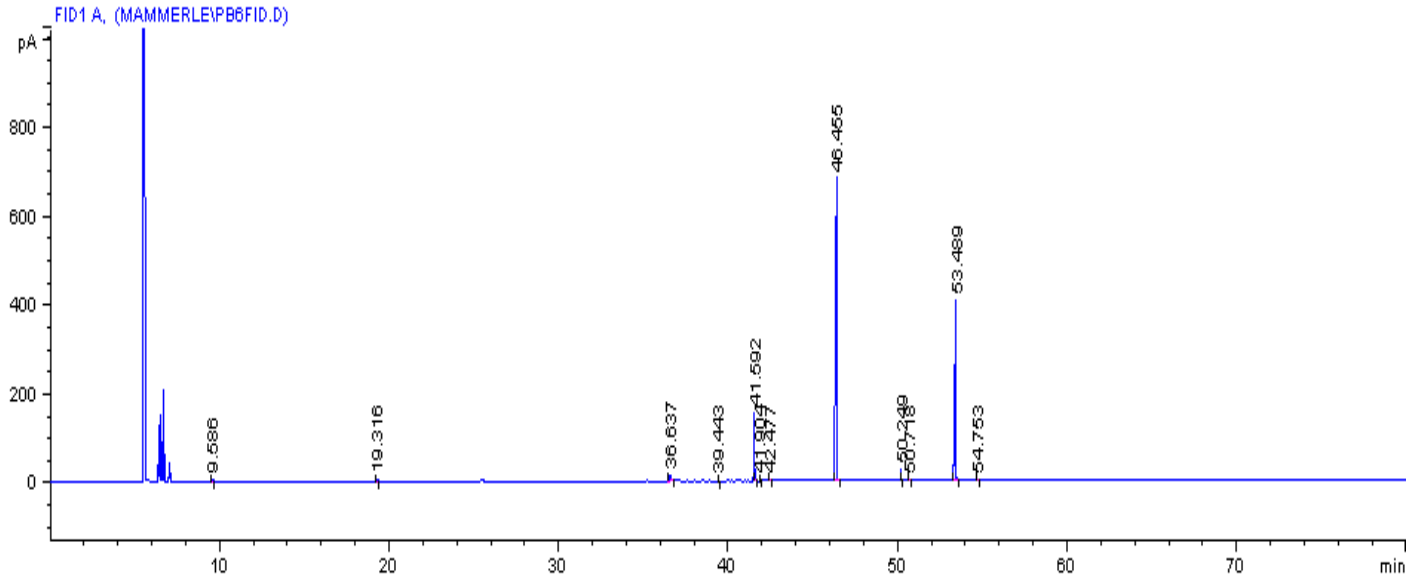

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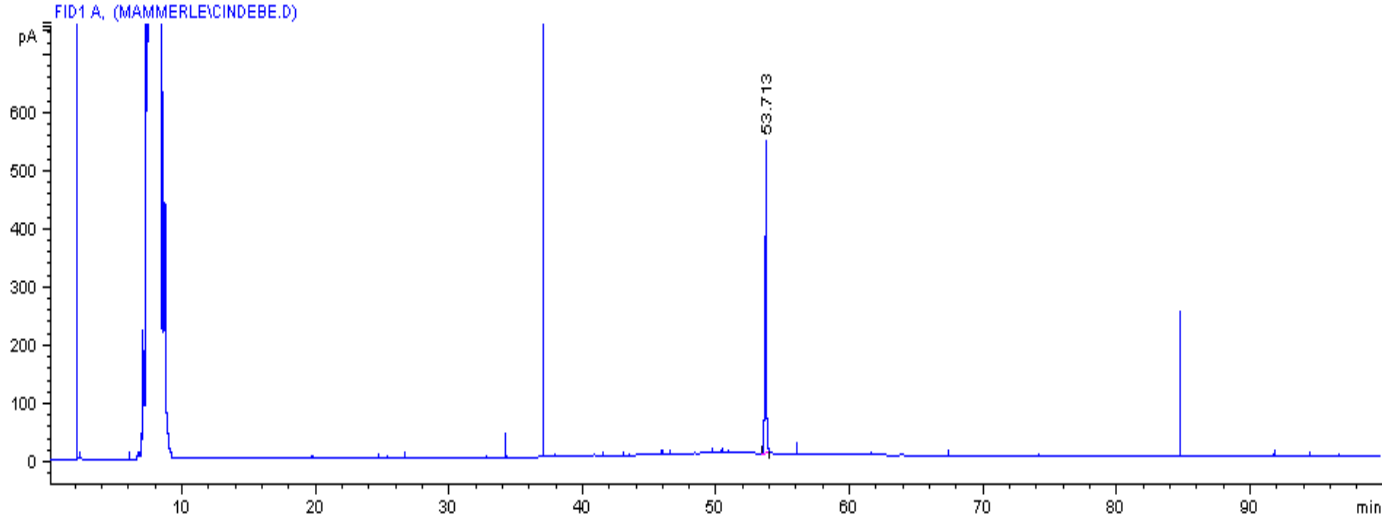

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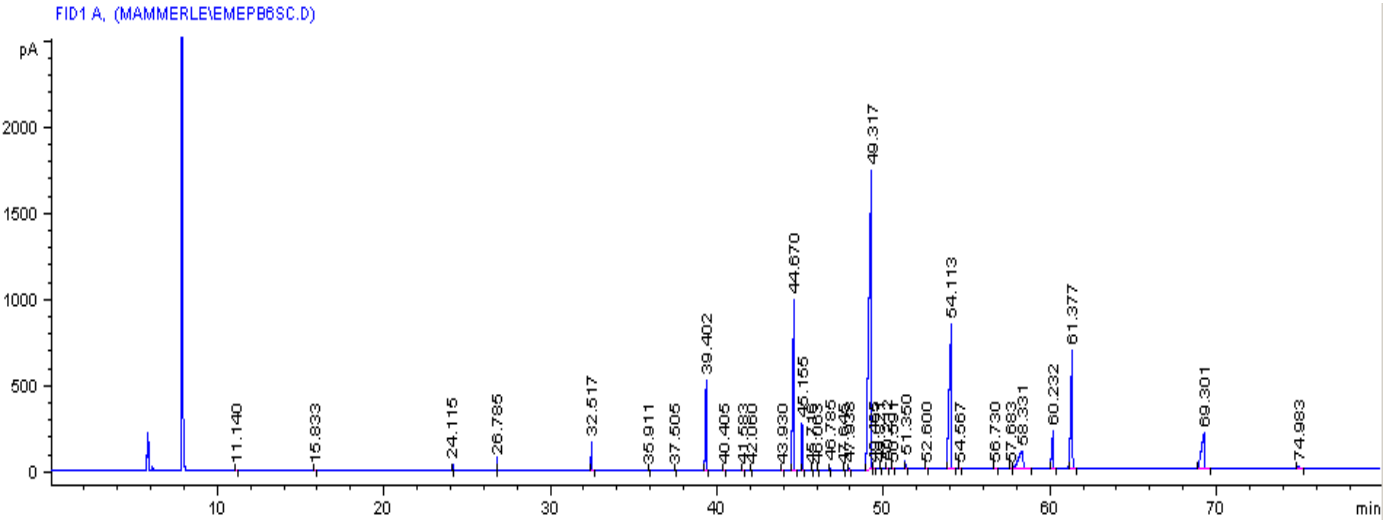

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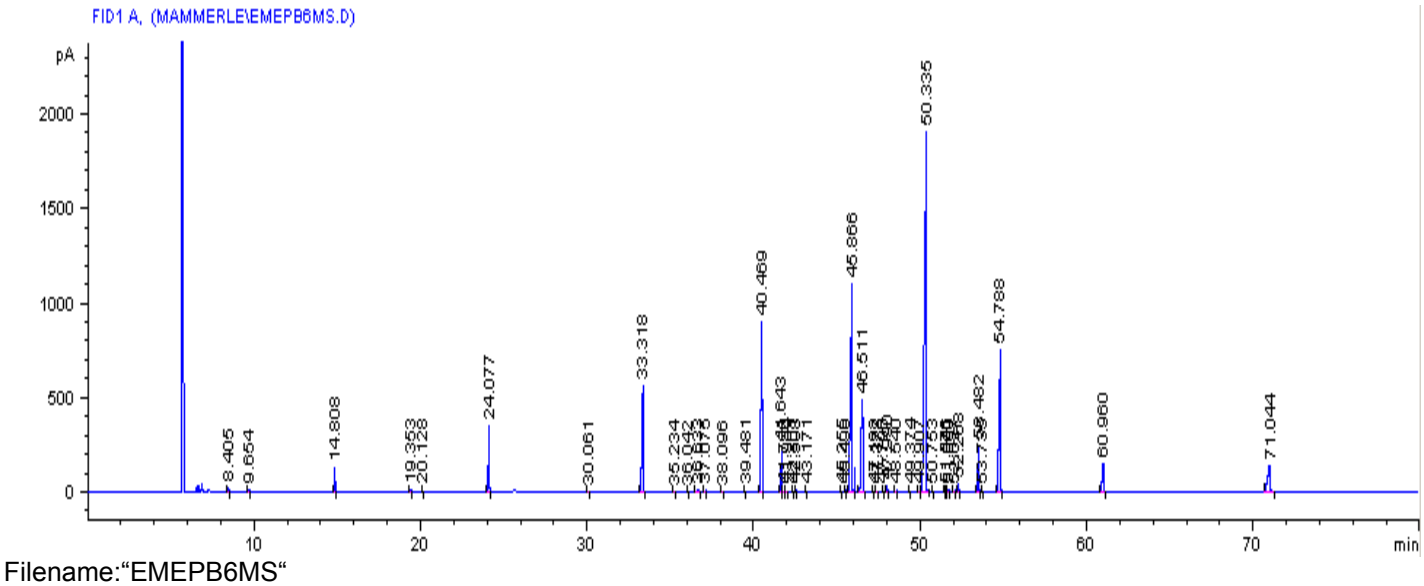

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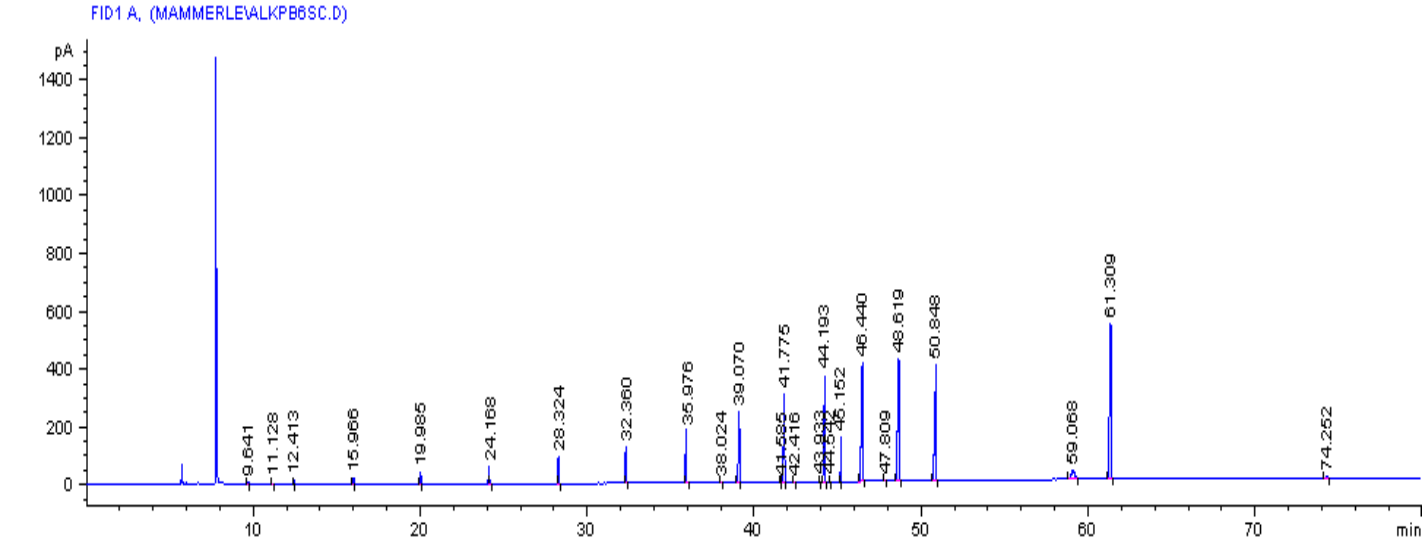

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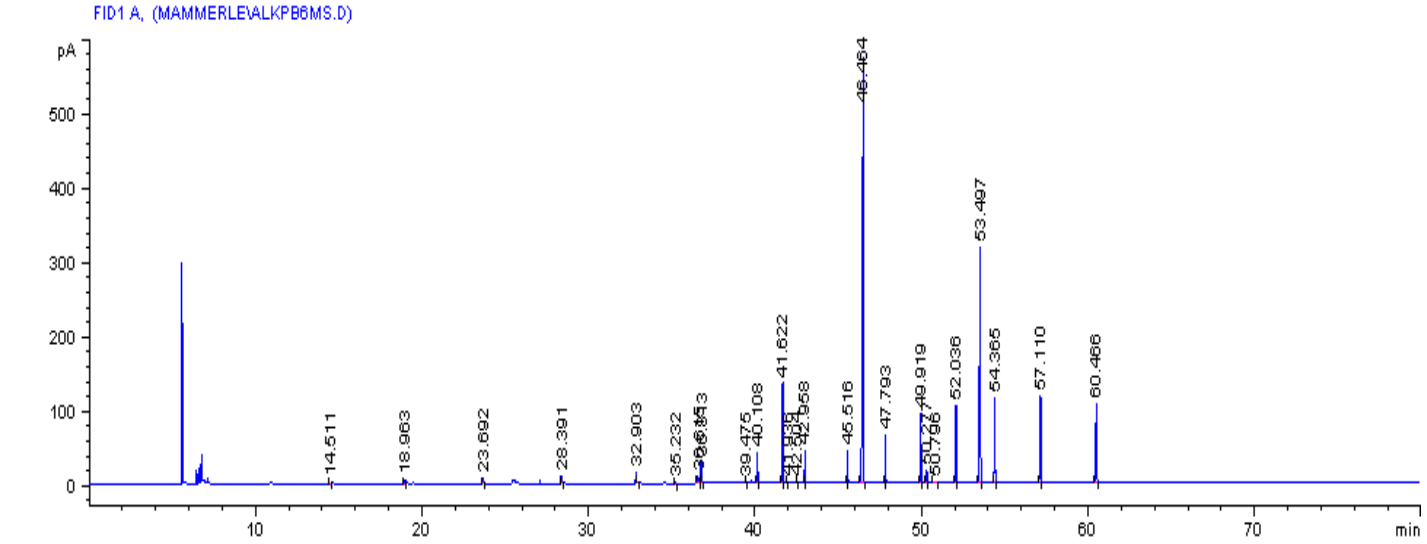

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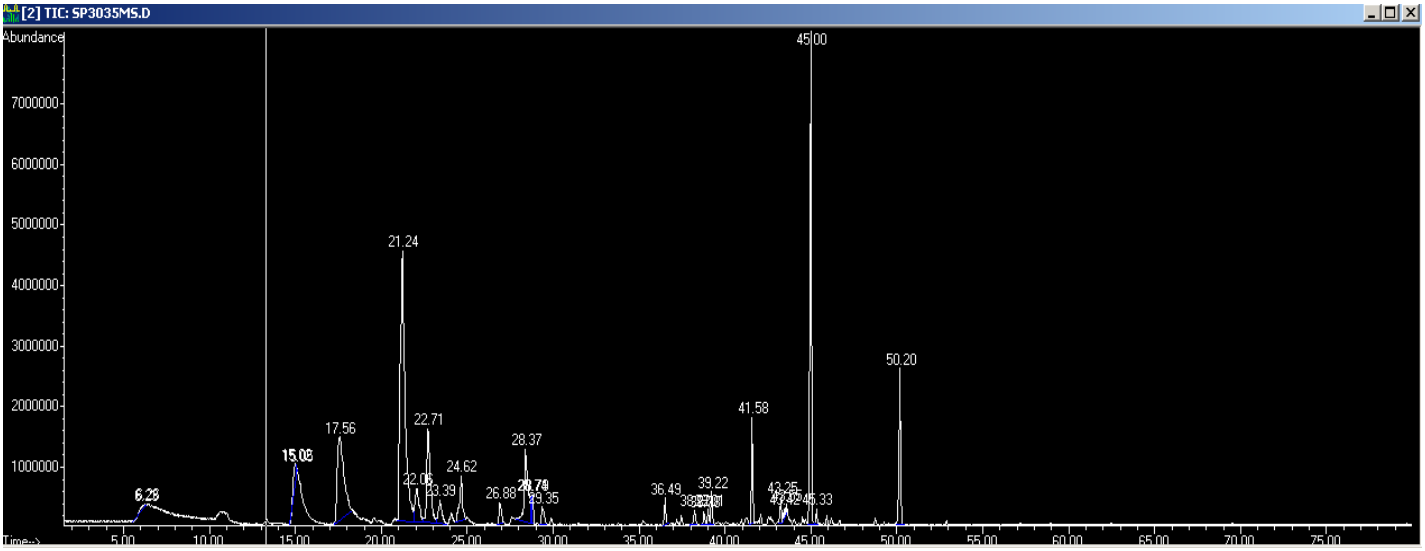

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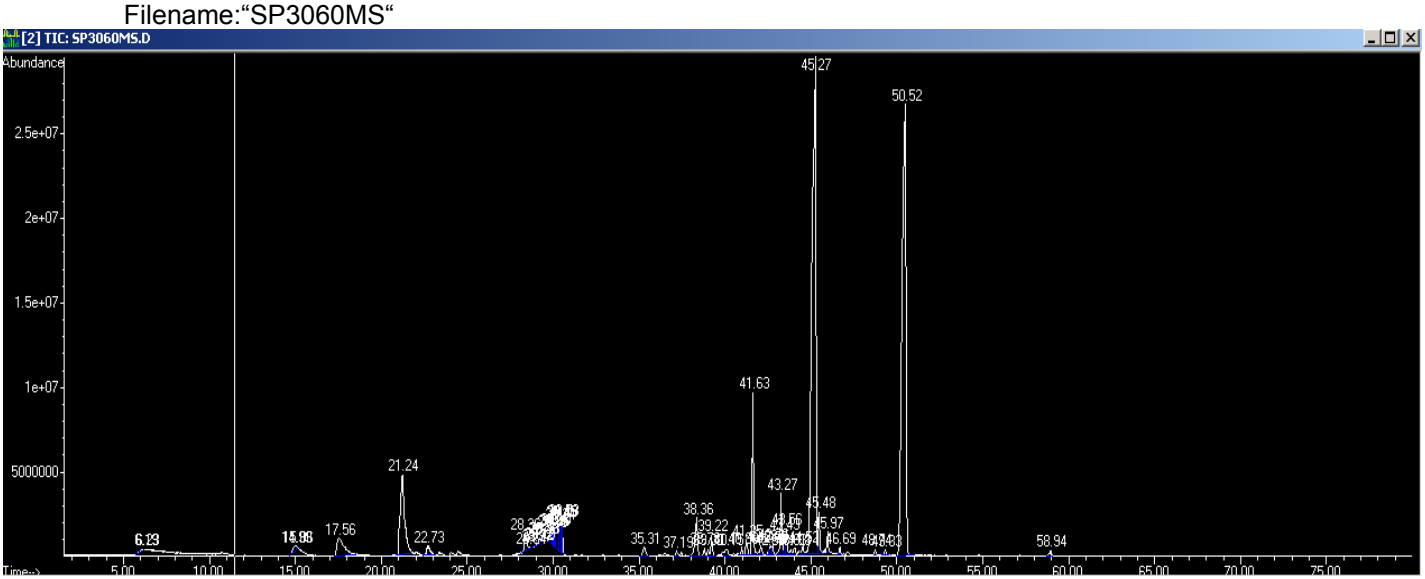

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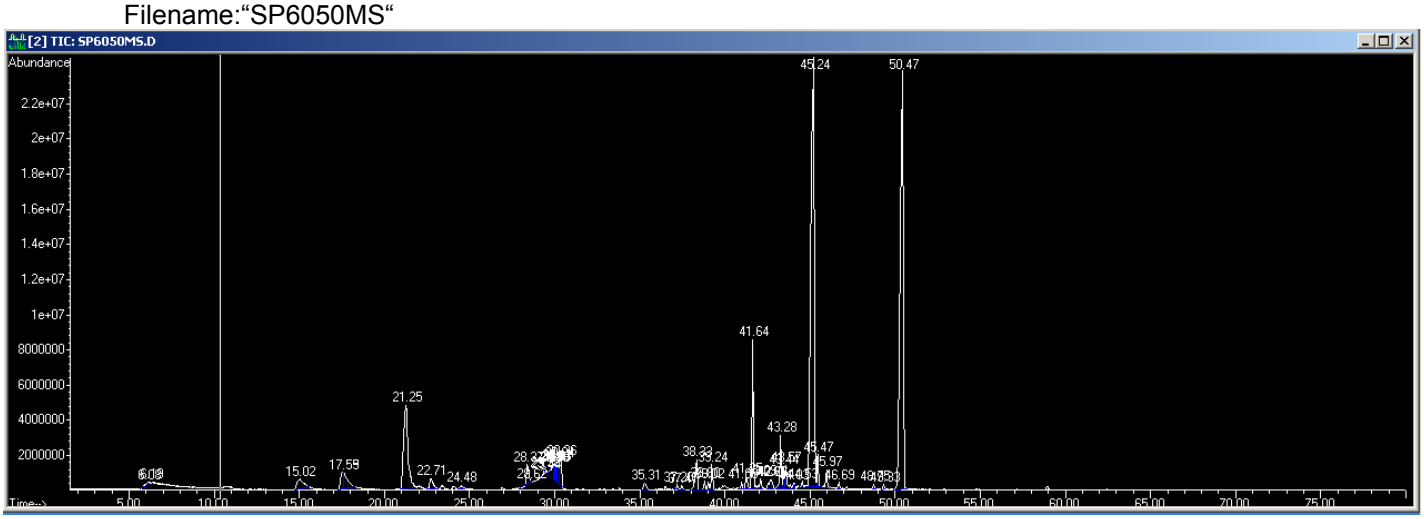

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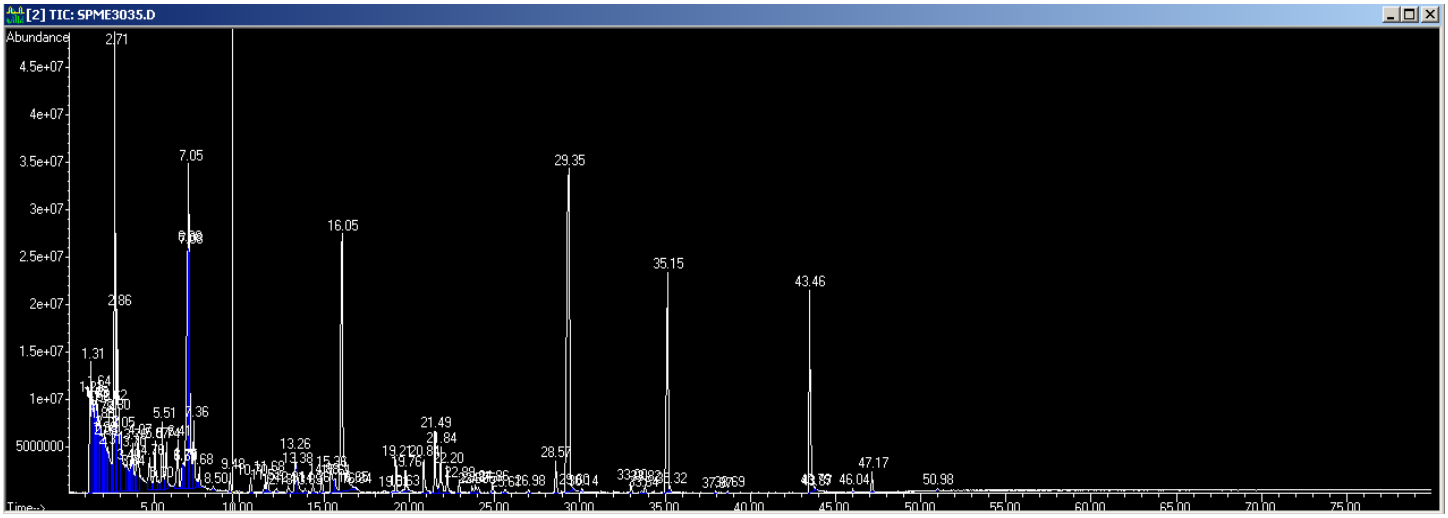

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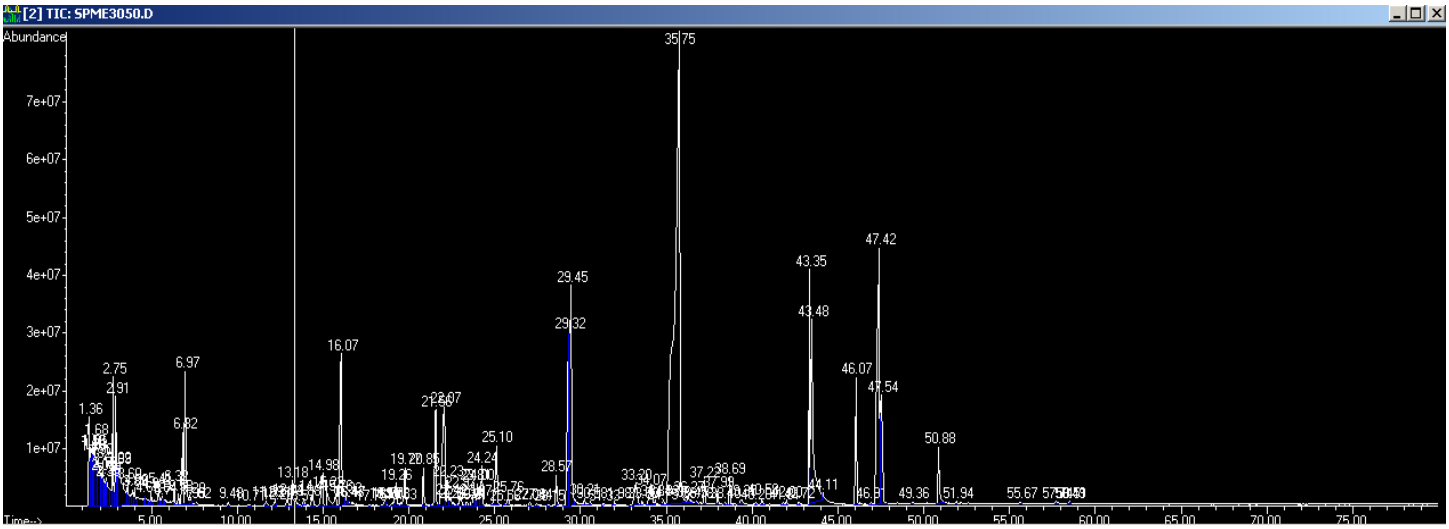

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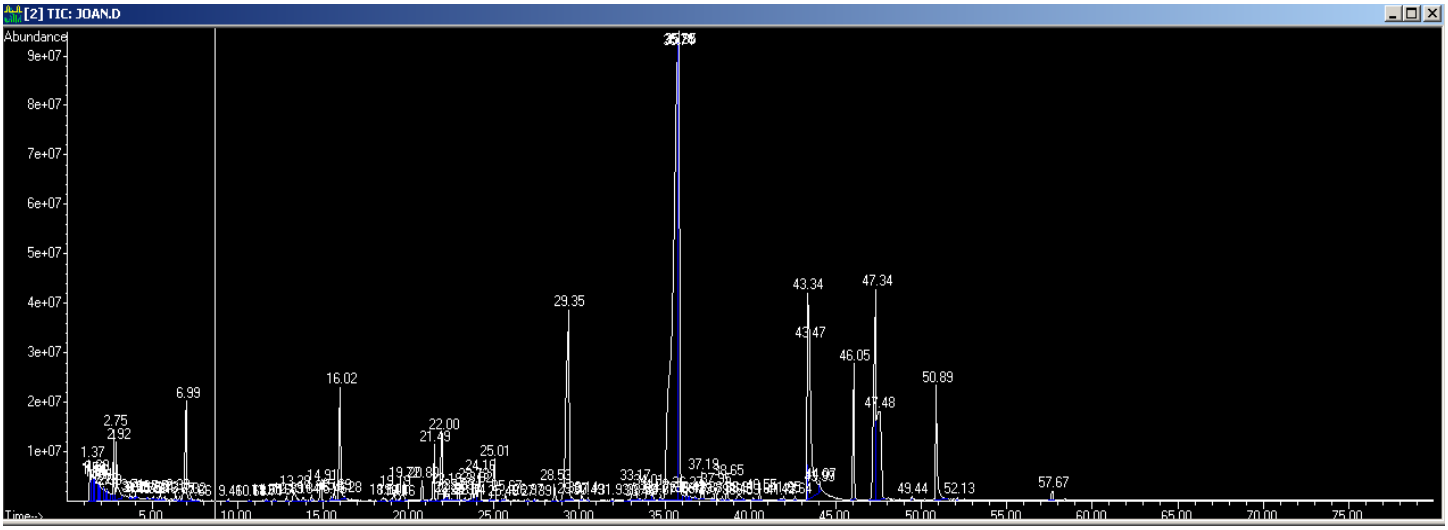

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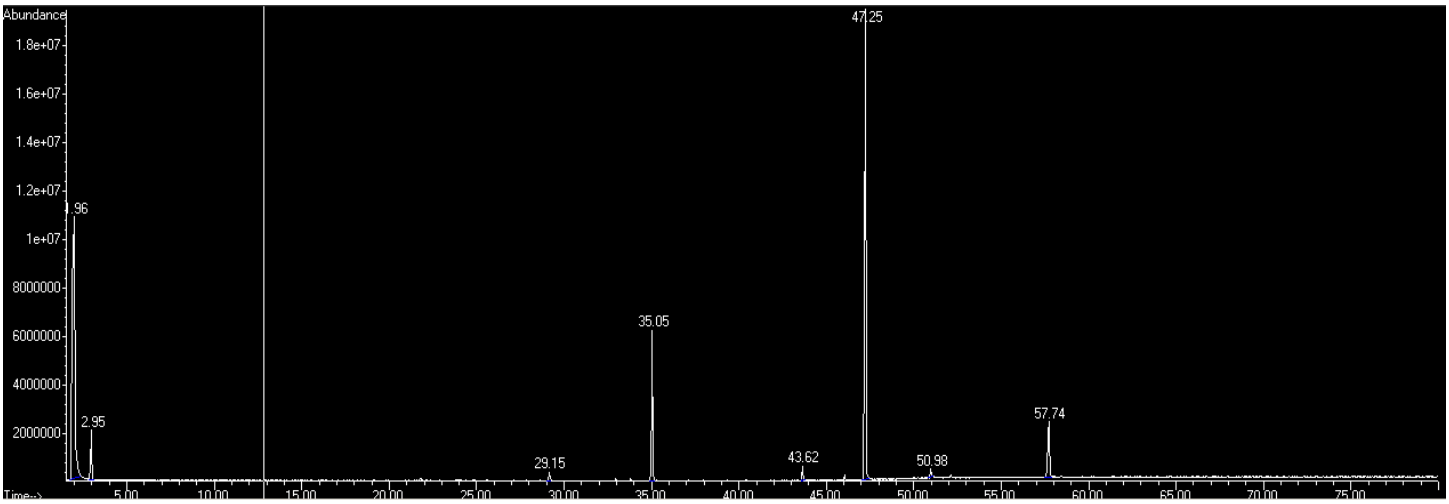

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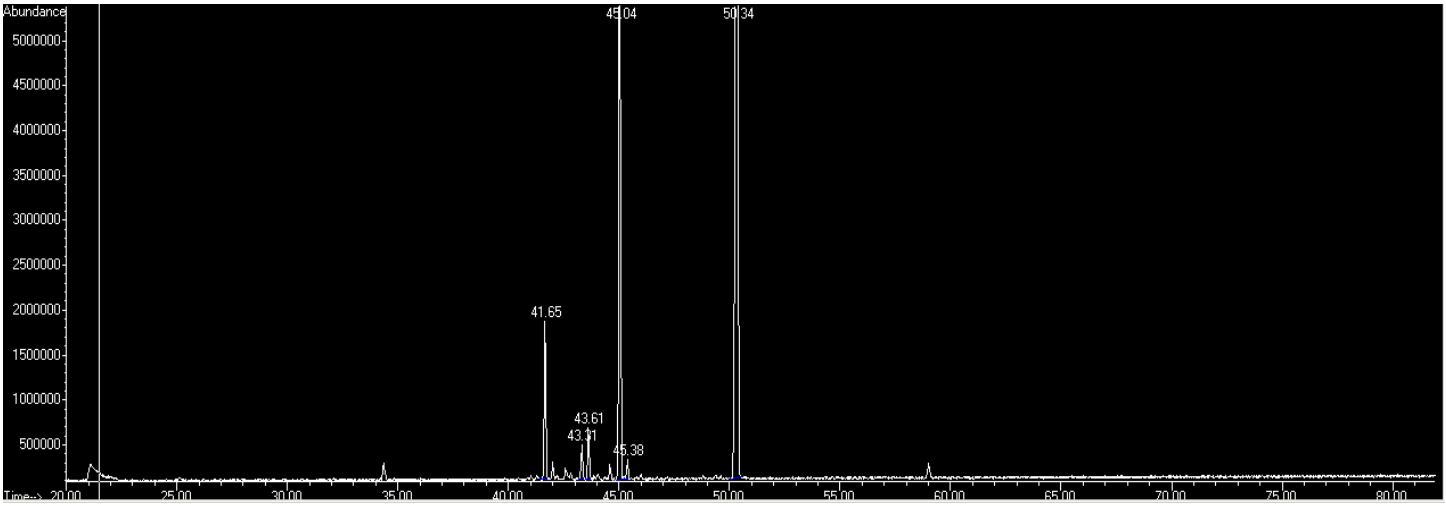

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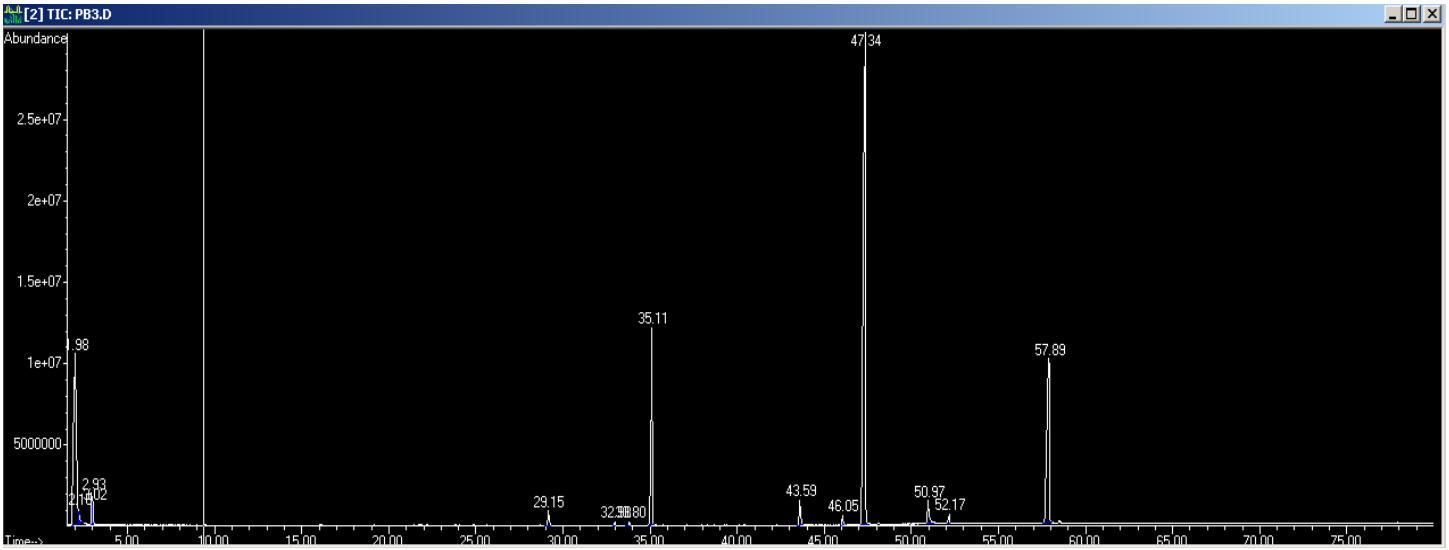

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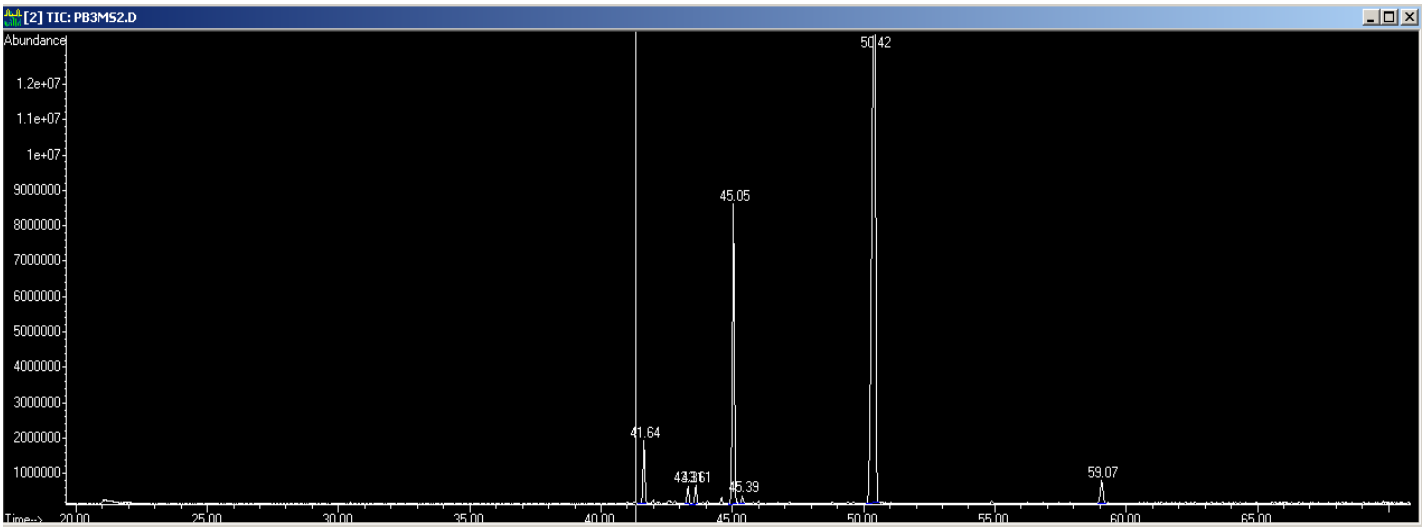

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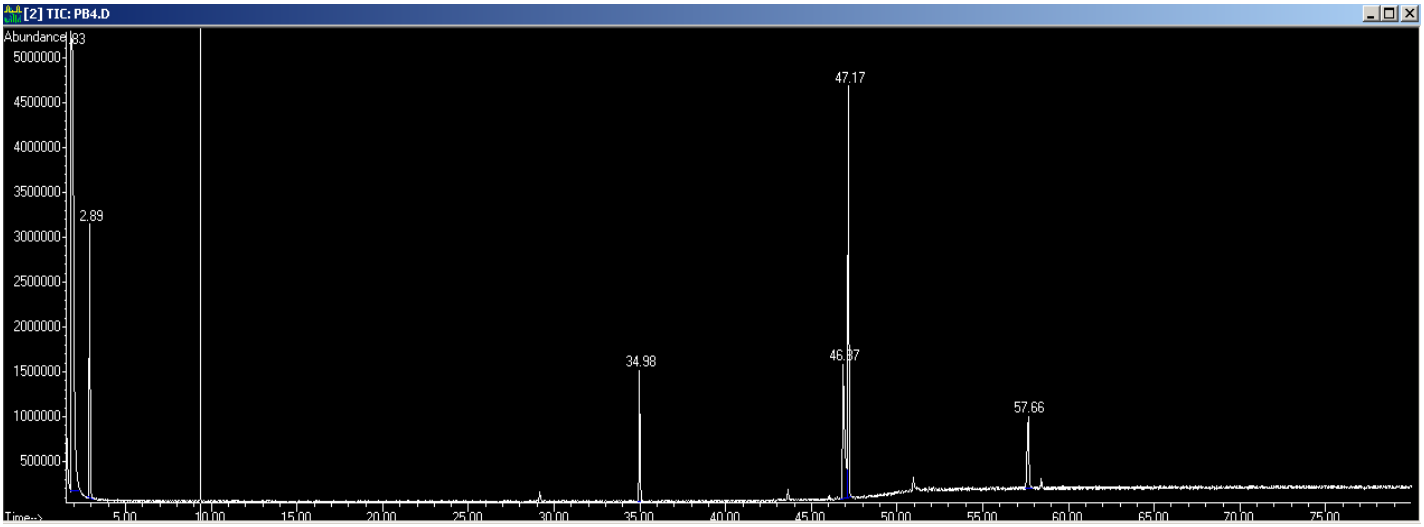

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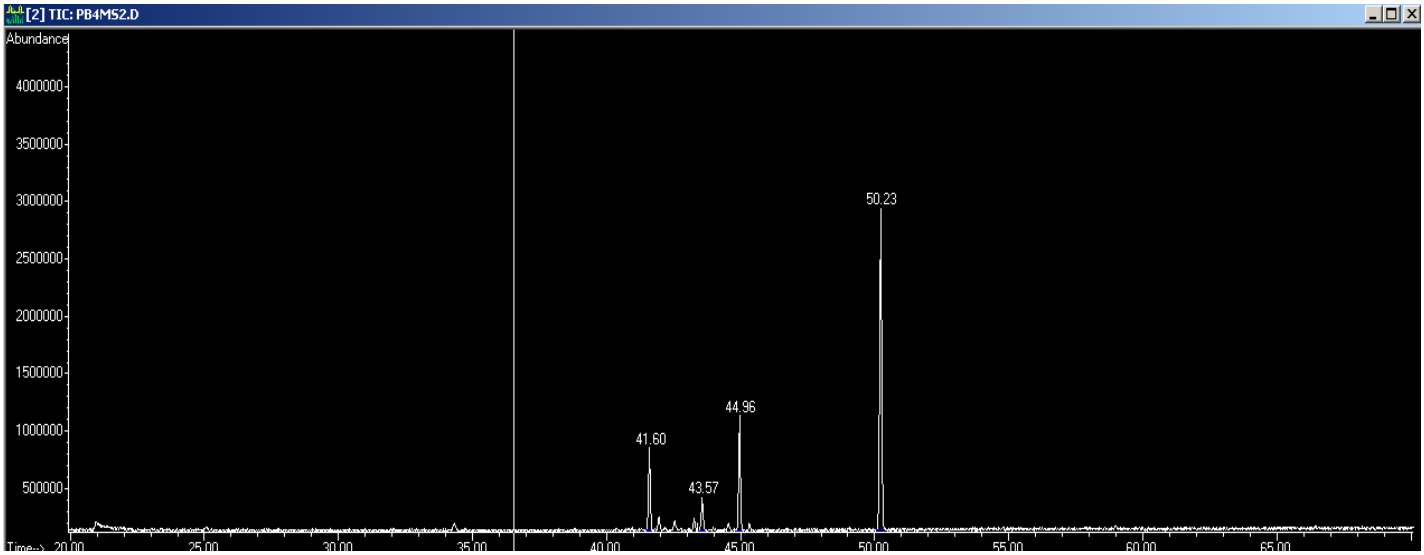

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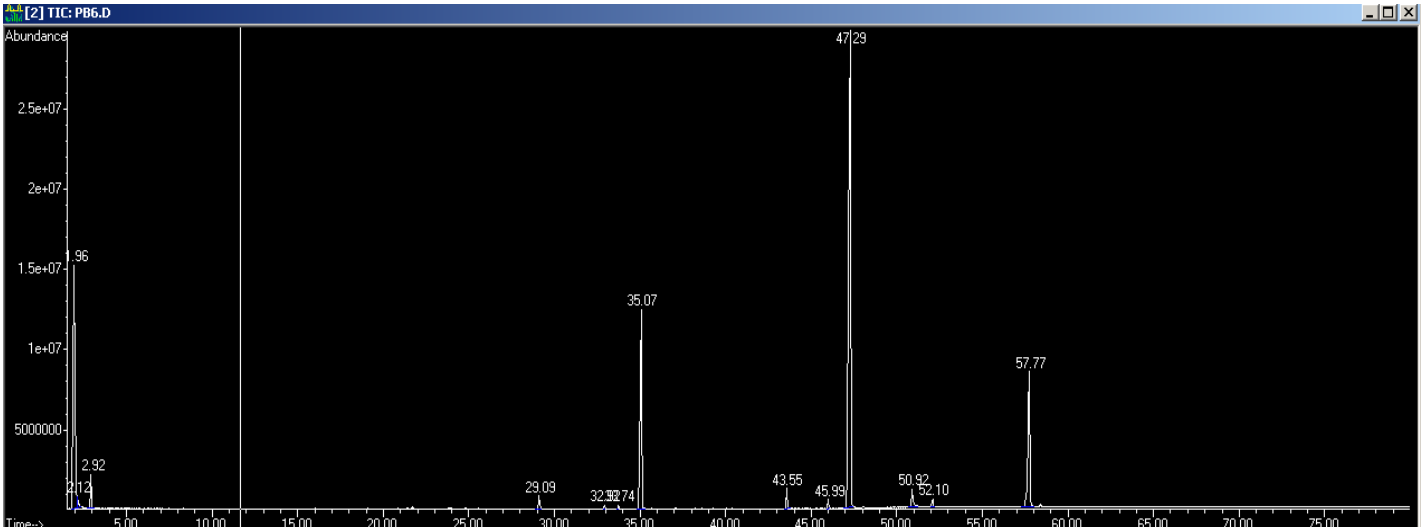

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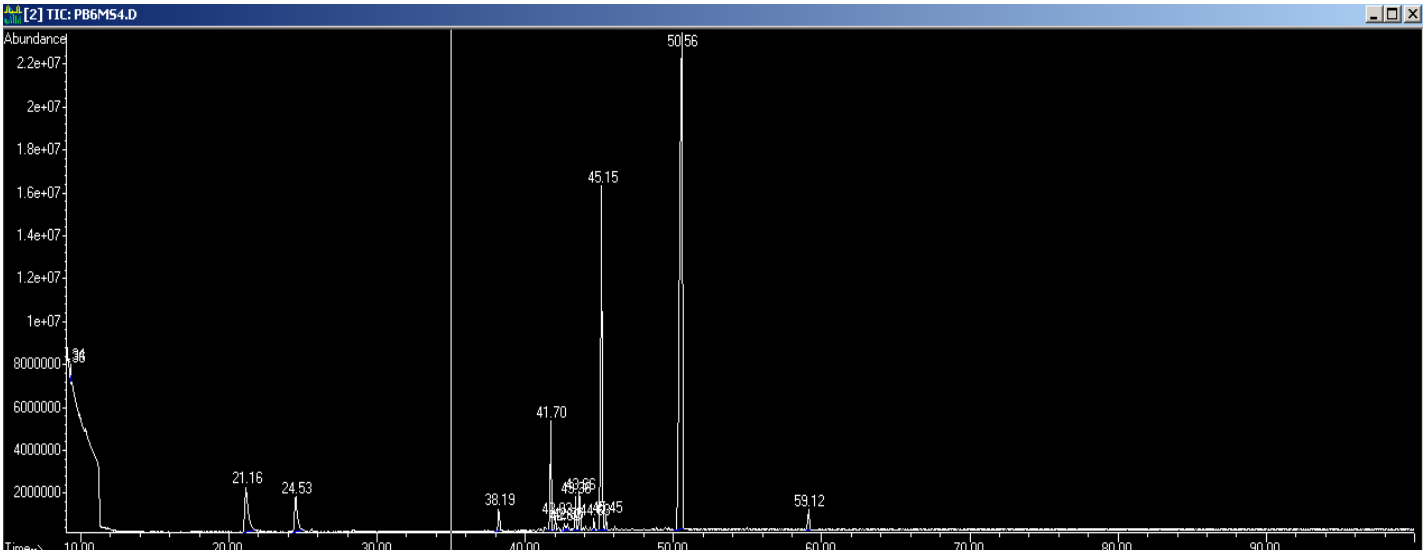

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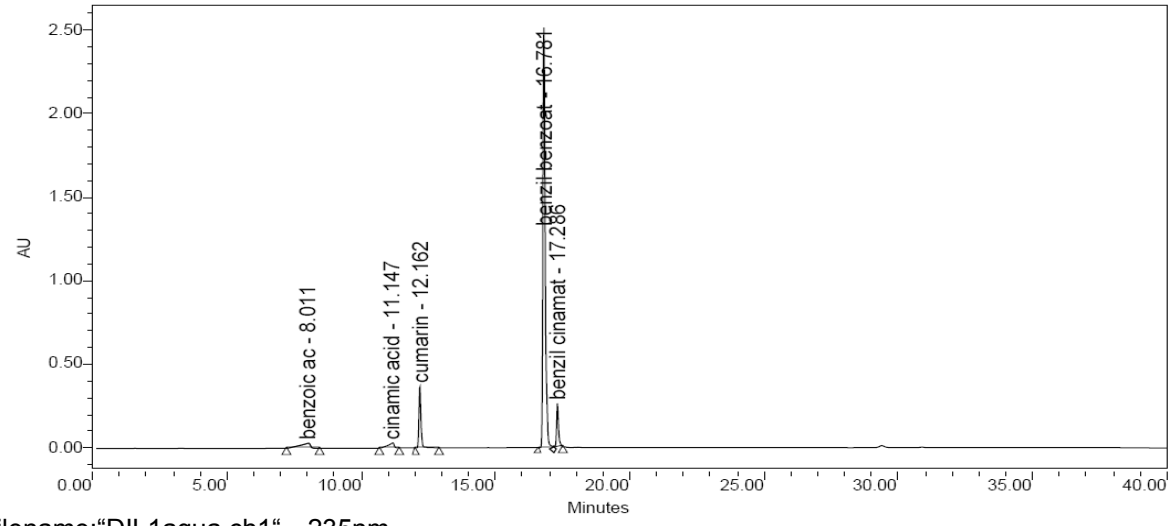

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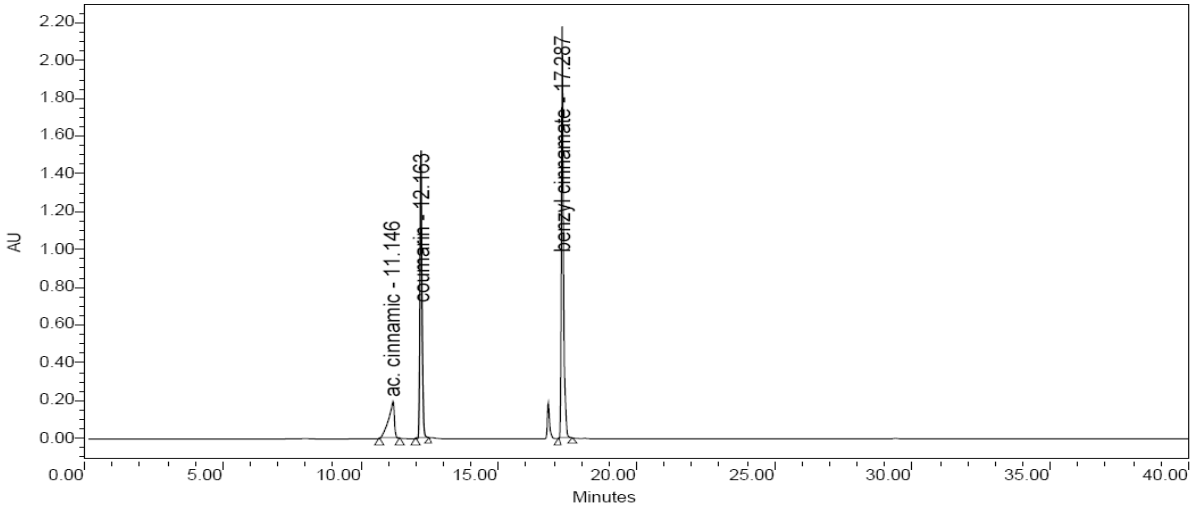

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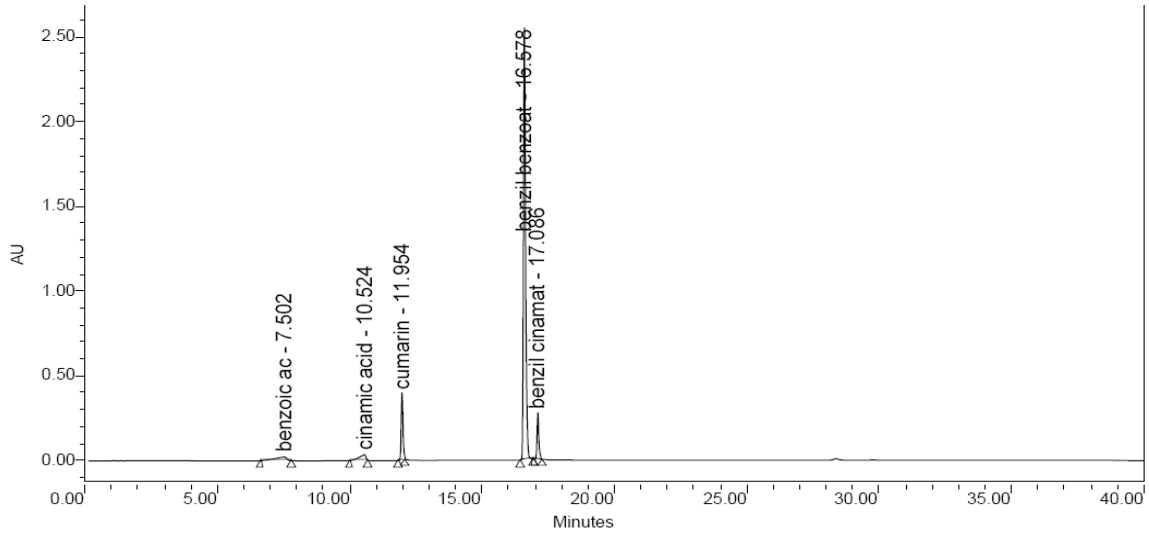

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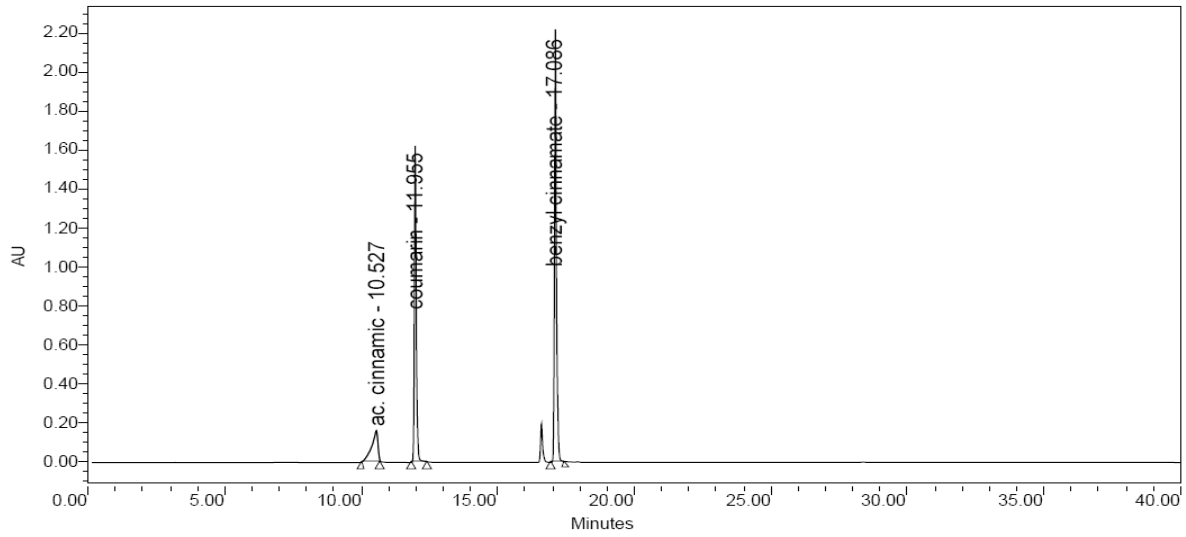

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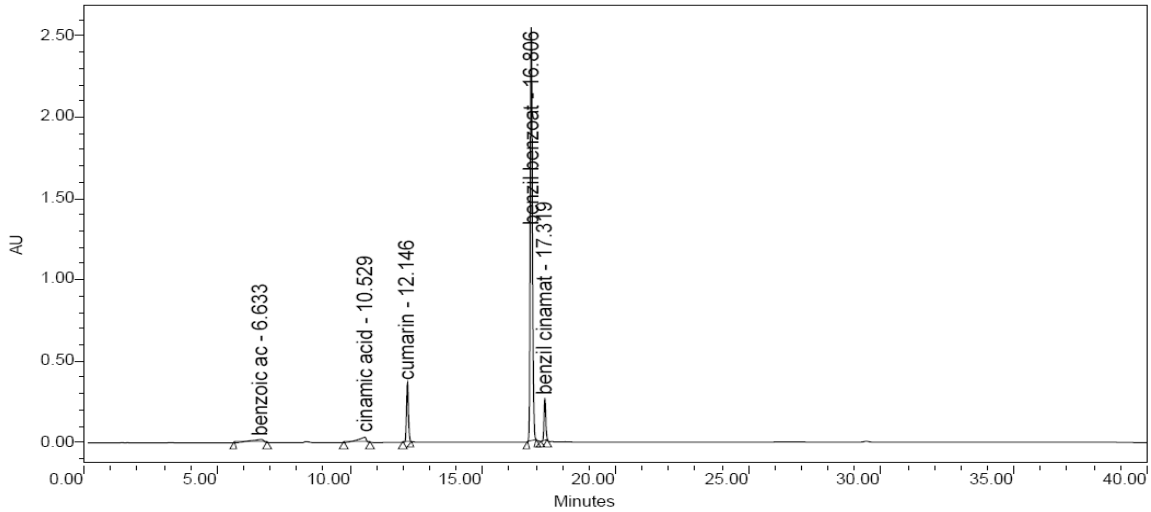

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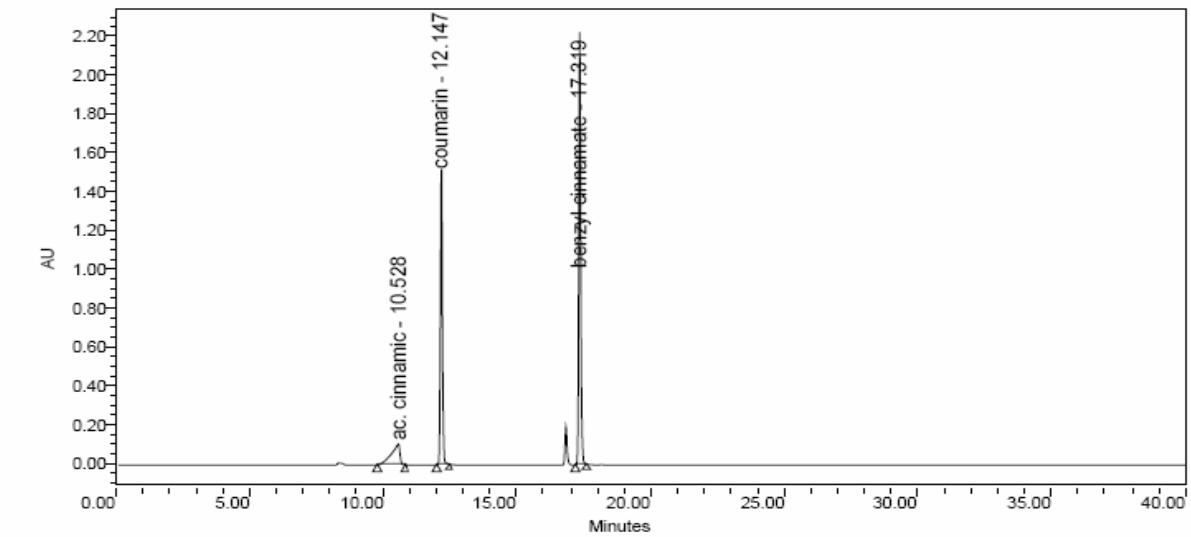

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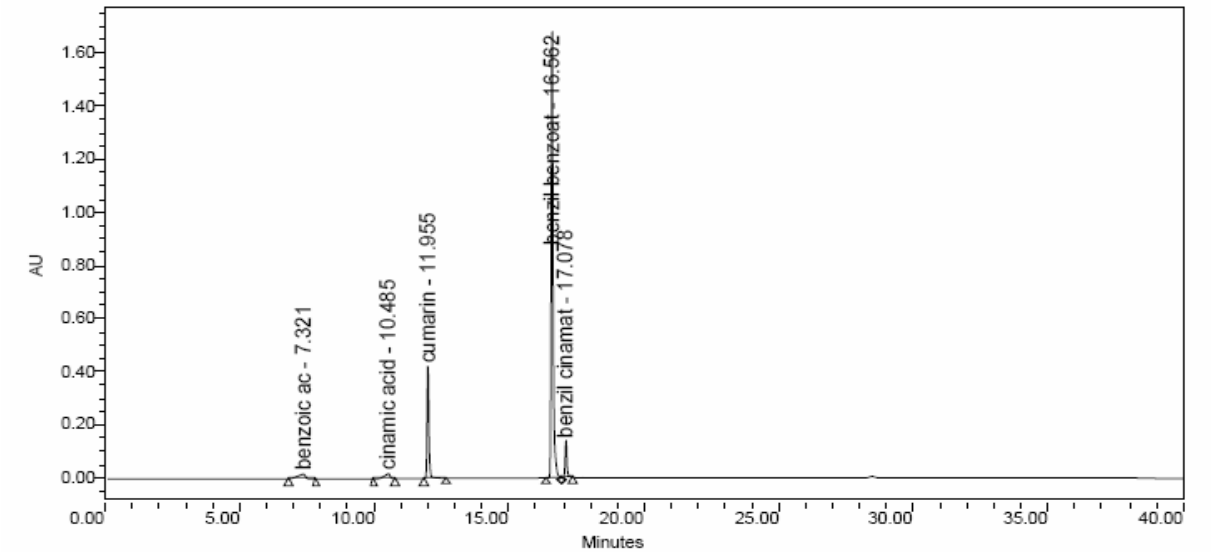

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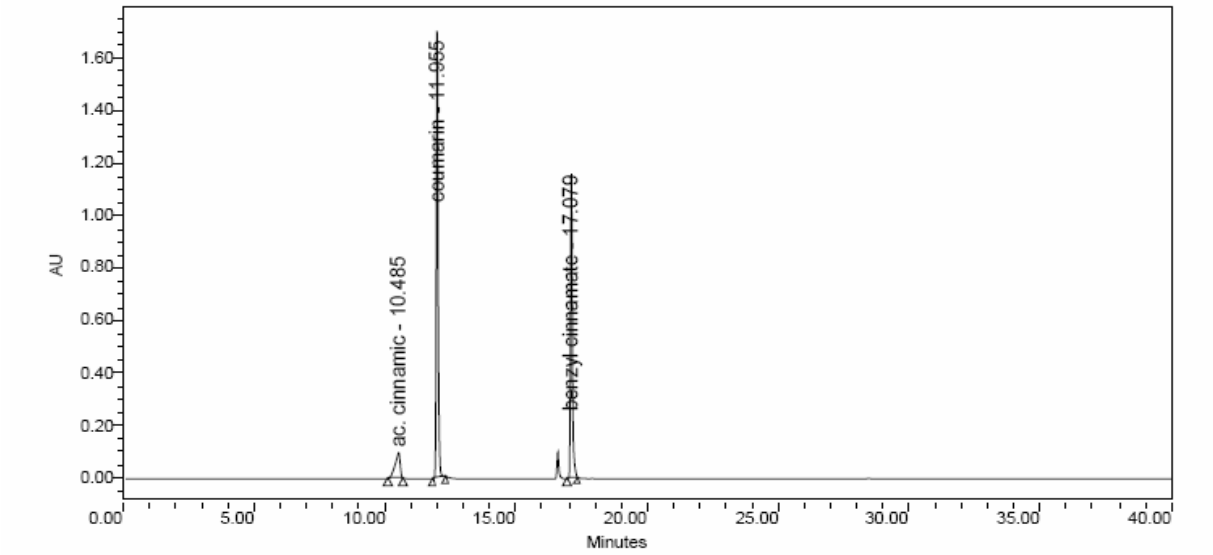

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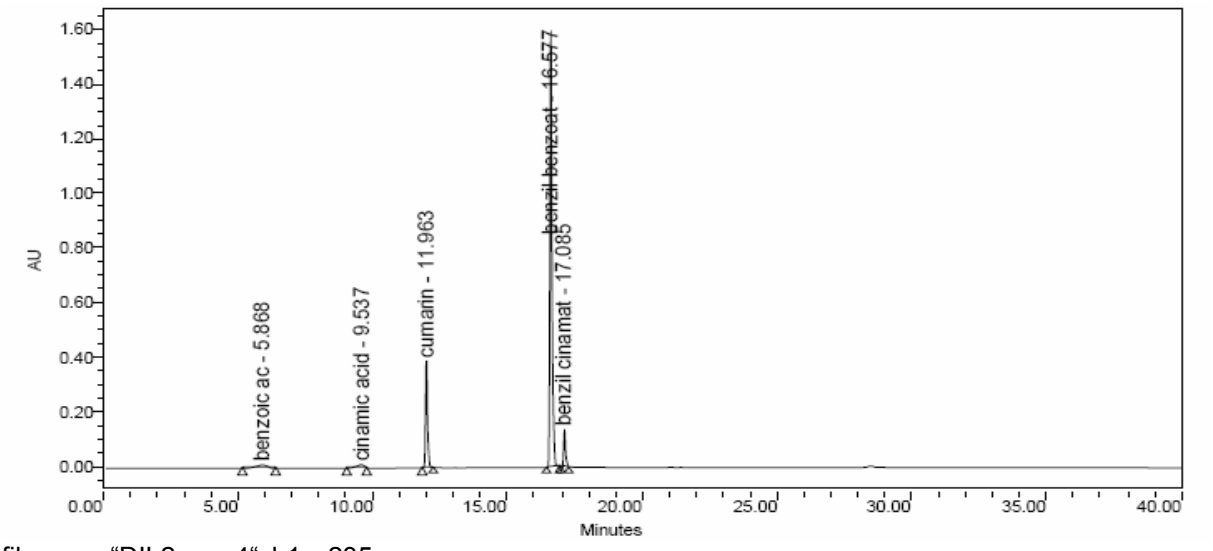

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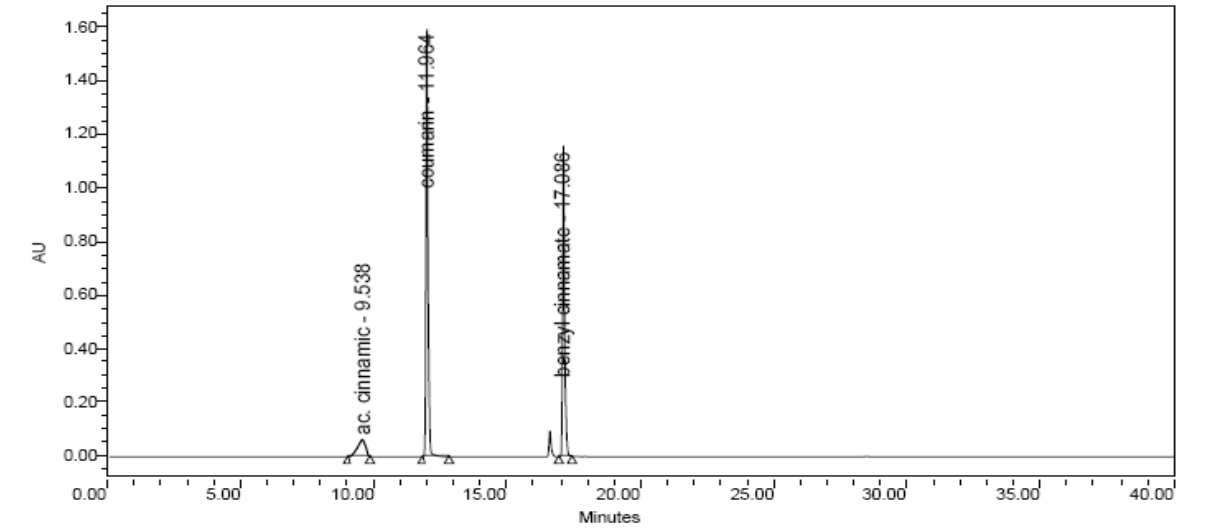

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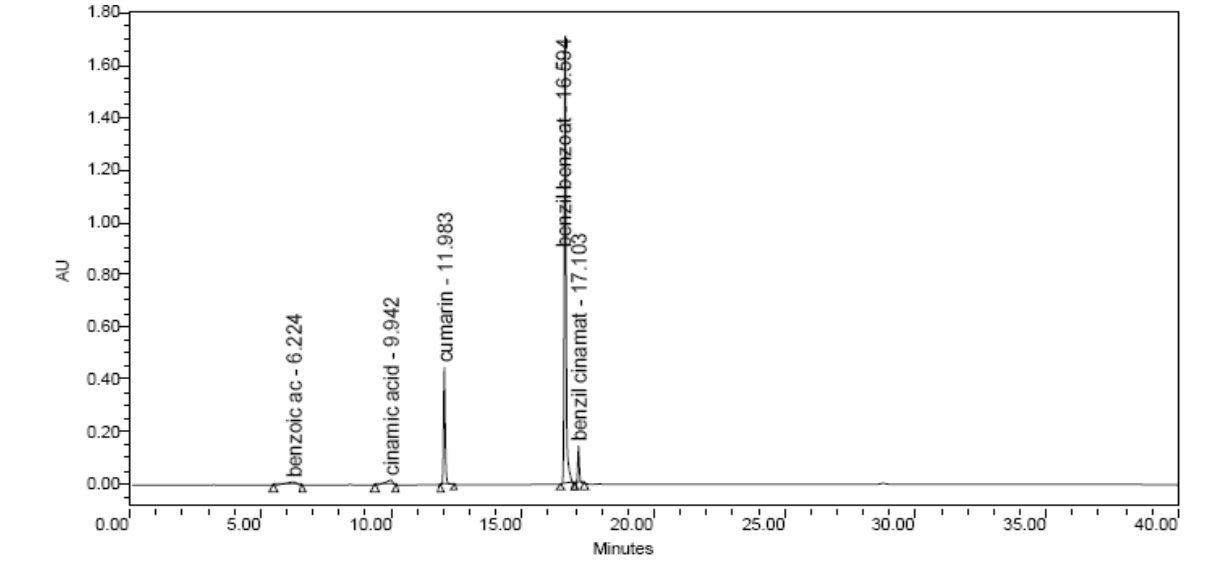

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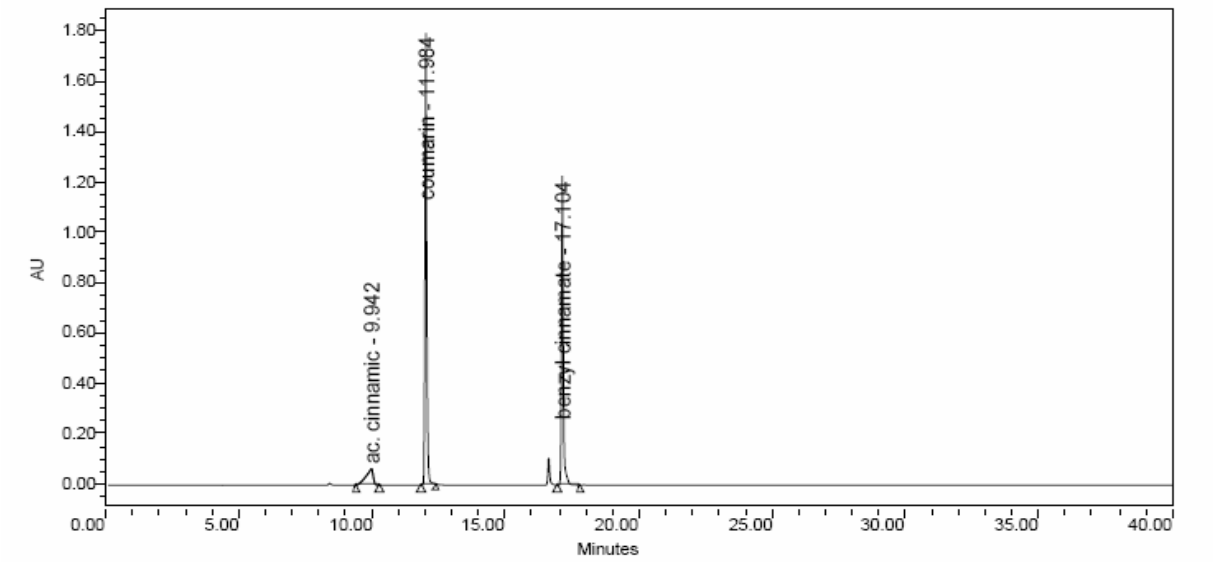

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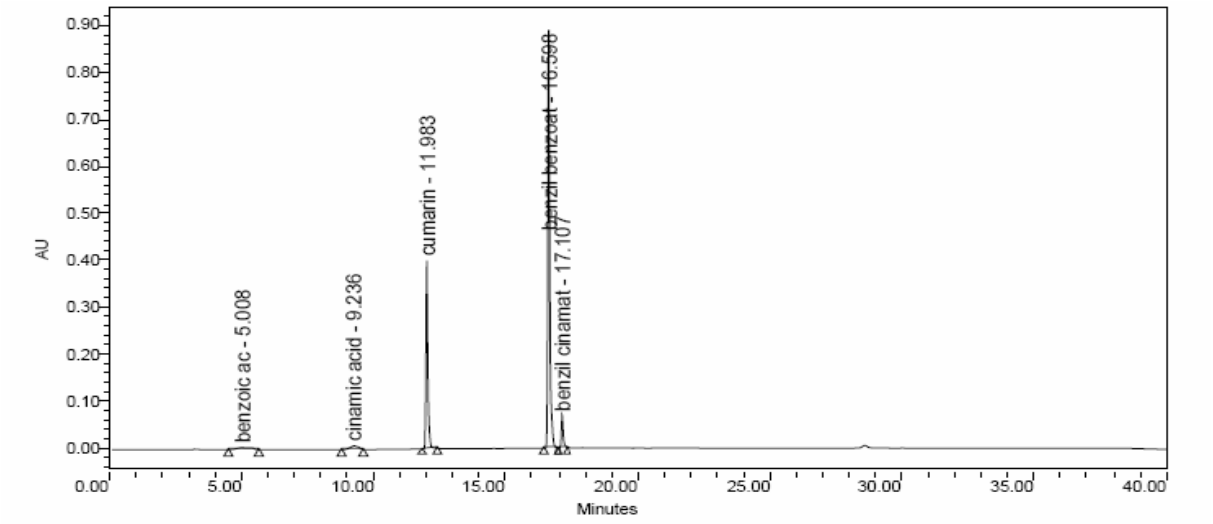

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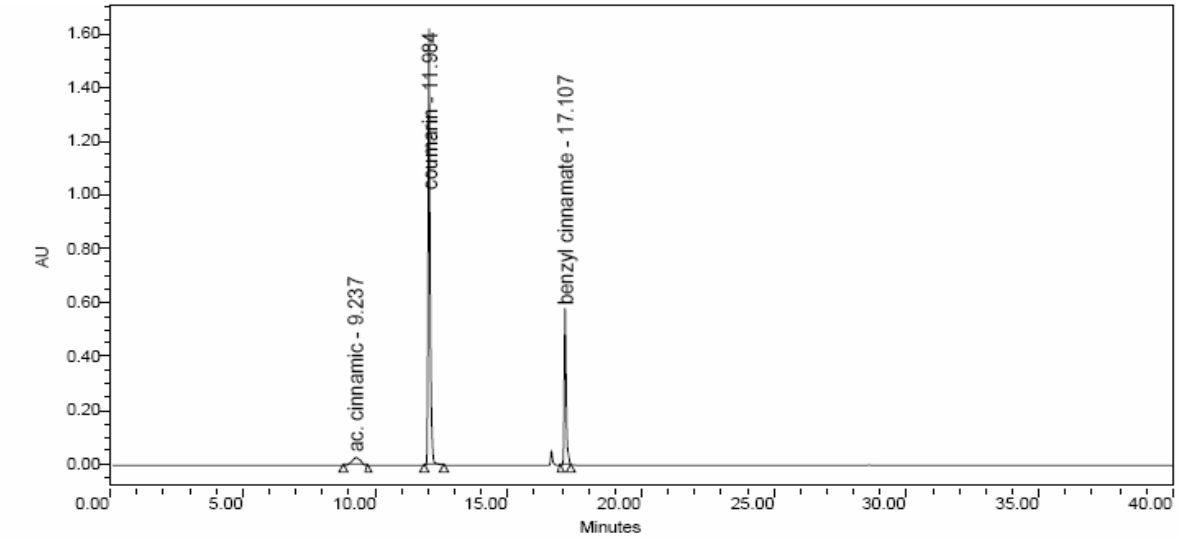

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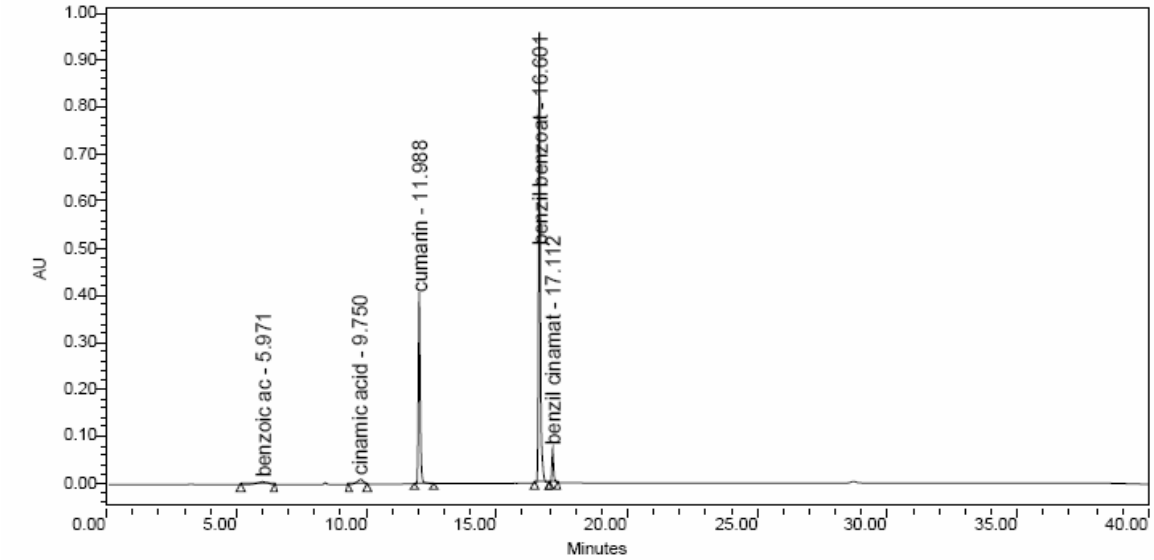

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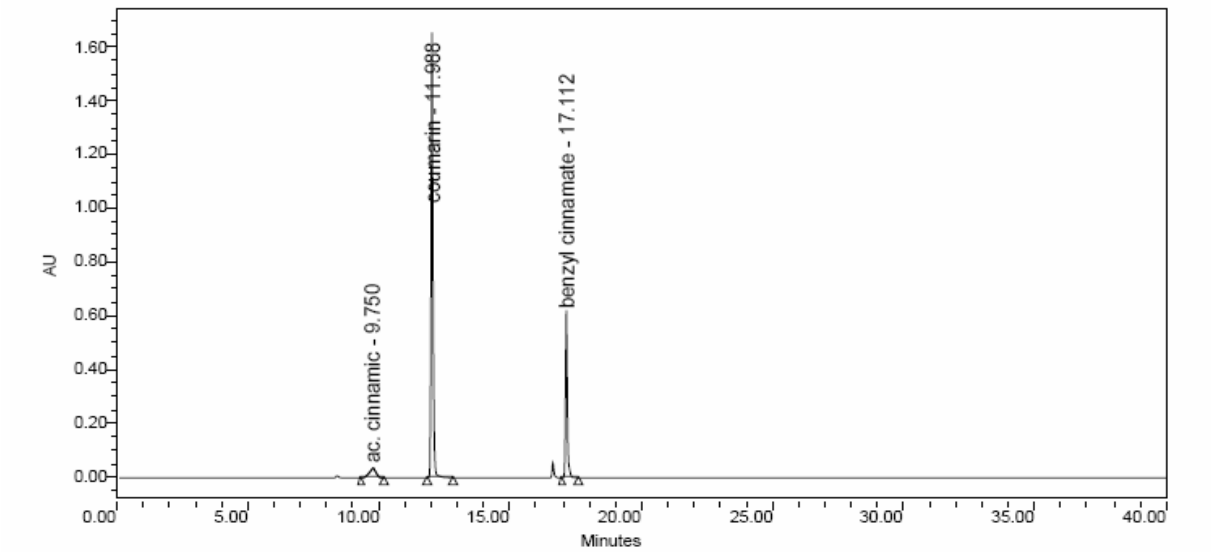

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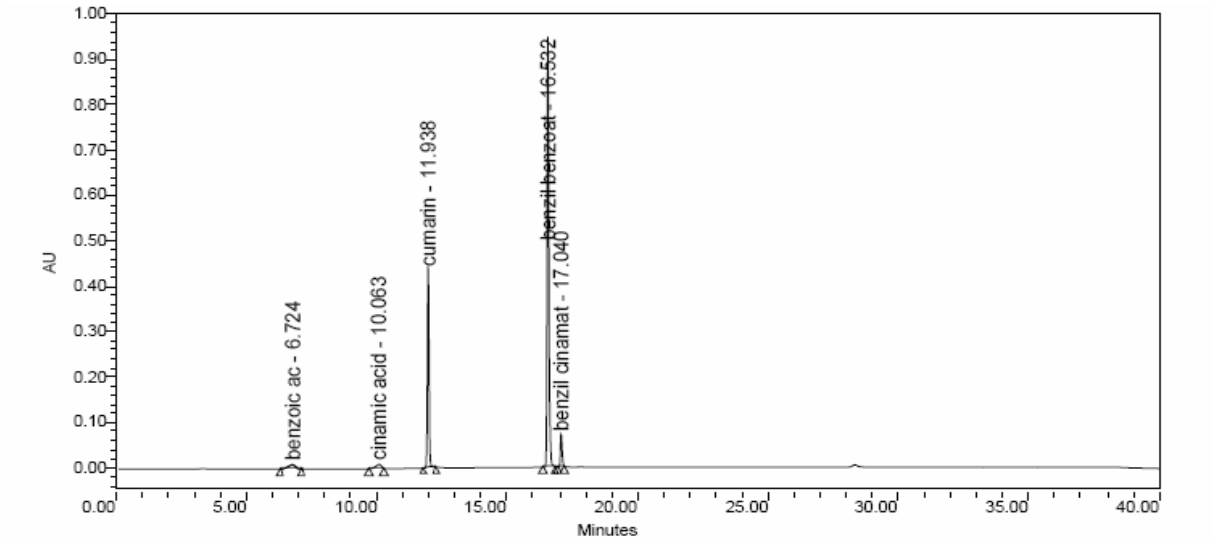

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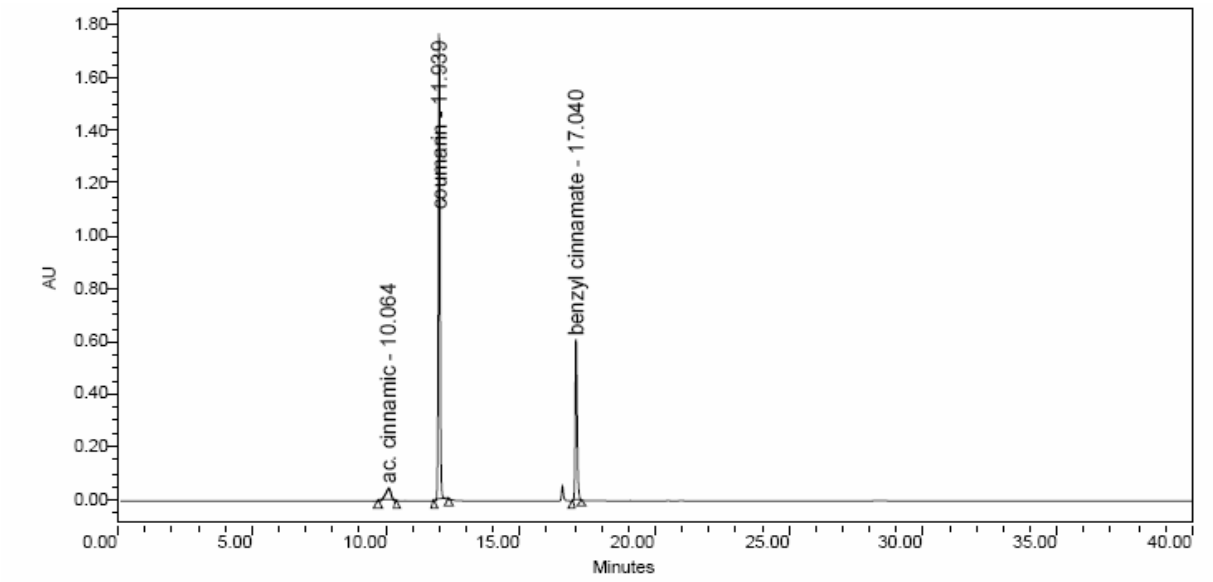

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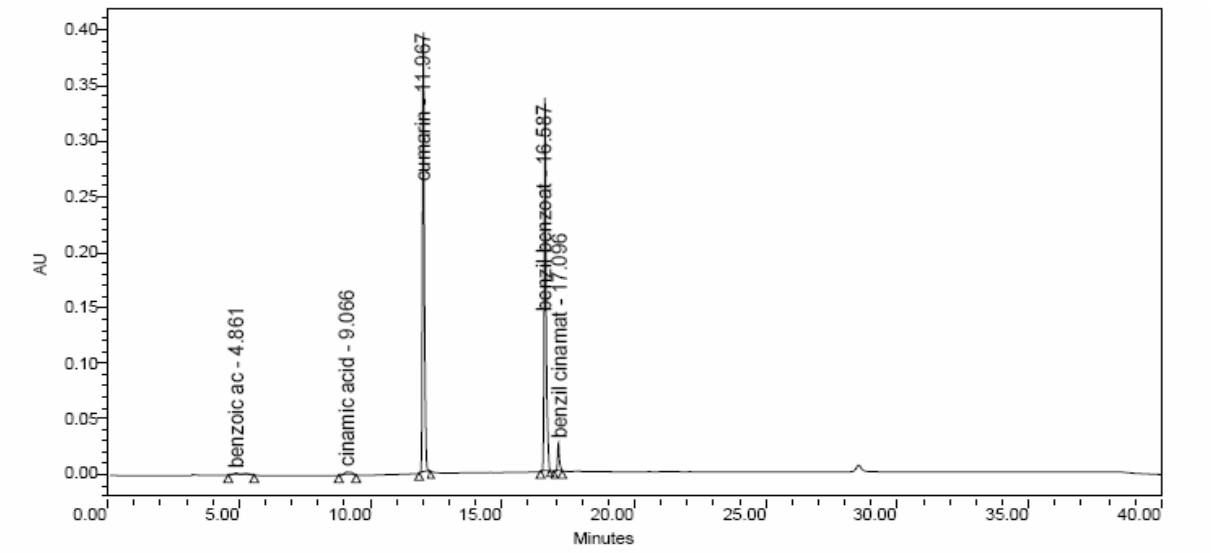

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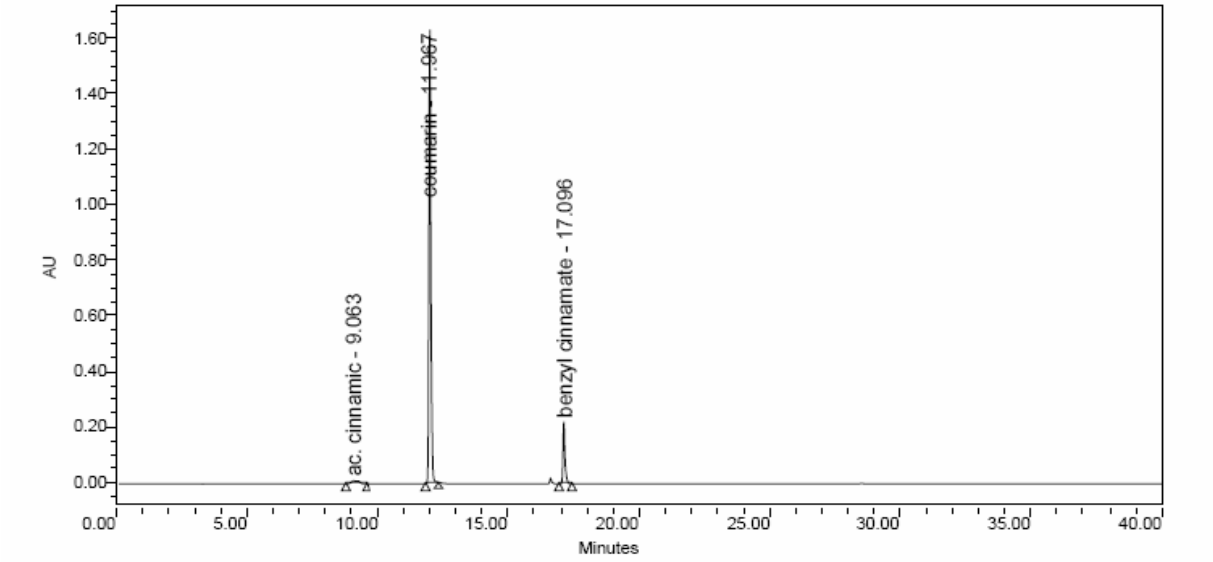

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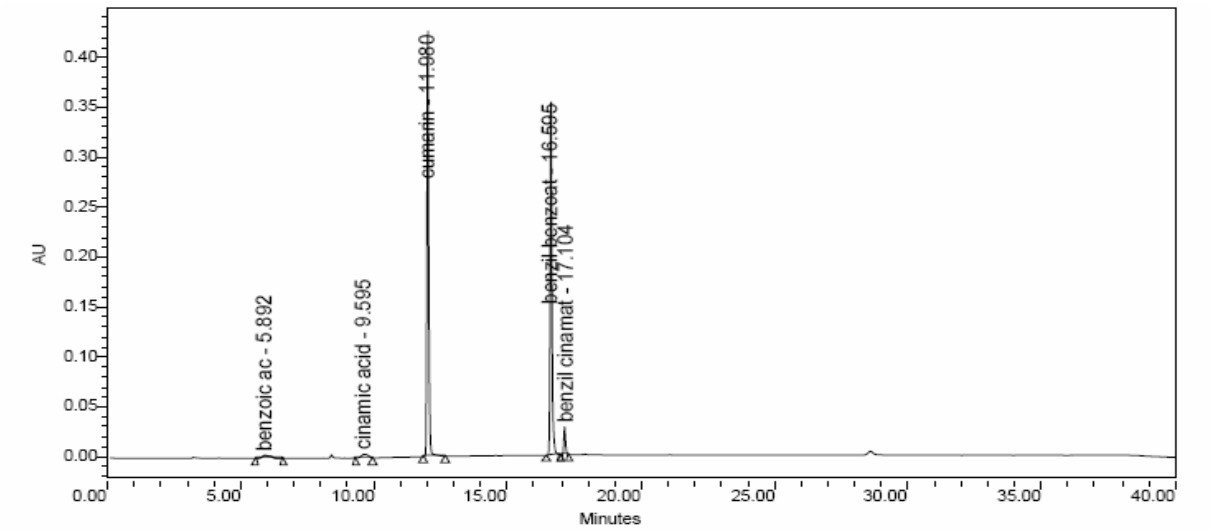

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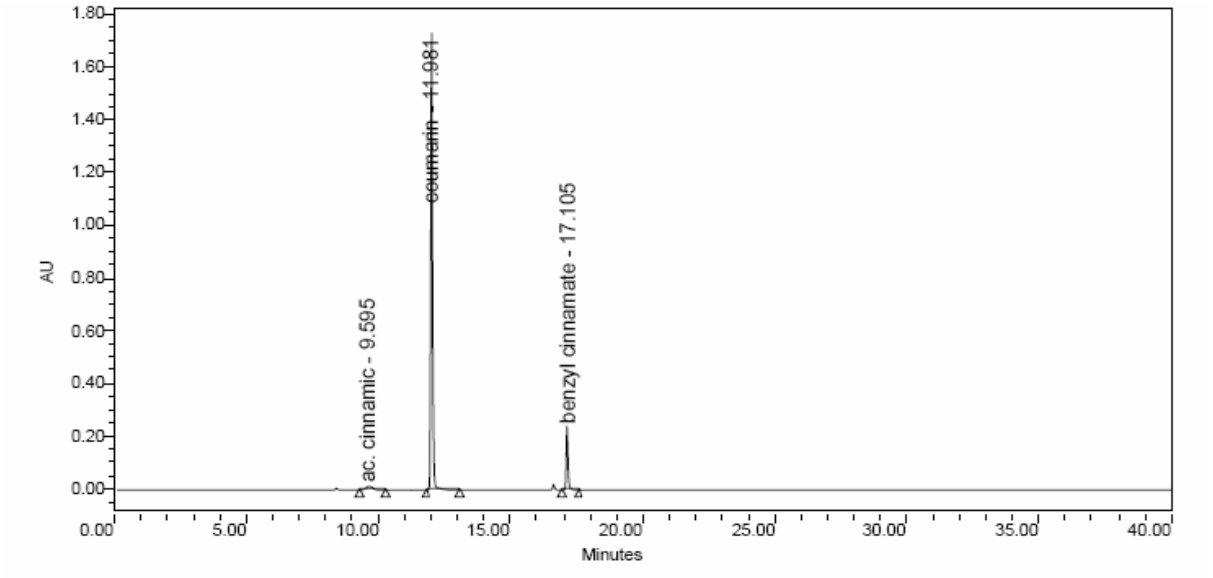

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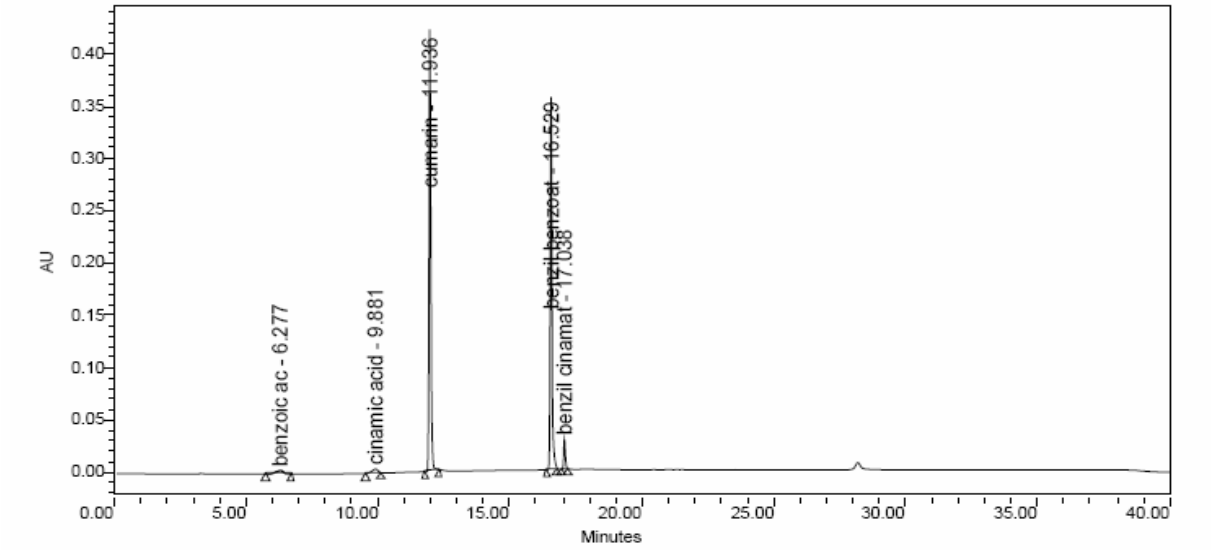

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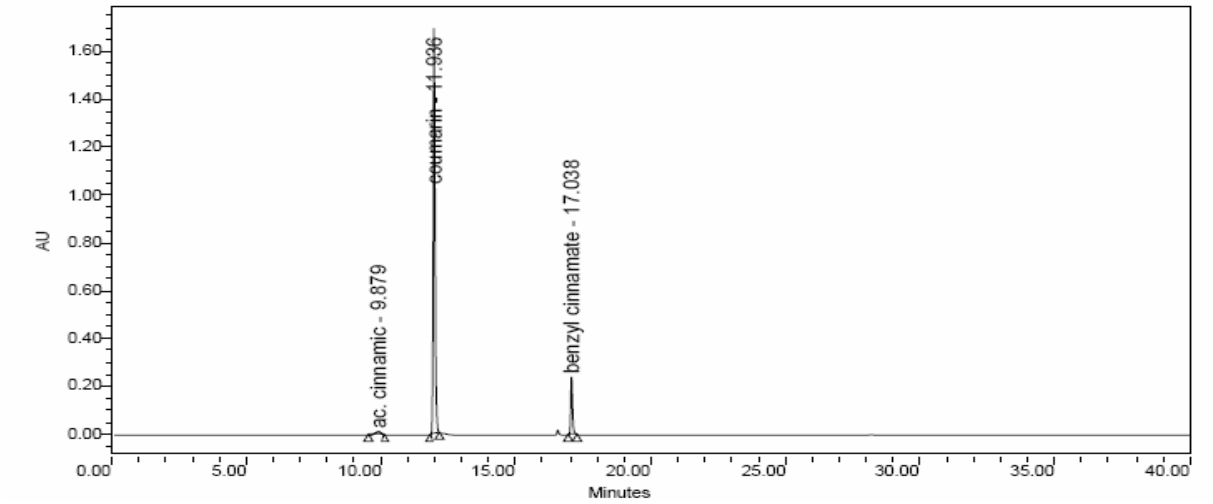

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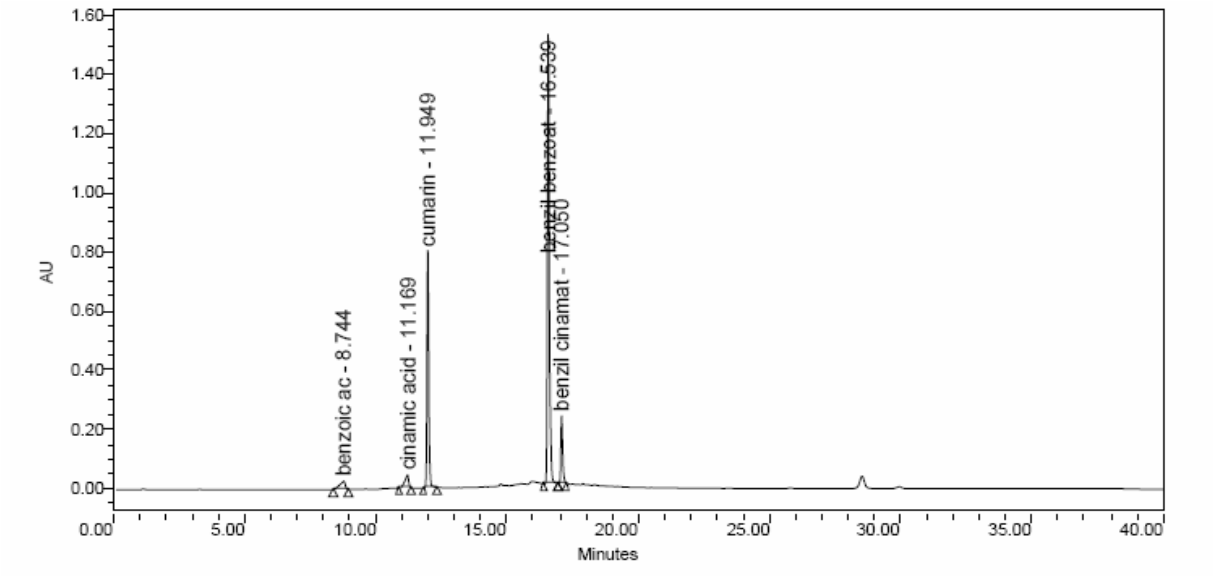

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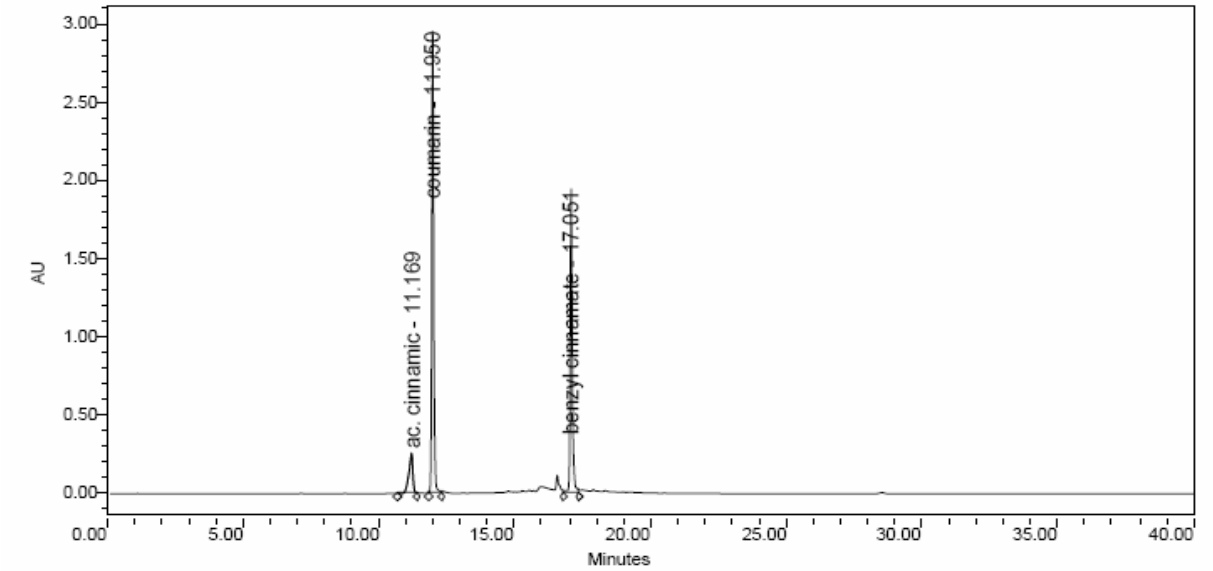

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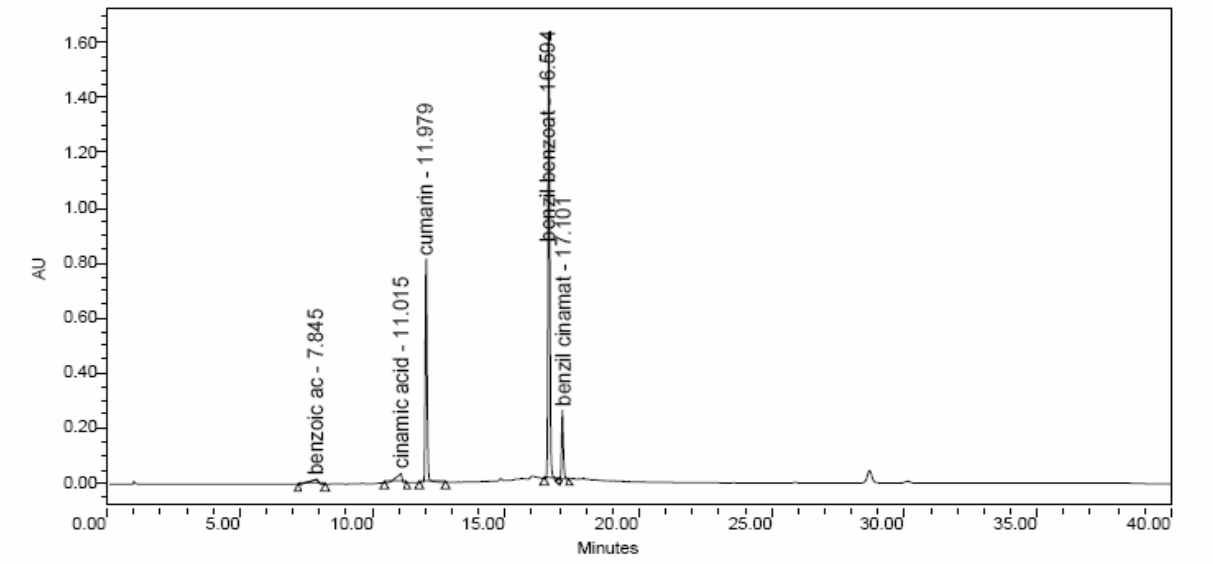

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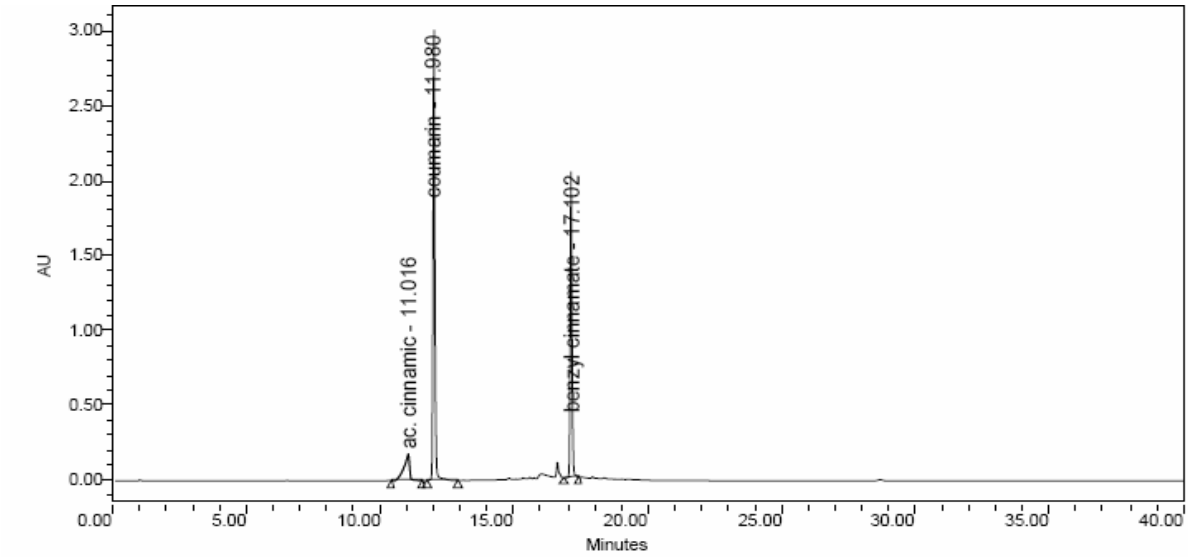

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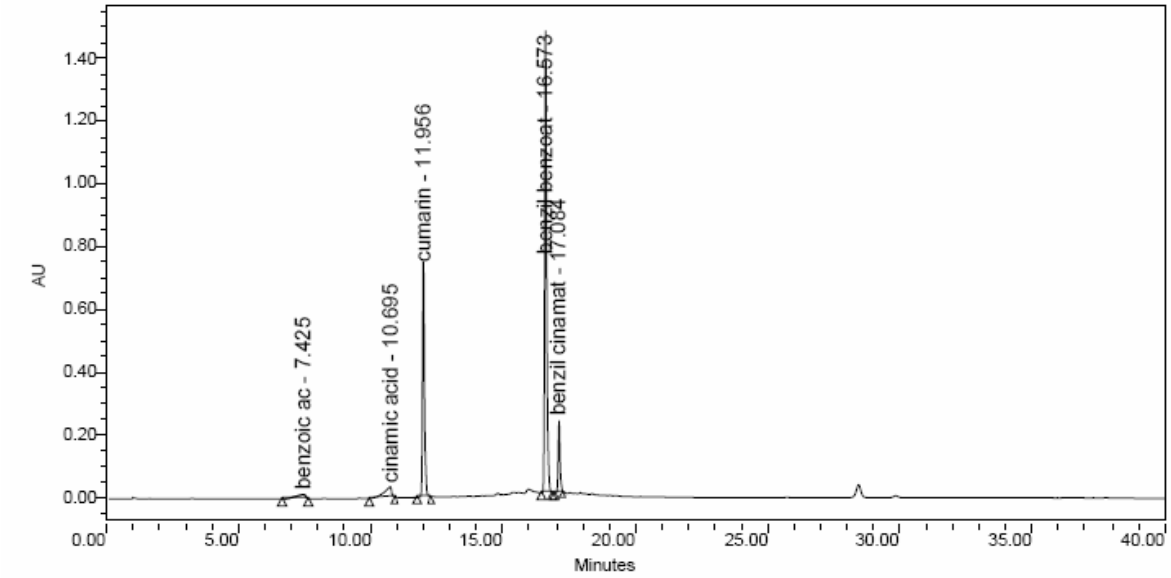

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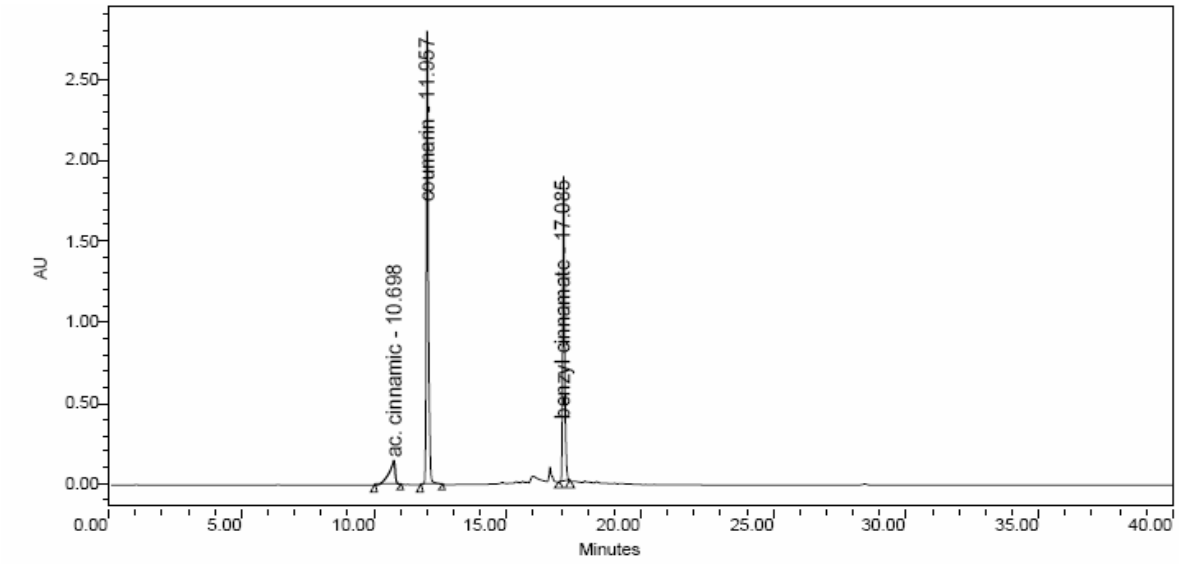

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Curriculum vitae

Name: Verena Mammerler

Date and place of birth: 28.6.1984, Wien

Parents: Mag.pharm. Adelheid Mammerler
Karl Mammerler

Education:
Primary school “Mater Salvatoris”
Primary school attendance from the years 1990 until 1994
BG/BRG Gänserndorf attendance from 1994 until 2002

Inscription to the University in 2002
Course of study: Pharmacy

SS 2007 course of studies abroad at the Facultat de Farmacia / Universitat de Barcelona. The experimental work for this diploma was elaborated during my stay abroad.