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DIPLOMARBEIT

„Chemical analysis of volatile components of

Juniperus communis L. with the newest methods of GC-MS“

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Ich widme diese Diplomarbeit
meinen Eltern und Großeltern

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ABSTRACT

In the context of this master thesis, *Juniperus communis* L. samples are analysed with recent preparation and analytical techniques. A comparison of traditional essential oil preparation techniques such as hydrodistillation to the modern extraction technique of headspace solid phase microextraction (HS-SPME), for sample characterisation, is made. In order to speed-up the analysis times, HS-SPME technique is coupled with fast gas chromatography (FastGC). Furthermore, the response of two different detectors, flame ionisation detector (FID) and mass spectrometry (MS), are compared.

ZUSAMMENFASSUNG

Im Rahmen dieser Diplomarbeit wurden *Juniperus communis* L. Proben mit den neuesten Präparations- und Analysentechniken untersucht. Für die Charakterisierung der Proben wurde ein Vergleich von traditionellen Herstellungstechniken ätherischer Öle, wie Wasserdampfdestillation, mit der modernen Extraktionstechnik, head space solid phase microextraction (HS-SPME), durchgeführt. Um die Analysenzeiten zu verkürzen, wurde HS-SPME mit Fast Gaschromatographie (FastGC) kombiniert. Des Weiteren, wurden die Resultate zweier verschiedener Detektoren, Flammenionisationsdetektor (FID) und Massenspektrometriedetektor (MS), miteinander verglichen.

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1. INTRODUCTION

Juniper is a plant belonging to coniferous order and family of *Cupressaceae*. It is widespread throughout the world and is used in several fields such as food and pharmaceutical industry. The high variability of the genus *Juniperus* and in particular the vast complexity of the plants that may be referred to the species *Juniperus communis* L. renders the taxonomic classification of the species and subspecies difficult. A clear and uniform distribution by morphological and geographical analysis has not been found yet particularly because of the significant influence of soil and climatic conditions. Therefore, an extensive study with a high number of "standardised" samples of *Juniperus communis* L. was started in order to determine a characterisation by the chemical composition of the plants. "Standardised" samples means that either a statistical or significative number of samples are collected and all samples are accurately known in their collecting places (locality and altitudes) and collecting times. Literature and preliminary results showed different chemical characterisation for main compounds also between the same subspecies that suggest the possible presence of "chemotypes". This research project has the aim to differentiate "chemotypes" based on main compounds of the samples and to establish if they are distinguishable into taxonomic classification effectively as "chemotypes" or as different subspecies. Furthermore, the editorial board of the Flavour and Fragrance Journal (reference journal in this field), based on the recommendation of the IOFI international body (International Organisation of the Flavour Industry), gave brief rules about the sample preparation and the detectors which are used for the characterisation of plant materials in general and in particular for the definition of the taxa classification. These Flavour and Fragrance rules state that the headspace sorptive techniques are not accepted for the discrimination between "chemotypes" or species. In addition, for the characterisation of a plant in quantitative terms MS detectors, considering one target and two qualifiers in SIM (single ion monitoring) mode, are not accepted and in methods that assume response factors equal to unity only FID or TCD detectors can be used. However, to screen a huge number of

samples, fast analytical methods, which are able to recognise the differences between samples, are required. For that reason both, suitable techniques and appropriate method conditions, are selected to reach a high number of sampling throughput in fast analysis times.

In the context of this research project, the purpose of this thesis work was to investigate 25 samples of *Juniperus communis* L., collected at Lillehammer National Park (Norway 2008), in order to sort out the Juniper (*Juniperus communis* L.) taxa classification. Additionally, this work deals, in view of more samples to analyse, with the comparison of different techniques both by different sample preparation techniques and gas chromatographic separations with different detectors:

- Preparation of essential oils *vs.* headspace solid phase microextraction

and

- Fast gas chromatography / flame ionisation detector *vs.* mass spectrometry

This dual stage investigation has been performed to speed-up and to automatise the analytical process and to reach more quality results, due to the MS detector, in order to analyse a high number of samples in saving time and to increase the throughput of the laboratory.

2. THEORETICAL PART

2.1. The vegetable matrix: Juniper

2.1.1. Introduction

Juniper, a woody-plant spread throughout the world, has several areas of application. In particular, "Juniper-berries" of *Juniperus communis* L. are used as a spice in a large variety of culinary dishes as well as a main ingredient at the well known alcoholic beverage gin. This coniferous plays not only an important role in food industry but also in perfumery and pharmaceutical or medical sciences.

The first chapter deals generally with the genus *Juniperus* and in particular with *Juniperus communis* L., which is studied in this thesis. Furthermore, it contains a description of different applications and gives an overview of chemical compounds.

2.1.2. The genus Juniperus

The genus *Juniperus* belong to the family of *Cupressaceae*[1], also called the cypress family. It is the second most varied genus of the conifers and has the largest distribution of any woody plant. Juniper grows from sea level to above timberline throughout the cool temperate northern hemisphere from the Arctic to North America, Europe and Asia.[2]

Most of the evergreen shrubs or trees are dioecious, which means that female and male cones are on separate trees. The scales of the female seed cones are fused together and form a fleshy berry-like structure, therefore they are often called "pseudofruits" or "berries". These "fruits" differ in form, size and colour depending on species and varieties. After 1 to 3 years the green cones mature and are consumed

by birds or small animals. The birds swallow the whole cones, digest the fleshy scales and eliminate the seeds, thus *Juniperus* is established on the Atlantic islands as well, for example in Bermuda, and on the Canary or Caribbean Islands.[2]

The classification of the genus *Juniperus* and the number of species are still in dispute. Alois Farjon (2001) categorised the genus *Juniperus* into 52 different species[1], whereas Robert P. Adams (2004) separated out 67 species and divided them into three sections:[2]

- *Caryocedrus*
- *Juniperus*
- *Sabina*

Caryocedrus is the smallest section, since only one species, named *Juniperus drupacea* Labill., is included. This species grows in the region between Greece and Turkey and its characteristics are very similar to those of the section *Juniperus*. Trees up to 30m or more are dioecious. Leaves are acicular, acuminate and linear lanceolate with two white bands above. Cones are 18-25mm tall and at maturity they are purple or bluish-black, with 3 cone scales and 3 united seeds per cone forming a drupe.[3]

Section ***Juniperus***, also called *Oxycedrus*, consists of 10 species and 10 varieties. All species in this section are dioecious and can be either trees or shrubs. Leaves are acicular, narrow, linear, with a basal abscission zone and whorled in groups of 3. Cones are medium sized (8-25mm) and have usually 3 separate seeds. The Section *Juniperus* can be divided into two groups: a northern and a Mediterranean group. The northern group, to which *J. communis* L. (figure 1.1) belongs, has blue or blue-black seed cones and one stomatal band. Whereas, the Mediterranean group, which includes *J. oxycedrus* L. (figure 1.2), is characterised by two narrow leaf stomatal bands and red or reddish-brown cones.[2]

Figure 1.1 *Juniperus communis* L.

Figure 1.2 *Juniperus oxycedrus* L.[4]

The third section ***Sabina*** contains remaining species (56 species with 24 varieties) and shows the greatest diversity.[2] These trees or shrubs are not only dioecious, but also rarely monoecious, for example *J. californica*, *J. chinensis* and *J. convallium*. The leaves are scaly or decurrent, without a basal abscission area. In some species, such as *J. carinata*, *J. pingii* or *J. squamata*, the juvenile leaves are borne throughout the tree´s lifespan. *Sabina* has the smallest cones (6-15mm) which are blue, brown, red or pink coloured. A fertile cone is comprised with 1-4 scales and 1-12 seeds, which are not fused together.[3]

2.1.3. Juniperus communis L.

The coniferous plant *Juniperus communis* L., part and main representative of the northern group in section *Juniperus*, is also called Common Juniper.

In addition to the morphological description in 2.1.2, all species of section *Juniperus* have insignificant, green or green-brown inflorescences, female and male on different trees. Seed cones are ovoid to globose, green in the first year and ripening into cones with various colour tones of blue. There are usually 3 separated seeds in one cone. A summary of all main characteristics of *Juniperus communis* L. is shown in figure 2.

Figure 2 *Juniperus communis* L.[5]

Juniperus communis L. itself is subdivided into the following varieties or subspecies:[2]

Juniperus communis L.	*var. communis*
"	*var. charlottensis* R.P. Adams
"	*var. depressa* Pursh
"	*var. jackii* Rehder
"	*var. megistocarpa* Fernald & H.St. John
"	*var. nipponica* (Maxim.) E.H.Wilson
"	*var. saxatilis* Pall.

The subspecies *charlottensis*, *jackii*, *megistocarpa* and *nipponica* grow as shrubs in very specific zones, therefore they can easily become threatened. There are no known uses of these varieties. *Juniperus communis sub. depressa* Pursh. is

widespread in Canada and the USA and no traditional uses are reported. The most common subspecies are *communis* and *saxatilis*. The subspecies *saxatilis* has many synonyms: *alpina*, *montana*, *nana* and *sibirica*. Both subspecies grow in the same areas from Europe to Russia and Asia. It is very difficult to classify them because of their similar morphological appearance. For example, both have acicular, lanceolate or linear leaves in whorls of 3. Only the size is a significant trait to distinguish subsp. *communis* because its leaves can become larger (15-20mm). Also the seed cones of both are globose and have nearly the same colour and diameters when they fully ripe, subsp. *saxatilis* brownish-blue, 4-7mm and subsp. *communis* blue-black with 6-9mm. The only distinct characteristics in which they are distinguishable are the appearance of the bark and the size of the plants. Subsp. *communis* grows as tree (4-5m) or shrub and its brown bark exfoliates thin strips. Whereas, subsp. *saxatilis* is only a procumbent shrub, 70cm tall and scales off wide strips. This classification of subsp. *communis* and *saxatilis* is therefore often controversial. Both have several uses, for instance, their "fruits" are the basis of the gin flavour.[2]

Gin, Jenever or Genova, which are older names of gin, was developed in Europe in the 17th century. At that time gin was used as a digestive beverage and as a remedy for kidney problems. Two different techniques are used for production: distillation and aromatising alcohol. In the Netherlands gin is made by distillation of cereal alcohol, like barley or corn, and admixed with juniper "berries" and other aromatic plants. In Great Britain the production of "London dry gin" starts with neutral ethyl alcohol. The alcohol is subsequently flavoured with juniper "berries" coriander, lemon and orange peel, fennel and anise seeds as well as angelica, cardamom, almonds and other aromatic herbs. This aromatised mixture is finally distilled.[6] Today gin is one of the most used spirits for cocktails and several different qualities are available on the market.

Further applications of *Juniperus communis* L. are found in cooking, aromatherapy and perfumery. Ripe, whole or crushed "berries" are a spice for meat dishes, sauerkraut or dark sauces. For aromatherapy and perfumery essential oils or cedar oils are used, they are often created from other species, for example *Juniperus sabina* L., as well[7].

In conventional medicine, ripe and dried "juniper berries" of *Juniperus communis* L. are used as a remedy for dyspeptic complaints, to enhance the renal water elimination and to stimulate the appetite. The German Commission E lists dyspepsia as the only therapeutic indication, because juniper "berries" and its oil are reputed to be toxic and could irritate renal epithelium or cause hematuria.[7] Moreover, the diuretic effect has not been clearly confirmed by experimental investigations. They present either positive or negative results.[8] Another proven effect is the contraction of smooth muscles. These are all effects of internal applications, however, the essential oil of *Juniperus communis* L. is often used for external applications. For instance, it is a main component of liniments, like *Juniperi spiritus* or *Linimentum salicylatum* PhHelv, which have skin-irritation effects.[9] In alternative medicine juniper "berries", wood and essential oil are used externally and internally as a remedy for arthritis, rheumatism, abdominal pain and coughing, but these effects are disputed.

The drug "juniper berries", defined by the *Pharmacopoea Europea*, contains at least 2% or minimum 10ml/kg essential oil. The main compounds of juniper oil are monoterpene hydrocarbons, in particular α-pinene, β-pinene, sabinene, myrcene, limonene and monoterpene alcohols, for example α-terpineol and terpinen-4-ol, the latter is most responsible for the diuretic effect. Sesquiterpenes such as β-caryophyllene, germacrene-D and β-elemene are also present.[8,10] The chemical structures of the main compounds are shown in figure 3.

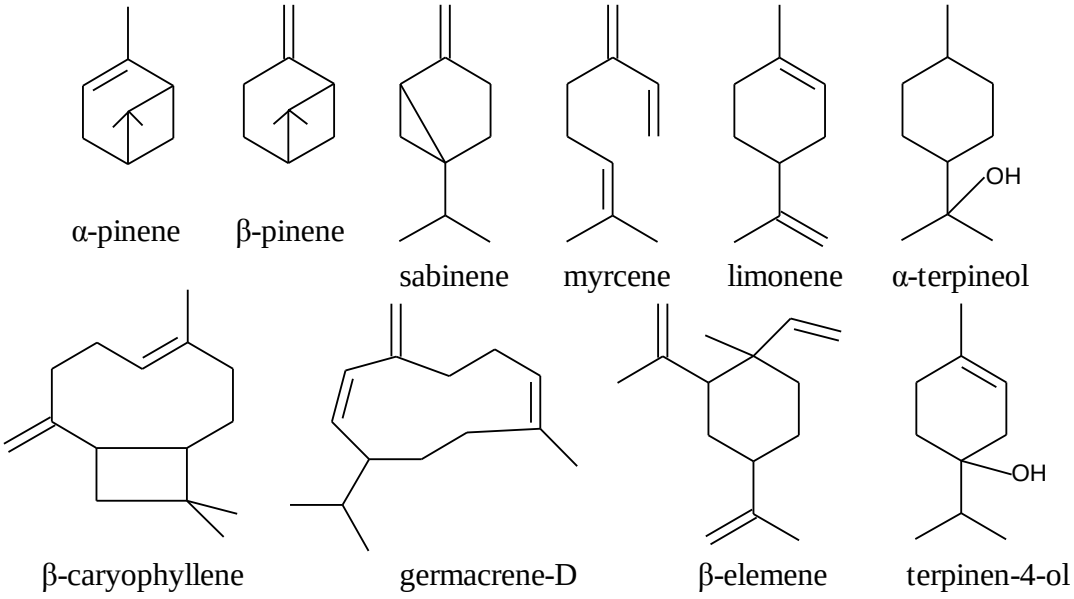

Figure 3: Chemical structures of some juniper oil components

The qualitative and quantitative composition of the "berry" oil depends mainly on the degree of maturity and the geographical origin. For example, unripe "fruits" in general present a higher content of α-pinene than mature "fruits". Some monoterpenes occur enantiomerically pure, for instance (+)-sabinene or (-)-β-pinene, whereas either the (+)- or (-)-enantiomer of α-pinene can prevail.[8]

The composition of leaf essential oil even within the subspecies of *Juniperus communis* L. presents differences as reported by Adams. For example the essential oil of subsp. *saxatilis* was dominated by sabinene and moderate amounts of camphene, α-phellandrene and β-phellandrene. In contrast subsp. *communis* had large amounts of α-pinene, β-pinene and β-phellandrene but low ones of sabinene.[2]

However, even a clear taxonomic distinction between the subsp. *communis* and *saxatilis* has not yet been found, because of the complexity and the high variations of the essential oil composition. Filipowicz et al., in 2006, has tried to distinguish several samples of subsp. *saxatilis* and *communis* on the basis of the chemical composition of leaf essential oils. They have distinguished three chemical groups: an α-pinene type with a high content of α-pinene and low of sabinene; a

sabinene type, high sabinene percentage and low α-pinene percentage and a third type with intermediate amounts of these two monoterpenes. However, a direct relation between these chemical characterisations and the classification of the subspecies has not been found yet. All three "chemical types" have occurred in both subsp. *communis* and *saxatilis*.[11]

Furthermore, there are significant differences among the essential oil composition from needles, berries, wood and roots within the same subspecies. Gonny et al. investigated plant material of subsp. *saxatilis*, also called *alpina* which was collected from several bushes growing in the centre of Corsica. The results of this study showed that limonene was the main component of the berry, needle and wood oil. The needle oil was dominated by the content of α-pinene as well. Sesquiterpenes, in particular farnesol, β-elemene and α-cadinol were present in high amount in wood oil as well. In contrast, the root essential oil exhibited a high content of sesquiterpenes and very low amounts of monoterpenes.[12]

In summary, the species *Juniperus communis* L. varies not much in its morphological characteristics, but more in its chemical composition. In particular, the subsp. *saxatilis* and *communis* are very difficult to classify and identify, because of their similar characteristics. For that reason a clear uniform classification has still to be found and is nowadays in discussion.

2.2. Techniques for extracting volatile compounds

2.2.1. Introduction

Today the pharmaceutical, cosmetic and food industry have a considerable interest in the research of plants and their components. Therefore, many different techniques have been developed for the extraction of plant components, each of them having advantages as well as disadvantages.

The next chapter deals mainly with the theory of two techniques:

- Preparation of essential oils
- Headspace solid phase microextraction

2.2.2. Essential oil and its preparation

According to *Pharmacopoea Europea*, an essential oil or *Aetherolea* is defined as an "odorous product, usually of complex composition, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or 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."[10]

Essential oils are also known as volatile oils, or simply "oil of the plant" from which it is extracted. The oil is "essential" because it is based on a distinctive scent of the plant. A pure essential oil vaporises completely in contrast to fat oil and does not leave grease stains on a paper. It consists of many different chemical compounds and is liposoluble, but it does not contain fat. In addition, essential oils have a high refractive power and their density is below 1, except for the volatile oil of cinnamon and cloves.

An essential oil mainly consists of a mixture of terpenes, in particular monoterpenes, for example geraniol, limonene, terpinene, cineole, sabinene, pinene, camphor, and sesquiterpenes, but phenols and phenylpropanoids can also be present. Some oils, such as mustard oil, contain nitrogen or sulphuric components which are present in their water-soluble, glycosidic form in the plant. They are released by enzymes, for instance while the plant is reduced into small pieces. Most volatile oils, however, are mixtures of hydrocarbons, or their oxygenated components originated from the hydrocarbons.

Due to the numerous complex compounds of essential oils, their usage is also very wide-ranging. They are important raw materials for many industries, such as perfumeries, and the cosmetic and food industries. Furthermore, volatile oils are used in aromatherapy, scented candles or diluted in carrier oils for massages. Pharmaceutical and medical applications depend on the respective composition of the essential oils of different plants. For instance, the essential oils of rosemary or lavender are used as a remedy for rheumatism, the oil of cloves and eucalyptus show antiseptic effects and some components of chamomile or yarrow oils have anti-inflammatory effects. Only small doses of essential oil can be taken, because use at higher doses can induce intoxication. In addition, some oils are used externally for skin irritations.

For the preparation of an essential oil three methods are differentiated:

- Expression
- Distillation
- "Solvent Extraction"*

*Solvent extraction is not a technique to prepare an essential oil because other components of the plant are extracted as well. However, it is an approach to obtain oil representing the composition of plant. Thus is the reason why it is described in this chapter as well, although it disagrees with the definition of the *Pharmacopoea Europea.*

Expression is the most ancient of the three techniques, although it is still applied mainly for citrus fruits. This manual or mechanical process is based on a very simple principle. Odorants of fruits or vegetables, in particular of their pericarp, are forced out by physical pressure. Most citrus oils are prepared in this way, which is also known as "cold-pressed".[10]

Three methods are known for the **distillation** of essential oils: steam distillation, dry distillation or hydrodiffusion. In steam distillation, also called hydrodistillation, a still pot containing raw material of a plant and water at the base is submitted to steam and heated by steam coils. The oil co-distills with the steam and recondenses by means of a cooling coil. The final distillate, a mixture of oil and water, is collected in a Florentine flask with one outlet near the top and another at the base. The oil is separated from the water in the Florentine flask, because of their different densities and it can be withdrawn by the upper outlet. The water, however, is mostly discarded or recycled to the still pot. During the heating process some degradation of components can occur. For instance, tertiary alcohols present in plant material often dehydrate in the still pot and appear as the corresponding hydrocarbons in the final product. However, in steam distillation the temperature is always below 100ºC, because of the boiling point of water.[13]

In dry distillation much higher temperatures can be achieved and therefore more degradation may occur. Dry distillation is a process, through which solid plant material is heated to produce gaseous products without the addition of water or steam. In most cases a direct flame is used to reach the high temperatures. It is used for the production of some wood oils, for example birch tar oil.[13]

The third method hydrodiffusion is somewhat like steam distillation, but it is carried out upside down. That means that steam is added at the top of the pot and water and oil are withdrawn at the bottom, although it is not distillation in *sensu stricto*. The steam in hydrodiffusion is used to break open the plant cells which include the oil.[13]

In many cases plants contain little amounts of volatile oil or their chemical components are too sensitive to heat, therefore it is not possible to obtain the essential oil by expression or distillation. In these cases another technique, called **solvent extraction** can be used to determine the composition of the volatile fraction of a plant. Strictly seen, it is not a technique to "extract" the essential oil, because other non-volatile compounds are released as well. This technique adopts a solvent such as petrol ether, ethyl acetate, hexane or acetone to extract the plant components but resins, waxes and other lipophilic plant material can be extracted additionally. For that reason this mixture, also called "concrete", can be leached out again with ethanol to yield an "absolute" or distilled to obtain the extract oil. A further option for solvents is supercritical carbon dioxide. Supercritical fluid extraction benefits from the fact that when the gas is under pressure, it behaves like a normal extraction solvent that can be completely removed when extraction and separation are finished, without leaving residue in the oil.[13]

Enfleurage is another technique of solvent extraction, but nowadays there is no commercial significance for it. In enfleurage, a large glass plate is covered with a layer of animal fat and plant material, for example flowerheads, is placed on the fat. The scent of the flowerheads diffuses into the fat over time. The flowerheads are constantly replaced until the fat is saturated with fragrance. Then the perfume oil, also called "pomade", can be washed out in alcohol and separated from fat and other extracted waxes.[13]

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The preparation techniques of essential oils are very costly and the yield of essential oils is often low, thus they can be very expensive and other substances with similar properties can be added.

However, the following parameters are adopted to determine the quality of an essential oil:[10]

- Boiling or freezing point
- Refractive index
- Relative density
- Optical rotation
- Thin layer chromatography
- Spectrometry UV and IR
- Acid value
- Saponification value
- Peroxide value
- Foreign esters
- Solubility in alcohol
- Gas chromatography

2.2.3. Headspace Solid Phase Microextraction

Pawliszyn and Arthur introduced solid phase microextraction (SPME) in 1990 as a solvent free sampling technique which unifies extraction, concentration, and cleanup into a single step.[14] After few years, in 1993, Pawliszyn and Zhang introduced headspace solid phase microextraction (HS-SPME) as a further extension of SPME.[15] In HS-SPME, the volatile components can be analysed without interference by the non volatile sample matrix.

Since the introduction of headspace analysis, several HS-techniques have been established. They can be divided into three groups: static headspace, dynamic headspace and high concentration capacity technique of headspace.

In **static headspace** (SHS) a liquid or solid sample is placed in a vial so that it occupies only a small part of its volume and is closed immediately. The vial is heated at a constant temperature until a thermodynamic equilibration between volatile components of the matrix and the gas phase is obtained. After that, an aliquot of the vapour phase is taken for further analysis. This technique is very simple in handling but it can only be applied for samples with high concentration.[16]

Dynamic headspace (DHS) is based on a continuous removal of the gas phase in equilibrium with the matrix. That means that a carrier gas is either passed directly over the surface or it goes through the sample stripping the volatile components that are accumulated into a trap that adsorbs and concentrates the volatile compounds.[16] Compared with SHS the DHS technique is more complex and involves longer analysis times, but it can successfully be used when analytes are present in very low amounts.

The third technique, called **high concentration capacity headspace** (HCC-HS) links the other two groups together.[17] It allows reaching a high capacity of enrichment as with the dynamic head space and concentrates the analytes on a polymer, while it remains as simple and reproducible as the technique of static headspace.

Solid phase microextraction belongs to the HCC technique group, its name signifying that the amount of the extraction is very low compared to the volume of the sample. As a consequence, an exhaustive release of analytes into the extracting phase does not occur, rather a thermodynamic equilibration among three phases is obtained. Two of these phases are the same as described in SHS, but additionally a fused silica fibre coated with an accumulating polymer is used as the third phase. This optical fibre is chemically inert and part of a syringe needle assembled on a holder. During sampling the fibre comes out by moving the plunger and is introduced directly into the sample matrix. In case of **headspace solid phase microextraction,** the coated fibre is exposed to headspace of the matrix which can be a gas, solid, semisolid or liquid sample (figure 4). During this time, the volatile analytes pass from the sample matrix through the headspace on the polymer coating to the fibre, where they are accumulated.

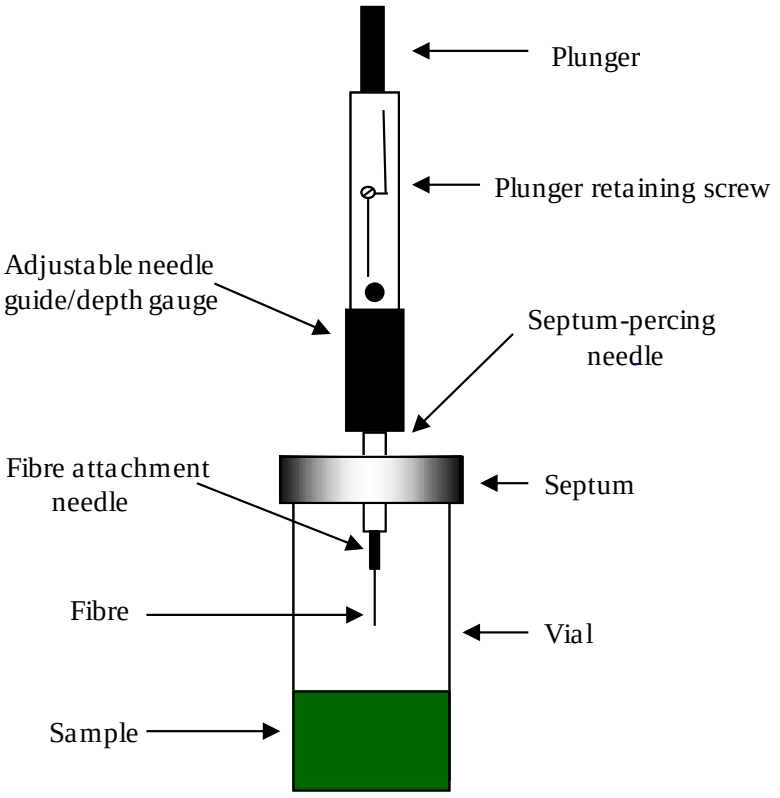


Figure 4: Sampling of HS-SPME

new drawn according to http://ulceet.com/piclib/460.gif

After an appropriate sampling time, the plunger is moved up and transferred to a gas chromatograph injector where the analytes are recovered by thermal desorption. During this desorption the fibre is cleaned as well and ready for a further use afterwards. The target analytes in the gas chromatograph are separated on a column and identified and quantified by means of a detector (figure 5). In addition, SPME techniques can be coupled with GC-MS, HPLC and LC-MS systems as well.[18]

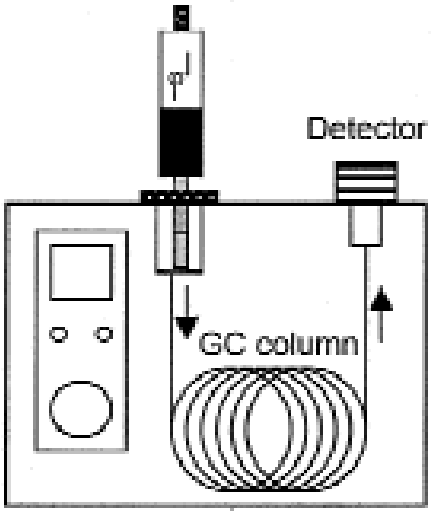


Figure 5: Thermal desorption on GC[18]

To reach a suitable recovery by HS-SPME it is necessary to choose the most effective fibre as well as to develop appropriate sampling conditions. Several factors must be considered to select a suitable fibre, because recovery of the target analytes depends on their polarity and volatility, the physicochemical characteristics of the fibre coating and the affinity between polymeric fibre and analyte. In addition, it depends on the physical state and the composition of the sample matrix as well as on equilibration time and temperature. Another significant factor is the nature of the fibre or rather of its coating.[17]

The most frequently used fibre coatings for HS-SPME are the followings:

- Polydimethylsiloxane (PDMS)
- Polyacrylate (PA)
- Polydimethylsiloxane-Divinylbenzene (PDMS/DVB)
- Carbowax-Divinylbenzene (CW/DVB)
- Carboxen-Polydimethylsiloxane (CAR/PDMS)
- Divinylbenzene-Carboxen-Polydimethylsiloxane (DVB/CAR/PDMS)

The recovery capabilities of PDMS and PA are based on the phenomena of sorption, whereas the others exploit sorption and adsorption to capture target analytes.[17] PDMS is liquid or highly viscous and is available in different film thickness (7μm, 30μm and 100μm). The PDMS coating supports a temperature of 280ºC, it is non-polar and therefore more suitable for apolar compounds and less for polar ones. The PA coating is solid at room temperature and is used to recover polar compounds, for instance phenols, because of its polar nature.

The other fibres consist of two or three different components which extend the range of polarity and efficiency. The characteristic of PDMS/DVB is to be non-polar therefore it absorbs non-polar analytes and uses hydrophobic interactions with lipophilic compounds. The fibre CW/DVB consists of carbowax (polyethylenglycol) and divinylbenzene including insaturations because of the aromatic rings and support π-π interactions with double bonds of analytes, e.g. terpenes. This coating is also very effective to adsorb large compounds, whereas CAR/PDMS, composed of porous carbon, favours recovery of small molecules. The polymer fibre with three phases (1 or 2cm length) supports an operating temperature of 270ºC. The DVB/CAR/PDMS fibre as well as PDMS/DVB and CAR/PDMS are the most effective fibres for sampling monoterpene and sesquiterpene from several plant matrices.[19]

A criterion function, introduced by Zuba, in 2002, can be used for the selection of the suitable fibre coating as well as of the sampling conditions. This function is able to describe the concentration capability of a given fibre within a set of different fibres:[17]

$$F_j = \frac{1}{n} \sum_{i=j}^{n} \frac{H_{ij}}{\frac{1}{k} \sum_{j=1}^{k} H_{ij}} \qquad \text{eq. 1}$$

F_j is the concentration capability factor of the fibre j, H_{ij} is the height of the peak of analyte i, k is the number of fibres and n is the number of marker components characterising the sample matrix. This equation was recently simplified by Hamm:[17]

$$F_{ij} = \frac{\sum_{i} H_{ij}}{\frac{1}{k} \sum_{i,j} H_{ij}} \qquad \text{eq. 2}$$

These two equations allow the expression of a fibre´s sampling capability in a single number, which is very useful for choosing the most effective fibre coating and the best sampling conditions.[17]

In summary, HS-SPME is a solvent free sampling technique which is based on two steps: equilibration between headspace, sample matrix and fibre, and thermal desorption of the concentrated volatile compounds in an analytical instrument. Although volatile components are required, HS-SPME has several advantages compared to other traditional sample preparations: No solvent residuals disturb the interpretation of the chromatograms, sample throughput can be increased, because the desorption and cleanup step is unique and it is fast and easy to automate. Another advantage is that the small size of the fibre makes the extraction technique portable and allows in field sampling of volatile components.[20]

Compared to hydrodistillation, less degradation of components occur because of mild sampling conditions. During hydrodistillation the sample matrix is exposed to hot water for hours, therefore degradation can occur and the behaviour of each component can be different. A significant advantage of HS-SPME is that the sample preparation time can be shortened while keeping high repeatability and reproducibility. However, the sorption of concentrate analytes is limited due to the small surface of the fibre. As a consequence, the two methods of sample preparation under discussion are based on a different approach and thus certain variations in quality and quantity are expected.

Furthermore in HS-SPME, the fibre recovers the volatile components without any contact with the non volatile components of the matrix.[15] Therefore, the sample conditions are nearly non-influent and more complex samples with a high molecular weight such as sludge or soil and hostile samples such as those with high or low pH can be processed as well.[20]

The praxis of HS-SPME, however, has also shown some limits, such as the problematic of quantitative analysis of headspace components, especially of solid samples, because of the difficult construction of a calibration curve. Another disadvantage is that some fibres need longer sampling times than the manufacturers recommend and fibre coatings, in particular carboxen can produce artefacts.[17]

Nevertheless, headspace solid phase microextraction is used in several fields, e.g. to study the composition of medicinal or aromatic plants or to determine toxic impurities in the environment, in the food industry, because it simplifies the monitoring of purity and freshness of flavoured products and helps to optimise harvest time, or in clinical or forensic laboratories, e. g. to monitor drug residues in blood or urine.[15,20]

2.2.3.1. Theoretical aspects of HS-SPME

As previously explained, the HS-SPME technique is based on two steps: equilibration distribution and desorption of volatile components. In particular, there are several factors involved, such as the nature of the matrices (e.g. solid or liquid) or fibre coatings. However, to better understand the theory of the equilibrium it is necessary to consider the three-phase system, i. e. polymeric coating, headspace and sample matrix. As a consequence, the amount of target analyte is related to its distribution in the three-phase system whose total amount during the process is constant:[15] We have

$$C_0V_2 = C_1^{\infty}V_1 + C_2^{\infty}V_2 + C_3^{\infty}V_3 \qquad \text{eq. 3}$$

where

C_0 is the initial concentration of the analyte in the sample matrix,

C_1^{∞}, C_2^{∞} and C_3^{∞} are the concentration of the analyte in the coating, sample matrix and head space during equilibrium,

V_1, V_2 and V_3 are the corresponding volumes of the polymeric coating, the matrix and the headspace.[15]

The polymeric coating/headspace (K_1) and headspace/ matrix (K_2) distribution coefficients are defined as:

$$K_1 = C_1^{\infty} / C_3^{\infty} \qquad \text{eq. 4}$$

and

$$K_2 = C_3^{\infty} / C_2^{\infty} \qquad \text{eq. 5}$$

therefore, the amount of the analyte extracted by the polymeric coating, $n = C_1^{\infty} / V_1$, can be calculated with following equation:[15]

$$n = \frac{C_0 V_1 V_2 K_1 K_2}{K_1 K_2 V_1 + K_2 V_3 + V_2} \qquad \text{eq. 6}$$

As a consequence, the amount of a target analyte extracted by HS-SPME depends on two equilibriums:

- the equilibrium between sample matrix and headspace which is responsible for the headspace composition depends on the conditions of the sample matrix and the volatility of its compounds (measured by its distribution coefficient, K_2,) and
- the equilibrium between polymeric coating and headspace which is responsible for the interactions between target analytes and polymeric coating as well as for diffusion of the volatile analytes (measured by its distribution coefficient, K_1).[15,17]

HS-SPME equilibrium is established when the concentration of the analyte is constant in the three phases and when the concentration in two adjacent phases is equal to their distribution coefficient. Therefore, sampling time is defined as the time needed to the absorbed analyte to obtain 90% of its final concentration. Further, the concentration of the target analyte depends on physical parameters, such as the agitation of the sample matrix, sampling temperature and time. Therefore, the equilibration time for less volatile compounds can be significantly reduced by agitation of the sample matrix and its headspace, an increase of the sampling temperature as well as a reduction of headspace volume. [15,20]

However, when several analytes with different polarities and volatilities have to be simultaneously extracted from a complex matrix, it is difficult to reach the equilibrium of each of them simultaneously. Non-equilibrium sampling conditions are therefore often adopted to obtain a suitable sampling within reasonable times. As a consequence, rigorous and reproducible standard sampling conditions must be applied for consistent results (see 3.1.6).

2.3. The analytical technique of chromatography

2.3.1. Introduction

Chromatography, invented by the Russian botanist Mikhail Tswett at the beginning of the 20th century, is an analytical approach for separation of closely related components of complex mixtures. This principle is based on different partitioning of the components between a stationary and a mobile phase. The stationary phase is immobilised in a column or on a surface. The mobile phase transports the chemical mixture through the stationary phase, thus the components are distributed and because of their different migration finally separated. In general, there are three different categories of chromatography: liquid chromatography (LC), gas chromatography (GC) and supercritical fluid chromatography (SFC).[21]

Gas chromatography is an applied technique for separation, identification and determination of volatile compounds and its applications and improvements have increased since the introduction of the concept of GC by Martin and Synge in 1941. Currently, there are almost a million of gas chromatographs in use throughout the world.[22]

The next chapter describes the equipment and the technique of gas chromatography. In addition, it deals with a description of detectors, in particular the flame ionisation and mass spectrometry detectors and their advantages and disadvantages.

2.3.2. Gas chromatography

There are two types of gas chromatography: gas liquid chromatography (GLC), also called gas chromatography (GC) and gas solid chromatography (GSC). In gas solid chromatography, the stationary phase is a solid, such as silica gel or graphite, in which retention and thus separation occurs because of adsorption of the

volatile compounds. However, this type of chromatography is not often used because of semipermanent retention of polar or active components. In gas liquid chromatography, the stationary phase is a liquid, as the name implies, which is immobilised on the capillary inner surface. The mobile phase in both techniques is a gas which carries the components of a vaporised sample through the stationary phase. This carrier gas has to be chemically inert. Mostly helium but also nitrogen, argon and hydrogen are used as a mobile gas phase. The flow rates of the carrier gas are controlled either by a two-stage pressure regulator or directly at the column by an electronic flowmeter.[21]

A gas chromatograph consists basically of an injector, a column and a detector, as shown in figure 6. The analysis starts when an aliquot of a sample (liquid or gas) is inserted by a micro syringe or a SPME fibre into the inlet port (injector). The injector has two functions, first to vaporise the sample analytes and second to mix the analytes with the mobile phase. The mobile phase sweeps the vaporised analytes into the column which is situated in an oven and contains the stationary phase. Further, the mobile phase carries the mixture of the analytes through the column. Each molecule, depending on its type (volatility, polarity) and on the stationary phase material, is retained for different times. This process is based on adsorption, mass distribution and size exclusion, thus each molecule is separated and comes to the end of the column at different times (retention times). The sample molecules are determined by a detector at the end of the column. The signals of the detector are transmitted either to a recorder or a chromatographic data system which displays the results as a chromatogram.[10,22]

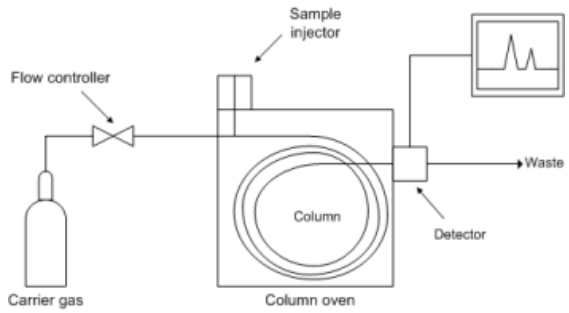


Figure 6: Schematic of a gas chromatograph[23]

To obtain a high level of efficiency in the column the sample has to be an appropriate size and injected as a "plug" of vapour, otherwise band spreading or poor resolution can occur.[21] Therefore, calibrated micro syringes and often fast autosamplers are used to provide reproducibility and time-optimisation. The temperature of the injector is about 50ºC above the boiling point of least vaporised analyte. Further, different modes of injection, depending on the column used can be selected, such as split/splitless, direct vaporisation injection and cold on-column injection.[22]

A split or splitless injector is often needed for capillary columns, because they require very small sample volumes. The carrier gas either sweeps the totality (splitless) in the case of diluted samples or only a part (split) of the sample into the column. Direct vaporisation injection is often used for packed columns. The whole sample is directly vaporised and injected through a septum into the column in a few seconds. Cold on-column injection is an approach for capillary columns where the sample is cold injected as a liquid and is vaporised by a temperature program of the injector or the column. It is useful for thermolabile samples or for separating analytes from the solvent by the thermal effects.[21,22]

As mentioned before, two types of columns are differentiated: open tubular, also called capillary columns and packed columns. Packed columns are 1 to 10m in length and there are inert particles coated with stationary phase inside the column. Capillary columns vary in length from a few meters to 60m and their walls are impregnated of stationary phase. Currently, for most applications, packed columns have been replaced by the more efficient capillary columns. These columns are composed of fused silica tubes which are coated with stationary phase. They are formed as coils and placed in an oven at the right temperature by means of an oven program. This program improves the separation of samples with a broad boiling range by increasing the temperature continuously or in steps.[21,22]

In the case of Fast-GC, several factors of conventional GC are improved to speed up analysis times. One approach is to reduce the length of the column or/and use a column with narrower diameter or a thinner film of stationary phase. For

instance, narrow bore columns can decrease retention times with almost same separation efficiency as in conventional GC. Furthermore, a higher carrier gas flow rate and/or a rapid temperature program can reduce analysis times as well.

At the end of the column, there is always a detector system which produces an electronic signal of the eluate. This signal depends on the time since injection (retention time) and is shown as a 2D graph, called chromatogram by means of data system software.

There are several detectors available, each with its own characteristics. Some are universal, meaning that they are sensitive to nearly all separated components, however, most detectors are more sensitive only to selected components. In addition, GC can be coupled with spectroscopic instruments, for example infrared spectrometers (IR) or mass spectrometers (MS).[21]

An ideal detector system should have the following characteristics:[21]

- Sufficient sensitivity
- Reproducibility and stability
- Reliability and easy handling
- High range of temperature
- Large linear response range
- Short response time
- Non-destructive
- Similar response of all analytes

Unfortunately, there is no detector which possesses all of these traits at once. Each one has its own advantages and disadvantages and the application depends on the purpose of the analysis. The most common detectors are, the flame ionization detector (FID), the thermal conductivity detector (TCD), the electron capture detector (ECD), the nitrogen phosphorus detector (NPO), the flame photometric detector (FPD), the mass spectrometer (MS) and, to a lesser extent, the Fourier transform infrared spectrometer (FTIR).[21,22] This thesis work focuses mainly the evaluation of the response given by FID and MS detectors.

2.3.2.1. GC-Flame ionisation detector

Organic compounds such as food, cosmetics, pharmaceuticals, volatile pollutants and volatile components of plants include a major content of carbon. The detection process of flame ionisation requires flammable components, therefore GC/FID is one of the most commonly applied methods for analysing organic and biochemical compounds. As the name flame ionisation detector implies, the detection is based on ions which are produced by organic compounds via a hydrogen/air flame. In particular, the effluent from the gas chromatograph column is carried into this small hydrogen/air flame which burns at a high temperature and pyrolyses the organic compounds. As a result, positively and negatively charged ions are produced and generate a current between two electrodes. The positive electrode is the burner tip and the negative electrode is a tubular collector located above the flame. Finally, the resulting current (~10^{-12} A) is collected, amplified and measured by a picoamperemeter.[21,22] Schematic of FID is shown in figure 7.

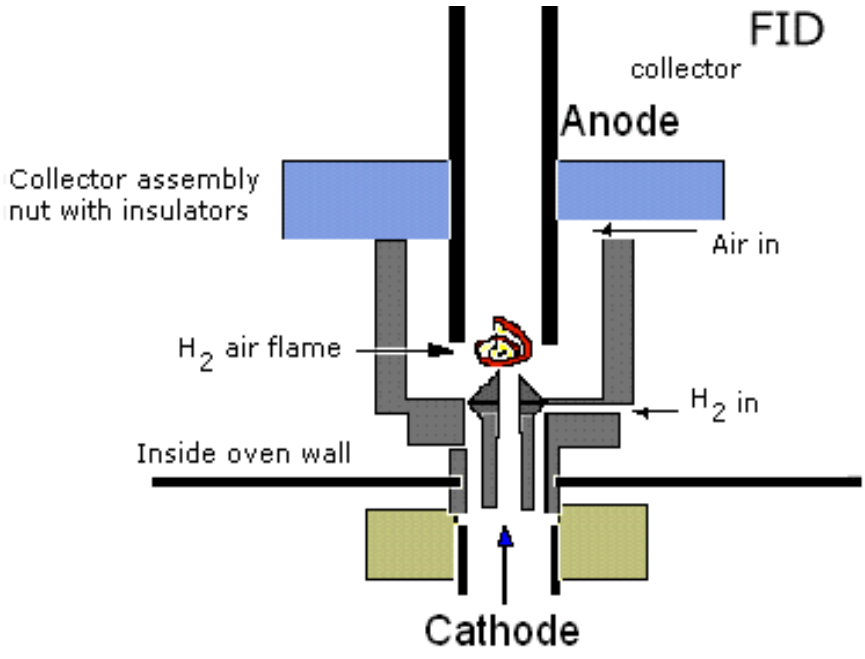


Figure 7: Schematic of a flame ionisation detector[24]

The ionisation process of carbon components in FID is still not completely clear, however, the number of generated ions is approximately proportional to the number of carbon atoms in the flame. Therefore the signal depends on the immediate mass flow and changes in the flow rate of the mobile phase have almost no effects on how the detector responds.[21]

In summary, the flame ionisation detector is prior sensitive to hydrocarbons, however, insensitive to H_2O, SO_2, CO_2, CO, NO_2 and noble gases, since they can not be ionised by the flame. For that reason samples can be analysed even if they are contaminated by these non-combustible gases. Other advantages are that FID has a high sensitivity (~10^{-13}g/s), it is a rugged device and easy to handle. However, a significant disadvantage is that FID destroys the components, therefore they cannot be used for other measurements afterwards and must always be the last detector in case of multiple-detector-analysis-systems. Furthermore, it is not a diagnostic detector for determining the matrix qualitative composition. Therefore, authentic standards and/or retention indices must be used to identify and confirm the detected components.[21,22]

2.3.2.2. GC-Mass spectrometry

Mass spectrometry coupled with gas chromatography is one of the most powerful tools to separate, identify and quantify biochemical, organic or sometimes inorganic compounds in a mixture. The principle is based on the measurement of the ratio of the mass to the charges of ions (m/z) in the gas phase. This ratio is declared in atomic mass units (amu) or in daltons (Da). In general, GC-MS consists of three main parts (except for the GC device described in section 2.3.2): **Ion source**, **analyser** and **detector**. All these elements are placed in a high vacuum chamber.[21,22]

In GC/MS, a capillary column is directly introduced into the ionisation chamber. The separated components from the end of the column are directly fed into the ion source where they are converted into ionised ones by collision with electrons, ions, photons and molecules. After the component fragmentation, ions are focused by

several electronic lenses and accelerated as well. The analyser separates the ions according to their mass/charge ratio (m/z). After filtration, the ions conclude their path in a detector where the electrical charges are measured.[21,22] The scheme of a gas chromatography mass spectrometry is shown in figure 8 and an example for an instrumental setup is presented in section 3.1.7.

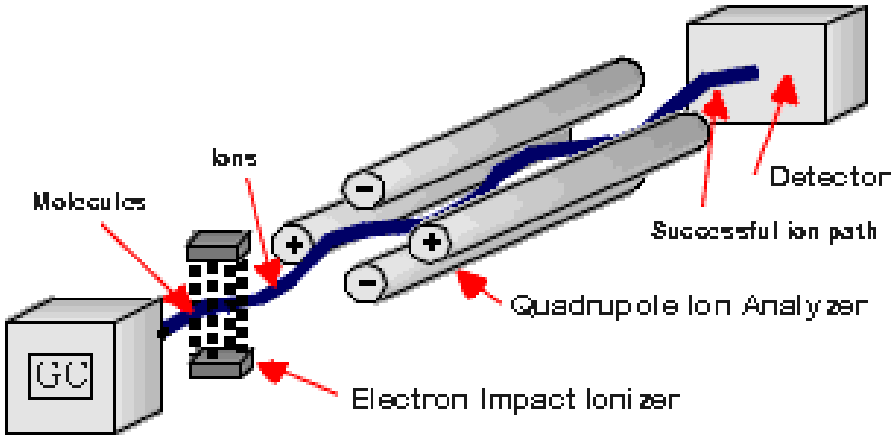


Figure 8: Scheme of GC/MS[25]

The first step of the eluate of GC in a MS-device is **ionisation**, therefore several methods are available: Electron impact (EI), chemical ionisation (CI), fast-atom bombardment (FAB), Matrix-assisted laser desorption ionisation (MALDI), electrospray, atmospheric pressure chemical ionisation (APCI) and thermospray.[10]

The electron impact is the most widely used technique for the analysis of volatile organic compounds. The ionisation process occurs by a beam of electrons which collide on the sample components with an energy of 70eV. The following reaction describes the process:

$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

where M is the sample molecule, e^- is the electron and $M^{+\bullet}$ is the resulting ion.[21]

After ionisation of each sample component, the ions are separated with an **analyser** such as a quadrupole, ion trap or time of flight analyser. An ion-trap analyser is based on the same principle as quadrupoles but with a three dimensions electric field. Whereas the time of flight analyser measures the time which each mass needs to travel through a field free fixed pathway. That time is proportional to the square root of the m/z ratio. The most often applied analysers coupled with GC are the quadrupoles. They have been developed because they are more compact, rugged and less expensive than other types of mass spectrometry; furthermore, they achieve very high scan rates.[21] The heart of a quadrupole consists of four parallel hyperbolic or cyclindrical rods. They are arranged in opposite pairs and each pair is connected electrically. A potential, which is a result of a constant component and an alternating component, is applied across these pairs. The ions of the source are transmitted into the space between the rods. Their separation occurs by increasing the voltages applied to the rods while the ratio of continuous voltage to alternating voltage keeps constant.[10] In addition, only ions with a certain value of m/z can reach the ion transducer.

For the **detection** of the separated ions several transducers can be applied, however, the electron multipliers are mostly selected. There are two types of electron multipliers, discrete-dynode electron multiplier and continuous-dynode electron multiplier. Both collect the separated ions and convert them into an electrical signal by the means of an increasing voltage applied to a resistive conductive surface. Other detectors of mass spectrometry are the Faraday cup and array transducers such as microchannel plates.[21]

The signal acquisition of the ion abundances can be achieved in two different modes:

- the continuous spectrum which considers an interval of masses or a selected mass. In this spectrum, ions are displayed as peaks of different abundances, depending on the particular instrument. High performance devices can discover the masses with a accuracy about 10 parts in a million (10^{-5} Da); and

- the fragmentation spectrum. It is generated by summing up the intensities of ion masses which are expressed as a percentage of the base peak (the most intense peak). Therefore, these intensities represent a nominal mass which is closest to the exact mass of the determined ions.[22]

Furthermore, mass spectrometry is a very suitable method for identification of compounds. The structure of molecules can be reconstructed from the ion fragmentation pattern as a fingerprint of the specific compound. Either a library of mass spectra or retention indices can be used for the identification of each compound detected by MS.

In summary, although GC/MS requires a high vacuum and purchase and preservation are very expensive, it has been applied for the identification of thousands of components which occur in natural systems. In particular, MS plays an important role for the characterisation of flavour components of food and studies of plants. The reason is its special ability to separate signals from noisy background or signals of coeluated compounds when different specific ions are available.

3. EXPERIMENTAL PART

3.1. Materials and Methods

3.1.1. Introduction

The preparation of samples and the development of method conditions are very important and usually the longest stage of the analytical process. In order to obtain repeatable and reproducible results, it is necessary to standardise the preparation. For that reason a sample preparation was developed from the very beginning of the *Juniperus communis* L. plant as matrix. Also several HS-SPME conditions were tested and compared with the essential oil preparation, in order to apply a sample preparation easy to automatise. Finally, the method conditions of HS-SPME were optimised to be coupled with FastGC. Because of these improvements, the whole analytical process was speeded-up, which was particularly useful for analysing a large number of samples.[26]

The next chapter describes the preparation of the samples, conditions and methods which were used for the analyses.

3.1.2. The samples

25 samples of *Juniperus communis* L. were provided by Prof. J. Karlsen, Oslo (Norway). They were collected in the year 2008 from different bushes growing at several altitudes in the Lillehammer National Park. Table 1 reports details for names of the samples and altitudes of the collecting places.

Table 1

Samples	Altitude in meter
N1-1 to N1-5	1400
N2-1 to N2-5	1100
N3-1 to N3-5	900
N4-1 to N4-5	sea level
N5-1 to N5-5	600

3.1.3. Preparation of the samples

The morphological structure was first described to get an impression of the phenotypic differences. Then the needles were separated from twigs and berries and finally the needles were crushed three times for 4 seconds with a coffee grinder. Every sample was conserved in a vial in the refrigerator between 4-5 ºC.

3.1.4. Hydrodistillation of the essential oil

For the preparation of the essential oil a special microapparatus, developed in the phytochemical analysis laboratory[27], was used. The apparatus comprised a distillation flask, an overflow pipe, a condenser, a pear-shaped swelling where the distillate was collected and a heater (see figure 9).

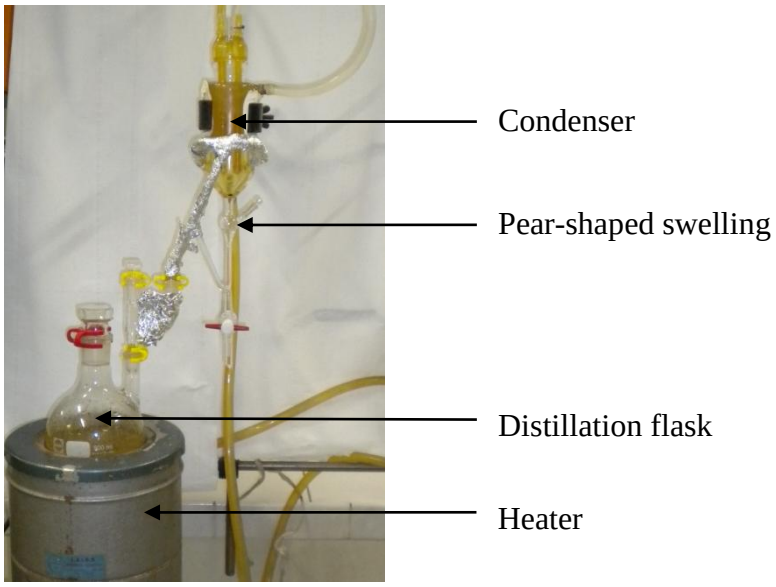


Figure 9: The apparatus for the distillation

Before distillation, 1.0g of a sample was weighted into the flask and 200ml of distilled water were added. This mixture was macerated for 30 minutes. After distillation, in agreement with the *Pharmacopoea Europea*[10], 300µl of cyclohexane were introduced into the pear-shaped swelling to collect the essential oil. For the analysis of FastGC/FID a concentration of 5µg/µl of the essential oil was prepared.

3.1.5. Preparation of the samples for HS-SPME

For each sample 60 mg of milled needles were exactly weighted in a 20 ml vial. 5µl of dodecane at a concentration of 2mg/ml was added to the wall of the vial as internal standard. The vials were closed with a silicone septum and an aluminium screw top. Two vials of the same sample were always prepared.

3.1.6. Method conditions of HS-SPME

Each sample was equilibrated for 5 minutes at 50 ºC. The SPME device was then introduced into the vial either manually for FastGC/FID or automatically for FastGC/MS and the fibre was exposed to the headspace of the matrix for 10 minutes at 50ºC. After sampling the fibre was withdrawn into the SPME device and transferred to the GC-injector, from where the analytes were thermally desorbed for 5 minutes at 250ºC. The development and the selection of the sampling time are reported elsewhere.[26] The fibre core was consisted of DVB/CAR/PDMS (Divenylbenzene / Carboxen / Polydimethylsiloxane) with the thickness of 50/30μm and the length of 1cm. For FastGC a fibre of 1cm was necessary in order to avoid the overloading of the narrow bore column used for the chromatography separation.

3.1.7. Chromatographic conditions

All investigated samples were analysed by both FastGC/FID and FastGC/MS and a method translation software was used to transform the analytical conditions of Conventional-GC (C-GC) into FastGC. This software package allowed to translate the original optimised analytical method to a true real method monitoring the same separations.[28,29]

The analyses were carried out on GC-2010 SHIMADZU equipped with a FID or a MS detector. The instrument of FastGC/MS was coupled with a COMBI PAL autosampler with HS-SPME option, whereas the HS-SPME of FastGC/FID was run manually. The same chromatographic conditions were applied for FastGC/FID and FastGC/MS. See details of the instrumental setup on table 2 (on the next page).

Table 2: Instrumental setup

FAST GC/FID and FAST GC/MS	
Carrier Gas	Helium
Flow Rate	0,8 ml/min
Column Type	SE52 10m; 0,1mm ID; 0,1µm d_f
Injector Temperatur	250°C
Injector Mode	Split
Split Ratio	100
Oven Program	40°C(0.78min)-19,22°C/min-250°C(0,78min)
FID Temperatur	270°C
MS Transfer Line Temperatur	270°C
MS Ionisation Mode	Electron Impact, Ionization 70 eV

3.2. Results and Discussion

3.2.1. Introduction

In a routine laboratory, time, costs and equipment are unfortunately important factors. In theory, reducing the analytical times should increase the sample throughput and reduce the costs of analysis. In addition, obtaining results of the highest quality with less equipment would mean more productivity of the laboratory.

The next chapter deals with the results of analysis and characterisation of the 25 *Juniperus communis* L. samples. Furthermore, it presents the results of hydrodistillation compared to those of HS-SPME focussing on the time saved. It continues reporting the results obtained by FastGC/FID compared to those by FastGC/MS.

3.2.2. The samples

The main differences of results of the analysis carried out by Conventional-GC/MS, FastGC/MS and FastGC/FID of the 25 *Juniperus communis* L. samples, involve the area percentages of two characteristic compounds, α-pinene and sabinene. Whereas the other components cannot be easily distinguished, because of their low quantity.[30]

Figure 10 and figure 11 demonstrate the differences in abundance of α-pinene and sabinene compared to terpinen-4-ol and α-cubebene.

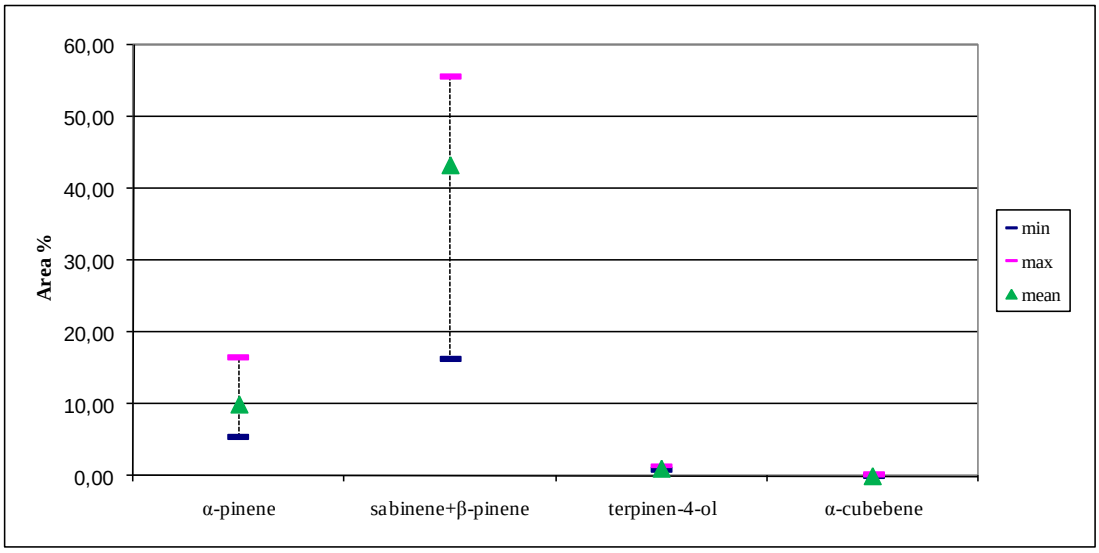


Figure 10 presents the abundance of 4 compounds of 5 samples collected at 1400 m (N1_1 to 5)

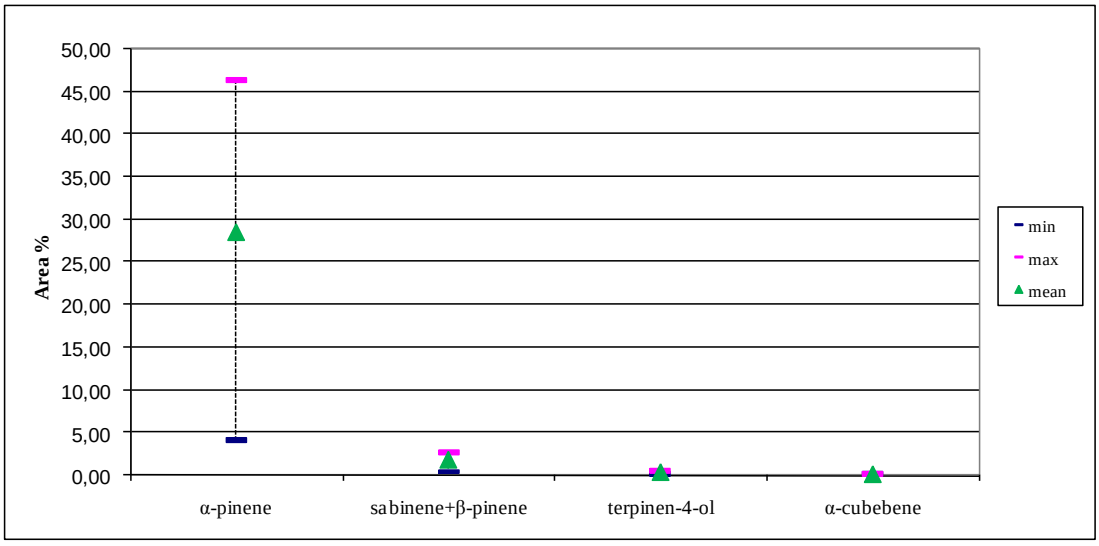


Figure 11 shows the abundance of 4 compounds of 5 samples collected at sea level (N4_1 to 5)

Because of the abundance of α-pinene and sabinene, the 25 samples of *Juniperus communis* L. can be divided into two different "chemotypes", the α-pinene type and the sabinene type. During the study sabinene was co-eluted with β-pinene, but the quantity of β-pinene was always below 3%, not interfering with the chemical type assignment.

In addition, the results reveal that the distribution of the supposed "chemotypes" is independent on the altitude to which they have been harvested. Some samples of series N1, collected at 1400 meters or of series N2 at 1100 meters, or else of N3, collected at 900 meters have been found to be outliers, as it appears in figure 12.

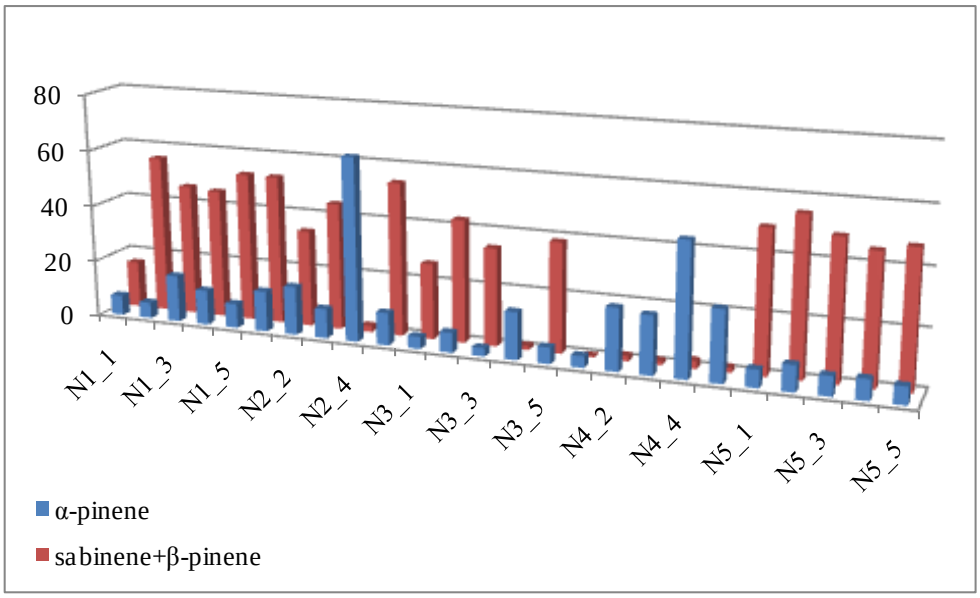


Figure 12 shows the area percentage of α-pinene and sabinene of the 25 samples analysed with FastGC/FID.

3.2.3. HS-SPME vs. Hydrodistillation

HS-SPME as well as hydrodistillation is a technique applied to extract the volatile fraction of plant matrices, but the basic principles of both are completely different. As reported before, in chapter 3.1.4 and 3.1.6, the two approaches are also based on different conditions and procedures. During the hydrodistillation the vegetable matrices are under the influence of hot water for two hours. That means that some compounds, such as sesquiterpenes, are better recovered than by HS-SPME. Therefore it is more interesting to compare their results.

In the context of the investigations, 22 marker components are chosen to illustrate the results. Table 3 compares the area percentage of each marker between the essential oil and the headspace sampled by SPME markers of a sample taken as a reference. The abundances of the essential oil are very similar to those of same sample extracted by HS-SPME and the ratio among the compounds remains nearly the same.

Table 3: Results of sample N1-1 analysed with FastGC/FID

COMPOUND	RETENTION TIME	AREA % OIL	AREA % HS-SPME
tricyclene	2.44	0.02	0.04
α-thujene	2.49	0.40	0.72
α-pinene	2.56	14.02	25.21
camphene + α-fenchene	2.67	0.11	0.16
sabinene + β-pinene	2.90	10.51	17.53
β-myrcene	3.04	2.33	4.55
Δ-2-carene	3.11	0.18	0.33
α-phellandrene	3.15	2.16	2.13
Δ-3-carene	3.19	0.79	1.39
α-terpinene	3.25	0.14	0.15
limonene + βphellandrene	3.37	16.82	26.29
γ-terpinene	3.60	0.40	0.42
α-terpinolene	3.84	1.11	1.88
trans-sabinenene hydrate	3.93	0.10	0.06
terpinen-4-ol	4.56	0.77	0.11
bornyl acetate	5.38	0.23	0.17
α-cubebene	5.83	0.04	0.04
β-elemene	6.13	0.45	1.09
γ-muurolene	6.71	0.12	0.03
germacrene D	6.77	17.50	9.16
bicyclogermacrene	6.85	3.74	1.74
germacren D-4-ol	7.36	4.11	0.32

Even if some deviations of the area percentage of the same compounds can be recognized in table 3, the relative abundance of the compounds within a sample is the same, as it appears from the following three figures.

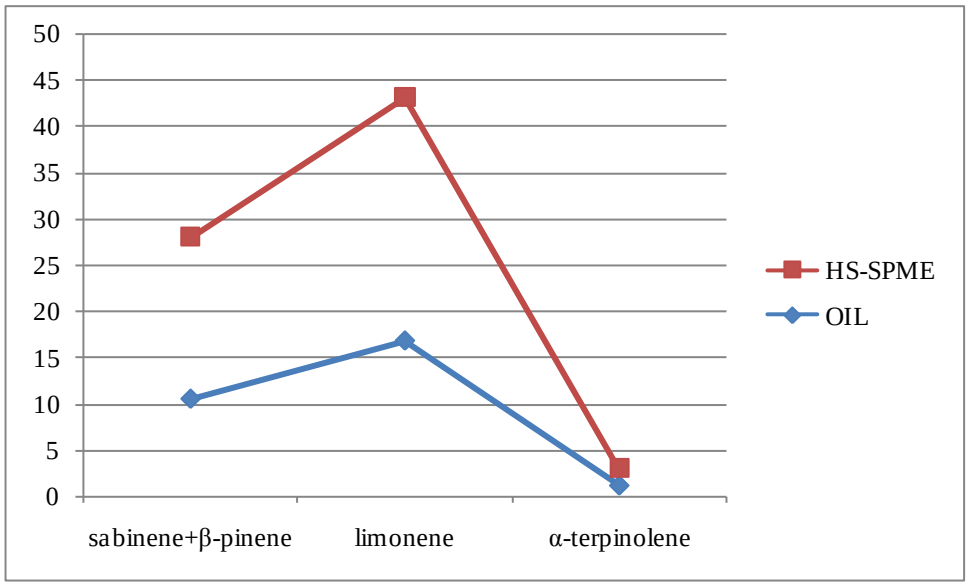


Figure 13: Area percentage of three components of the sample N1_1

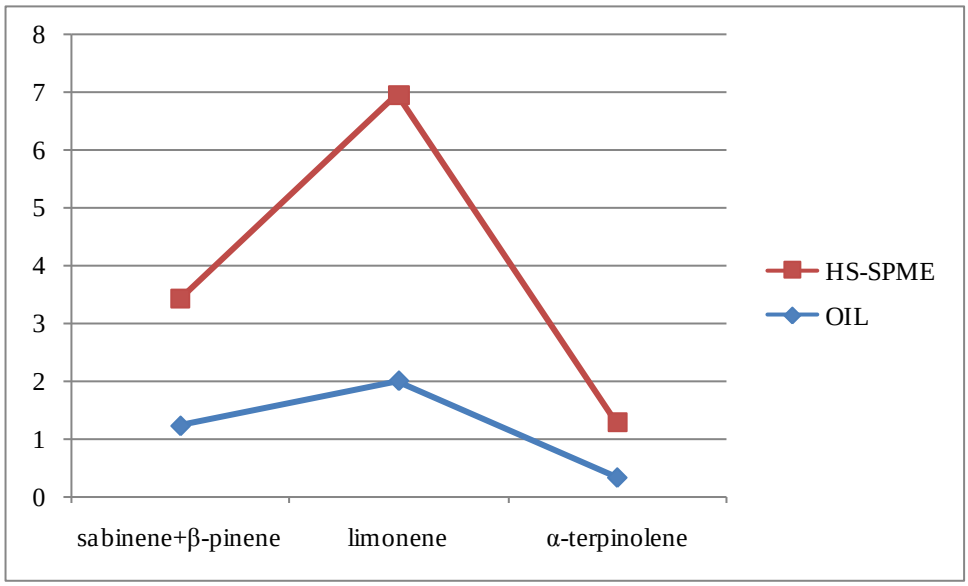


Figure 14: Abundance of three components of the sample N2_4

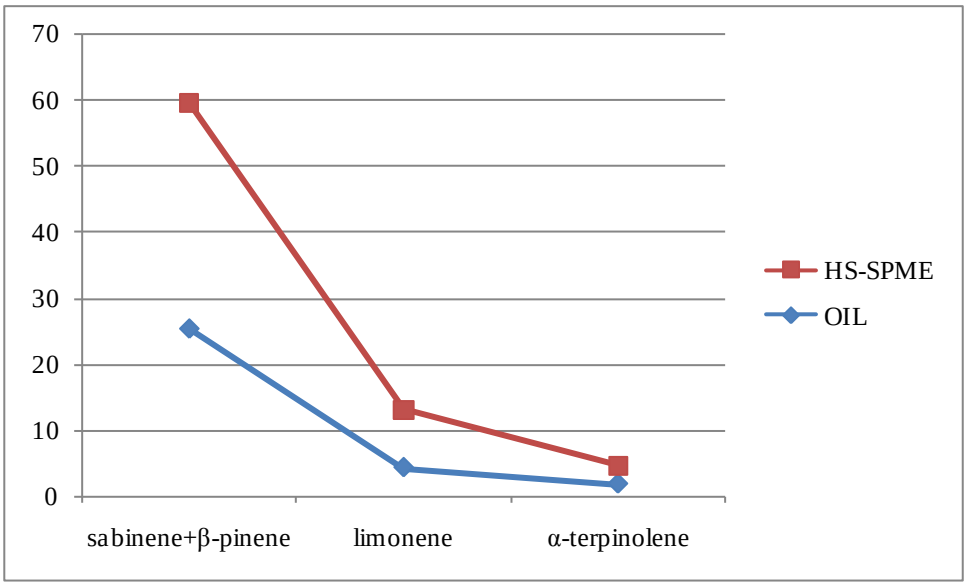


Figure 15: Area percentage of three components of the sample N3_3

Figures 13-15 demonstrate also that it does not make a difference with the relative ratios of minor or major components. In addition, they make it clearer that the two approaches are not comparable for quantitation although they are quite comparable, if the different behaviour of the compounds is considered. These results are confirmed by the chromatograms of the several *Juniperus communis* L. samples. Each profile of an essential oil corresponds to that obtained by HS-SPME, as shown on figures 16-18. They present the profiles of three different samples of *Juniperus communis* L. analysed with FastGC/FID.

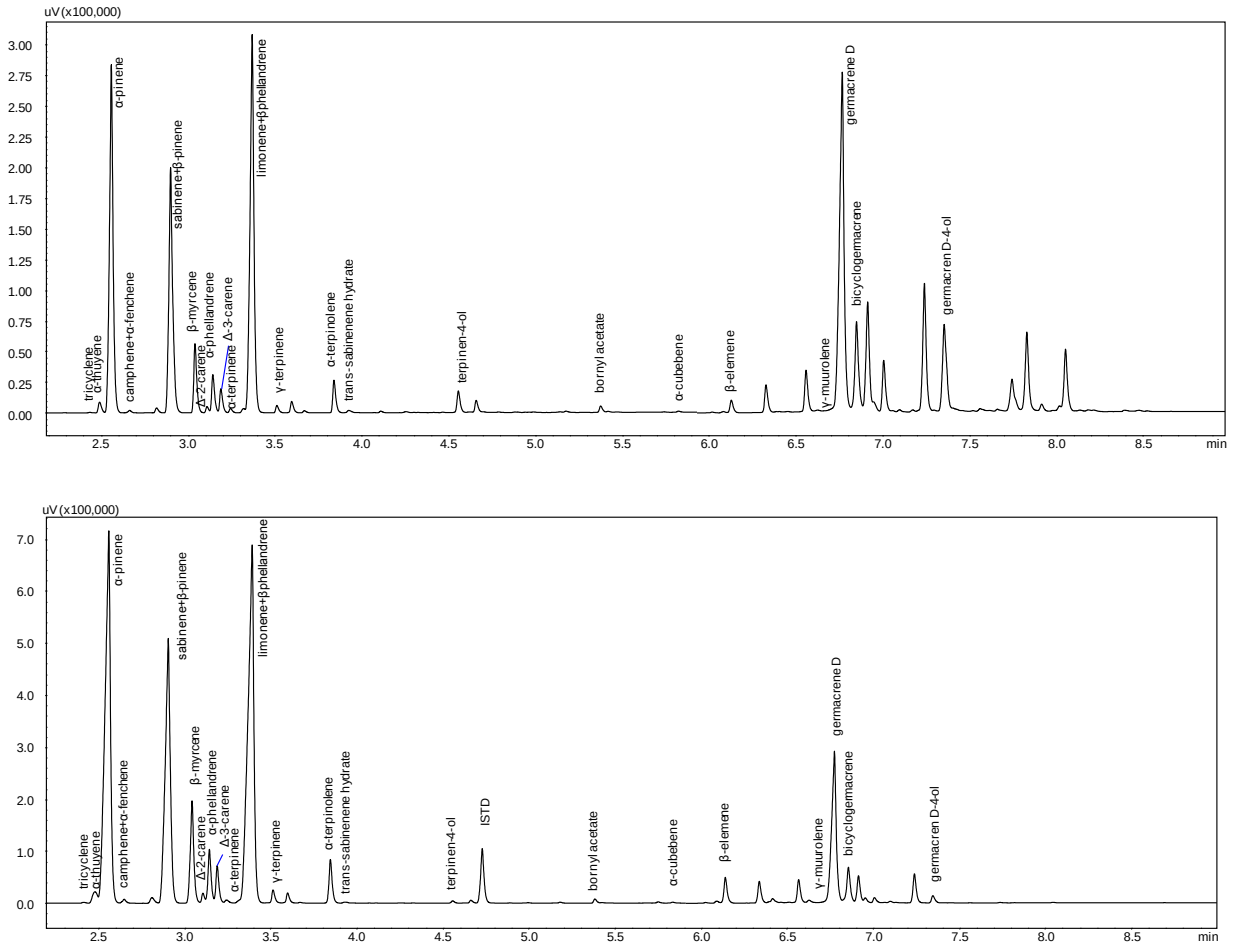


Figure 16 presents profiles of sample N1_1; “intermediate type”; on top an essential oil and below the same sample extracted by HS-SPME

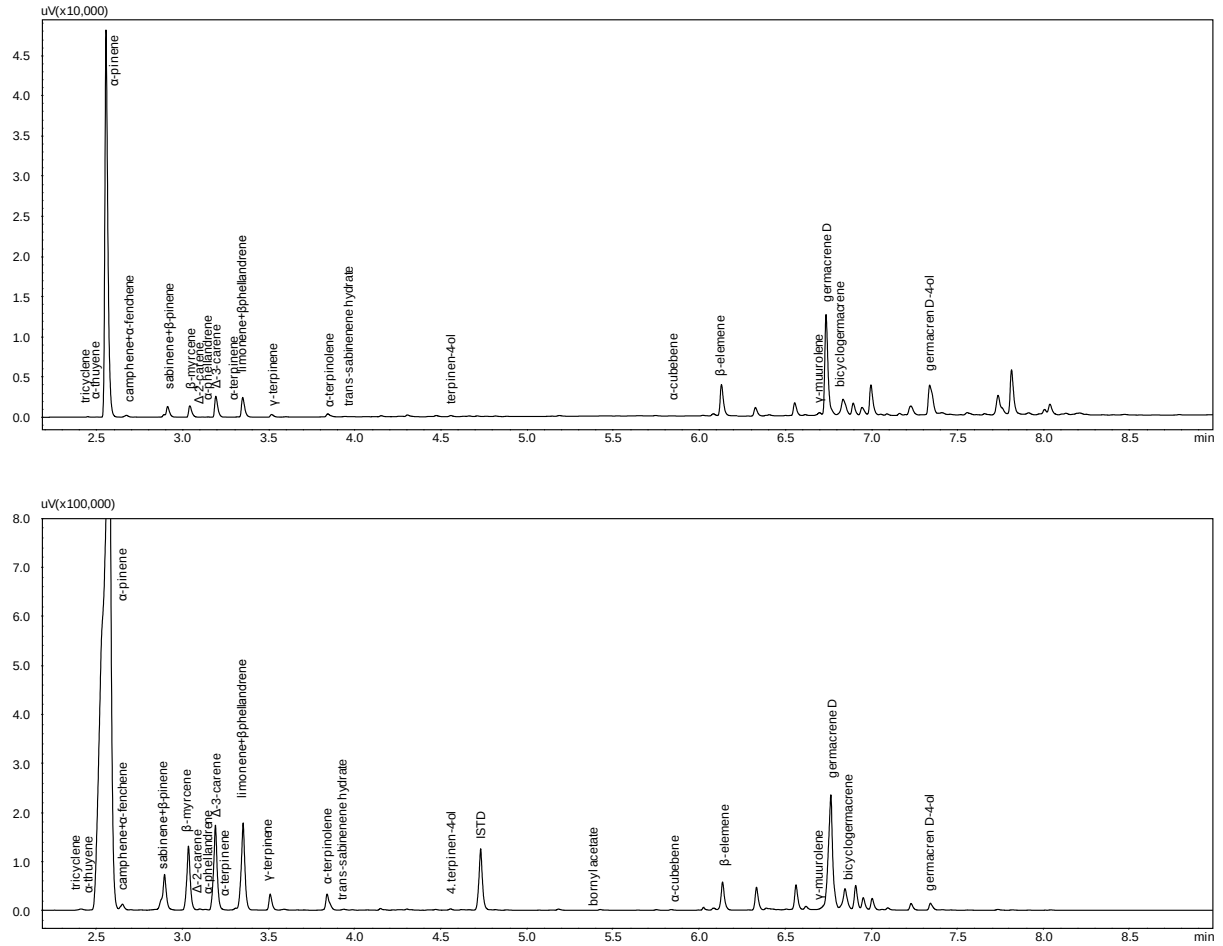


Figure 17 shows profiles of sample N2_4; "*α*-pinene type"; above an essential oil and below the same sample analysed by HS-SPM

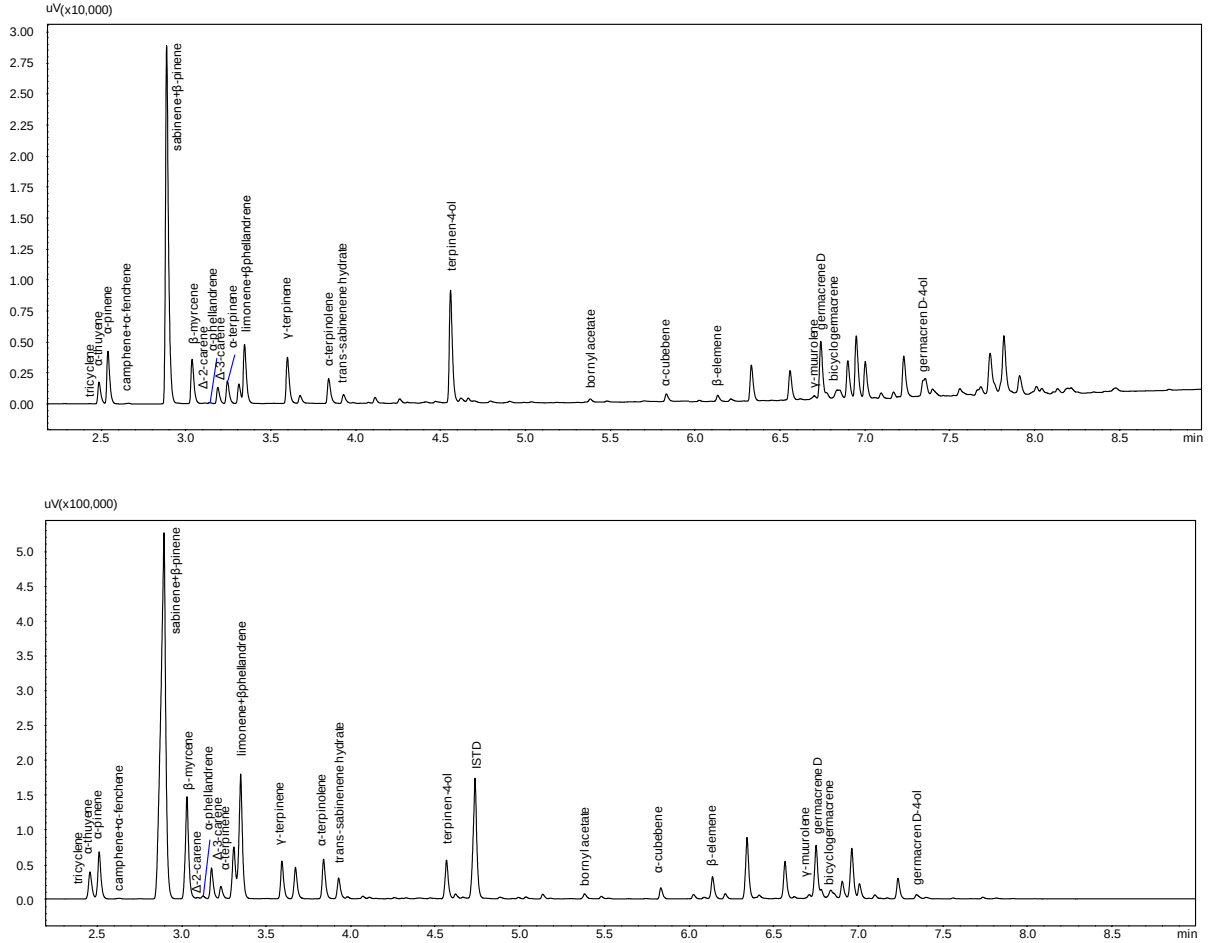


Figure 18 presents two profiles of sample N3_3; “sabinene type”; on top the essential oil and above the same sample extracted by HS-SPME

The profiles of each chemical type are reported on figures 16 to 18. In particular, figure 16 shows an "intermediate chemical type" which is characterised by the same area percentage of α-pinene and sabinene. Within the 25 samples, which were collected in 2008, this type was handled like an outlier. But newest analyses of *Juniperus communis* L. samples collected in 2009 and carried out on FastGC/MS provide the third "chemotype" which is the "intermediate type".

3.2.3.1. Comparison of the analysis times

As previously explained, speeding up the analysis time is as important as the quality of analysis. Hydrodistillation even when coupled with FastGC results in a method that is still time consuming. For this reason, it would be useful to adopt an equally effective sample preparation method which is quicker than hydrodistillation. Table 4 presents an overview of the times needed to prepare and to analyse a sample of *Juniperus communis* L., also compared to the conventional method according to the *Pharmacopoea Europea*[10].

Table 4: Comparison of total analysis time of processing one sample

	Hydrodistillation + C-GC	Hydrodistillation + FastGC	HS-SPME + FastGC
Preparation of one sample	30 min maceration	30 min maceration	5 min equilibrium
	120 min distillation	120 min distillation	10 min adsorption
GC/FID Analysis	58 min	12 min	12 min
Total	208 min	152 min	27 min

These times enable analyses of maximum three samples per day by hydrodistillation with GC/FID, since one sample takes more than 4 hours, considering the cleaning time of the apparatus as well. The replacement of C-GC with FastGC alone does not make a difference, because during the GC process the next sample can be prepared. However, if hydrodistillation can be replaced alternatively with the HS-SPME, as the results revealed, the speed of the analytical step can be increased dramatically. As a consequence, a larger number of samples can be screened.

3.2.4. Fast-GC/FID vs. Fast-GC/MS

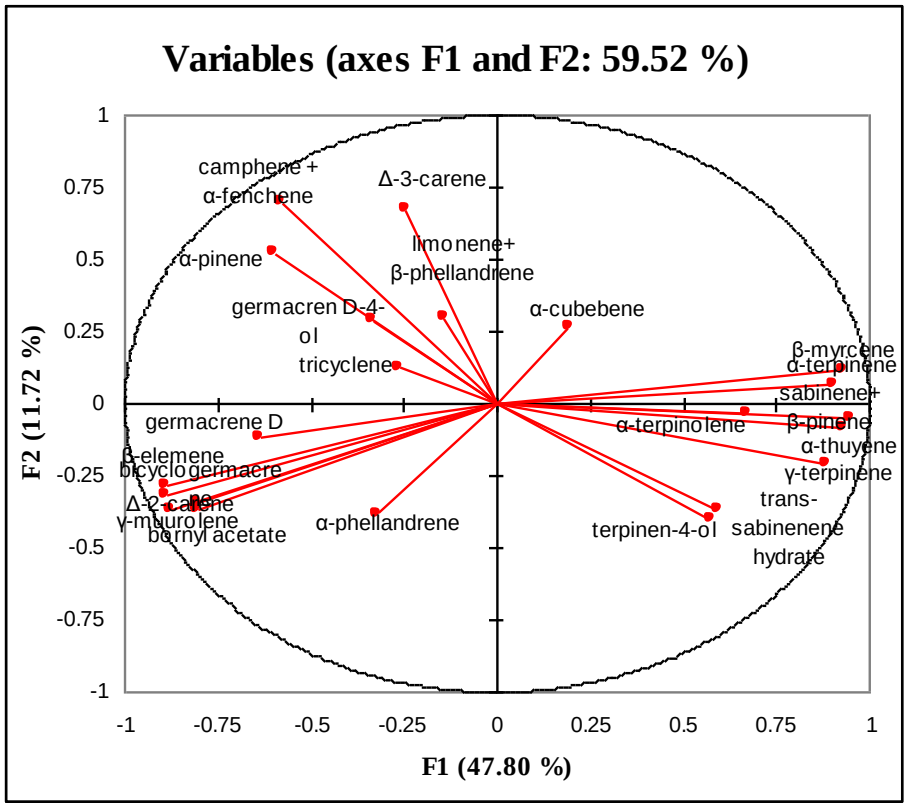
As explained in chapter 2.3., the detection mechanism of both, FID and MS, are completely different. In order to demonstrate the response of both detectors the data sets of the HS-SPME analyses were submitted and the principal component analysis (PCA) and the ratio of the absolute areas were calculated.

3.2.4.1. Statistical elaborations

XLSTAT 2009 software was used for the statistical elaborations of principal component analysis (PCA) to compare the results between FastGC/FID and FastGC/MS. It was based on the 22 marker compounds as variables and 25 samples as objects. The PCA applied to the FastGC/FID results (figure 19) represents a total variability of 59% and to the FastGC/MS results (figure 20) 60%, meaning that although the two detectors work differently, the variability of the samples is the same.

The statistical elaboration of the results of both detectors reveals a positive correlation among α-pinene, tricyclene, camphene and Δ-3-carene, while α-thujene, sabinene, β-myrcene, α-terpinolene and trans-sabinene-hydrate are negatively correlated. The reason for the opposite correlation between α-pinene and sabinene may be the different biosynthetic pathway.

Figure 19 and figure 20 show that the location of the samples as well as the correlation among them is quite similar. Both figures display three groups of samples. The first group includes the series N4 together with two other samples, N2_4 and N3_4. All these samples reveal a high content of α-pinene. The samples N2_2 and N3_1 are in the second group while the third group contains the residual samples, which are all belonging to sabinene type.



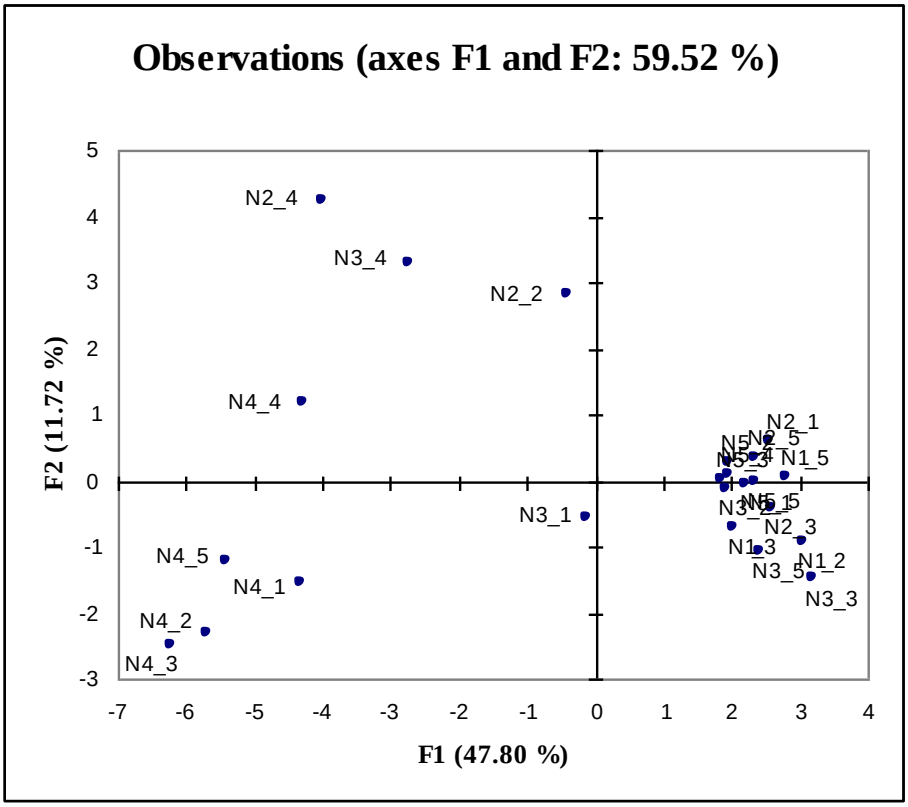
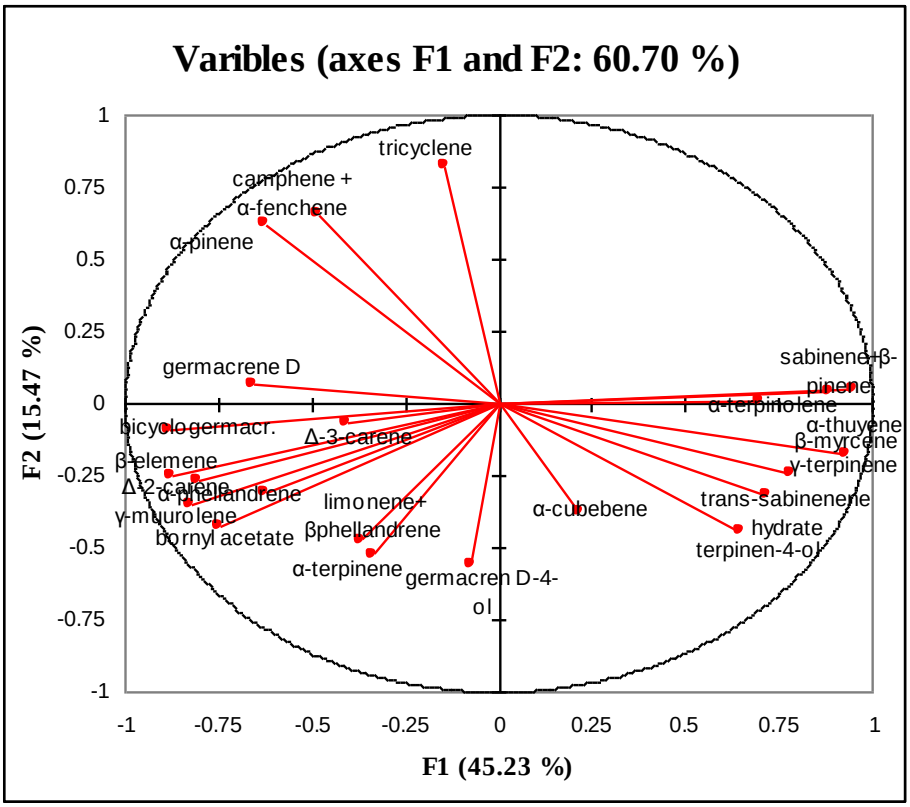


Figure 19: Score plot and loadings of the samples analysed with FastGC/FID



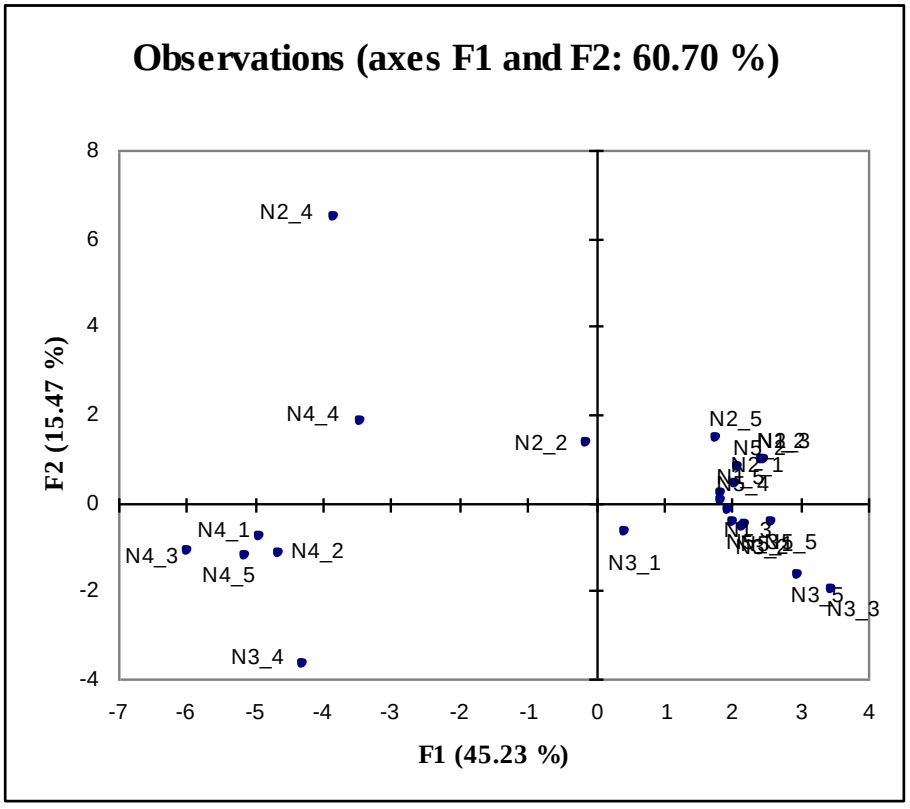


Figure 20: Score plot and loadings of the samples analysed with FastGC/MS

The only difference between the two plots of figure 19 and 20 is the position of the sample N3_4. That is because the sample N3_4 on the third principal component shows a high value for α-cubebene, limonene and β-phellandrene in both detectors (see figures 21).

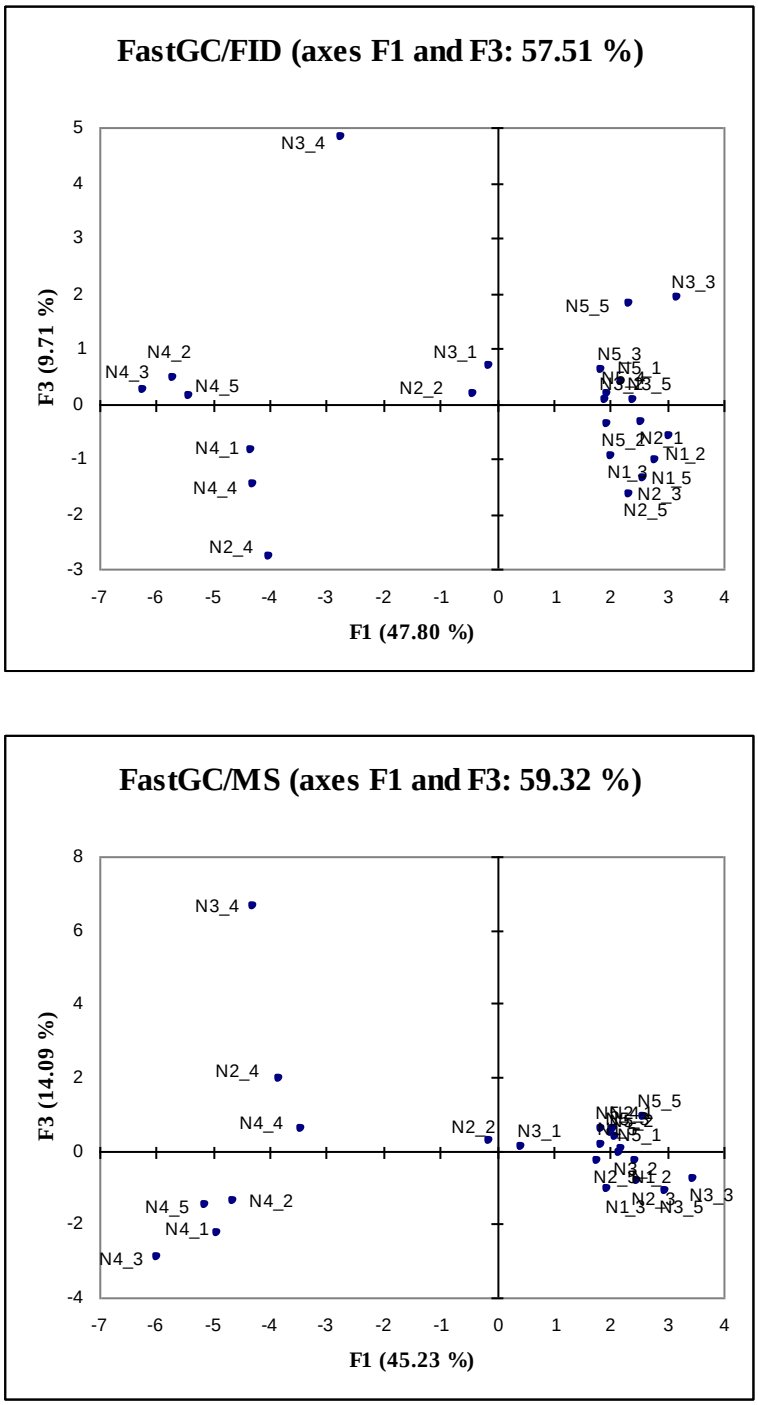


Figure 21 shows the scores of axes F3 versus F1

The PCA shows the relation among the samples and gives an overview for the comparison between the results obtained by FastGC/FID or FastGC/MS. The next table of ratio between the absolute area of FastGC/FID and FastGC/MS is even more detailed. It represents the ratio of the series N5 samples as an example of all analysed Juniper samples.

Table 5 shows the ratio between absolute area FastGC/MS and absolute area FastGC/FID

COMPOUND	**N5_1**	**N5_2**	**N5_3**	**N5_4**	**N5_5**
tricyclene	16.0	11.7	11.0	11.9	15.3
α-thujene	9.1	9.3	10.1	8.4	11.8
α-pinene	8.9	8.7	10.1	9.1	11.5
camphene + α-fenchene	9.6	11.7	10.9	11.1	14.0
sabinene + β-pinene	8.6	8.4	9.4	8.9	10.9
β-myrcene	11.1	11.0	11.9	12.3	13.4
Δ-2-carene	20.1	21.8	20.9	19.6	59.8
α-phellandrene	6.3	6.2	6.8	4.7	12.5
Δ-3-carene	10.4	10.4	11.0	11.6	12.9
α-terpinene	5.2	4.9	5.7	4.0	8.2
limonene + βphellandrene	9.0	8.8	9.4	9.3	11.0
γ-terpinene	6.2	5.6	6.4	5.0	9.4
α-terpinolene	9.1	8.8	9.6	10.4	11.2
trans-sabinenene hydrate	10.5	8.5	10.0	9.5	11.2
terpinen-4-ol	9.8	8.9	9.4	10.4	11.1
bornyl acetate	6.7	7.6	9.2	9.1	9.7
α-cubebene	9.5	8.4	9.3	9.9	9.8
β-elemene	7.9	7.0	7.9	5.2	8.5
γ-muurolene	7.4	7.0	7.9	7.4	3.9
germacrene D	7.4	6.8	7.6	8.4	9.3
bicyclogermacrene	10.6	8.9	10.6	11.9	12.5
germacren D-4-ol	6.3	7.2	10.1	11.1	7.7
ISTD C12	9.8	9.8	9.4	7.8	11.8

Table 5 shows that the absolute areas of FastGC/MS are higher than those by FastGC/FID in nearly all samples. This observation is also confirmed by results of the internal standard. As a consequence the ratio reveals the different sensibilities because of their different detection modes, however, the ratio of the absolute area is always nearly equal as well.

In summary, all these results show that even if the response of the two detectors is different in absolute quantitative terms, because of the different mechanism, the results are very similar in relative terms. This means that these two detectors can be used alternatively for the qualitative screening of a large number of samples, as usual in a quality control laboratory.

4. CONCLUSION

In this thesis, *Juniperus communis* L. samples were analysed with different preparation and analytical techniques. A comparison of two sample preparation techniques, namely HS-SPME and hydrodistillation, is performed to determine their differences and to present the possibility to speed-up the analysis times. In addition, gas chromatography analyses either with flame ionisation detector or mass spectrometry are applied for the investigation of the samples to compare their different analytical abilities.

Thus the results of the present thesis may be concluded by following remarks:

- the analytical approaches allows to distinguish samples of *Juniperus communis* L. in three different "chemotypes": α-pinene type, sabinene type and intermediate type. At the moment it is not possible to define exactly if they are "chemotypes", as reported by Filipowicz et al.[11], or if they are different subspecies. Multidisciplinary approaches like botanical investigations and genetic analyses are needed to combine chemical data for a deeper investigation.

- HS-SPME, carefully optimised and standardised, is shown to be an alternative sample preparation technique for samples discrimination compared to hydrodistillation of essential oils. This approach is a fast and easy to automate tool for screening a high number of samples. Furthermore, it is a suitable tool to discriminate between "chemotypes" or species despite of the indications of some international bodies;

- although the process of flame ionisation detector and mass spectrometry is based on completely different principles, the comparison of the two different detectors reveals the same results. As a consequence we are able to use FID or MS detector for the screening of samples alternatively, the advantage of MS detectors however is that they allow us to obtain also a chemical recognition of the matrix components which means high quality data.

In summary, all techniques which were applied in this thesis are appropriate to determine the qualitative composition of *Juniperus communis* L. samples, but the approach of HS-SPME coupled with FastGC/MS separation reduces analysis times with the same effectiveness or better than the other techniques for qualitative sample discrimination.

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