Alternative Green Extraction Methods for Natural Products

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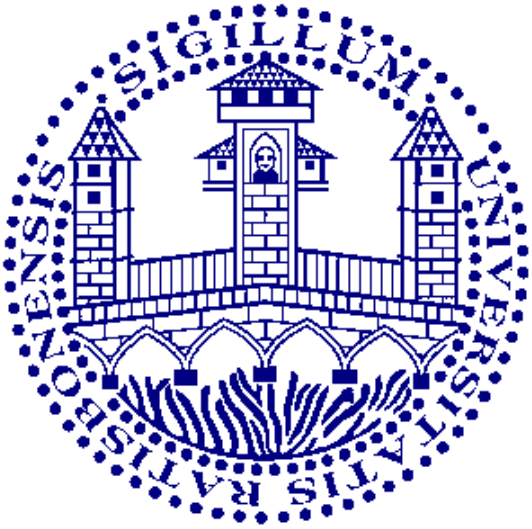

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Abstract

The aim of this thesis was the invention of alternative green extraction methods for the isolation of fragrance compounds from plants paying particular attention to the concepts and principles of Green Chemistry. Conventional extraction methods, such as hydro distillation and solvent extraction, exhibit several drawbacks such as long process duration, high energy consumption and the use of flammable and toxic solvents like hexane.

Therefore, a simple, efficient and mild extraction method for fragrance compounds with natural, biocompatible and biodegradable soap solutions was developed and patented (EP 3 130 655 A1, 2017). Iris butter obtained from iris rhizomes (*Iris germanica* L. and *Iris pallida* Lam.) is one of the most luxurious raw materials for the perfume industry. The attractive violet-like fragrance is due to irones, i.e., terpenoids formed by oxidative degradation of iridals during rhizome aging. By using aqueous soap solutions, especially a myristate solution, an almost complete extraction of the desired irones was possible within a short time and at moderate temperatures. This gentle method prevents the degradation and volatilization of the fragrances at high temperatures, which are disadvantages of conventional extraction methods. Furthermore, various fatty acids, particularly myristic acid, are naturally occurring in the rhizomes. Thus, this extraction method uses an intrinsic plant substance as a highly efficient extraction medium and solubilizer. After the removal of the rhizomes from the aqueous soap solution, myristic acid containing the desired nonpolar fragrance molecules was precipitated by neutralization and separated from the remaining aqueous phase. Excess myristic acid can be recovered by crystallization in cold ethanol or by molecular distillation. As myristic acid is already a frequent ingredient of formulations like crèmes, lotions, or perfumes, the final extract with its remaining fatty acid represents an ideal basic raw material.

In a second part, this newly invented extraction method was transferred to rose blossoms to evaluate its extraction power and applicability. The most important ingredient in roses is the essential oil, which contains around 400 substances such as citronellol, geraniol, and

2-phenylethanol. Rose oil obtained by steam distillation is a key ingredient in cosmetics and fine fragrances. Since 2-phenylethanol is soluble in water, its content in the distillate is very low. Consequently, the rose absolute, which is obtained by solvent extraction commonly with hexane, is of high interest for perfume industry due to its fragrance composition similar to the rose petals. Extracting rose blossoms with an aqueous soap solution prevents the risk of handling large quantities of flammable and toxic solvents as well as the danger of potential petrochemical residues in the extract. In collaboration with a major international perfume company, it could be demonstrated that the experimental procedure of the micellar extraction of *Rosa x centifolia* L. is less complicated compared to iris rhizomes due to the different nature of the plant material. The odoriferous extract is completely soluble in ethanol and with its remaining fatty acid an ideal basic raw material for cosmetics or perfumes. However, compared to the extraction of the non-water-soluble irones, the addition of an inorganic salt to the aqueous extraction solution is necessary to decrease the solubility of partially water-soluble substances such as 2-phenylethanol but also geraniol. Adding a harmless salt, for instance potassium carbonate or sodium chloride, is still in accordance with the principles of Green Chemistry. As a result, an extract with a scent similar to the pure rose blossoms is achieved, but without toxic residues as obtained by the production of rose absolute.

In a third part, the extraction of plant material solely with the pure fatty acids without using water was examined. The direct extraction of the fragrances, which are located on the surface of the rose petals, should be possible with a liquid mixture of lipophilic fatty acids. Therefore, a ternary mixture of lauric acid, myristic acid, and palmitic acid was used as extraction solution since a low melting system with a melting point around 30 °C is formed. Thereby, the advantages of classical enfleurage and solvent extraction can be combined. Also, the extraction of plants respectively plant materials, which continue their physiological activities after picking, is conceivable under these mild conditions. The focus of this study was mainly the isolation of the fragrance compounds from the fatty acid mixture. Therefore, molecular distillation, which is a continuous thermal separation process with only short exposure to increased temperature and decreased pressure, was investigated. In contrast to solvent extraction, not the extraction medium but the fragrance compounds were distilled. As a result, a product free of toxic residues with a

composition similar to the origin was obtained, which was not feasible applying simple vacuum distillation. Moreover, the separated extraction medium can be reused for further extraction cycles due to the chemical and thermal stability of fatty acids. For the recycling of the extraction medium, the solution does not even have to be purified or saponified in contrast to micellar extraction. Instead, the solution can be reused directly. So far, a proof of concept regarding the isolation of the fragrance compounds was accomplished. The aim is to continue the investigation of the extraction power of the fatty acid mixture in future. With the approach “modern enfleurage” a solvent-free, non-toxic and sustainable extraction process for fragrances from plant material is possible without thermal degradation of sensitive compounds.

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1. General introduction

Extraction of natural products has been used probably since the discovery of fire.[1] Leaves, flowers, fruits, resins, bark, and wood were treated to gain the desired fragrance compounds, which were interesting for many different reasons. In earlier civilizations, these perfume compounds were extracted and used for religious ceremonies, for instance. Fragrances and perfumes today represent a mainstream business in the cosmetic and toiletries industry. The global market of fragrances accounts for around 152 Billon US$ every year. It is dominated by six international companies which comprise 57 % of the total market: Givaudan (Swiss), IFF (American), Firmenich (Swiss), Symrise (German), Quest International (Anglo-Dutch) and Takasago (Japanese).[2] The fragrances created by these companies are incorporated by manufactures into shampoos, conditioners, laundry products, and cleaning products as well as in fine fragrances for perfumes or aromatherapy.[3] To this purpose, more than 500 natural raw materials are available beside of many synthetic fragrance molecules. Examples of extremely valuable natural ingredients for fragrance industry include rose oil, jasmine absolute, tuberose absolute, iris butter and orange flower oil.[4]

Depending on the amount of waxes in the extracted product, the extracts are either defined as essential oils, absolutes, concretes or butters.[5] The principle of distillation already applied hundreds of years ago, is still the main technique for the production of a variety of fragrances. However, this method requires high amounts of energy for heating and cooling and moreover the quality of the obtained essential oil is reduced by the formation of undesired side products by distilling at high temperatures.[6] Another possibility to extract sensitive fragrances from plants, especially flowers, was to press the plant material into solid animal fat coated on glass plates. The flowers were regularly replaced until the fat was saturated with fragrance compounds. Although this method, known as enfleurage, was carried out at ambient conditions, perfume molecules get lost since the fat was subsequently treated with alcohol to isolate the fragrances.[7] More recently, the enfleurage method was replaced due to the availability of petrochemical solvents. Hexane, for example, is nowadays a common solvent for extracting fragrances from plant material. Due to its relatively low boiling temperature, it can be easily

recovered by distillation afterwards. However, traces of the solvent remain in the extracts and are often not accepted in products due to their toxicity.[6]

Considering the drawbacks which are linked to the conventional extraction techniques distillation and extraction, the demand to find new alternative extraction processes is very high. In 2012, concepts and principles based on Green Chemistry had been developed by Chemat *et al.* as a strategy to design green and sustainable extraction methods of natural products. Its definition can be summarized as follows: "*Green Extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product.*".[1, 6]

Within these principles, the aim of this work was the invention of alternative green extraction methods to isolate fragrance molecules from plants. Based on iris rhizomes, an aqueous extraction medium containing soap surfactants was established to extract and isolate the valuable nonpolar irones (see chapter 3: micellar extraction of *Iris germanica* L.). Subsequently, this newly invented extraction method was transferred to another plant material, here rose petals, to evaluate its extraction power and applicability. The achieved results are presented in chapter 4: micellar extraction of roses. The gained experiences led to a further attempt to extract fragrances from plant material solely with pure fatty acids and without using water. In chapter 5: modern enfleurage, this approach is outlined.

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2. General information

2.1. Chemistry of natural products

Plants produce a variety of compounds, which can be divided into two groups: primary and secondary metabolites. Primary metabolites are essential to the growth, catabolism and proliferation of the cells and are produced continuously. They are identical in most organisms and include sugars, amino acids, polysaccharides, and lipids. In contrast, secondary metabolites are not essential to sustain the life of cells or organisms, but usually have important ecological functions and can be specific to a plant or fungi species. Secondary metabolites often play an important role in plant defense against attractants, herbivores and microbial infections.[1] Because of their interesting properties, they are used by humans in numerous applications such as in the pharmaceuticals, cosmetic and food industry. Secondary metabolites can be classified according to their technological role: coloring agents, functional foods and nutraceuticals, preserving agents, flavors, fragrances and edible oils.[2] Another possibility to classify secondary metabolites is based on their biosynthetic origin. Among higher plants, they can be divided into (poly)phenolic compounds, terpenoids, and alkaloids, which are discussed in the following briefly.[3]

2.1.1. Polyphenols

Phenolic compounds consist of a hydroxyl group attached to an aromatic ring and can be further divided into flavonoids and non-flavonoids. Flavonoids are (especially as glycosides) mostly water-soluble dyes, which are involved in plants as UV protection, stimulation of nitrogen-fixing nodules and disease resistance, for instance. They comprise two aromatic rings connected by a three-carbon bridge and can be subdivided into flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins (compare Figure 1). Flavonoids are estimated by humans due to their anti-inflammatory, anti-allergic and anti-cancer activities.[4]

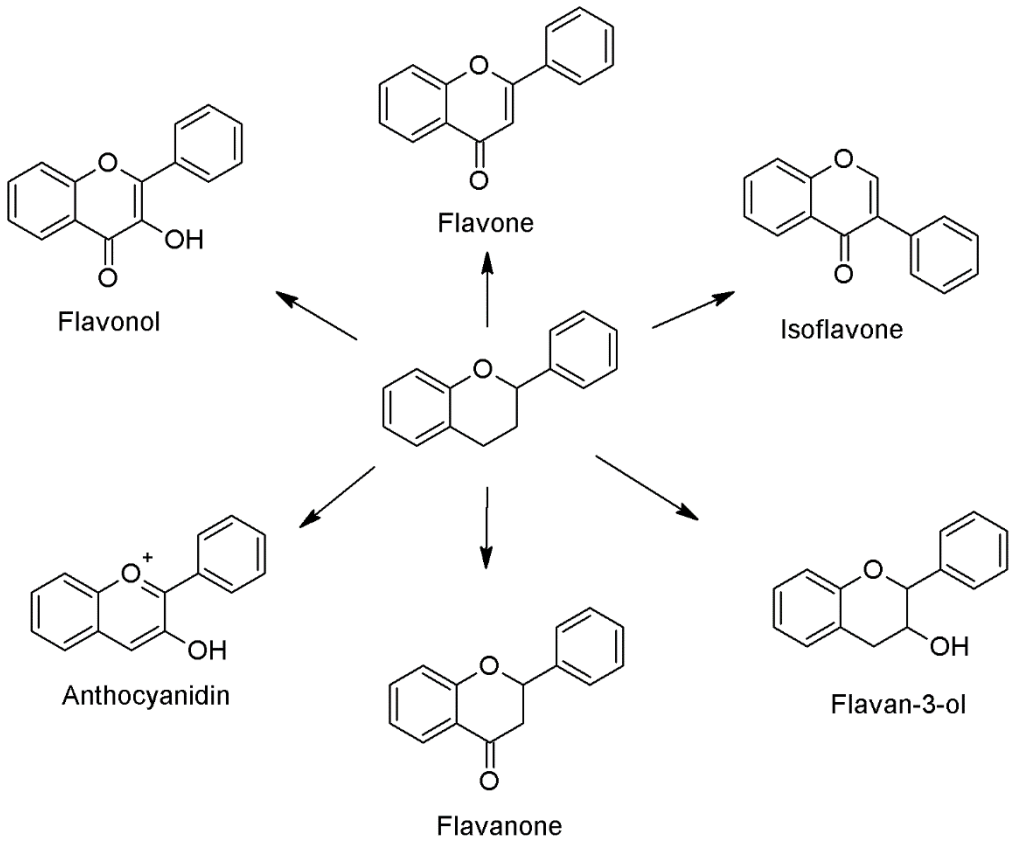


Figure 1: Chemical structure of the major flavonoids.

The main non-flavonoids are the phenolic acids and their conjugated derivatives as well as the acylphloroglucinols, polyphenolic stilbenes and anthranoids. Phenolic acids contain a carboxylic acid group with one or more hydroxyl substitutions on the benzene ring. They have important biological and pharmacological properties due to their potential antioxidant activity.[5]

Phenylpropanoids are common components found in essential oils and represent the majority of naturally occurring phenolic compounds, respectively their biosynthetic precursor molecules. Formally, they are derived from phenylpropane consisting of a benzene ring and a chain of three carbon atoms. Anethole and eugenol are well-known representatives of this group and are used as fragrances and flavor materials.[6]

2.1.2. Terpenoids

Terpenoids are the largest class of secondary metabolites and are found in all plants. They are built of isoprene units and classified according to the number of these units (Table 1). Because of their flavoring properties, terpenoids, especially mono- and sesquiterpenes, are mainly known as major components of essential oils.[2] Due to the large variety of terpenoids and the different chemical and physical properties, no general statements regarding their significance can be made.[7]

Table 1: Classification and examples of terpenoids.[2]

Classification	Basic structure		Example
	Isoprene units	Carbon atoms	
Monoterpene	2	C10	Linalool, limonene
Sesquiterpene	3	C15	Farnesol
Diterpene	4	C20	Phytol, retinol
Sesterpene	5	C25	Geranylfarnesol
Triterpene	6	C30	Amyrin
Tetraterpene	8	C40	β-Carotene

The acyclic monoterpene alcohols geraniol, linalool, and citronellol, but also the sesquiterpene alcohols farnesol and nerolidol are the most important terpenoids used for perfume compositions. Often, these fragrances are also produced synthetically. However, the quality or the enantiomeric purity of the synthetic products differs significantly from that of the compounds isolated from natural products. In addition, traces of compounds with similar physical properties but different odor also contribute to the scent.[6]

2.1.3. Alkaloids

To be complete, also the class of alkaloids should be mentioned briefly, although they do not represent typical fragrance molecules. Alkaloids are a group of chemical compounds which are biogenetically derived from amino acids. They differ from the other secondary metabolites as they neither possess a common structural element such as the polyphenols nor a common biosynthetic origin as the terpenoids. Nevertheless, alkaloids in the narrow sense are always derived from a biogenic amine resulting from an amino acid after decarboxylation. Due to the nitrogen contained in the molecules, alkaloids have particular physicochemical properties and often show pronounced pharmacodynamic effects in mammals. Some of them are the most biologically active natural products. At appropriate dosage, they show medical effects but they can be toxic among uncontrolled administration. About 10 to 20 % of the higher plants possess alkaloids.[1, 7]

For example, indole and quinoline are fragrance molecules within this class. However, due to their toxicity, they are only used sparely.[6]

2.2. Green Extraction

Extraction of natural products finds application in almost every production process in the perfume, cosmetic, pharmaceutical, food or fine chemicals industries. In order to protect both environment and consumers, it is nowadays necessary to expand and modify the traditional extraction techniques. Ideally, the extraction process is exhaustive, reduces or eliminates petrochemical solvents and is combined with moderate energy consumption. Within these constraints, Green Extraction has been introduced on the basis of Green Chemistry published by Paul Anastas and John C. Warner in 1998.[8] Green Chemistry is focused on reducing environmental and health impacts. Based on these twelve principles, Farid Chemat and Jochen Strube adapted the six principles of Green Extraction, which should be viewed for industry and scientists as a direction to establish an innovative and green label, charter and standard.[2, 9]

Principle 1:	Innovation by selection and use of varieties of renewable plant resources.
Principle 2:	Use of alternative solvents, principally water or bio-based solvents.
Principle 3:	Reduction in energy consumption by energy recovery, using innovative technologies.
Principle 4:	Manufacture of co-products instead of waste to include the bio- and agro-refining industries.
Principle 5:	Reduction in unit operations, favoring safe, robust and controlled processes.
Principle 6:	Aiming for a non-denatured and biodegradable extract without contaminants.

One of the six principles of Green Extraction (Principle 2) implies the usage of alternative solvents. Most of the solvents currently used for plant extraction are derived from crude oil, with the exception of water and ethanol produced by fermentation.[2] The used lipophilic solvents are flammable, volatile and often toxic and are responsible for environmental pollution and the greenhouse effect.[9] Additionally, some volatile organic solvents (VOCs), like dichloromethane or benzene are also known to be carcinogens.[10] In

spite of the danger, the world demand for solvents, including hydrocarbons and chlorinated solvents, is currently growing at 2.3 % per year and is approaching 20 million tons annually.[10] The in-house recycled solvents are excluded in this statistics, which leads to a global use in solvents far higher.

The introduction of legislation by the United States Food and Drug Administration (FDA) resulted in the prohibition of some solvents in the pharmaceutical industry, such as benzene (class 1 solvents). Toluene, hexane and further class 2 solvents should only be used if unavoidable.[11] Nevertheless, according to the Environmental Protection Agency (EPA) Toxic Release Inventory, more than 20 million kg of hexane are released into the atmosphere each year through these processes resulting in environmental pollution.[10] Therefore, less toxic, renewable and biodegradable solvents are in high demand to substitute dangerous solvents like hexane and thus, the topic of this thesis is of high importance.

A short explanation of conventional extraction techniques such as solvent extraction and distillation as well as already existing alternative extraction methods is given in the following chapter.

2.3. Extraction techniques

2.3.1. Principles and fundamentals of plant extraction

The extraction of plant material can be described as a mass transfer process of one or more components of the solid plant matrix to the extracting solvent. The first step involved in the extraction of natural products is the sample preparation. The plant material often has to be washed, dried and ground before to obtain a homogeneous material and to increase the contact area with the extraction medium.[12] The solutes are more or less uniformly distributed in seeds, fruits, and roots, whereas in leaves and flowers the solutes are inside fragile glandular trichomes. In general, the extraction process follows these steps:[13]

1. The solvent permeates the solid surface of the plant material.
2. The solvent penetrates into the plant matrix by molecular diffusion.
3. The soluble material of the plant is solubilized by desorption into the extraction medium.
4. The solution containing the solutes returns to the surface of the plant matrix by molecular diffusion.
5. The solution is transferred from the plant material to the solvent by natural or forced convection.

Three primary methods are used to obtain plant extracts: mechanical expression, solvent extraction, and distillation. Mechanical expression or pressing is suitable for plants with high oil contents, for example, citrus fruits. The extracted oil is free of solvent residues and is not exposed to heat. Consequently, these extracts have a high quality close to the original. Solvent extraction is applied in the separation of heat-sensitive plant materials. According to their method of preparation, it is distinguished between concretes, absolutes and resinoids. Essential oils are obtained by distillation with water or steam. As a result, only volatile components are found. Thus, the products of distillation and extraction differ concerning the sensory properties as the non-volatile components can also have complexing and fixing properties.

Before the different extraction methods are explained in more detail a short explanation of the various plant extracts is given:[6, 14]

Concretes are prepared by extracting fresh plant material (flowers, herbs, leaves) with nonpolar solvents such as hexane and petroleum ether. Concretes are waxy, semisolid, dark-colored compounds, which are not completely soluble in alcohol. For this reason, they find limited use as perfume ingredients.

Absolutes are prepared by extracting the concrete with alcohol. After the concrete is immersed in the solution at slightly increased temperature, the solution is cooled down to precipitate the waxy compounds, since waxes are insoluble in alcohol below -1 °C. Absolutes are completely soluble in alcohol and therefore used in perfume industry.

Resinoids are prepared by extracting dried plant material with solvents such as methanol, ethanol or toluene. The products are usually highly viscous and consist mainly of non-volatile compounds. Resinoids are primarily used for their fixative properties.

Essential oils are obtained by water or steam distillation. They consist of volatile, lipophilic substances such as hydrocarbons, esters, terpenes, lactones, phenols, aldehydes, acids, alcohols, and ketones. Most of the essential oils are used directly. However, some of them are concentrated by distillation or crystallization.

2.3.2. Solvent extraction

Solvent extraction can be carried out applying different methods, which are all based on the solid/liquid interaction to dissolve the desired compounds. Mainly sensitive plant material such as flowers of jasmine, bitter orange (neroli), tuberose, narcissus and roses are extracted with solvent. It can be distinguished between non-exhaustive (see 2.3.2.1) and exhaustive (see 2.3.2.2) solvent extraction methods.

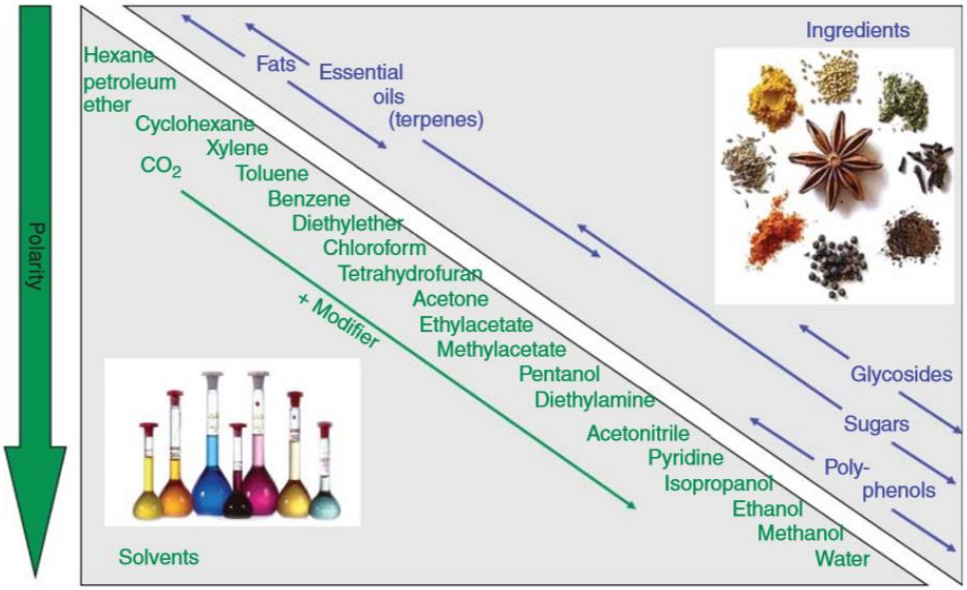


Figure 2: Solvent selection for solid/liquid extraction of plant material.[2]

The solubility of a compound in the extraction medium depends mainly on the polarity and is the key aspect of the whole extraction process (compare Figure 2). As a general rule, non-polar solutes are dissolved by non-polar solvents, whereas polar solutes are dissolved by polar solvents. The primary goal of the extraction process is to achieve a high yield of the desired compounds with a high selectivity, respectively purity. In this process, the mass transfer kinetic is very important, which can be described by the diffusion phenomenon based on Fick's law. The central mechanism responsible for the mass transfer is convection. Thus, agitation as well as ultrasound or microwave irradiation (see 2.3.4.1) enhances the extraction efficiency. The rate of mass transfer decreases as the concentration of solutes in the solvent increases until an equilibrium is reached. Since the

mass transfer also depends on its solubility, heating the solvent can enhance the extraction efficiency further.[12-14]

Besides the solubility, further criteria for solvent extraction have to be considered: the solvent should be highly selective in order to prevent further purification steps; the solvent recovery should be easy in order to avoid loss or degradation of the desired compounds implying a low boiling point and to prevent toxic residues of solvent in the extract; the solvent should be low viscous to enable mass transfer and to facilitate the filtration of plant material; the solvent should have a low surface tension in order to allow a fast wetting of the plant material; the solvent should be thermally and chemically stable to be recycled during the solvent recovery; the solvent should be readily available and nontoxic as also environmental friendly.[11]

For the extraction of plant material, hexane has been the most commonly used solvent despite its toxicity. Hexane is an excellent solvent for hydrophobic compounds and can be easily recovered by distillation due to its low boiling point around 65 °C.[13] Nevertheless, there is a strong tendency towards the use of green and alternative solvents as the environmental impact, and the economic aspects gain more and more importance. Not only the risk of handling large quantities of flammable and often toxic solvents, but also the danger of potential residues in the extract led to the fact that solvent extraction, especially with the most commonly used *n*-hexane, is increasingly questioned (discussed in more detail in chapter 4).[9, 10] Recently, 2-methyltetrahydrofuran was introduced as a green alternative solvent for extraction processes. It is biodegradable and can be derived from renewable resources. Thus, 2-methyltetrahydrofuran is in accordance with several principles of Green Chemistry. Due to its physical and chemical similarities such as boiling point or water immiscibility, it is a possible solvent to substitute hexane.[10, 15, 16]

2.3.2.1. Maceration

Maceration describes the soaking of untreated or powdered plant material in an appropriate solvent at room temperature. The plant material stays in contact with the solvent for several hours until days, with occasional shaking. Agitation is provided to

increase the mass transfer rate and to accelerate the extraction process. If maceration is carried out at increased temperature, called digestion, the extraction process can be enhanced further. However, heating should be avoided when extracting thermosensitive compounds. The advantage of maceration is that sensitive molecules can be extracted under mild conditions. At the end of the soaking process, the remaining plant material is pressed and usually re-extracted with fresh solvent. The liquid phases are combined from the various maceration steps, and the solvent is removed by evaporation or drying. Despite the above-mentioned advantage, maceration also has disadvantages as it is a very time-consuming method, which requires large volumes of hazardous solvents. Furthermore, the separation of the solvent afterwards is energy-consuming, and due to the degradation of thermosensitive compounds, there can still be a loss in quality, at least in a digestion process.[6, 13, 14]

2.3.2.2. Percolation and Soxhlet extraction

In contrast to maceration, percolation is an exhaustive method to extract plant material. To this purpose, usually hot solvent is poured on top of the solid plant material and allowed to percolate through the bed. Generally, this process is driven by gravity from the top to the bottom. It is, for example, applied in the preparation of coffee. A benefit of this method is that the used extraction medium can be recycled directly without additional filtration and the solvent can be passed through the bed several times until the extraction of the desired compounds is completed.[2, 11, 14]

In laboratory scale, a Soxhlet apparatus is used for this procedure. It consists of a flask, a Soxhlet extractor, and a reflux condenser. The raw material is placed in a thimble made of filter paper in the middle of the extractor. The solvent in the flask is then heated to reflux and percolates the solid material. When the level of extract reaches the top of the syphon tube, the solvent is rinsed back into the flask. As a result, the plant material is extracted several times with fresh solvent, whereby only small amounts are required. The extracted compounds are collected in the bottom flask. However, disadvantageous of this method is that the solutes are always at the boiling temperature of the solvent, which may cause damage of thermo-labile compounds.[11, 13, 14]

2.3.3. Steam and hydro distillation

For highly volatile compounds, distillation with water or steam is applied. The gained essential oils usually have boiling points ranging from 150 °C to 300 °C. When the plant material is subjected to heat, either with steam or hot water, these oils are separated from the plant. The basic principle of steam and hydro distillation is that almost non-volatile substances form low boiling azeotropes with water.[2] These azeotropes evaporate and are transferred through the vapors to the condenser, where the liquid separates into water and oil phase. Consequently, for steam and hydro distillation the essential oils must be immiscible with water to ensure subsequent phase separation. Moreover, the essential oils must be sensitive to the action of heat and water.[13, 14, 17, 18]

The pressure within the distillation process can be described by means of Raoult's law:

$$p = p^*_{water} + p^*_{essential\ oil}$$

where p = total pressure of the system and p^*_{water}, $p^*_{essential\ oil}$ = saturation steam pressures of the single compounds.

If the temperature of the mixture rises to such an extent that p becomes equal to the atmospheric pressure, the liquid starts to boil. Since the total vapor pressure is composed of the partial pressures p^*_{water} and $p^*_{essential\ oil}$, the boiling point is lowered. As a result, the boiling point of the mixture is below the boiling points of the single compounds and can be extracted without reaching the initially high boiling temperature.[18, 19]

In industrial scale, distillation with cohobation is used.[14] In this process, the distilled water turns back once it has been separated and is re-boiled again. By returning the condensate water, the loss of oils which are slightly soluble in water can be reduced. This problem is often known by distilling rose petals. Some of the valuable ingredients of rose oil, such as 2-phenylethanol, are partly dissolved in water. Therefore, distillation is often combined with simultaneous extraction of the volatiles into a small quantity of water-immiscible organic solvent, since first reported by Likens and Nickerson in 1964.[20] Using n-butyl acetate as co-solvent, also most of the water-soluble constituents of rose oil can be recovered.[21]

Distillation with water and steam is largely used because solvent-free products are generated and no subsequent separation steps are necessary. In contrast, very long extraction times and a high energy consumption is required to gain the desired oils. The duration of the distillation depends strongly on the quality and size of the plant material. The diffusion of essential oils and hot water through crushed plant material takes shorter distillation times than through uncrushed material. Furthermore, the essential oil of fragile rose blossoms is gained much faster compared to dried iris rhizomes. In the latter case, the plant material has to be soaked with water first to facilitate the permeability of the vapor. Another drawback of distillation is the high temperature, which can induce thermal degradation and hydrolysis of the products. Certain constituents of essential oils like esters, tend to react with water to form acids and alcohols, especially at high temperatures. As a result, the yield is decreased and unwanted off-flavors can contribute to the scent of the essential oil.[13]

2.3.4. Alternative extraction methods

To sum up, the major drawbacks of conventional extraction methods are long extraction times, the requirement of volatile, flammable and often toxic solvents and the thermal decomposition of thermo-labile compounds. To overcome these limitations, non-conventional extraction techniques have been introduced, which include ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) as well as alternative solvent extractions.

2.3.4.1. Ultrasound- and microwave-assisted extraction

Ultrasound, ranging from 20 kHz to 100 MHz, facilitates the release of metabolites from plant material into the extraction solvent due to the disruption of plant cell walls. Thus, the mass transfer and diffusion of the solutes is increased. The operative conditions are closely related to the plant matrix. Harder woody plant material needs much higher power density compared to the external glands of sensitive flowers, for instance. In addition to the reduction in extraction time compared to conventional extraction methods, UAE

enables saving of energy and the consumption of solvents. A further significant advantage is the reduced thermal exposure to the plant material, which also allows the extraction of thermo-labile compounds. UAE is a simple and low cost process that can be used in laboratory as well as in industrial scale.[2, 22, 23]

Microwaves are electromagnetic fields in the frequency range from 300 MHz to 300 GHz. They interact with dipoles of polar and polarizable materials causing heating near the surface of the materials. Owing to the increased temperature, the solvent ability to penetrate the plant material, and thus the mass transfer of the solutes, also increases. In non-polar solvents only poor heating occurs. Consequently, MAE can be considered as a selective method which favors polar solvents and molecules. MAE has been used for the extraction of polyphenolics from tea leaves and flax seeds, for instance. Generally, power and extraction time are in the range of 25 – 750 W and 30 s to 10 min. Apart from the relatively high capital costs and possible thermal decomposition of sensitive compounds, MAE enables an immense reduction in extraction time and additionally achieves often higher extraction yields.[2, 22, 24]

2.3.4.2. Supercritical fluid extraction

Supercritical fluids share the physical properties of a gas and a liquid at their critical point. They behave more like a gas but have the solvating properties of a liquid. A typical supercritical fluid is CO_2, which becomes supercritical above 31.1 °C and 73.8 bars. CO_2 is readily available at low cost and is non-toxic. It is an excellent solvent for nonpolar analytes and can be adapted for the extraction of polar substances as well by adding small amounts of ethanol, for example. Thus, the extraction efficiency can be modified by changing temperature, pressure or by adding additives. Due to the higher diffusion coefficient and lower viscosity compared to conventional solvents, supercritical carbon dioxide extraction leads to a better penetration of the plant material and to an enhanced mass transfer. Consequently, the extraction time can be reduced extremely. Moreover, there are no residues of solvent in the extract due to the volatility of carbon dioxide. However, the initial investment costs of the SFE unit are very high.[22, 24-27]

2.3.4.3. Alternative solvent extraction

It is often recognized that water is a suited solvent for Green Extraction because it is environmentally friendly, nonflammable and nontoxic. Due to its polar character, it is favored for the extraction of polar plant compounds such as oligosaccharides, glycosides or amino acids. However, by changing parameters such as temperature or pressure or by adding additives this can be overcome.[2] The addition of ionic liquids or surfactants to water enables the extraction of also lipophilic molecules and is presented in the following.

2.3.4.3.1. Ionic liquid-based extraction

Ionic liquids (ILs) are solvents consisting entirely of ions and at least one organic ion. The ions are poorly coordinated leading to a melting point below 100 °C. It is possible to design tailor-made ILs by the appropriate choice of anions and cations, which leads to a high selectivity during the extraction process. Due to their low or negligible vapor pressure and non-flammability ILs are often referred as green solvents.[11, 28]

Several applications have shown that ILs are suited for the extraction of biomolecules from plant material.[11, 29] The best developed example is the extraction of artemsinin from *Artemisia annua*.[30] But also the extraction of lignin from sugar cane plant waste using aqueous ILs should be mentioned.[31] Moreover, in a previous work it was shown that it is possible to extract fragrance compounds from plant material with aqueous choline carboxylates.[32] An almost quantitative extraction of the valuable irones from *Iris pallida* Lam. rhizomes was possible within short times and at room temperature.

However, simple evaporation of the ILs cannot be applied as known from organic solvents. Other methods of separation have to be developed to make ILs attractive as an alternative solvent on production scale.[11]

2.3.4.3.2. Micellar extraction

Surfactants are amphiphilic compounds consisting of a hydrophilic and a lipophilic part. At a certain concentration in water, surfactants self-assemble and form aggregates, called micelles (discussed more detailed in 2.4.2.1). The structure and size of these micelles are dependent on the nature and the concentration of the surfactant molecules. In water, typically spherical shapes are built. Thereby, the hydrophobic tail orientates towards the center creating a non-polar core, as it can be seen in Figure 3.

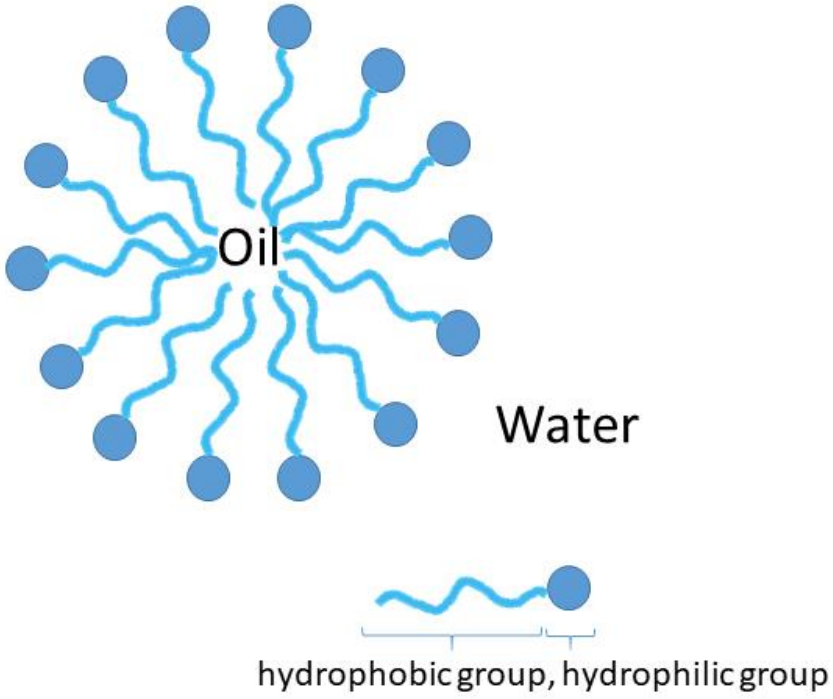


Figure 3: Schematic presentation of a micelle with a hydrophobic core and a hydrophilic shell.

Under defined conditions involving pH value, temperature, other solutes and the nature of the surfactant molecules, these aggregates are thermodynamically stable. Consequently, they provide an oil-soluble phase in water enabling hydrophobic compounds to be dissolved.[2, 33]

Nonionic surfactants have a specific character because at a certain temperature the micelles are no longer stable and break down. At this so-called cloud point, two phases are formed. Dependent on the nature of the extracted compound it can be found either in the surfactant-rich or the water-rich phase. This “clouding” is reversible and occurs from the competition between entropy, which favors the miscibility of micelles in water and the enthalpy, which favors the phase separation. In cloud-point extraction (CPE), the first step involves the micellar extraction of the plant material below the cloud temperature. After the hydrophobic molecules are extracted, phase separation is performed by a temperature increase to separate the surfactant-rich phase from the matrix.[2, 11, 33] Various examples for CPE of biomolecules like proteins, polyphenols and triterpenes are described in literature.[34-36]

However, CPE is only possible with nonionic surfactants. Extraction of plant material with ionic surfactants is hardly found in the literature due to its complicated extract removal and solvent recovery. Therefore, economically feasible isolation methods have to be established to make micellar extraction of natural compounds from plants more interesting. Apart from the difficulty to recover the desired compounds from the extraction medium, micellar extraction shows various advantages, as the micelles, for example, offer a high capacity to concentrate analytes with almost quantitative yields. Moreover, a lot of surfactants which are environmentally friendly and inexpensive are available. Only small amounts of surfactants are necessary compared to the need of organic solvents in plant extraction. Also the extraction of thermally sensitive compounds is feasible due to relatively mild extracting conditions.[33] Since micellar extraction is a big part of this thesis, the following chapter gives a deeper insight into the field of surfactants.

2.4. Surfactants

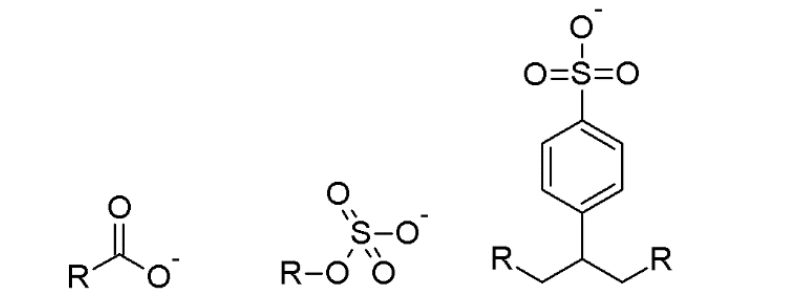
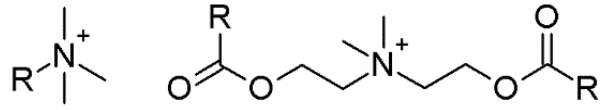
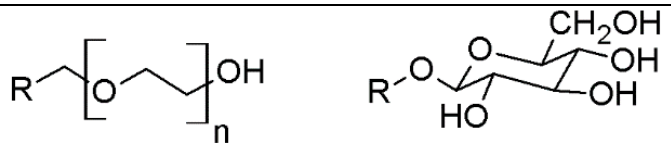
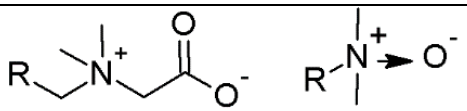
Surfactants are omnipresent and indispensable in daily life. They find application in personal care, cleaning and washing products or in emulsifying food. Moreover, they are used in pharmaceutical products, in the paper industry, in the textile sector and many others.[37] The global surfactant market comprised 30 billion US$ in 2015. It is expected to reach 40 billons US$ in 2021 triggered by the rising demand from the personal care industry.[38]

2.4.1. Classification

As already described, surfactants are amphiphilic molecules consisting of a hydrophobic and a hydrophilic part. The hydrophilic part has a polar group with an affinity for polar solvents, whereas the hydrophobic part has a nonpolar group with an affinity for nonpolar substances.[11] Concerning the charge of the polar hydrophilic group surfactants can be divided into ionic and nonionic surfactants. The ionic surfactants can further subdivided into anionics, cationics and zwitterionics, as it can be seen in Table 2.

This dissertation is focused on anionic surfactants, especially soaps. Soaps are sodium or potassium salts of fatty acids and are produced by saponification of natural oils and fats. They are the oldest surfactants, but still part of modern detergents. Soaps show excellent detergency, good wetting ability, and good foaming. However, soaps are water-insoluble at neutral or acidic pH values and furthermore, very sensitive to hard water. The precipitation of insoluble lime soaps by polyvalent ions is undesirable in some applications, but can be prevented by adding additives.[37, 39-41] Moreover, soaps are readily biodegradable under anaerobic and aerobic conditions.[42] With regard to their easy and cheap production, their low toxicity and the presence of fatty acids in the human body as well as in animals and plants, soaps are the ultimate green surfactants.[43]

Table 2: Structures of some important ionic, nonionic and zwitterionic surfactants.

Surfactant class	Structure examples
Anionic	 Alkyl carboxylate Alkyl sulfate Alkyl benzenesulfonate
Cationic	 Alkyl quat Alkyl ester quat
Nonionic	 Alcohol ethoxylate Alkyl glucoside
Zwitterionic	 Alkyl betaine Alkyl amine oxide

2.4.2. Surfactant micellization

The amphiphilic nature of surfactant molecules is responsible for their tendency to concentrate at interfaces or surfaces at low concentrations. Thereby, the free energy of the system is reduced. However, when all interfaces are saturated, another mechanism to reduce the free energy of dissolved surfactants in water occurs. The surfactant molecules start to form micelles, as illustrated in Figure 4.[39, 41]

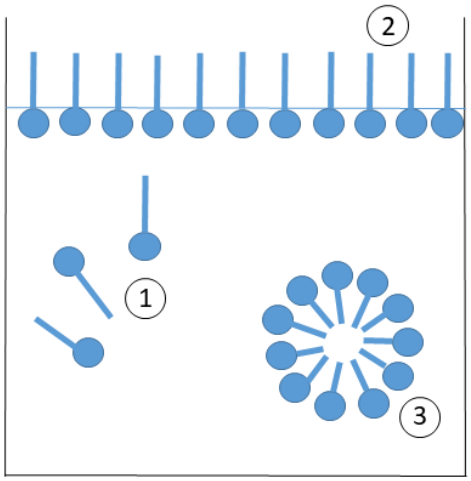


Figure 4: Formation of self-assembly (3) from dissolved (1) and surface-adsorbed (2) surfactants by increasing concentration.

2.4.2.1. Critical micellar concentration

The surfactant concentration at which self-assembly appears is called the critical micellar concentration (CMC). At this concentration, two opposite forces arise. On the one hand, there is the hydrophobic interaction, because hydrocarbons in water have strong driving forces to transfer their tails out of water or into the interior of the micelles. On the other hand, there are the repulsion forces of the head groups. Thus, the surfactant molecules in micelles are in a dynamic equilibrium. Consequently, micelles are changing their size and shape permanently.[39, 44]

The CMC is strongly dependent on the chemical structure of the surfactant and its charge. Several general remarks can be made.[37, 39, 44, 45]

1. The CMC decreases strongly with increasing alkyl chain length of the surfactant due to the hydrophobic interactions mentioned above.
2. Branching or introduction of double bonds increases the CMC due to decreased hydrophobic interactions.
3. The CMC values of nonionic surfactants are much lower than those of ionic surfactants of comparable chain lengths because of missing electrostatic repulsions.

2.4.2.2. Surfactant solubility and Krafft temperature

The solubility of ionic surfactants is dramatically temperature dependent. At low temperatures, the solubility of surfactants is very low and then increases strongly within a narrow temperature range. This behavior is called Krafft phenomenon and is determined by the interplay of two opposing thermodynamic forces. One is the energy of the solid crystalline state and one the energy of the micellar solution. The latter varies only slightly by changing the chain length of the surfactant or by varying the counter ions, for instance. However, the free energy of the crystalline state can vary strongly due to packing effects. Thus, the Krafft temperature can be lowered by a hindered crystalline packing or by changing the counterion.[39, 41]

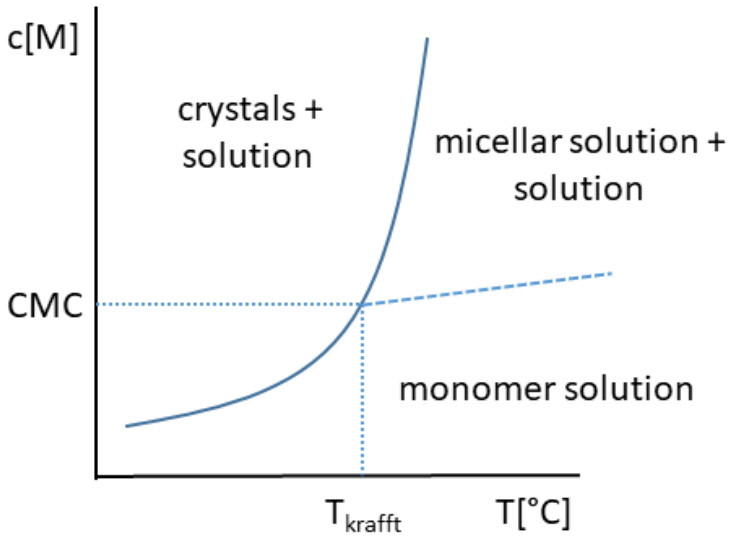


Figure 5: Temperature dependence of the CMC (Krafft temperature).

The Krafft temperature is often measured as the temperature at which turbidity of a 1 wt. % aqueous surfactant solution disappears.[46] In a more strict definition, it is defined as the intersection of the solubility curve and the CMC curve as plotted in Figure 5.

2.4.2.3. Aggregate structure

The driving force for micellization is the reduction of water-hydrocarbon contacts. Thus, the larger a spherical micelle, the more efficient is the elimination of these contacts since the volume-to-area ratio increases. The geometrical packing and the preferred aggregate structure can be described by the critical packing parameter N_s.[39, 41, 44]

$$N_s = \frac{v}{a_0 * l}$$

N_s is defined as a ratio between the volume v of a surfactant molecule and its cross sectional area a multiplied with the length l of the surfactant molecule. Small values of N_s imply higher curved aggregates, whereas planar bilayers are formed when $N_s = 1$ (compare Table 3).

Table 3: Aggregate structure in relation to the surfactant critical packing parameter N_s.

Packing parameter N_s	Aggregate structure
0.33	Spherical or ellipsoidal micelles
0.5	Elongated cylindrical of rod-shaped micelles
1	Planar bilayers
> 1	Reversed micelles

Nevertheless, the formation of a certain aggregate in solution is also dependent on the surfactant concentration. By increasing the surfactant concentration, the spherical micelles turn into rod-shaped micelles.[11, 39] Consequently, also the solubilizing capacity increases and an improved extraction efficiency is possible.[41]

In order to use a micellar medium for the extraction of plant material, several further tasks have to be considered. For example, a strategy to recover the desired compounds from the micellar media has to be established. Moreover, a selective enrichment of the target substances can be helpful. For this purpose, a possible approach is described in the following.

2.5. Effects of salts

In 1888, it was first noted by Hofmeister that inorganic salts and ions showed different abilities of precipitating proteins.[47] He reported the effect that some ions are able to precipitate proteins in water (salting-out) and other ions enhance their solubilization (salting-in). A typical ordering of the ions according to the Hofmeister series is shown in Figure 6.

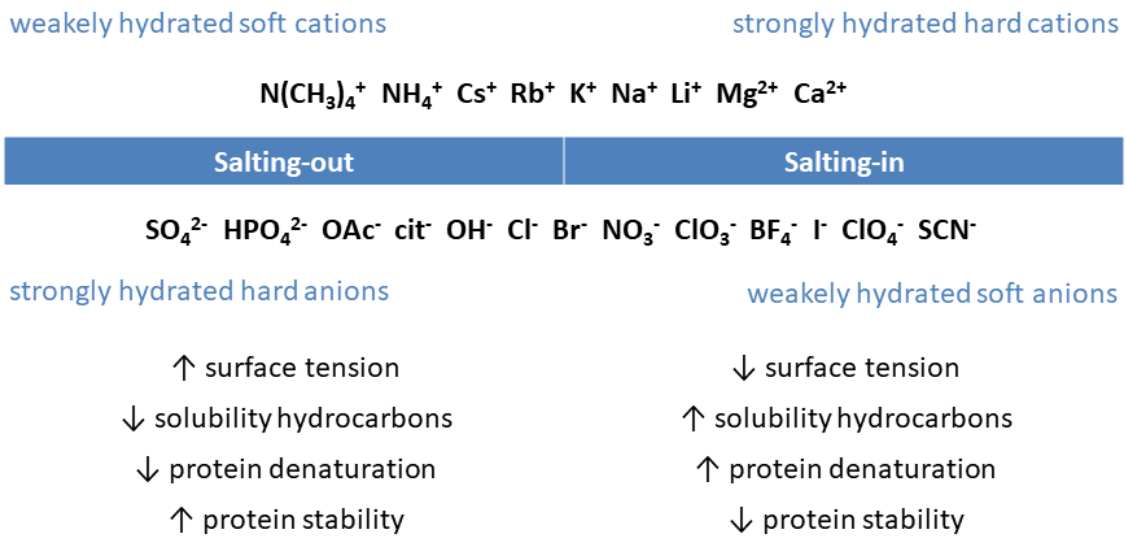


Figure 6: Hofmeister series of anions and cations.

This specific ion effect correlates with the charge density of the ions. All anions of the left side of the series exhibit a high charge density and remain highly hydrated in the bulk. Anions on the right side have a low charge density and are less strongly bound to their hydration shell. In the case of cations, it is the opposite effect. The series of cations go from soft weakly hydrated ions on the left side to hard, strongly hydrated ions on the right side. The borderline is usually set at the chloride ion for anions and the sodium ion for cations.

Indeed, not only in biochemistry the Hofmeister series is useful, but also in the field of physical, colloid, polymer and surface chemistry.[48] In general, salting-out ions tend to

decrease the solubility of hydrocarbons. When a salt is dissolved in an aqueous media, its ions are surrounded by a layer of water molecules. When those water molecules are tightly bound to the ions, their role as solvents to other molecules is reduced. On the contrary, salting-in ions increase the solubility of hydrocarbons.[49, 50]

Commonly, the salting-out effect is applied for the purification of proteins.[51] However, it is also widely used to separate and purify organic compounds from aqueous mixtures. Observations were made for a number of water-miscible organics such as acetone[52], ethanol[53] and acetonitrile[54]. Moreover, the salting-out effect can be used to enhance the extraction efficiency of analytes, which are extracted only poorly. Nikolić *et al.*, for example, investigated the salting-out extraction of the phenolic compounds catechol and hydrochinone in aqueous solution with diethyl ether and diisopropyl ether.[55] Generally, the salting-out effect depends on the analyte and on the type of the salt. The higher the number of carbon atoms in a compound, the higher the effect of salting-out resulting in a lower solubility of the compounds in water. In most cases, an increase in salt concentration also increases the concentration of the analyte in the organic phase. Potassium pyrophosphate, phytic acid sodium salt, and ammonium sulfate are, for example, effective salts.[56, 57]

2.6. Thermal purification processes

2.6.1. Basic concepts

As in steam or hydro distillation (compare 2.3.3), every distillation process has the goal to separate volatiles from less volatile substances. For the thermal purification of a substance mixture, it is required that the liquid and the vapor phase have different compositions. Often this is achieved by a sufficiently large difference between the boiling temperatures of the pure substances. Raoult's law describes the relative volatility of an ideal mixture (also known as separation factor). Thereby, the molecular interactions of the different components are the same or very similar to the pure substances. Thus, the partial vapor pressure p_i of each component is equal to the vapor pressure of the pure component p_{0i} multiplied by its mole fraction in the mixture x_i.

$$p_i = p_{0i} * x_i \qquad \text{(ideal)}$$

For real systems with additional interactions between the molecules, such as van der Waals interactions or hydrogen bonds, the Raoult's law is modified by an activity coefficient γ_i, which is concentration dependent.

$$p_i = p_{0i} * x_i * \gamma_i \qquad \text{(real)}$$

For example, if the hydrogen bonds in a mixture are weakened, the vapor pressure against the pure component rises. This represents a positive deviation of Raoult's law and the activity coefficients are higher than 1. An azeotrope with a boiling point minimum is built. In contrast, if the hydrogen bonds in a mixture are increased, the vapor pressure against the pure components decreases. Thus, a negative deviation of Raoult's law and activity coefficients smaller than 1 are the consequence and an azeotrope with boiling point maximum is created. The so-called McCabe-Thiele diagram, based on Raoult's law, reflects this relationship (see Figure 7).[58-60]

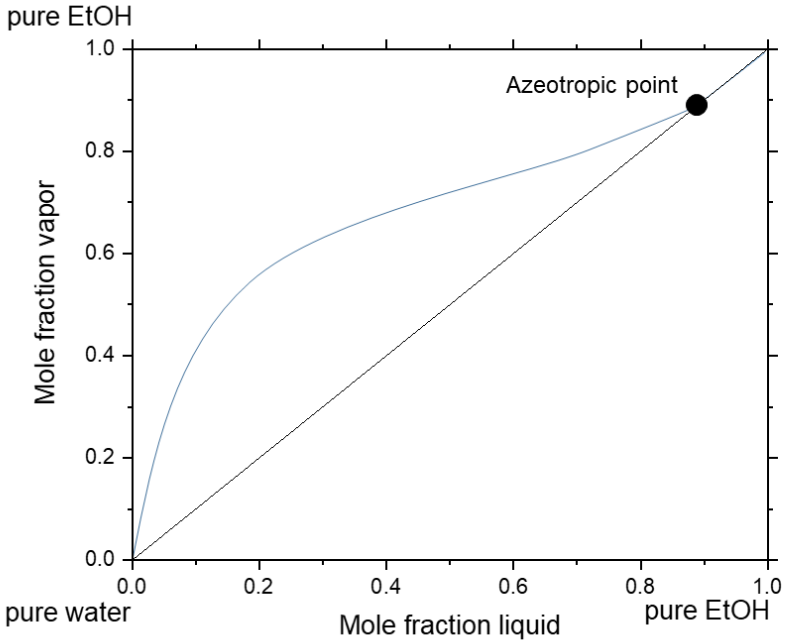


Figure 7: McCabe-Thiele diagram for a EtOH-water-mixture at ambient pressure, redrawn from [58].

In the figure above, the vapor-liquid-equilibrium of an ethanol-water mixture is shown. At a concentration with a low ethanol content, the vapor has a much higher ethanol concentration as the liquid. Consequently, the distillative separation is easily possible. With increasing ethanol concentration, the separation factor decreases and at the azeotropic point, at which the liquid and the vapor concentration are equal, separation is impossible. Hence, the McCabe-Thiele diagram is very important for the calculation and optimization of technical distillation processes.[58]

2.6.2. Rectification

For many processes, especially in systems with high boiling point differences, simple vacuum distillation provides acceptable results. But if substances are needed of high purity or in large quantities, distillation under rectification is recommended. Thereby, evaporation and condensation of a mixture are repeated continuously. In a rectification column, the produced vapor pressure is passed in countercurrent to the refluxing condensate. Due to the increased contact between vapor and liquid phase, an elevated

heat- and mass transfer occurs. Raschig rings or structured sheet metals expand the phase boundary. On these so-called plates, the equilibrium between the vapor and the liquid phase is adjusted. The concentration of the low-boiling component in the liquid reflux is greater and the concentration of the high-boiling component in the vapor phase is smaller. Thus, low-boiling substances will evaporate and high-boiling substances will condensate. How many theoretical plates, i.e. how many repeated distillations, are necessary for thermal separation depends strongly on the substances and on the operating conditions. It can be determined by using the McCabe-Thiele method.[58, 59]

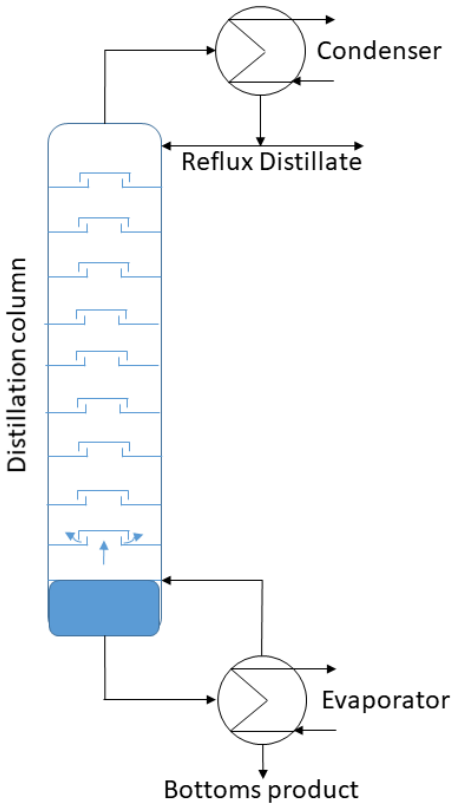


Figure 8: Schematic presentation of a rectification column.

Figure 8 shows the basic construction of a rectification plant. It consists of an evaporator at the bottom of the column, a rectification column and a condenser at the top of the column. A part of the condensate is taken as distillate and the other part is returned to the column as reflux. This reflux has two indispensable tasks: first, it has to enrich the

more volatile components at the top of the column to guarantee the vapor-liquid-equilibrium and secondly it has to remove the less volatile components to the bottom of the column.

To sum up, for the thermal separation of a mixture applying distillation under rectification the following criteria have to be fulfilled:[61]

- The composition of the vapor phase must differ sufficiently from the composition of the liquid phase.
- The mass transfer from one phase to the other phase must be fast.
- The components must be thermally stable.
- The boiling points of the respective components must differ from each other.

2.6.3. Molecular distillation

In the case of thermally very sensitive substances, a gentle separation process under reduced pressure must be applied. Molecular distillation is characterized by a high vacuum in the distillation column and a small distance between evaporator and condenser. The distance is less or at most equal to the mean free path of the molecules. At a vacuum around 10^{-3} mbar, this corresponds to a few centimeters. Thus, the distillation technique is also known as short-path distillation. A schematic presentation is given in Figure 9.

The liquid mixture, which has to be distilled, is spread on the heating surface in a thin, constantly circulated film. The low-boiling substances leave the evaporator as residue, whereas the high-boiling substances get in a short path to the condenser and are drawn off as distillate continuously.[60]

Due to high investment and operational costs, this technique is only used for particularly high-quality products, such as for perfumes.

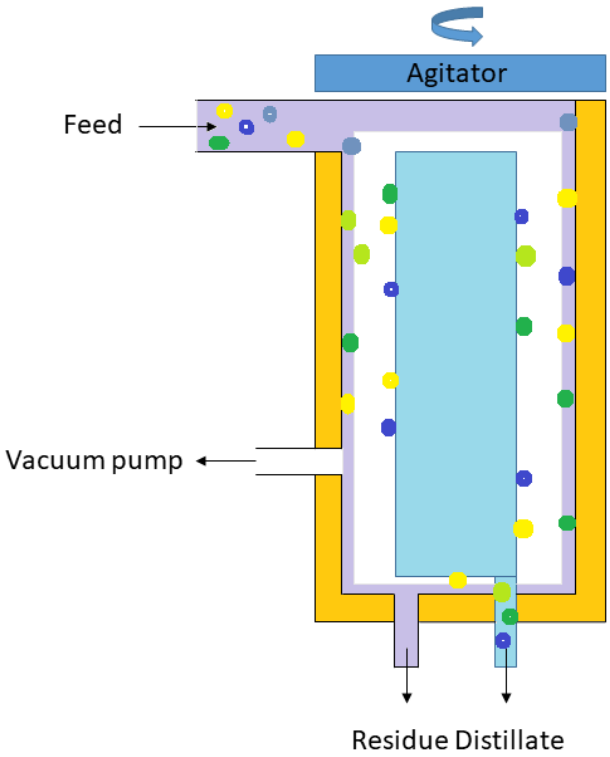


Figure 9: Schematic presentation of a molecular distillation apparatus.

2.7. Chromatography

2.7.1. Principles of chromatographic separation

2.7.1.1. Basics

Chromatography is the main technique for the separation of a plant extract in order to analyze the fragrance content or the presence of certain compounds, for instance. Chromatographic separation of chemical compounds is carried out by passing the mobile phase, containing the plant extract to be analyzed, through a stationary phase. The separation is based on different partitioning between the both phases. In general, chromatography can be divided depending on the state of aggregation of the mobile phase into liquid chromatography (LC, chapter 2.7.2) and gas chromatography (GC, chapter 2.7.3). In order to quantify and qualify the separated compounds, the process has to be coupled with an appropriate detection method.[62, 63]

2.7.1.2. Separation

Chromatography can be classified according to the mechanism of separation as: adsorption chromatography, partition chromatography, ion exchange chromatography, size exclusion chromatography and affinity chromatography. Thereby, the separation is always based on the continuous transition of the solute between the stationary and mobile phase.[64]

Van Deemter *et al.* considered that four spreading processes are responsible for the separation: multi-path dispersion, longitudinal diffusion, resistance to mass transfer in the mobile phase and resistance to mass transfer in the stationary phase.[65] In Figure 10, the Van Deemter equation and its associated curve is plotted. Parameter A and B correlates to the diffusion effects, whereas parameter C describes the equilibrium between mobile and stationary phase. In order to achieve a high separation performance, these terms must assume a low value. The larger the linear velocity u, the greater the diffusion, but the more hindered is the equilibration.[66] Consequently, the minimum of the curve indicates the optimal flow rate for GC and HPLC measurements.[67]

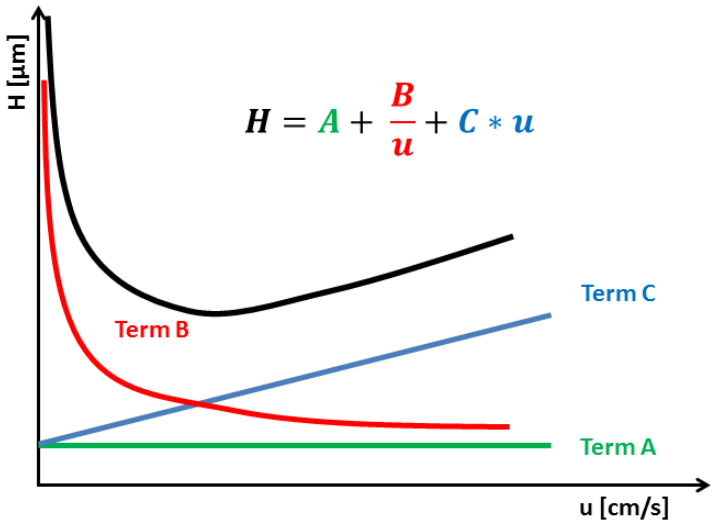


Figure 10: Presentation of the curve according to the equation of Van Deemter depending on the height of a theoretical plate H and the linear velocity u of the mobile phase.

2.7.1.3. Definition of a chromatogram

A chromatogram can be achieved by plotting the signal of the detector against the analysis time, as it can be seen in Figure 11. The most important parameters are the retention time of the compound $t_r(x)$ and the peak area from the associated peak. Substances can be assigned qualitatively considering the specific retention time by coupling an MS detector or through the injection of a reference sample.

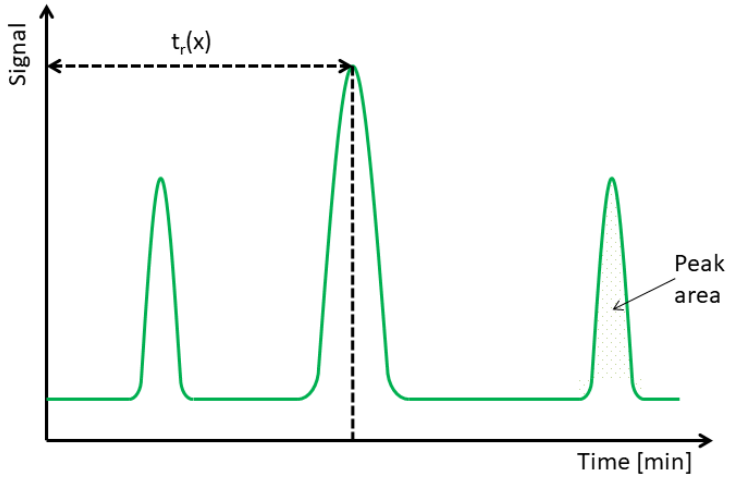


Figure 11: Typical chromatogram with the retention time of a compound ($t_r(x)$) and the integrated peak area.

Moreover, the chromatogram can be used to make quantitative statements. To this purpose, a calibration solution containing the analyte in a known concentration has to be injected. The peak area is proportional to the amount of the injected analyte.

2.7.2. High-performance liquid chromatography (HPLC)

A typical HPLC system, as it can be seen in Figure 12, includes a pump to push the mobile phase at moderately high pressure through the column containing an adsorption material (normal phase or reversed phase), an injector to insert the solution to be analyzed, and a detector with a data integration system.

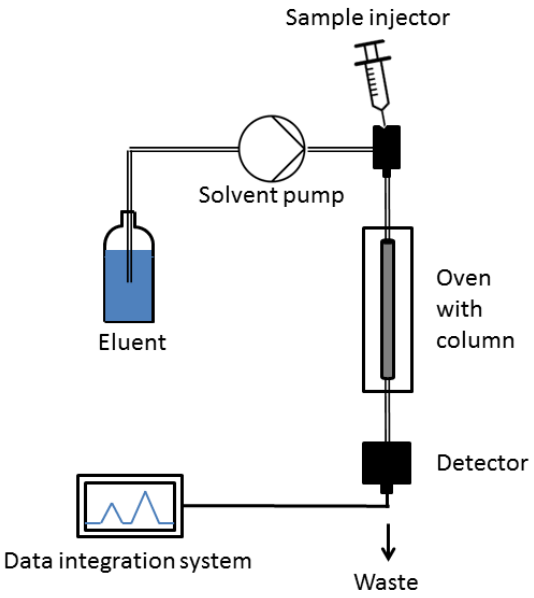


Figure 12: Simplified scheme of a HPLC system.

Most separations are carried out on reversed phase columns consisting of silica gel particles, which are chemically modified with nonpolar alkyl chains, usually C18. The choice of an appropriate solvent as mobile phase is decisive for the separation efficiency, since the technique of HPLC is based on the polarity of the analytes and their partition between the mobile and stationary phases. Solvent gradients can be used to improve the

resolution and separation. To reveal the presence of the analytes, ultraviolet detectors of fixed wavelength, dual wavelength or variable wavelength (photodiode array detector) are most frequently used. Other options are refractive index detectors, conductivity detectors, electrochemical detectors and evaporative light scattering detectors.[14, 64]

Although HPLC is used in the fragrance industry to examine also the non-volatile fractions of essential oils, for instance, it is much more widely used in other industries such as the pharmaceutical industry.[62]

2.7.3. Gas chromatography (GC)

Gas chromatography is one of the most important and widely used analytical techniques in the fragrance industry, as it is ideally suited for volatile compounds.

The chromatographic separation is based on the partition of analytes between the mobile phase (a gas such as helium, hydrogen or nitrogen) and the stationary phase. Substances which are increasingly located in the gaseous phase are transported faster than those which have a lower vapor pressure. The separation process is similar to fractional distillation, since the separation process is based on boiling point differences. By coating the inner capillary surfaces with different materials (internal diameter usually 0.1 – 0.5 mm, column length usually between 10 and 100 m), it is possible to adapt the separation properties. The basic design of a gas chromatograph can be seen in Figure 13.[68]

The most commonly used detector is the flame ionization detector (FID). Thereby, the eluted compounds are ionized in a hydrogen-air flame. To detect these ions, two electrodes are used to measure the current flow depending on the present amount of analyte. Furthermore, the FID detector can be combined with a mass spectrometer. With this coupling, almost all compounds in a complex mixture, such as an essential oil or a perfume, can be identified and quantified.[64, 68]

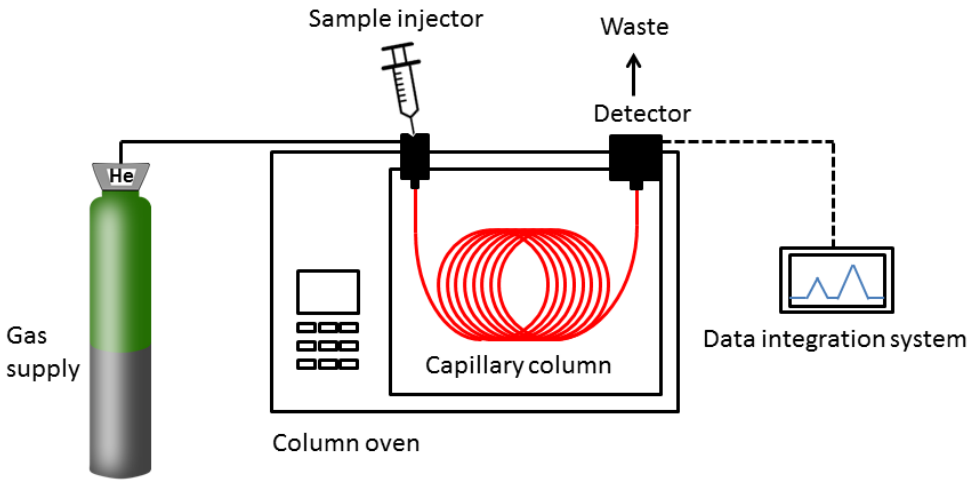


Figure 13: Simplified scheme of a GC system.

GC-sniffing, a coupling of gas chromatography and olfactometry, is also of high importance in the fragrance industry. This technique combines the chemical information of GC-MS with the sensory evaluation of the human nose.[69]

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3. Micellar extraction of *Iris germanica* L.

The results of this section are part of the European Patent EP 3 130 655 A1 “Process for Isolation of Odoriferous Agents” published by Theresa Höß, Marcel Flemming, Didier Touraud and Werner Kunz in 2017 (University of Regensburg).[1]

3.1. Introduction

The essential oil of *Iris germanica* L. is one of the most valuable natural products, called iris butter. Due to its high price (approximately 15.000 €/kg), it is used only in smallest doses in the perfume and cosmetic industry.[2] The iris butter is characterized by a violet-like scent. The main fragrance molecules are irones (*cis*-α-, *trans*-α-, *cis*-γ- and β-irone), which are not present in the rhizomes initially. These odorant compounds are formed by oxidative degradation of iridals during a long-lasting drying and aging process of the rhizomes.[3] Commonly, the iris butter is obtained by hydro distillation with an extraction yield of 0.1 – 0.25 %.[4] Drawbacks of the long-lasting hydro distillation are the high energy consumption, the often required wastewater re-distillation, and the high temperatures during the distillation, which often induce thermal degradation of the odorous compounds. Another possibility to isolate the desired irones is solvent extraction. But there, large amounts of flammable, volatile and often toxic solvents are required, which are responsible for environmental pollution and the greenhouse effect.[5]

Soaps are molecules having both a hydrophobic and a hydrophilic component. Therefore, an aqueous soap solution provides a suitable system to solve also nonpolar components from plant material without organic and hazardous solvents and thus, offers a convenient alternative to the conventional extraction methods. The aim of this research was to investigate whether it is possible to extract the desired irones with salts of the fatty acids, which are contained in iris rhizomes in non-negligible amounts. Substances extracted with this method can be safely used in foods or cosmetics.[6]

First of all, an analytical method to quantify the irone content in the rhizomes and in the extract was established. Then, various aqueous extraction mediums based on fatty acid

salts were tested to find the optimum parameters for a gentle and selective extraction of irones. Thereby, parameters such as the particle size of the rhizomes, the extraction time and temperature, the solid to liquid ratio, the concentration of the respective salt solutions, the influence of chain length of the soap surfactant as well as the influence of the counterion were examined. Moreover, the aim of this part of the thesis was also to find a strategy to isolate the desired irones from the extraction medium. Several approaches including the neutralization of the myristic acid, the precipitation of poorly soluble lime soaps, breaking the micelles by adding ethanol as well as the combination with hydro distillation were investigated and are described in the following. In cooperation with Phytotagante S.A.S. (France), a scale-up of this invented extraction method was explored.

3.2. Fundamentals

3.2.1. Iris germanica L.

Iris is the largest genus of the family *Iridaceae* with approximately 300 species. The main plants for perfume use are *Iris germanica* L., *Iris florentina* L. and *Iris pallida* Lam. and are domiciled in the whole Mediterranean area and south-west Asia. *Iris* was introduced in Germany as a popular garden flower with sword-shaped linear leaves. The plant needs only little maintenance and prefers medium moisture, well-drained soils in full sun. *Iris germanica* L. is flowering from May until July. The flowers have six petals with three upright petals and three hanging petals in many different colors including blue, pink, purple, reddish, white, yellow and bi-colors (compare Figure 14). *Iris germanica* L. grows up to 100 cm and 30 cm wide and is a hemicryptophyte with a thick, highly branched rhizome. The plant is sterile and reproduction occurs by rhizome division. The divided rhizomes are planted in fields in September at a distance of about 25 cm and 10 cm deep with the cut surfaces facing upward. Harvesting is done in July or August after two or three years of growing.[7-9]

Figure 14: Field of various iris species in Bavaria.

With 120 tons of produced dry *Iris germanica* L. rhizomes, Morocco is the leading global producer of iris rhizomes. In the past, Italy harvested more than 200 tons of rhizomes. But, in 2008, the farmers walked away from iris agriculture claiming unsustainable prices. This makes France a new reliable source of quality material with 40 tons per year. Finally, new players appear also in Bavaria, Germany, because of its high demand and profitability. With 100 tons of iris, China also represents an important supplier but the quality is often assessed as inferior.[10]

3.2.2. Ingredients of the rhizomes

The rhizomes of *Iris germanica* L. find application mainly in the perfume and cosmetic industry (see 3.2.3), but are also used for the aromatization of food such as fine liqueurs like Benediktiner or Danziger Goldwasser. In the USA, iris rhizomes are added to the fermentation process of tobacco leaves to flavor cigarettes. Over time, the pharmacological application of iris rhizomes have become less important.[7]

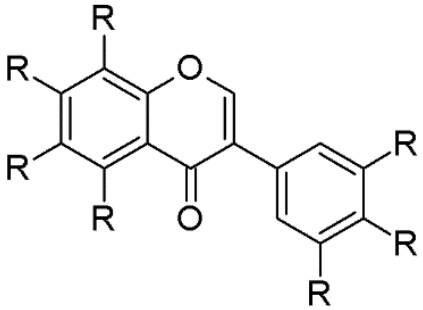


Figure 15: Basic chemical structure of isoflavone, c.f. Figure 1.

The rhizomes consist largely of carbohydrates with 20 – 50 % of starch. Other ingredients are resins (< 3 %), tanning agents and waxes.[7] Isoflavones build another important class of secondary metabolites contained in iris rhizomes (see Figure 15 for the basic chemical structure). Ten different isoflavones with a content around 15 % are documented.[2, 11, 12] Especially irigenin is abundantly present in various *Iris* species. The amount of other isoflavones, like irilone, irisolon, and iriflogenin, is lower and varies according to the

species. These plant dyes are secondary phytochemicals and defend the plant against pathogens. They are widely examined for their ability to provide health benefits.[13] Isoflavones contained in iris rhizomes are chemopreventive, anti-bacterial and anti-inflammatory.[2, 14] Other phenolic compounds like acetovanillone, piceol, protocatechuic acid and sinapinic acid are also found in iris rhizomes.[7, 15, 16]

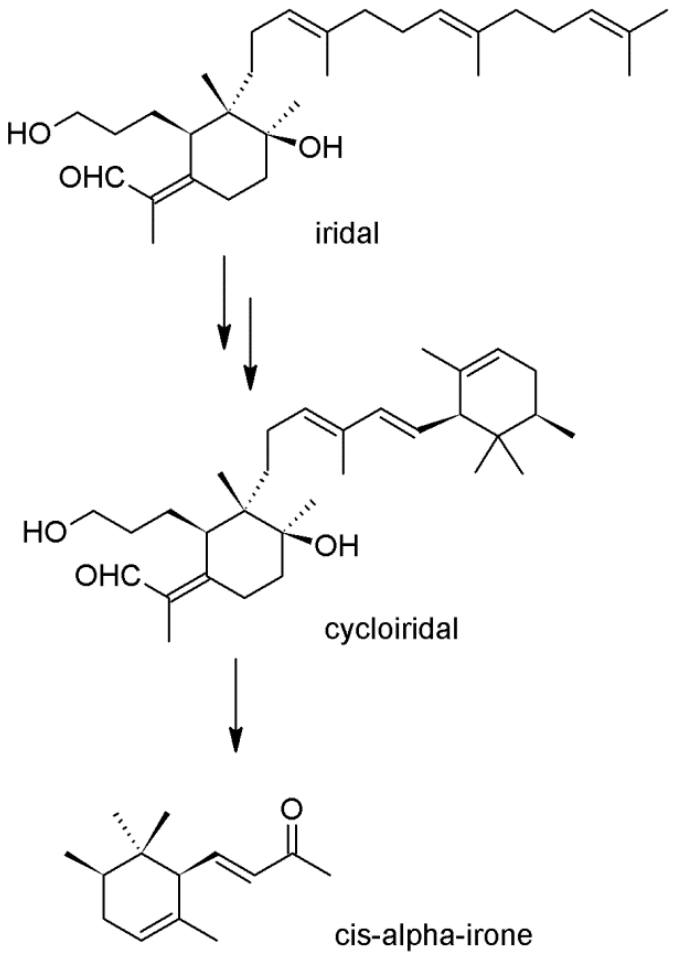


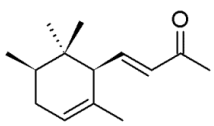
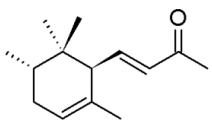
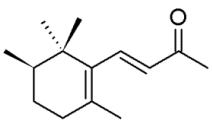
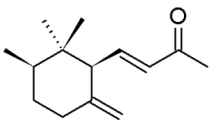
Figure 16: Transformation of iridals to irones exemplified by *cis*-alpha-irone.

Iris germanica L. and also *Iris pallida* Lam. rhizomes contain up to 1 % iridals related to the fresh weight.[3, 7] The iridals, a class of triterpenoids, are present in many different variations, like monocyclic and bicyclic or esterified with fatty acids. 70 % are present as free iridals in fresh rhizomes. The ecological background of iridals in iris rhizomes is still not clearly known. The amphiphilic molecules are very bitter tasting, and it can be

assumed that they may act as deterrents.[3] Moreover, Marner and Kept assumed that the iridals have the task to prevent dehydration and to produce a protective polymer during wounding.[17] The total amount of iridals is the highest in young rhizomes, whereas the amount decreases slightly with age.[4] After the harvest and drying of the rhizomes, an oxidative degradation of the iridals occurs with time, as it can be seen in Figure 16. These irones are the most important and valuable ingredients in iris rhizomes due to their pleasant smell. They can be described as softly sweet, warm and reminiscent with slight woody undertones. Due to the different isomeric irone distribution in *Iris pallida* Lam. and *Iris germanica* L. rhizomes, a difference in smell can be observed. In *Iris pallida* Lam. rhizomes, *cis*-γ-irone predominates, and the scent is described as powerful, woody, floral and powdery. *Cis*-α-irone is the predominant isomer in *Iris germanica* L. Here, the iris butter has a much fruiter note and offers a more diverse range of flavors.[10]

The formation of the cycloiridals is initiated by the addition of an active CH_3 group to the terminal double bonds of iridals.[3, 18, 19] The reaction does not lead to the formation of a single irone isomer but to a mixture of three regioisomers α, β, and γ. The isomeric distribution of the irones is dependent on the iris species from which they have been isolated. Mainly the *cis*-isomers are formed, whereas the *trans*-isomer can be found only in minor amounts. The isomeric distribution of the irones contained in *Iris germanica* L. and *Iris pallida* Lam. with the corresponding odor is given in Table 4.[2, 20]

Table 4: Isomeric distribution of irones in *Iris germanica* L. and *Iris pallida* Lam. rhizomes.

	Proportion of irone isomers (% of total irone content)			
	 cis-alpha-irone	 trans-alpha-irone	 beta-irone	 cis-gamma-irone
***Iris germanica* L.**	61	1	1	37
***Iris pallida* Lam.**	34	5	0	61

Odor description	very intense and rich odor, woody, ionone, floral, dry, fruity	floral, ionone, violet chamomile effect, sweet	iris family, sweet, anis, liquorice, green	woody, ionone, sweet, dry, floral

Several technological attempts have been made to accelerate the transformation of iridals to irones. Buono *et al.* established a method to oxidize iridals by treating the rhizomes with $KMnO_4$ in an organic solvent (FR-2 620 702, 24/3/89). Moreover, Baccou *et al.*[21] patented a proceeding with ionizing radiation. Bioconversions of lipophilic extracts with fungi or peroxidizing enzymes have been proposed by Gil *et al.*[22, 23] and bioconversions by bacteria in the presence of a plant cell medium by Belcour *et al.*[24] In addition, treatment of the rhizomes with nitrite salts was invented by Ehret *et al.*[25] However, the mentioned methods are not implemented in practice. To produce pure and natural, organic certifiable essential oils, the rhizomes must be distilled without prior treatment.[26] A good alternative oxidation method without chemicals or enzymes represents the recently patented method of Flemming.[27] Storing the rhizomes under an atmosphere containing oxygen, an elevated pressure and an elevated temperature leads to an increase of irones within days to weeks to a level higher than in conventionally altered rhizomes.

These irones are the most important and valuable ingredients in iris rhizomes due to their pleasant smell. They can be recovered by hydro distillation of the dried and ground rhizomes (see 3.2.3). The essential oil obtained this way, called iris butter, contains about 15 % of irones.[2] Other ingredients are discussed in the following chapter in more detail.

3.2.3. Iris butter

Iris butter is produced by distilling the iris rhizomes in water. To be suitable for distillation, the rhizomes must be stored for at least three years to guarantee that the irones are formed completely by oxidative degradation of the precursor molecules. Freshly

harvested rhizomes contain very low concentrations of irones, whereas 50 % of the irone content is formed during the first six months of storage. The irone formation rate then slows down to reach a final irone content of about 340 mg/kg dried rhizomes after the fourth year, for *Iris germanica* L. rhizomes. Further storage of the rhizomes only gives an increase of 10 % irones. Therefore, rhizomes are usually stored between two and four years before distillation.[26] Regarding *Iris pallida* Lam. rhizomes, the amount of irones per dried rhizomes is higher with around 640 mg/kg.[28]

After an adequate storage time, the rhizomes are milled and distilled for 36 h. Often the rhizomes are soaked in aqueous acetic acid solution over night before hydro distillation to gain a better access to the valuable ingredients.[4] With this long-lasting process, 0.1 – 0.25 % essential oil can be achieved.[7] Irones, with a content around 15 %, are the most relevant group of odorous compounds in the essential oil. But other ketones like acetophenone, acetovanillone, and acetoveratrone are also partly responsible for the attractive smell. Benzaldehyde, furfural, guajen and naphthalene contribute to the scent in traces. Due to its buttery consistency, the extract is called iris butter. It is a pale yellow mixture with a melting point around 40 °C.[7] Iris butter contains a large amount of myristic acid and other fatty acids, as it can be seen in Table 5.[29] Partially esterified fatty acids are also found in iris butter.[4]

Table 5: Composition of fatty acids contained in *Iris germanica* L. rhizomes.[29]

Fatty acid (carbon atom:double bond, position of the double bond in the chain)	Relative proportion [%] (in sum 100 %)
Caprylic acid (8:0)	1.5
Capric acid (10:0)	2.3
Lauric acid (12:0)	3.2
Myristic acid (14:0)	47.7
Palmitic acid (16:0)	13.3
Linoleic acid (18:2 n-6)	14.0
α-Linoleic acid (18:2 n-3)	4.1
Oleic acid (18:1 n-9 cis)	4.7

Elaidic acid (18:1 n-9 trans)	3.4
Stearic acid (18:0)	2.0
Arachidic acid (20:0)	2.0
Behenic acid (22:0)	1.6
Lignoceric acid (24:0)	Traces

The price of iris butter containing about 15 % irones is about 15.000 €/kg.[2] Through the addition of natural myristic acid, it is possible to achieve irone standardization. Iris butter has a long durability and is perfectly soluble in vegetable oil as well as in alcohol.

Moreover, it is feasible to produce essential oils with an irone content of 80 % and higher. This is referred to iris absolute, which is traded at prices up to 100.000 €/kg.[30] The starting material to produce iris absolute is the iris butter obtained by hydro distillation with a high amount of myristic acid. To concentrate the desired irones, the iris butter is dissolved in ethanol. By cooling the solution to -20 °C, the fatty acids precipitate and can be removed by filtration. After evaporation of the solvent, an amount of around 20 % of the initial butter is achieved. However, the irone content amounts to 80 % compared to the initially approximately 15 %. Also, distillation or vacuum rectification of the iris butter can be done to eliminate the fatty acids. The received highly odorant yellow liquid represents one of the most expensive products for perfumery, priced five times more than gold.[10]

Iris butter is widely used in the perfume industry. Especially, it is contained in luxury perfumes like Dior Homme™ (Dior), Iris Silver Mist™ (Serge Lutens), Insolence™ (Guerlain), Chanel No. 5™ (Chanel), and No. 1 Iris™ (Prada).[31] Among more than 200 known species of the genus *Iris*, only the rhizomes of *Iris pallida* Lam. and *Iris germanica* L. are primarily used for odorant and flavoring applications in perfume and cosmetic industry.[4] Synthetically produced irones do not satisfy from a sensory point of view. Several synthesis have been reported in literature, but none of them has found practical application. One possible way to produce racemic irone isomers is the acetic catalyzed cyclisation of methyl-3-pseudo ionone, yielding mainly α-irone and in minor quantity β-irone.[19, 32]

3.2.4. Iris resinoid

An alternative way to gain the desired irones is to extract iris rhizomes with solvents. The produced extract is called resinoid. Using a volatile solvent mixture such as *n*-hexane/ethyl acetate, yields an extract outcome ten times higher than the essential oil obtained by hydro distillation. The resinoid is much less expensive, and its odor is quite different. It is described as chocolate, woody, leathery and grassy.[2] Resinoids are pasty masses at room temperature and brown in color. The irone content is usually between 1 – 3 %. The total extraction yield with respect to the mass of dried rhizomes is 3 – 5 %.[10] The solvent extract contains both, volatile and non-volatile compounds in contrast to the iris butter. Thus, the resinoid is also rich in flavonoids. Flavonoids have very interesting pharmacological activities, as they are chemopreventive, anti-bacterial and anti-inflammatory, for instance.[2]

Nevertheless, solvent extraction is always associated with the risk of handling large quantities of combustible and often toxic solvents. Solvent residues in the resinoids can often not be prevented. Moreover, the high temperature, which is necessary to remove the solvent, can induce thermal degradation of the sensitive fragrance compounds as it also may occur during hydro distillation. To avoid the drawbacks of conventional extraction methods, an alternative and green method to gain fragrance molecules from plant material was examined and is presented and discussed in the following.

3.3. Results and discussion

3.3.1. Analytics

3.3.1.1. Identification of compounds

The easiest way to examine the presence of irones in the extract is to apply thin-layer chromatography (TLC). For visualizing the spots, an anisaldehyde/sulfuric acid solution was sprayed onto the plate. The irone isomers became visible in a dark violet color. This fast screening was carried out due to its simplicity and its possibility to make first predictions about the extraction efficiency.

Because irones are volatile and UV/VIS light absorbent compounds, they can further be analyzed by GC-FID and HPLC-UV. A typical chromatogram from a Soxhlet extract with methanol measured by HPLC-UV and GC-FID can be seen in Figure 17 and Figure 18 below (irones are marked with “4”).

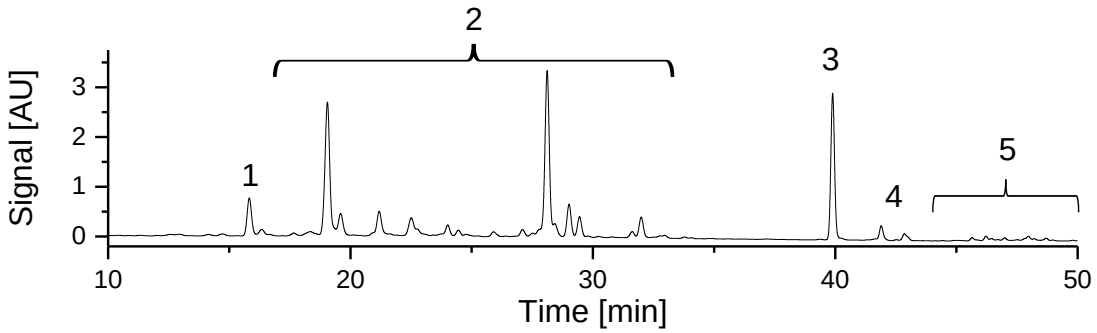


Figure 17: HPLC-UV chromatogram of a Soxhlet extract with methanol obtained from iris rhizomes (see description of extraction and HPLC-UV method in Experimental p. 117 and p. 125, respectively). Peak assignment: (1) acetovanillone, (2) flavonoids, (3) internal standard α-ionone, (4) irone isomers and (5) iridals and iridal esters.

HPLC-UV is recommended for extracts, which contain volatile and non-volatile compounds. The only requirement for HPLC measurements is that the sample is soluble in an appropriate solvent and the analytes are detectable. In Figure 17, it can be seen that the Soxhlet extract with methanol contains a large number of compounds identifiable by a lot of peaks. By comparing the retention times of the pure substances and including

literature data, a classification of the peaks was possible.[29] The retention time, according to the instrumental method described in the Experimental section 3.5.11.2, of acetovanillone (1) was determined to be 16.5 min. Between 18 and 37 min flavonoids (2) are eluted. The internal standard α-ionone (3) and the irone isomers (4) are followed at 40 min, respectively 42.5 min. Iridals and iridal esters (5) are eluted at higher retention times.

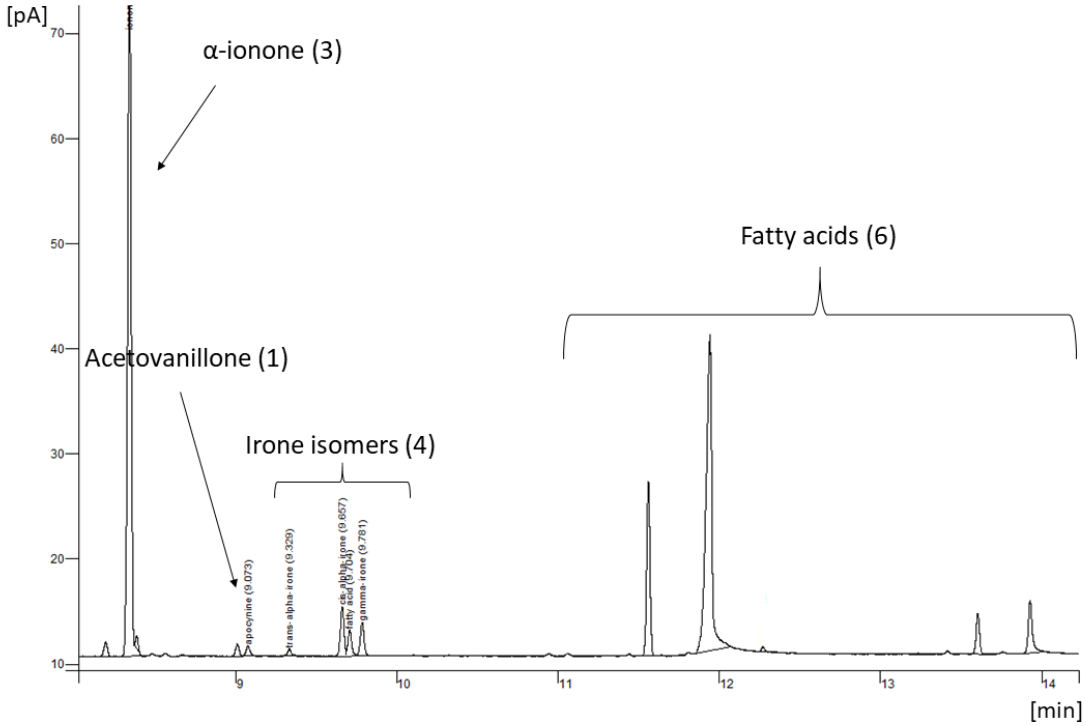


Figure 18: GC-FID/MS chromatogram of a Soxhlet extract with methanol obtained from iris rhizomes (see description of extraction and GC-FID/MS method in Experimental p. 117 and p. 124, respectively). Peak assignment: (3) internal standard α-ionone, (1) acetovanillone, (4) irone isomers and (6) various fatty acids.

By applying GC-FID/MS, only undecomposed volatile compounds can be detected. But this method is also suited to determine and identify fatty acids, which could not be detected via HPLC-UV. In Figure 18, a GC-FID chromatogram of a Soxhlet extract with methanol is shown. The assignment of the peaks was done with the coupling of a MS detector. The first eluted compound, according to the instrumental method described in the Experimental section 3.5.11.1, is α-ionone (3) at 8.3 min. This compound is not contained preliminary in the extract, but is added as internal standard for irone quantification.

Acetovanillone (1) can be found at 9.0 min, whereas the irones (4) are eluted around 9.6 min. Various fatty acids (6) are eluted afterwards. Myristic acid, which is the fatty acid with the highest content in the rhizomes, is eluted at 12.3 min.

This comparison demonstrates that with HPLC-UV it is possible to analyze more compounds and as a result, better conclusions about the selectivity of the extraction can be made. Therefore, HPLC-UV measurements were primarily applied to investigate the selectivity of the micellar extraction. The obtained yields of irones were almost equivalent using HPLC-UV and GC-FID. Nevertheless, to ensure better comparability of the individual extractions, the irone yields were largely determined by GC-FID/MS. Thereby, the results could be achieved faster.

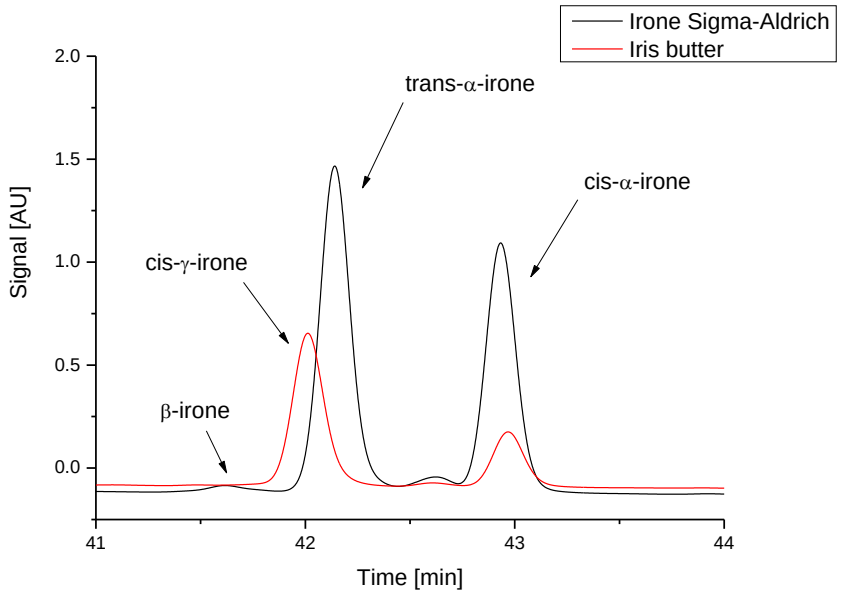


Figure 19: HPLC-UV chromatograms of irones obtained from Sigma-Aldrich and the reference iris butter (performed as described in Experimental p. 125).

The irones contained in iris rhizomes always consist of various isomers. Also, the synthetic irones, purchased from Sigma-Aldrich, consist of different isomers mainly *trans*-α-irone and *cis*-α-irone. By comparing the HPLC-UV chromatogram of the irone mixture from Sigma-Aldrich with the HPLC-UV chromatogram of an iris butter from *Iris germanica* L.

rhizomes, it was possible to identify the different isomers (see Table 6 and Figure 19). First of all, the β-irone was eluted, which was difficult to detect. Because of its low presence in iris rhizomes, this isomer was not included in the determination of the total irone yield. The retention time of *cis*-γ-irone was determined to be 41.98 min. This isomer was present in the reference iris butter but not in the technical mixture. *Trans*-α-irone, which only exists in the technical mixture, was eluted at 42.20 min. At 42.98 min, the peaks of *cis*-α-irone were overlapping. Therefore, *cis*-α-irone was present in the iris butter as well as in the irone standard. For the irone quantification of the extracts, the peak areas of *cis*-γ- and *cis*-α-irone were summed up.

Table 6: Assignment of the irone isomers in relation to the retention times achieved via HPLC-UV (as described in Experimental p. 125).

	Retention time
***cis*-γ-irone**	41.98 min
***trans*-α-irone**	42.20 min
***cis*-α-irone**	42.98 min

The assignment of the irone peaks applying GC-FID/MS was much easier, because of the coupling with a mass spectrometer and matching with the NIST data base (see Table 7). The retention times of the isomers differ in comparison to the HPLC chromatogram. In a HPLC chromatogram, the sequence is *cis*-γ-irone, *trans*-α-irone, and *cis*-α-irone with increasing retention time. Against that, in a GC-FID chromatogram trans-α-irone occurs first, followed by *cis*-α-irone, and *cis*-γ-irone.

Table 7: Assignment of the irone isomers in relation to the retention times achieved by GC-FID/MS (as described in Experimental p. 124).

	Retention time
***trans*-α-irone**	9.3 min
***cis*-α-irone**	9.6 min
***cis*-γ-irone**	9.8 min

At this point, it should be mentioned again that the isomeric distribution of irones in *Iris germanica* L. and *Iris pallida* Lam. rhizomes differs (compare Table 4, 3.2.2). The *cis*-α-irone peak is always higher regarding *Iris germanica* L. rhizomes, whereas the *cis*-γ-irone peak is higher considering *Iris pallida* Lam. rhizomes. Thus, by comparison of the peak heights, a distinction of the rhizomes can be made.

3.3.1.2. Validation method

For HPLC-UV as well as GC-FID/MS, internal calibration was applied. Therefore, α-ionone, which is not present in the rhizomes, was used as internal standard. Chemically, it is very similar to the irones possessing only one methyl group less (compare Figure 20).

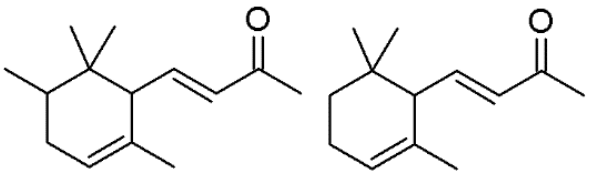


Figure 20: Chemical structure of irone (left) and the corresponding internal standard α-ionone (right).

To determine the response factor, a multipoint calibration was performed, and the ratio of the irone peak area (sum of the isomers) and α-ionone peak area against the ratio of irone concentration and alpha-ionone concentration was plotted (see Figure 21 for internal calibration using HPLC-UV and Figure 22 for internal calibration using GC-FID). The slope of the linear function represents the response factor (K), which is calculated to be K = 1.10 in the case of HPLC-UV and for GC-FID/MS K = 1.11.

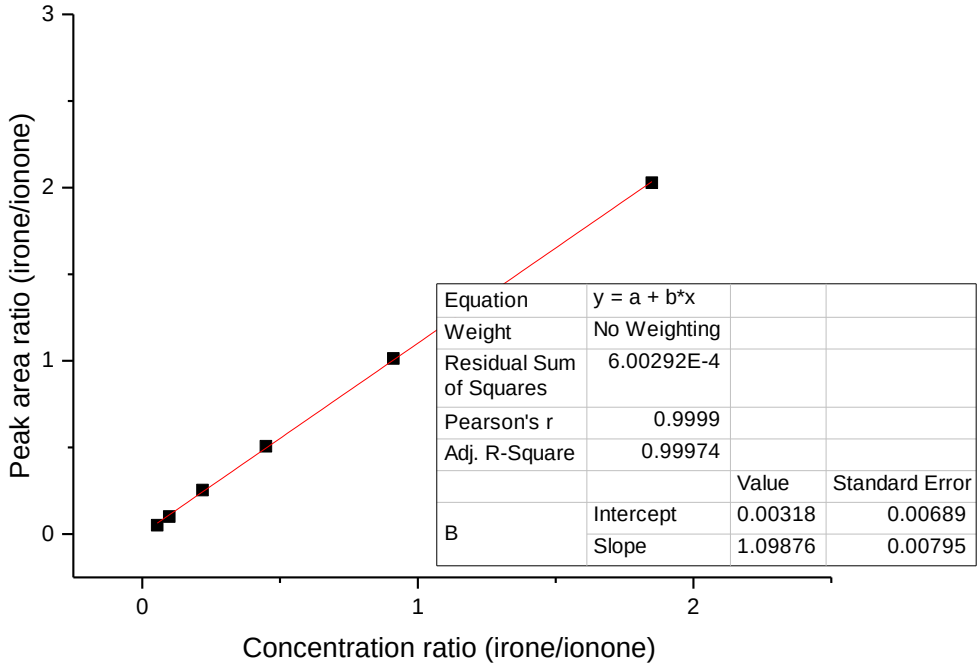


Figure 21: Determination of the response factor for internal standard calibration using HPLC-UV (as described in Experimental p. 125).

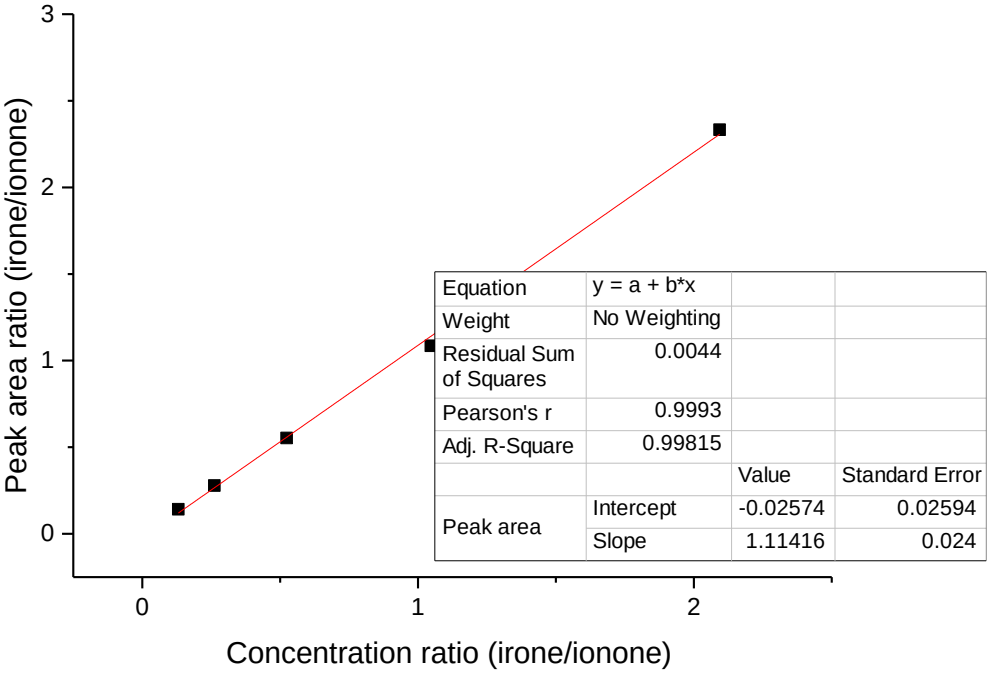


Figure 22: Determination of the response factor for internal standard calibration using GC-FID/MS (as described in Experimental p. 124).

For the analysis of the extracts, α-ionone was added to each sample in a known concentration. The amount of the desired irones was calculated according to the following equation:

$$m_{irones} = K * \frac{a_{irones}}{a_{ionone}} * m_{ionone}$$

where m_{irones} = mass [mg] of the irones, K = response factor of internal calibration, a_{irones} = peak area of the sum of the irone isomers, a_{ionone} = peak area of the internal standard, m_{ionone} = known mass [mg] of the internal standard.

3.3.1.3. Methylation of fatty acids

Usually, transesterification is applied to evaluate the fatty acid composition of glyceride fats and oils by gas chromatography. To this purpose, the triglycerides were converted into volatile fatty acid methyl esters by base catalyzed transesterification. Moreover, it is possible to convert free fatty acids which are present and interfere in the sample into their corresponding methyl esters. Trimethylsulfonium hydroxide (TMSH) is a very elegant and convenient methylation reagent.[33] It is just necessary to add the reagent to the sample solution. Removal of excess reagent is not required, since pyrolysis to methanol and dimethyl sulfide occurs in the injector of the gas chromatograph at 250 °C.[34] Thus, the only by-products are volatile and decompose during the reaction.

In Figure 23 (above), a GC-FID/MS chromatogram of pure myristic acid can be seen. The main fatty acid peak appears at a retention time around 12 min. More determining is the fact that around 9.7 min, an impurity of myristic acid is detectable. Comparison with the NIST database showed that this impurity refers to lauric acid, which is present in small amounts in the myristic acid purchased from Roth (Germany, purity ≥ 98 %). Considering a mixture of myristic acid and the technical mixture of irones, which consist mainly of *trans*-α-irone and *cis*-α-irone (see Figure 23 below), it can be seen that the impurity peak is nearly overlapping with the irone isomer. *Iris germanica* L. extracts consist mainly of *cis*-

α- and *cis*-γ-irones. *Cis*-γ-irone appears at 9.8 min and thus elutes very close to the impurity. In the case of a high content of myristic acid, this impurity is completely overlapping with the irone isomer as it can be seen in Figure 24. Therefore, a quantification of the irones was not further possible.

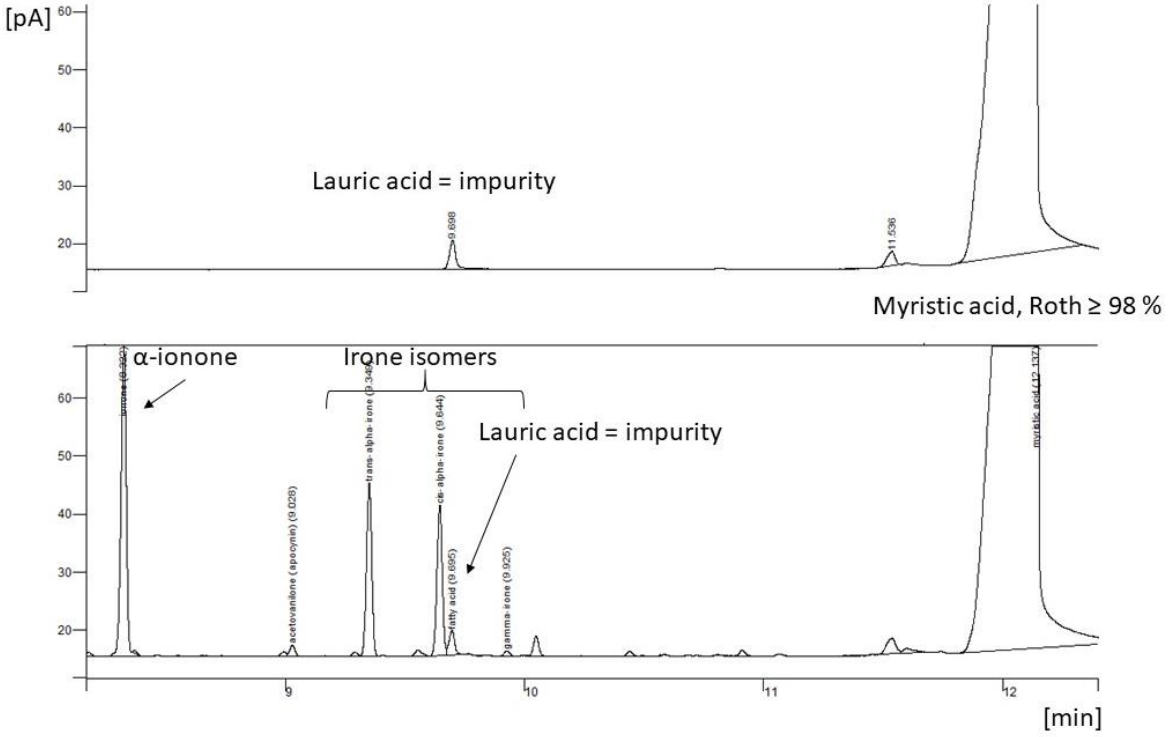


Figure 23: GC-FID/MS chromatogram of pure myristic acid (above) and a mixture of myristic acid, ionone and irones (technical mixture from Sigma-Aldrich) (below), performed as described in Experimental p. 124.

Changing the temperature program of the GC method to extend the separation failed. A modification always caused the disappearance of one isomer. Therefore, the methylation reagent TMSH was added to samples of high myristic acid content. This method was mainly applied carrying out the combination of micellar extraction with hydro distillation (see 3.3.8.5). Thereby, short-chained fatty acids are preferably distilled and thus occur in higher proportions. The impurity peak disappears by esterification of the contained free fatty acids in the range of the irone isomers. As a consequence, quantification was feasible without any effort.

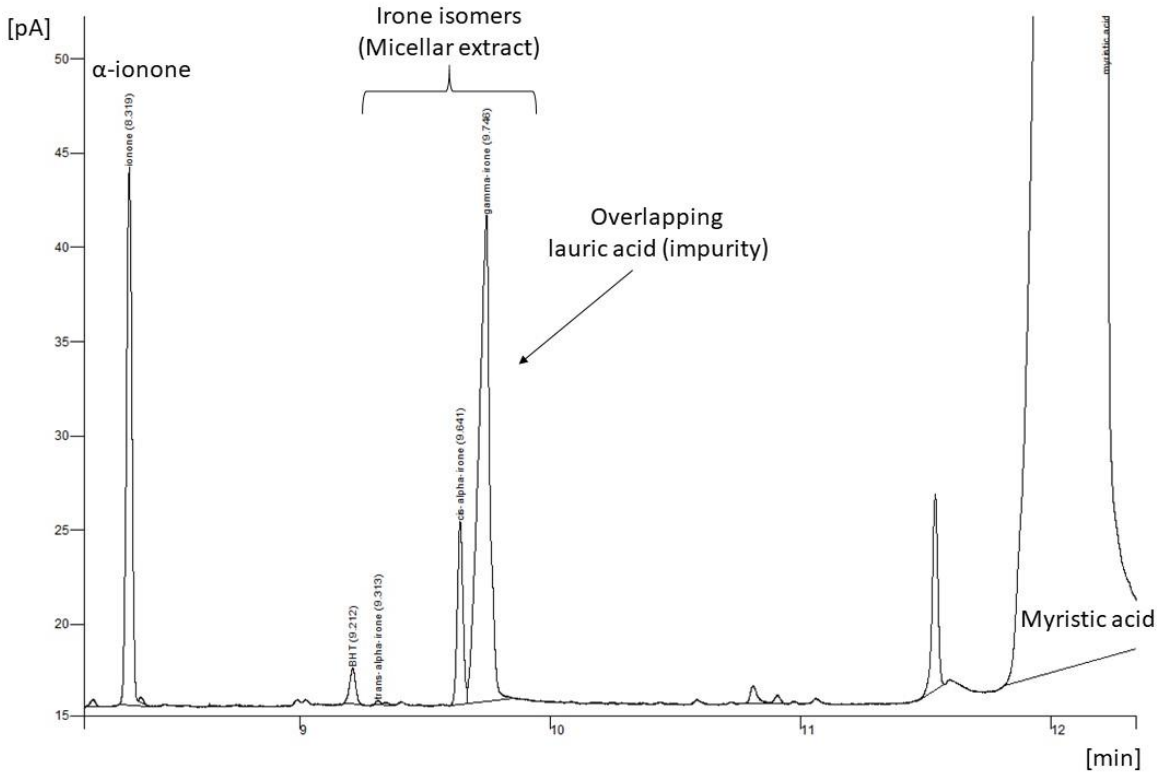


Figure 24: GC-FID/MS chromatogram of a micellar *Iris germanica* L. extract including ionone and myristic acid, performed as described in Experimental p. 124.

3.3.2. Determination of the maximum irone content

A solid/liquid extraction with methanol was performed in triplicate to determine the total irone content of iris rhizomes (n = 3). This method is already known in literature, where Soxhlet extraction for 6 h is applied.[26] Considering that the extraction was exhaustive, the irone content in the rhizomes was calculated to be 290.5 ± 5 mg/kg for *Iris germanica* L. rhizomes and 640.0 ± 40 mg/kg for *Iris pallida* Lam. rhizomes (see Table 8). The concentration is expressed in mg irones per kg dried rhizomes. These values were used as reference to examine the efficiency of the micellar extractions. *Iris germanica* L. rhizomes contain 56 % *cis*-α-irone, 38 % *cis*-γ-irone and 6 % *trans*-α-irone. *Iris pallida* Lam. rhizomes have a composition of 35 % *cis*-α-irone, 59 % *cis*-γ-irone and 6 % *trans*-α-irone determined by HPLC-UV, respectively GC-FID.

Table 8: Irone content and isomeric distribution of *Iris germanica* L. and *Iris pallida* Lam. rhizomes determined by Soxhlet extractions with methanol (n = 3), as described in Experimental p. 117.

	***Iris germanica* L. (3 years)**	***Iris pallida* Lam. (9 years)**
Total irone content	290.5 ± 5 mg/kg	640.0 ± 40 mg/kg
***cis*-α-irone**	56 %	35 %
***cis*-γ-irone**	38 %	59 %
***trans*-α-irone**	6 %	6 %

3.3.3. Optimization of the micellar extraction procedure

The easiest way to extract plant material is the maceration process. Thereby, the dried and powdered plant material is soaked in an appropriate solvent for several hours until days. In a first experiment, this procedure was applied to iris rhizomes. For this purpose, a micellar extraction medium consisting of aqueous sodium myristate was added to the ground rhizomes. It was necessary to work at slightly increased temperatures to ensure the solubility of the fatty acid salts in water. The mixture was stirred to improve the mass transfer of the desired molecules.

To determine the extraction yield, the irones had to be isolated from the extraction medium. To this purpose, the plant material was separated from the soap solution by centrifugation and the aqueous phase was re-extracted with an organic solvent. After solvent evaporation, the remaining compounds were analyzed via GC-FID/MS or HPLC-UV using internal calibration (see experimental section p. 124 and p. 125, respectively). Further isolation methods, which are also in agreement with Green Chemistry, are examined later in 3.3.8.

Often, the maceration process is combined with ultrasound radiation. Ultrasound can enhance the extraction efficiency and reduce the extraction time. The generated high shear forces break up the cell walls and increase the mass transfer.[35] However, the radiation indicated no improvement of the here investigated irone extraction compared with stirring for the same time. Probably most of the cells are already broken because of the drying and grinding process of the rhizomes. Therefore, ultrasound can only be seen

as a substitute for stirring and is better suited for fresh plant material than for dried rhizomes.

Microwave-assisted extraction was carried out using a SP Discover (CEM) with 100 W. Irradiated microwaves lead to the heating of the solvent and plant material, which increases the kinetic of the extraction process.[36] As the extraction of irones is already completed after a short time (see 3.3.6.2), no improvement using microwave power could be determined.

A further commonly used extraction method is the percolation process.[37] To this purpose, the plant material is mixed with sea sand in a percolation column with a glass frit and rinsed repeatedly with solvent driven by gravity until all desired molecules have been extracted. The aim of the percolation process was to reduce the amount of soap solution compared to the amount needed during the maceration process. Both extraction methods depend on the basic principle of diffusion. Thereby, percolation has the advantage of a continuous flow of fresh and unsaturated solvent. This process can lead to an exhaustive extraction. Compared to the maceration process, a further advantage of this technique is a shorter processing time because no additional filtering step is necessary. However, some drawbacks have been observed. First of all, a special equipment was needed to percolate the aqueous sodium myristate solution at increased temperature. Moreover, it was shown that the rhizomes have to be soaked for a certain time before the solvent passes through. Due to the swelling of the rhizomes a lot of sea sand was required and the soap solution still dripped slowly. It was not possible to accelerate the percolation using a vacuum pump due to the strong foaming of the soap solution. After a certain amount of solvent passed through the column, the yield of irones was stepwise determined by HPLC-UV. To get a comparable extraction yield, the amount of required solvent was at least as high as the amount used by maceration. Additionally, the procedure was complex and time-consuming. Commonly, the advantage of percolation is that volatile solvents are used, which can be recycled by evaporation and pass through the column several times (see 2.3.2.2). This recycling step is not working with aqueous soap solutions and thus no reducing of extraction medium was possible within this process.

To sum up, with an additional extraction force like ultrasound or microwave, it was not feasible to increase the extraction yield of the desired irones. A pressurized percolation process showed no improvement of extraction yield either. Therefore, a simple maceration process was used for extracting iris rhizomes in all further experiments.

3.3.4. Influence of the pH value on the extraction of irones

As it is known that soaps have a high pH-value, the stability of the irones was investigated to ensure that they do not decompose during the extraction. The pH value of the mainly used sodium myristate solution (c = 0.1 mol) was determined to be approximately 9.7.

First of all, the influence of the pH value was tested solely on the fragrance compounds without the plant matrix. Because of the chemical similarity to irones and the presence of only one spot applying thin-layer chromatography, α-ionone was used for this examination. A known amount of α-ionone was added to samples of different pH-values (adjusted with HCl and NaOH) and stirred for a certain time at increased temperature.

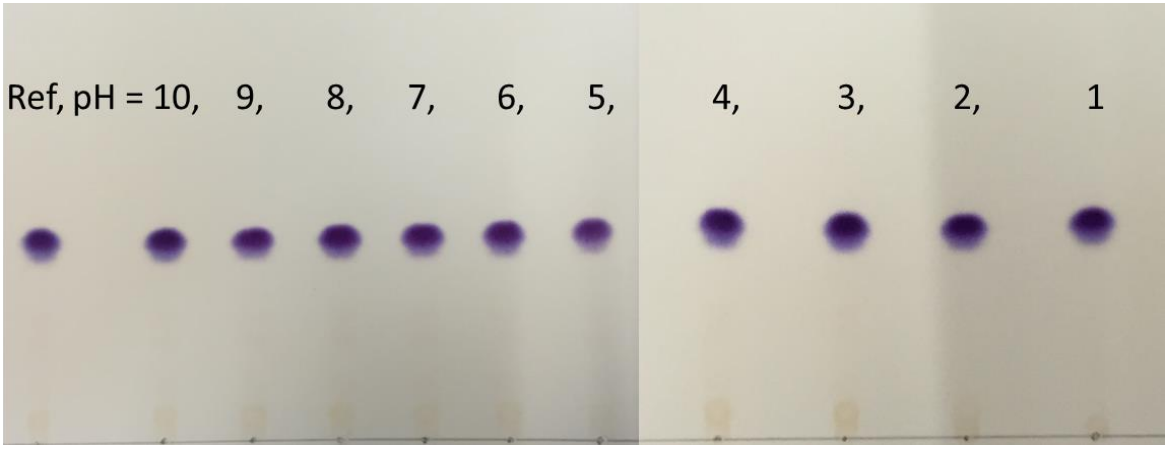


Figure 25: pH stability of α-ionone determined by TLC (as described in Experimental p. 125).

In Figure 25, the developed TLC plates of the stability tests are shown. By comparing the individual spots with the reference, it can be assumed that no decomposition of the α-ionone molecules occurs. Otherwise, further spots must be visible. Consequently, no

alteration of the irones during the extraction process can be expected. In addition, changes of the pH value do not show any influence on the irone stability.

In further tests, extractions of iris rhizomes with an aqueous sodium myristate solution (NaC14) (c = 0.1 mol/L, pH = 10), a sodium hydroxide solution (c = 1 mol/L, pH = 10) and pure water (pH = 6) was carried out under the same conditions (n = 3) in order to check the influence of the pH value on the extraction efficiency. The aim was to determine, if only the high pH value causes the extraction of irones or the micellar media is responsible for the irone recovery.

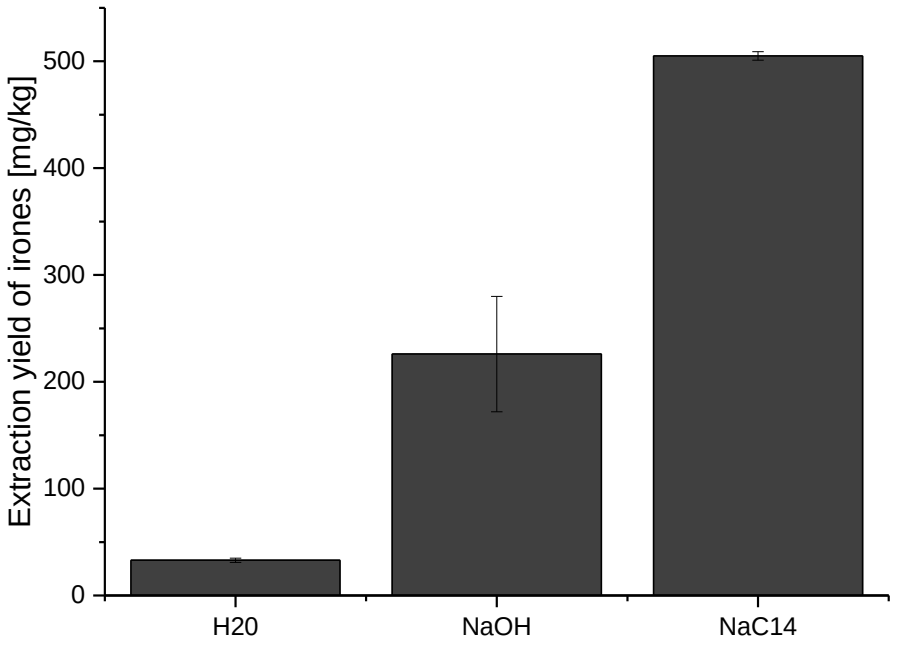


Figure 26: Influence on the extraction yield of irones while extracting with water, NaOH (1 mol/L) and NaC14 (0.1 mol/L) based on three independent experiments (n = 3) and determined by HPLC-UV (see description of extraction and HPLC-UV method in Experimental p. 118 and p. 125, respectively).

In Figure 26, the differences in extraction efficiency of the various extraction media can be seen. With water, only 33 ± 2 mg irones per kg dried rhizomes were extracted. Extracting with a sodium hydroxide solution led to a better extraction efficiency of 226 ± 54 mg/kg. But the highest extraction yield was reached by extracting iris rhizomes

with an aqueous solution of sodium myristate. 505 ± 4 mg/kg of irones could be extracted under the same conditions. In this experiment, *Iris pallida* Lam. rhizomes with an irone content of 640 ± 40 mg/kg were extracted and analyzed with HPLC-UV. The increased yield of the experiment extracting with NaOH compared to water is probably due to the saponification of the fatty acids, mainly myristic acid, contained in the iris rhizomes. Therefore, a slight micellar effect can be assumed, as a minor amount of soap surfactants are built by saponification. The still incomplete extraction can be further confirmed by the high error bar. The amount of irones solubilized in the micelles increases with increasing concentration of the fatty acid salts. As reported in literature, an increasing surfactant concentration results in a change of shape from spherical to elongated micelles.[38] Consequently, by increasing the number and size of the micelles the extraction capacity of hydrophobic compounds increases. This is indicated by the high extraction yield of irones obtained by extracting with an additional solution of soap surfactants (NaC14). Thus, if only the amount of fatty acid salts is increased, improved extraction of irones is possible. Consequently, a certain concentration of the soap solution is necessary to achieve a complete irone extraction from iris rhizomes. Thereby, the high pH value has no impact on the stability of the fragrance compounds.

3.3.5. Recovery experiment of irones

Moreover, the pH stability of the irones was tested in a more complex environment. A known quantity of analyte, here a technical mixture of irones from Sigma-Aldrich, was added to an aqueous myristate solution and stirred at slightly increased temperature. The irones were dissolved immediately. After stirring the solution for a certain time, the micelles were destroyed by neutralization with hydrochloric acid. Thereby, a white precipitate of myristic acid is formed, which is not soluble in the aqueous phase containing the resulting sodium chloride. The irones are no longer soluble either in the aqueous solution, due to their hydrophobic character. Thus, they migrate to the myristic acid precipitate, which can be separated from the aqueous phase by filtration. This experiment was repeated twice. The myristic acid containing the analyte was analyzed by GC-FID/MS as described in the experimental section, see p. 124. The peak area of the irones was

compared before and after the extraction. 94 ± 6 % of the initially amount could be recovered in the fatty acid phase without any decomposition product (n = 3). In contrast, no irones were found in the aqueous phase, as it was analyzed by GC-FID/MS after re-extracting the solution with an organic solvent.

This recovery experiment showed that the micellar extraction of irones is feasible without any losses in spite of strong pH changes. Moreover, a strategy to isolate the desired fragrances could be established, which is discussed in more detail in chapter 3.3.8.

3.3.6. Optimization of the micellar extraction parameters

The extraction of plant material is a complex process, which is influenced by several factors. The values of these parameters are not universal and have to be adjusted and optimized for each natural product.[35, 39] The basic parameters influencing the micellar extraction efficiency of irones form iris rhizomes are:

1. Particle size of the plant material
2. Extraction time
3. Extraction temperature
4. Solid to liquid ratio (s/l ratio)
5. Concentration of the soap solution
6. Type of aqueous soap solution
7. Influence of the counterion

In the course of the previously mentioned pre-tests, a high irone recovery was obtained by micellar extraction with a sodium myristate solution. Various aqueous soap solutions were studied regarding the influencing parameters. The rhizomes were soaked under stirring in the extraction medium under certain conditions. An aqueous solution of sodium myristate was specified in the beginning, as myristic acid is contained in iris rhizomes in high amounts. After soaking, the rhizomes were separated by centrifugation, and the soap solution was re-extracted with an organic solvent to determine the irone content by GC-

FID/MS (compare Experimental p. 118). The best parameter values for an almost complete extraction of the desired irones were selected based on three independent experiments (n = 3).

In literature, mainly micelle-mediated extractions of plant material using non-ionic surfactants are described. The extraction of antioxidants from elderberry blossom (*Flos Sambuci*) or three-part beggarticks (*Bidens tripartita*) and the extraction of lignans from *Schisandra chinensis* are mentioned as examples.[40-42] Thereby, it could be demonstrated that non-ionic surfactants might be an alternative for the preparation of plant extracts, which were commonly obtained by maceration with water or polar solvents. The extracts were directly analyzed without removing the surfactant. Only the extraction and not the isolation of the substances was in the foreground of the work of Slíwa *et al.* and Lee *et al.* (2013, 2016). Another example which should be mentioned is the micelle-mediated extraction of chlorogenic acid from *Morus laevigata* W. leaves using sodium dodecyl sulfate (SDS) as anionic surfactant.[43] A procedure for the extraction and also for the purification of the antioxidants has been successfully developed by optimizing parameters like surfactant concentration, solid to liquid ratio or the pH value of the extraction medium.

3.3.6.1. Influence of the particle size of the rhizomes on the micellar extraction

The initial point to start an extraction is the preparation of the plant material. In this case, dried and ground *Iris germanica* L. rhizomes were purchased from Phytotagante S.A.S. Approximately 5 % residual moisture is contained in these rhizomes, as it was determined by Wollinger (2016).[44] Since this amount was constant over a long period, the individual experiments can be compared with each other without further proceeding. The particle size of the plant material is a major factor to gain an efficient extraction. Lowering the particle size increases the surface contact between plant material and extraction medium. Thus, a smaller particle size results commonly in an increased extraction yield. In literature, particle sizes smaller than 0.5 mm are described as ideal for efficient extractions.[45]

Using DIN-standardized sieves of various pore diameters, the size distribution of *Iris germanica* L. rhizomes was determined in triplicate (n = 3, see Table 9). With 36.0 %, most of the particles have diameters of 1.0 mm. But also a high amount of powdered particles with a diameter around 0.2 mm are present in the sample (28.7 %), among others.

Table 9: Size distribution of *Iris germanica* L. rhizomes according to the method described in Experimental p. 116 (n = 3).

Diameter particle size	Proportion
1.68 mm	13.3 %
1.0 mm	36.0 %
0.841 mm	5.2 %
0.595 mm	12.9 %
0.177 mm	28.7 %
0.125 mm	3.9 %

To determine the influence of the particle size of iris rhizomes on the extraction efficiency of irones, extractions of three different particle sizes (Ø 1.0 mm, Ø 0.6 mm and Ø 0.2 mm) and an average sample of all particle diameters were carried out. Extraction time and extraction temperature were fixed to 30 min and 55 °C, respectively. The samples were extracted using an aqueous sodium myristate solution (c = 0.12 mol/L) with a solid to liquid ratio of 1/30. Every experiment was carried out in triplicate (n = 3). The results including the standard variance are plotted in Figure 27. The data were subjected to a one-way ANOVA followed by post hoc analysis (Scheffé procedure).

Compared to 1.0 mm, the extraction yields obtained with 0.2 mm and 0.6 mm diameter particle size are statistically significantly higher. According to the general principles underlying Fick's law: an increased surface area contributes to a higher mass transfer and thus to an increased extraction yield. Furthermore, as reported in literature and described above, particle sizes below 0.5 mm are ideal for efficient extraction. This statement can be confirmed with the extraction yield of particles with a diameter of 0.6 mm. With 261 ± 14 mg/kg, the highest extraction yield could be achieved with this particle size.

However, also extractions of mixed rhizome samples were carried out to check any differences. Without sieving the rhizomes before (average sample), an extraction yield of 268 ± 15 mg/kg was determined (see Figure 27). Consequently, no notable advantage of sieving the rhizomes could be noticed. Only a significant difference in comparison to the highest particle size (1.0 mm) could be ascertained regarding the average sample ($p \leq 0.001$, one-way ANOVA followed by post hoc analyisis). So, all further extractions were performed without sieving the rhizomes. Note that this is in accordance to the principles of Green Chemistry, whereby the entire plant material should be used completely to minimize waste and processing steps.[46]

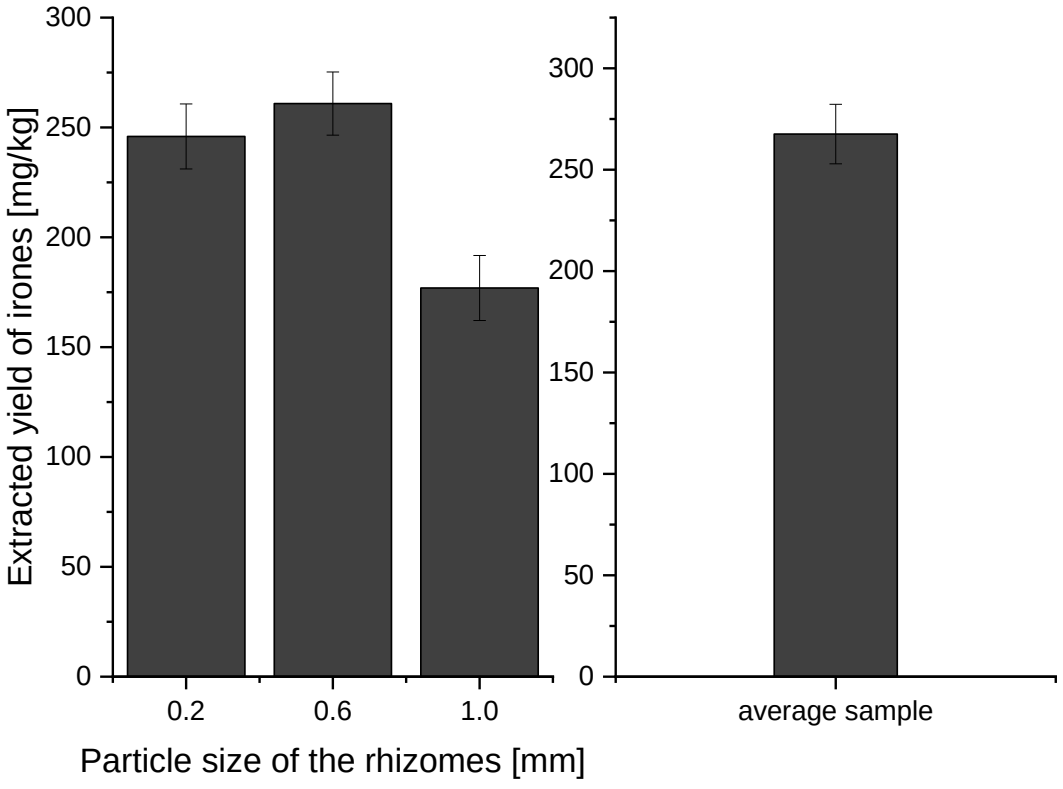


Figure 27: Influence of the rhizome particle size on the extraction yield of irones [s/l ratio 1/30, 55 °C, 30 min, c(NaC14) = 0.12 mol/L] based on three independent experiments (n = 3) and determined by GC-FID/MS (see description of extraction and GC-FID/MS method in Experimental p. 118 and p. 124, respectively). Statistical analysis was performed by a one-way ANOVA followed by post hoc analysis (Scheffé procedure): particle size 1.0 mm significant in comparison to 0.2 mm, 0.6 mm and the average sample ($p \leq 0.001$).

3.3.6.2. Influence of the extraction time on the micellar extraction

The extraction time is a crucial parameter in extraction processes. An appropriate extraction time can result in time and cost saving. Furthermore, a reduced extraction time minimizes the environmental exposures to the plant material like thermal stress, high pH values or light and oxygen.[47] Therefore, the influence of extraction time was determined by using an aqueous sodium myristate solution (c = 0.12 mol/L) with a solid to liquid ratio of 1/30 at 55 °C. Samples were extracted for 30 min, 1 h, 2 h, 3 h, 4 h, 5 h and 6 h in triplicate (n = 3). The effects of extraction time on the irone yields including the standard variation are shown in Figure 28. The data were subjected to a one-way ANOVA followed by post hoc analysis (Scheffé procedure).

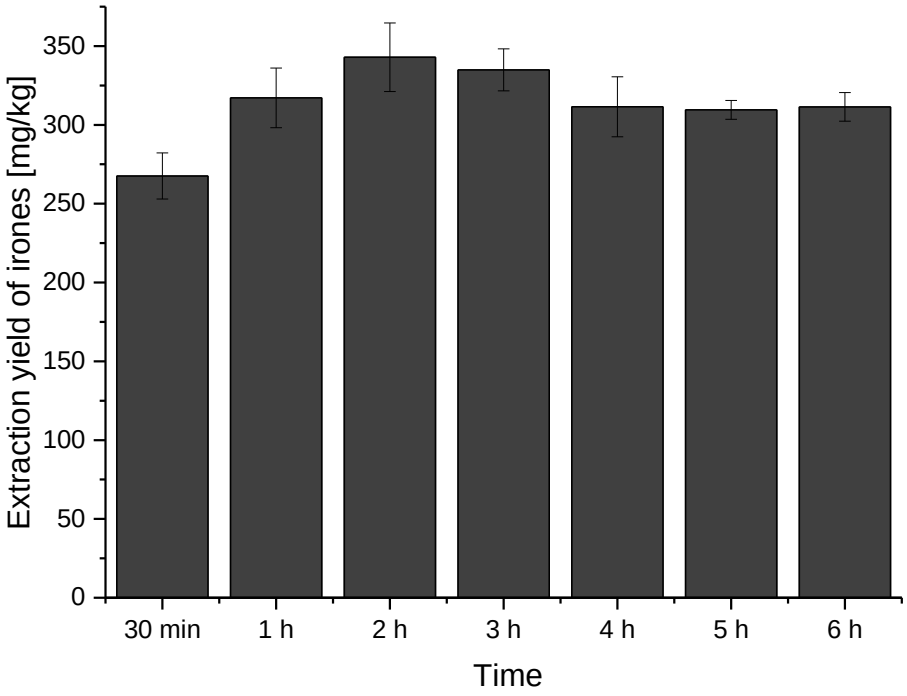


Figure 28: Influence of the extraction time on the extraction yield of irones [s/l ratio 1/30, 55 °C, c(NaC14) = 0.12 mol/L] based on three independent experiments (n = 3) and determined by GC-FID/MS (see description of extraction and GC-FID/MS method in Experimental p. 118 and p. 124, respectively). Statistical analysis was performed by a one-way ANOVA followed by post hoc analysis (Scheffé procedure): time 30 min significant ($p \leq 0.003$) from the maximum at 2 h.

At the beginning, a slightly increase in extraction yield could be observed by extending the extraction time. A small maximum was noticed at 2 h. After 2 h of extraction, no significant further enhancement of the extraction yield could be observed, according to one-way ANOVA and subsequent post hoc analysis. This effect can be explained by diffusion. First of all, the aqueous soap solution penetrates into the solid matrix of the rhizomes. The soluble compounds are solubilized by desorption. The solution containing the fragrances returns to the surface of the plant matrix by molecular diffusion. Then, the solution is transferred back to the aqueous extraction medium by stirring the mixture continuously.[35] After a certain time, an equilibrium between the irones in the plant matrix and the solvent is reached. Hence, prolongation of the extraction time does not result in a further increase in extraction efficiency. A prolonged extraction time even led to a small decrease in the irone yield, as it can be seen after 2 h of extraction. Presumably over time, more and more starch got dissolved additionally. As a result, the viscosity of the solution increased and the mass transport of the analyte was worsened.

Thus, extraction for 2 h was chosen as the optimal extraction time. From an economical point of view and also taking into consideration that the yield of irones after 30 min is only slightly lower, also an extraction time below 2 h is sufficient to extract irones from iris rhizomes. Compared with hydro distillation, this represents a significant saving of time.

3.3.6.3. Influence of the extraction temperature on the micellar extraction

The influence of the extraction temperature on the micellar extraction efficiency of irones was investigated, since the equilibrium and diffusion rate showed impacts on the experiments discussed before. An increase in temperature enhances the mass transfer and thus the diffusion of the extraction medium into the plant material.[48] Experiments at 45 °C, 55 °C, 65 °C and 75 °C were carried out in triplicate to investigate this effect (n = 3). An extraction time of 30 min, a solid to liquid ratio of 1/30 and an aqueous sodium myristate solution (c = 0.12 mol/L) were the fixed parameters for the extraction of iris rhizomes. The data were subjected to a one-way ANOVA followed by post hoc analysis (Scheffé procedure).

The water solubility of the sodium salts of fatty acids is dependent upon the carbon chain length and decreases with increasing length.[49] Therefore, sodium soaps of fatty acids with higher chain length require elevated temperatures for solubility.[50] For instance, sodium myristate (C14) requires a solubility temperature of 45 °C and sodium palmitate (C16) about 60 °C, whereas the short-chained sodium laurate (C12) dissolves in water at 25 °C.[51] This temperature is determined as Krafft temperature and is the criterion for the solubility of a surfactant in water. Since sodium myristate is not soluble in water at room temperature, experiments were carried out, starting at the Krafft temperature of 45 °C.

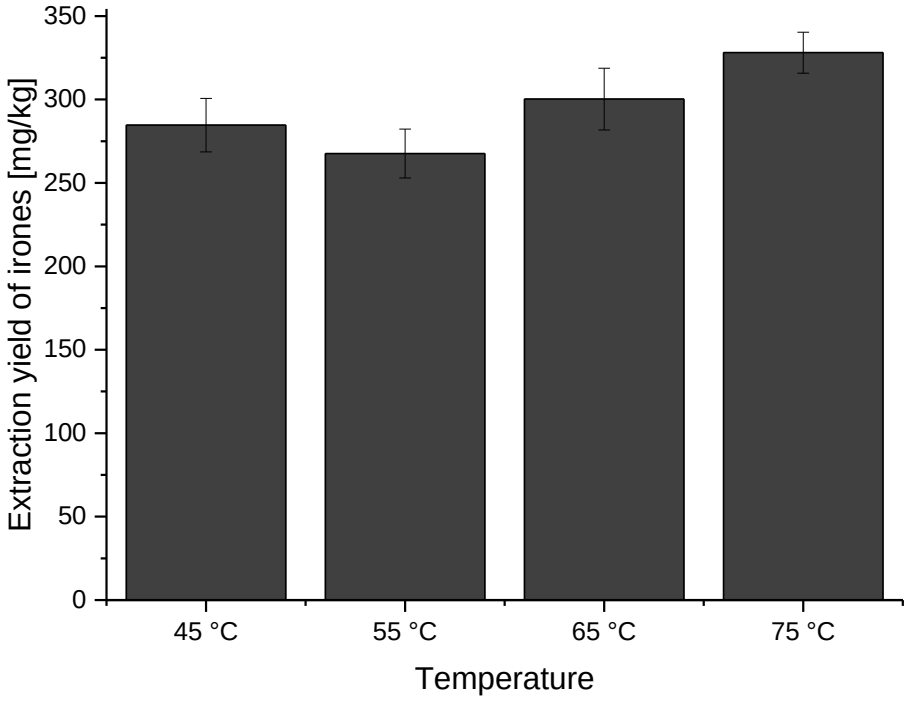


Figure 29: Influence of the extraction temperature on the extraction yield of irones [s/l ratio 1/30, 30 min, c(NaC14) = 0.12 mol/L] based on three independent experiments (n = 3)and determined by GC-FID/MS (see description of extraction and GC-FID/MS method in Experimental p. 118 and p. 124, respectively). No statistical significance according to a one-way ANOVA followed by post hoc analysis (Scheffé procedure).

Figure 29 shows that varying the extraction temperature only results in minor changes in extraction efficiency (no significant differences, $p \geq 0.05$, according to a one-way ANOVA followed by post hoc analysis). Extracting at 75 °C led to a minor increase. However, at

higher temperatures, the handling process was much more difficult. Because the solvent evaporates more quickly at high temperatures, the irone content in this sample is presumably enhanced. Only the temperature which is necessary to solubilize the fatty acid salt is the determining factor for micellar extraction. Consequently, in the case of a sodium myristate solution, an extraction temperature of 45 °C was sufficient to extract the irones from iris rhizomes. With an extraction yield of 285 ± 16 mg/kg, almost the total irone content in *Iris germanica* L. rhizomes (290 ± 5 mg/kg) is reached (compare 3.3.2.). Compared to the only slightly increased extraction yield of 300 ± 18 mg/kg achieved by extracting at 65 °C, this shows a good compromise between energy consumption and extraction efficiency. Moreover, working at moderate temperatures offers a decisive advantage compared to the high temperature applied by hydro distillation. Thermal degradation of the sensitive fragrances is excluded at 45 °C. Also “off-notes” by destroyed or burnt compounds, as often reported in literature, can be prevented this way.[5]

3.3.6.4. Influence of the solid to liquid ratio on the micellar extraction

The solid to liquid ratio (s/l ratio) is an essential parameter of plant extractions. On the one hand, it is necessary to prevent avoidable waste due to large solvent consumption, as it is also desired in Green Chemistry.[5] But on the other hand, small solvent contents may cause incomplete extractions. Ratios of 1:10, 1:20, 1:25; 1:30, 1:35, 1:40 and 1:50 grams of rhizomes per mL aqueous sodium myristate solution (c = 0.12 mol/L) were selected for comparison. Extractions were performed at 55 °C for 30 min in triplicate (n = 3). The data were subjected to a one-way ANOVA followed by post hoc analysis (Scheffé procedure).

Generally, a high solvent volume increases the extraction yield and a low solvent volume leads to incomplete extractions. This effect can also be recognized regarding Figure 30. The minimum s/l ratio to ensure complete wetting of the rhizomes was 1:10. Thereby, the yield of extracted irones was very low assuming that the extraction was not yet completed. By increasing the amount of solvent, the extraction yield rose significantly. Due to a facilitated mass transfer, a first extraction maximum at 1:30 was achieved. At a ratio of 1:40, the extraction yield of irones was slightly higher (288 ± 23 mg/kg). But taking the consumption of solvent into account, a solid to liquid ratio of 1:30 was selected to be

optimal. Considering this optimum, only the values of a solid/liquid ratio 1:10 are significantly lower according to the statistical analysis. With the mentioned extraction parameters above, a yield of 268 ± 15 mg/kg was achieved extracting with a solid to liquid ratio 1:30. Moreover, a further increase of solvent volume led to a decrease of extracted irones. The more solvent is used for the extraction, the more difficult is the subsequent isolation of the desired compounds from the extraction medium. Thus, not only a waste of solvent, but also a laborious recovery is the consequence. This difficulty is also reflected in the high error bar at the solid to liquid ratio of 1:50 and the deteriorated irone yield of 120 ± 119 mg/kg.

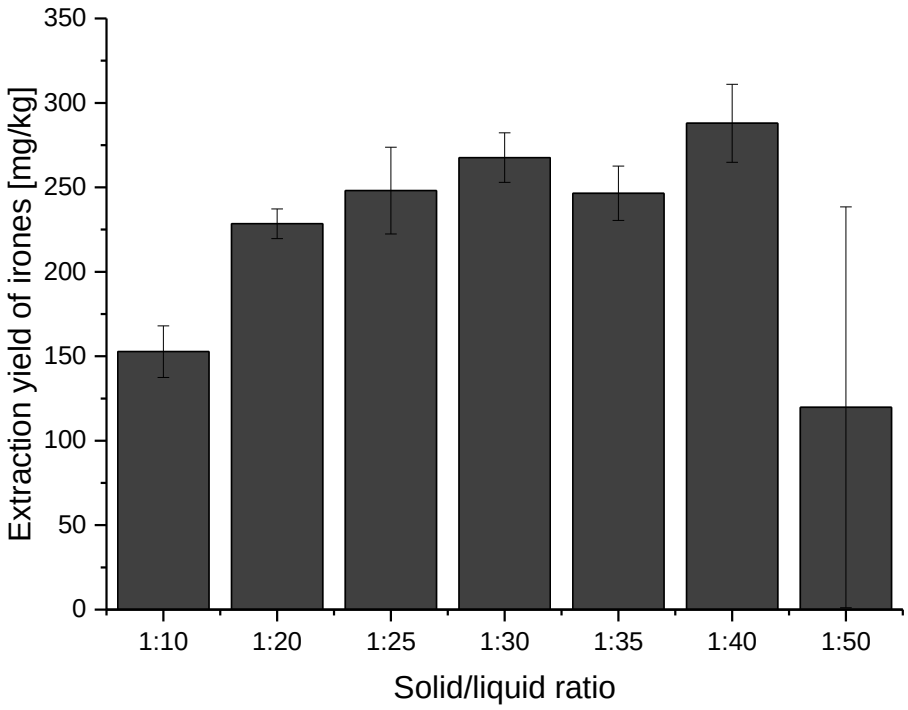


Figure 30: Influence of the solid/liquid ratio on the extraction yield of irones [30 min, 55 °C, c(NaC14) = 0.12 mol/L] based on three independent experiments (n = 3) and determined by GC-FID/MS (see description of extraction and GC-FID/MS method in Experimental p. 118 and p. 124, respectively). Statistical analysis was performed by a one-way ANOVA followed by post hoc analysis (Scheffé procedure): s/l ratio 1:10 significant ($p \leq 0.02$) from the optimum at 1:30.

3.3.6.5. Influence of the surfactant concentration on the micellar extraction

The surfactant concentration is a crucial parameter of micellar extractions as it influences the extraction yield immensely. Surfactants are freely solubilized in water at low concentrations, whereas upon a certain concentration, the CMC, surfactant molecules self-assemble and form aggregates.[38, 52] These micelles consisting of a hydrophobic core and a hydrophilic shell, enable hydrophobic solutes to solubilize in water. Therefore, a certain concentration of sodium myristate in water must be exceeded to guarantee the extraction of the irones from the plant material (see also 3.3.4).

By fixing extraction time and temperature at 30 min and 55 °C, respectively, samples were extracted using a solid to liquid ratio of 1/30 in triplicate (n = 3). Concentrations from 0 up to 0.18 mol/L of sodium myristate in water were tested regarding the extraction efficiency of irones. The data were subjected to a one-way ANOVA followed by post hoc analysis (Scheffé procedure).

Figure 31 shows the dependence of irone extraction on the surfactant concentration (including standard variation). As expected, the samples with concentrations below the CMC show low extraction yields. At a sodium myristate concentration of $5 \bullet 10^{-3}$ mol/L, only yields slightly higher (73 ± 4 mg/kg) than the extraction yields obtained with pure water (13 ± 4 mg/kg) were achieved. However, with increasing concentration, the extraction efficiency also increased. In literature, a CMC value of sodium myristate in water around $7 \bullet 10^{-3}$ mol/L is described.[53] As soon as the concentration reached the CMC, a significant increase in extraction yield could be observed (according to a one-way ANOVA followed by post hoc analysis). At $20 \bullet 10^{-3}$ mol/L, an extraction yield of 185 ± 7 mg/kg was already determined. Micelles are dynamic aggregates, which are approximately spherical at surfactant concentrations close to the CMC. An increasing surfactant concentration results in micellar growth and a change of shape from spherical to elongated micelles.[38] Because of the bigger hydrophobic core, the extraction capacity of hydrophobic compounds increases with this change in structure and size. Thus, the highest irone extraction yield of 296 ± 15 mg/kg could be achieved with a sodium myristate solution of 0.18 mol/L. Taking the high surfactant consumption into account, 0.12 mol/L was determined as sufficient and selected as an optimum concentration for

irone extraction. Thereby, an irone yield of 268 ± 15 mg/kg could be obtained, which is close to the complete irone content in iris rhizomes (290 ± 5 mg/kg). Moreover, according to the statistical analysis, the yields between the concentration range 0.06 mol/L – 0.18 mol/L are not significantly different.

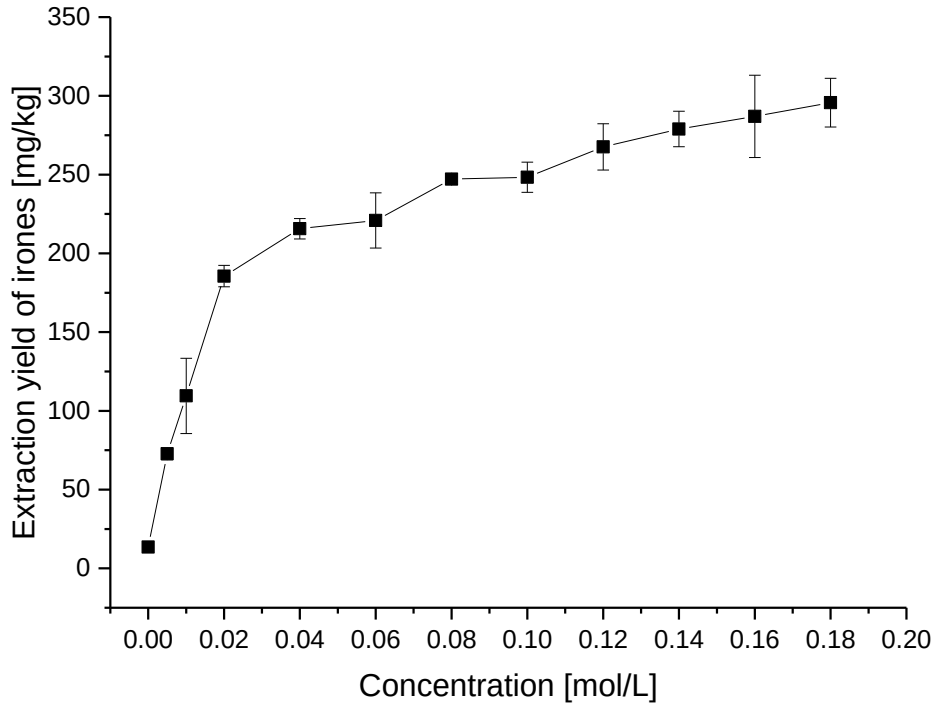


Figure 31: Influence of the NaC14-concentration on the extraction yield of irones [s/l ratio 1/30, 55 °C, 30 min] based on three independent experiments (n = 3) and determined by GC-FID/MS (see description of extraction and GC-FID/MS method in Experimental p. 118 and p. 124, respectively). No statistical significance according to a one-way ANOVA followed by post hoc analysis (Scheffé procedure) between the concentration range 0.06 mol/L – 0.18 mol/L, but statistically significant between 0 and 0.04 mol/L.

Referring to the work of Memon *et al.* (2010), who investigated the micelle-mediated extraction of antioxidants from *Morus laevigata* W. leaves, similar results regarding the surfactant concentration were determined.[43] Thereby, SDS as anionic surfactant was examined for the extraction of chlorogenic acid. The CMC value of SDS is $8.1 \bullet 10^{-3}$ mol/L. At this concentration, only small numbers of micelles are present in the solution. Thus, a complete extraction of the desired hydrophobic compounds was not feasible.

Consequently, the surfactant concentration is an important parameter while optimizing micellar extraction procedures. In the mentioned study, 0.1 mol/L of SDS showed the highest extraction efficiency and was set as optimum parameter for the extraction of *Morus laevigata* W. leaves. A concentration higher than 0.2 mol/L of SDS led to an increased viscosity of the extraction medium resulting in a worsened extraction efficiency of chlorogenic acid. To sum up, a concentration significantly higher than the CMC is typical for the micellar extraction of plant material.

3.3.6.6. Selection of different extractants and determination of their optimum concentration

Also, further fatty acids, which are present in iris rhizomes, were investigated in regard to the potential applicability of their fatty acid salts as a micellar extraction medium for the isolation of irones. To gain an overview, for each surfactant, the concentration of highest extraction efficiency was searched by performing several experiments (at least n = 3), respectively. The used concentrations were chosen to be higher than the respective CMC values found in literature from Mukerjee *et al.* (1971) (compare Table 10).[53]

Table 10: CMC values of sodium fatty acid salts at 25 °C according to Mukerjee *et al*. (1971).

Homolog	Formula	Molecular weight	CMC at 25 °C	
Sodium octanoate	$C_7H_{15}COO\ Na$	166.19 g/mol	$3.5 \cdot 10^{-1}$ M	5.73 wt. %
Sodium decanoate	$C_9H_{19}COO\ Na$	194.25 g/mol	$9.4 \cdot 10^{-2}$ M	1.83 wt. %
Sodium dodecanoate	$C_{11}H_{23}COO\ Na$	222.30 g/mol	$2.4 \cdot 10^{-2}$ M	0.54 wt. %
Sodium myristate	$C_{13}H_{27}COO\ Na$	250.35 g/mol	$6.9 \cdot 10^{-3}$ M	0.17 wt. %
Sodium oleate	$C_{17}H_{33}COO\ Na$	304.44 g/mol	$2.1 \cdot 10^{-3}$ M	0.08 wt. %

For the following experiments, *Iris pallida* Lam. rhizomes were used, and the analysis was carried out via HPLC-UV (compare Experimental p. 125) to investigate also the selectivity of the extraction. With this method, it is further possible to analyze non-volatile substances, compared to GC-FID/MS. The irone content of *Iris pallida* Lam. rhizomes was

calculated to be 640 ± 40 mg/kg, as can be found in chapter 3.3.2. Experiments with sodium octanoate, decanoate, dodecanoate, myristate and oleate with various concentrations were carried out by extracting iris rhizomes with a solid to liquid ratio of 1/30 for 30 min at 25 °C, except for sodium myristate at 45 °C. For a better comparability, the concentrations of the respective fatty acid salts are given in weight percent (wt. %).

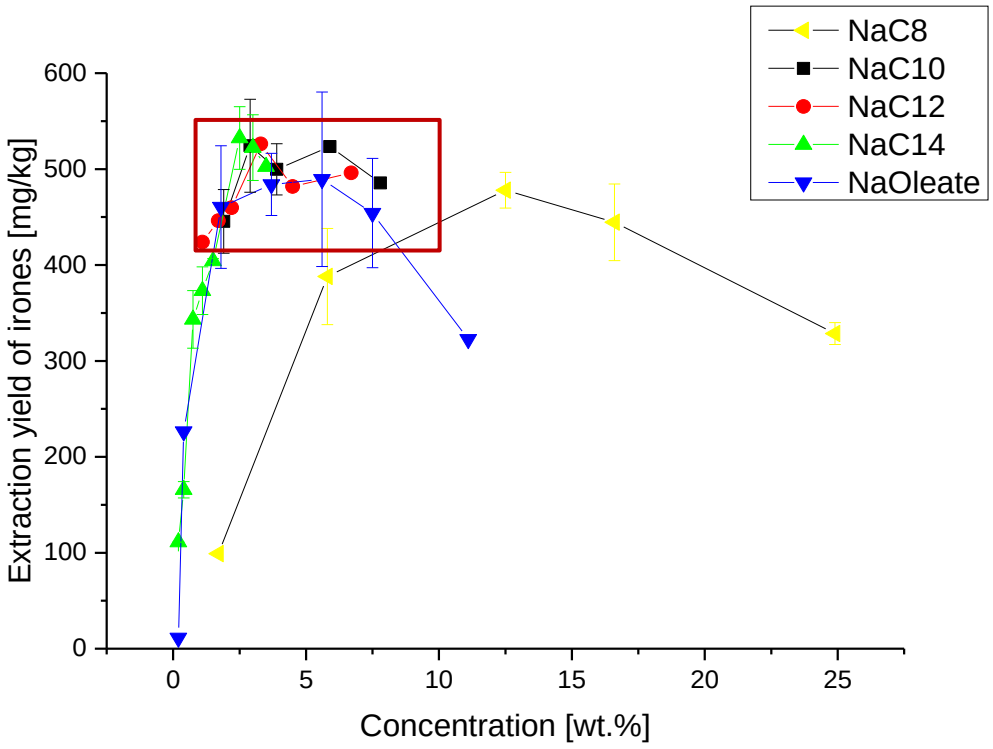


Figure 32: Influence of the concentration of various aqueous soap solutions on the extraction yield of irones [30 min, s/l ratio 1/30, 25 °C except for NaC14 45 °C] based on several independent experiments (at least n = 3) and determined by HPLC-UV (see description of extraction and HPLC-UV method in Experimental p. 118 and p.125, respectively).

An interesting correlation of the CMC of the sodium fatty acid soaps with the extraction yield of irones was found, as can be seen in Figure 32 and can be compared to the results investigated in 3.3.6.5. At low concentrations, only small amounts of the irones were extracted. As soon as the concentration reached the CMC value of the soap, a significant increase in the extraction efficiency was observed. The amount of solubilized irones in the micelles increased further at concentrations higher than the CMC. This is due to the

increasing size of the micelles by increasing the soap concentration. After a certain saturation, the extraction power decreases again as a result of the increasing viscosity of the soap solution. As a consequence, the mass transfer is deteriorated at high surfactant concentrations, as it is also reported in literature.[43]

For all surfactants, the extraction maximum leveled off at concentrations higher than 2 wt. %, except for sodium octanoate (NaC8). Thereby, a concentration of 12.5 wt. % is necessary to achieve approximately similar extraction yields. This relatively high concentration to obtain a sufficient extraction yield can be correlated with the chain length of the sodium carboxylates. A shorter chain length goes in line with a higher CMC. Therefore, a higher soap concentration for almost the same extraction efficiency is necessary (compare Table 10). In contrast, an increase in chain length leads to a lower CMC and a larger aggregation number so that more nonpolar compounds can be incorporated into micelles.[38] Thus, to achieve a similarly high yield of irones, the concentration of sodium octanoate had to be greatly increased compared to longer chained fatty acid salts. A comparable behavior showed Klevens (1950) in a study of the solubilization of ethylbenzene in a series of potassium carboxylates ranging from C8-C16.[38] He demonstrated that with increasing concentration of the surfactant, the amount of ethylbenzene solubilized in the micelles increases. Moreover, with a longer carbon chain, the amount of molecules incorporated in the micelles increases. Here, this trend can be seen clearly especially when comparing the results obtained with C8 and the longer-chained fatty acid soaps.

Accordingly, except the octanoate anion, all long-chained anions were highly suitable for the extraction of the fragrance compounds and gave comparable extraction yields between 2 and 6 wt. % of the fatty acid salts in water. In this range, sodium soaps as extracting agents extract almost completely the irones from iris rhizomes with a yield in sum of about 500 mg/kg (≙ 78 %).

Long-chained carboxylates (> C14) are no more appropriate for micellar extraction of irones because the handling and preparation step is too laborious if the surfactant solutions are not water-soluble at room temperature or moderately increased temperatures. Sodium palmitate (NaC16), for example, has a Krafft point about 60 °C.[51]

Thereby, the solution gets solidified very quickly at room temperature and the extraction cannot be processed any longer without additional expenses.

Short-chained carboxylates, like sodium octanoate, reaches the same extraction yield, but concentrations about 12.5 wt. % are necessary to compete with long-chained soaps. This represents a high consumption of soap solution. However, comparing the HPLC-UV chromatograms, interesting differences became apparent. The used reversed phase HPLC involves a non-polar stationary phase and a polar mobile phase. When a solution of the analyte is injected into the system, the components interact with the stationary phase in a different manner depending upon its polarity and hydrophobicity. As the polarity of the components decreases, the time spent in the column increases according to the principle "like dissolves like". Thus, separation is achieved, based on polarity and statements about selectivity can be made. For the identification of the compounds regarding a HPLC-UV chromatogram, it is referred to chapter 3.3.1.1. Briefly summarized, α-ionone (internal standard) can be found at a retention time of 40.0 min and the desired irone isomers are eluted at a retention time around 42.5 min. Moreover, the peaks in the beginning with the distinctive peak of acetovanillone (16 min) refer to polar components of iris rhizomes, whereas the later eluted peaks refer to nonpolar components like iridals and iridal esters.

Figure 33 shows that with a significant reduction in chain length it is possible to adjust the selectivity of the extraction. As reported in an earlier work, extractions of salts with a chain length of C4, C6 and C8 were implemented for the recovery of irones from iris rhizomes.[28] Extracting with a C4 salt showed a greater extraction of polar constituents. Nonpolar compounds at higher retention times were hardly observed and irone extraction was almost impossible. In contrast, extraction of acetovanillone and flavonoids (between a retention time of 18 and 34 min) was possible within this short-chain carboxylate. By using a C6 salt as extraction medium, extraction of polar and nonpolar compounds occurred. A C6 salt is less polar than a C4 salt due to the increasing chain length. Thus, nonpolar constituents could be extracted additionally. In contrast, C8 salt solutions possess nonpolar character and thus, mainly nonpolar components were extracted. A selective extraction with an almost complete irone yield could be achieved with this salt solution. Extraction of polar compounds was hardly feasible.

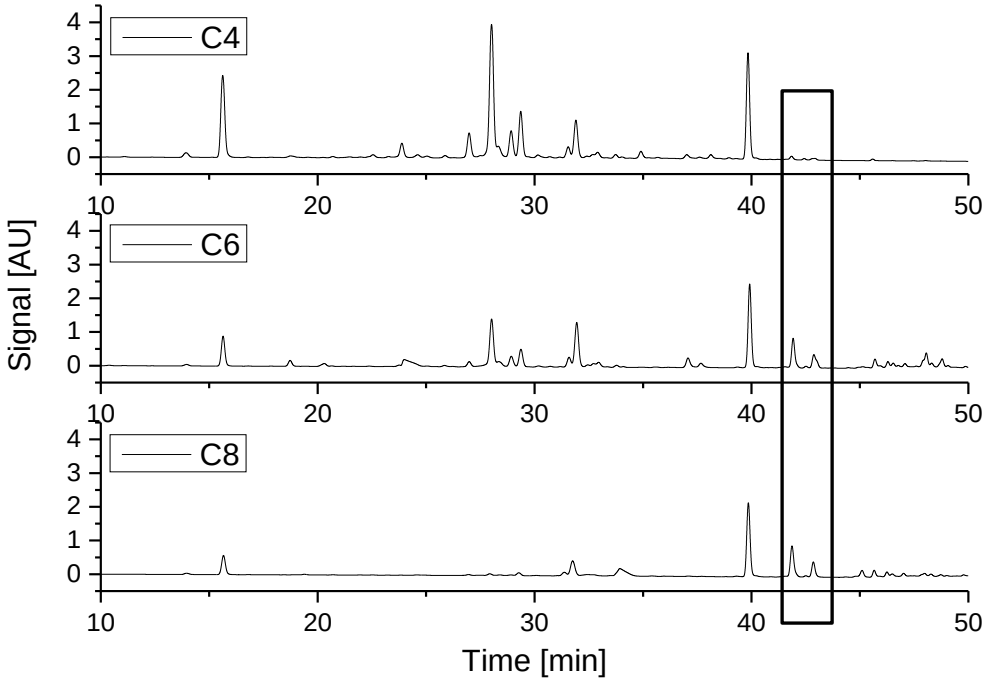


Figure 33: Comparison of HPLC-UV chromatograms of three iris extracts obtained with short-chained soap solutions: C4, C6 and C8 (see description of extraction and HPLC-UV method in Experimental p. 118 and p. 125, respectively). The irone isomer peaks are within the added frame and the peak at 40 min refers to the internal standard α-ionone.

To sum up, salts with a chain length of minimum C8 as extracting agent extract almost the total irone content of iris rhizomes. No differences in selectivity of the surfactant solutions were observed by increasing the chain length further (< C8). Consequently, upon a certain chain length, a rise in hydrophobicity of the surfactants has no influence on the extraction efficiency and the yield of the relatively nonpolar irones.

Branched extractants were not tested, since branching of the hydrocarbon chain usually results in a decrease of the solubilizing power of the micelles.[38] This is presumably due to geometric and packing restrictions, which limit the ability of the micellar core to incorporate nonpolar molecules. Therefore, only the saturated fatty acids, which are contained in iris rhizomes, were interesting and thus were investigated for the micellar extraction of irones. A further disadvantage of branched respectively unsaturated fatty acids is the fact that they become rancid very quickly and thus impair the odor of the extract.

3.3.6.7. Mixture of fatty acid salts

As described above, it is possible to influence the selectivity of the extraction by varying the chain length of the sodium fatty acid salts. Hydro distillation, in contrast, is limited to the isolation of volatile and nonpolar compounds. Potentially, with the combination of fatty acid salts with varying chain length, it is feasible to create a completely new method to extract plant material. Since polar constitutes can also contribute to a positive scent, various aqueous mixtures of sodium butanoate (NaC4), sodium octanoate (NaC8) and sodium myristate (NaC14) were examined and are discussed in the following.

For the extraction, rhizomes of *Iris pallida* Lam. with a total irone content of 640 ± 40 mg/kg (≙ 100 %) were used (compare 3.3.2). To make additional statements about the selectivity of the extraction medium, HPLC-UV was chosen (method see experimental section p. 125). The concentration of the respective aqueous soap solutions was applied as determined in 3.3.6.6. Each experiment was carried out twice (n = 2), whereby the solid to liquid ratio was set to 1/30, the extraction time 30 min, and the temperature 25 °C or 45 °C, respectively.

➔ Mixture of NaC4 and NaC14

Sodium butanoate is a very short-chain soap with polar character, as it could be seen in Figure 33. Mainly polar substances are retarded and nonpolar irones do hardly appear in the chromatogram. With a NaC4 concentration of 17.5 wt. % (≙ 1.6 mol/L) in water, it was possible to extract 65 ± 1 mg/kg of irones. This corresponds to only 10 % of the total irone content in the rhizomes. However, this yield is slightly higher compared with the extraction of pure water. In addition, a very high concentration of NaC4 was necessary to reach this small increase in irone extraction. This effect is due to the hydrotropic character of the extraction medium. Sodium butanoate as well as sodium hexanoate are able to form aggregates, but high concentrations to solubilize hydrophobic compounds are required, which is a typical behavior of hydrotropes.[54, 55] A hydrotrope possesses as well as a surfactant a hydrophilic and a hydrophobic part and above a certain concentration (minimal hydrotrope concentration, MHC) aggregation occurs also with a short alkyl

chain. In the case of sodium butanoate, a minimum concentration of 3.5 mol/L in water is reported at room temperature.[53] Here, NaC4 was only added to the solution of aqueous sodium myristate to extract polar substances additionally. However, despite the high concentration (17.5 wt. %) only a small amount of irones was extracted. Therefore, extractions with the combination of NaC4 and NaC14 were carried out with a concentration of 2.5 wt. %, respectively. The obtained irone yields and the related HPLC-UV chromatograms can be seen in Table 11 and Figure 34.

Table 11: Extraction yield of the aqueous solutions of NaC4, NaC14 and a mixture of both determined by two independent experiments (n = 2) and measured by HPLC-UV (see description of extraction and HPLC-UV method in Experimental p. 118 and p. 125, respectively).

Fatty acid salt	Extraction yield irone
NaC4 (17.5 wt. %)	65 ± 1 mg/kg
NaC14 (2.5 wt. %)	505 ± 4 mg/kg
NaC4 + NaC14 (for each respectively 2.5 wt. %)	508 ± 40 mg/kg

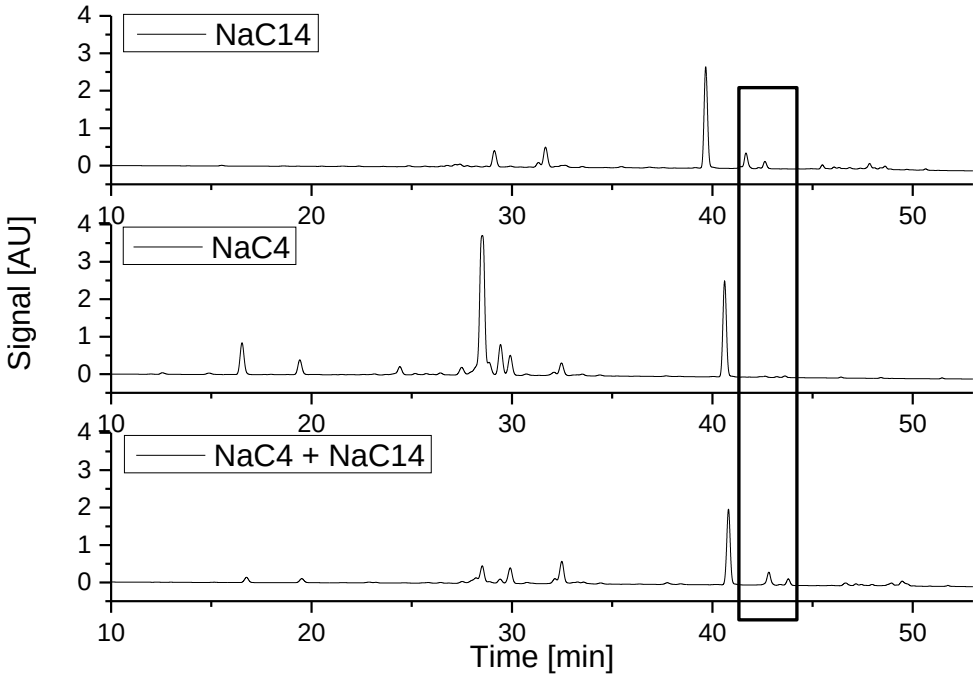


Figure 34: Comparison of HPLC-UV chromatograms of extracts gained by aqueous solutions of NaC4, NaC14 and a mixture of both (see description of extraction and HPLC-UV method in Experimental p. 118 and p. 125, respectively). The irone isomer peaks are within the added frame and the peak around 40 min refers to the internal standard α-ionone.

Besides the lower yield, the main difference in extracting with a solution of NaC4 in comparison to NaC14 is the extraction of acetovanillone, which is eluted at 16.5 min and the extraction of flavonoids (retention time between 18 and 37 min). With aqueous solutions of solely NaC14, it is not possible to extract polar compounds. However, if a mixture of both was used an extraction of polar odoriferous substances as well as the nonpolar irones was possible (compare Figure 34). Table 11 shows that a loss in extraction yield is not obtained using a mixture of NaC4 and NaC14. Thereby, 79 % of the contained irones in the iris rhizomes could be recovered. The smell of the extract was attractive and diversified. However, there is the risk that traces of the strong and unpleasant smell of butyric acid can remain in the extract depending on the isolation methods, described later in chapter 3.3.8. For industrial scale, a further drawback is reflected. The aqueous mixture of sodium butanoate and sodium myristate cannot be prepared at room temperature. Here, a temperature higher than 60 °C is needed for the extraction of iris rhizomes involving the problems discussed above regarding the Krafft temperature of longer-chained fatty acids in 3.3.6.6.

➔ Mixture of NaC6 and NaC14

Likewise, extractions using a mixture of sodium hexanoate (NaC6) and sodium myristate are also limited due to the same reasons described above. Hexanoic acid has a strong unpleasant odor, which can be hardly avoided in the extract. From a theoretical point of view, the extraction efficiency of this mixture was satisfactory with an extraction yield of 495 ± 32 mg/kg (≙ 77 % of the total irone content). Additionally to the irone extraction, also the extraction of polar compounds was possible. Sodium hexanoate has also hydrotropic properties like sodium butanoate and also permits the extraction of polar compounds.

➔ Mixture of NaC8 and NaC14

Sodium octanoate is a micelle-forming surfactant with a significantly lower critical concentration (0.4 mol/L) compared to sodium hexanoate (1.2 mol/L), which possess only hydrotrope properties.[55]

As it can be seen in Table 12, extracting with an aqueous solution of NaC8 leads to an extraction yield of 510 ± 7 mg/kg (≙ 80 %), whereas with an aqueous solution of NaC14 505 ± 4 mg irones per kg dried rhizomes were obtained (≙ 79 %). As already explained in chapter 3.3.6.6, a higher concentration of NaC8 is required, but the extraction yield is comparable with the longer chained fatty acid soaps. However, polar odoriferous substances are hardly extracted with NaC8, as it can be seen in the HPLC-UV chromatogram in Figure 35, NaC8.

Table 12: Extraction yield of aqueous solutions of NaC8, NaC14 and a mixture of both determined by two independent experiments (n = 2) and measured by HPLC-UV (see description of extraction and HPLC-UV method in Experimental p. 118 and p. 125, respectively).

Fatty acid salt	Extraction yield irone
NaC8 (12.5 wt. %)	510 ± 7 mg/kg
NaC14 (2.5 wt. %)	505 ± 4 mg/kg
NaC8 (12.5 wt. %) + NaC14 (2.5 wt. %)	489 ± 2 mg/kg

Thus, combining NaC8 and NaC14 results in a similar extraction yield as obtained with the pure fatty acid salts (489 ± 2 mg/kg≙ 76 %). Moreover, only a slight variation in selectivity was determined. However, a large benefit could be observed by mixing NaC8 and NaC14 regarding the extraction temperature. With NaC8, extraction at room temperature was possible, whereas NaC14 required an extraction temperature higher than 45 °C due to the Krafft point. A mixture of both allowed extracting without additional heating. This effect leads to a simpler handling of the extraction procedure and to the reduction of energy consumption. The lowering of the extraction temperature by mixing NaC8 and NaC14 offers a decisive advantage without loss in extraction yield.

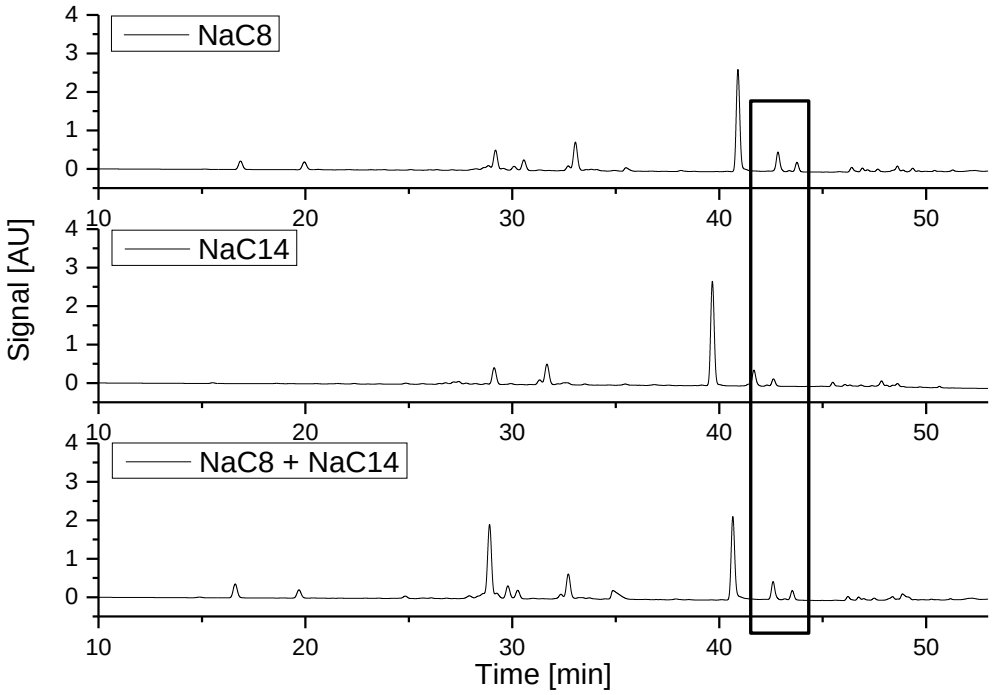


Figure 35: Comparison of HPLC-UV chromatograms of extracts gained by aqueous solutions of NaC8, NaC14 and a mixture of both (see description of extraction and HPLC-UV method in Experimental p. 118 and p. 125, respectively). The irone isomer peaks are within the added frame and the peak around 40 min refers to the internal standard α-ionone.

The same results were achieved using sodium oleate instead of sodium octanoate. Only the extraction of nonpolar compounds was possible, whereas an adjustment of the extraction selectivity failed. Nevertheless, sodium oleate should be avoided because of its unsaturated character in regard to oxidation sensitivity.

➔ Mixture of NaC4, NaC8 and NaC14

To sum up, by using a short-chained soap (NaC4), it is feasible to extract polar substances and by using longer-chained fatty acid salts, nonpolar substances, especially irones, are extracted. However, mixing both soap solutions (NaC4 and NaC14) results in a hardly compatible mixture, which requires high temperatures to get dissolved. In contrast, mixing NaC8 and NaC14 do not lead to a change in selectivity, but extracting at room temperature is possible. To combine the advantages of increased solubility at room

temperature and the extraction of also polar odoriferous substances, which cannot be extracted with common extraction methods, an optimum mixture of NaC4, NaC8 and NaC14 was searched.

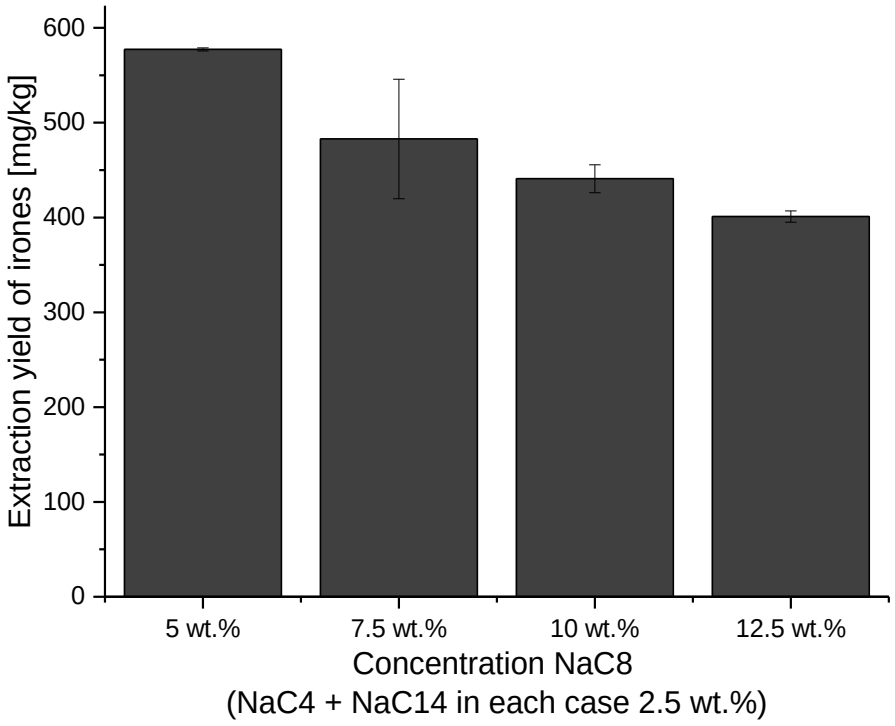


Figure 36: Extraction yield of an aqueous mixture of NaC4, NaC8 and NaC14 by varying the concentration of NaC8, based on two independent experiments (n = 2) and determined by HPLC-UV (see description of extraction and HPLC-UV method in Experimental p. 118 and p.125, respectively).

By adding NaC8 to the aqueous fatty acid salt solutions of NaC4 and NaC14, a better solubility in water was achieved, and extraction at room temperature was possible. The third component cooperates with the fatty acid salts and acts like a solubilizer. However, a high concentration of NaC8 was required to prepare the extraction mixture at room temperature. To determine the minimum necessary concentration of NaC8 to work at room temperature, extractions were carried out with the concentration of NaC4 and NaC14 set at 2.5 wt. % in each case (see Figure 36). The first effect observed was that by increasing the concentration of NaC8, also the viscosity of the extraction medium increased. The resulted worsened mass transport led to a deteriorated yield of the desired

irones. However, only with a NaC8 concentration of 10 wt. %, the mixture was soluble at room temperature. Therefore, a good compromise was to use an aqueous solution of NaC8 with a concentration of 5.0 wt. %. On the one hand, heating up to 50 °C is required once to solubilize the extraction medium. On the other hand, during the extraction, heating could be dispensed, since the solution remains dissolved at room temperature. With this composition, 577 ± 2 mg irones/kg dried rhizomes could be extracted at room temperature within 30 min. This corresponds to 90 % of the total irone content in *Iris pallida* Lam. rhizomes. Furthermore, a notable difference in the smell was perceived due to additionally extracted polar substances.

To sum up, mixing various short-chained and long-chained fatty acid soaps lead to an almost complete extraction of the irones. Furthermore, it is possible to create a tailor-made extract with polar and nonpolar odoriferous substances and extracting at room temperature is practicable. However, it is also important to note that the isolation of the odoriferous substances is not guaranteed without any contamination of smelly short-chained fatty acids.

3.3.6.8. Influence of the counterion

Up to now, only the effect of the anion was examined, and sodium as counterion was used all the time. Thereby, it was detected that the Krafft temperature above which surfactants form micelles, is elevated with increasing hydrophobic chain length. Thus, the long-chained fatty acid soaps cannot be used at room temperature. At the same time, however, it was shown that the longer-chained fatty acids salts were more appropriate for the extraction of irones. In fact, the Krafft point rises in the same manner as the solubilizing power of the micellar solution increases. In this chapter, the influence of the cation is investigated in so far as the cation can influence the Krafft temperature of surfactants.[56] Therefore, extractions of potassium myristate and sodium myristate were carried out in triplicate under the same conditions (n = 3) to evaluate the differences in irone extraction. For statistical analysis, a t-test was performed.

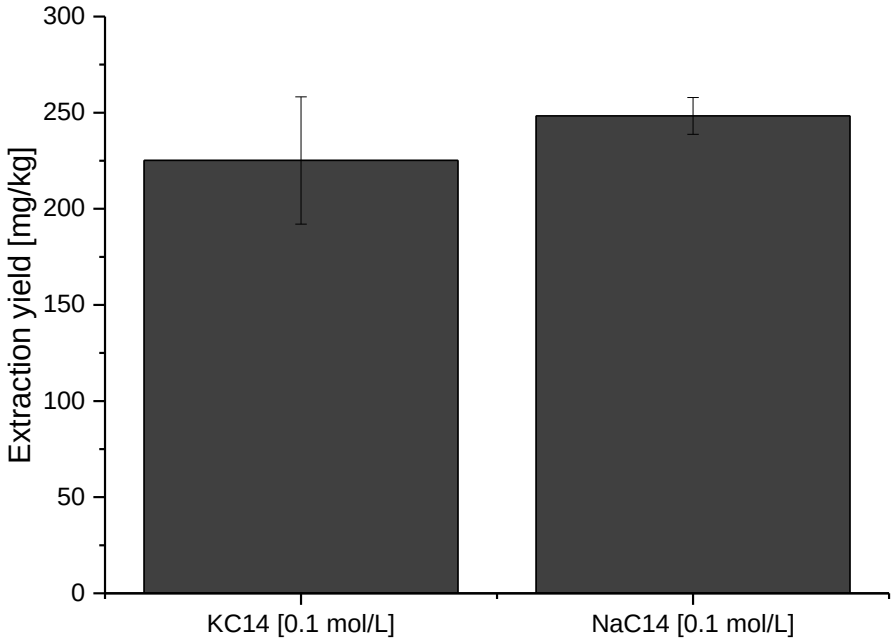


Figure 37: Influence of the counterion potassium and sodium on the extraction yield of irones [s/l ratio 1/30, 30 min, c = 0.1 mol/L, 25 °C respectively 45 °C] based on three independent experiments (n = 3) and determined by GC-FID/MS (see description of extraction and GC-FID/MS method in Experimental p. 118 and p. 124, respectively). No statistical significance according to a t-test.

The first distinction already occurred during the sample preparation regarding the solubility, respectively the Krafft temperature. As also reported in literature, potassium soaps are better soluble in water than sodium soaps.[50] The Krafft temperature of sodium myristate is 45 °C and can be reduced to 30 °C with potassium as counterion.[51] Obviously, the Krafft points of the myristate surfactants decrease with increasing size of the counterion. This effect can be further confirmed regarding choline as counterion. Choline chemically refers to (2-hydroxyethyl)trimethylammonium and is of biological origin. It is formerly known as Vitamin B_4 and is present in most foods.[54] With choline as counterion, the Krafft temperature of myristate can even be dropped to 0 - 1 °C.[51]

The Krafft temperature is determined by the interplay of two competing thermodynamic forces: the free energy of the solid crystalline state and the free energy of the micellar solution. The latter only varies slightly by changing the counterion, whereas the free energy of crystalline state changes dramatically.[52] Therefore, the Krafft temperature can

be reduced by the hindrance of regular surfactant packing, which increases the free energy of the surfactants' solid state. Thus, the bulky cation choline enables the formation of micelles at room temperature and even lower. However, it has to be mentioned that choline decomposes slightly into trimethylamine and aldehydes resulting in a fishy odor.[57] For this reason, only experiments with potassium as counterion were carried out. Thereby, the Krafft temperature is lower compared to sodium myristate and extractions can be performed at slightly increased temperature.

The type of counterion influences the Krafft point of a surfactant significantly. In contrast, the CMC is mainly dependent on the chain length of the surfactant and less on the type of the counterion.[51, 56] Regarding the almost equal CMC values of choline, sodium and potassium myristate, shown in Table 13, this effect can be confirmed. Moreover, by comparing the extraction yields of aqueous solutions of KC14 and NaC14, it can be approved that also the extraction efficiency of irones is only determined by the anion (see Figure 37). Taking the error bars into account, nearly no difference is observed between the extraction yield of potassium or sodium as counterion.

Table 13: CMC values of choline, sodium and potassium myristate.[51]

	Choline	**Sodium**	**Potassium**
C14	6.4 mmol/L	6.9 mmol/L	6.6 mmol/L

To sum up, the counterion markedly influences the Krafft temperature and therefore the temperature at which extraction is applicable, but the CMC and its solubilizing power is mainly affected by the chain length of the soap. Working with potassium myristate is a good possibility to avoid heating during the extraction process, but an improvement of irone extraction compared with sodium myristate (Krafft temperature 45 °C) is not given.

3.3.7. Combination of the optimal extraction parameters

Briefly summarized, micellar extraction of iris rhizomes enables the solubility enhancement of hydrophobic molecules, especially irones, in water. The chain length of the carboxylate surfactants and their concentration influence the structure and size of the micelles, which are thermodynamically stable concerning a defined pH value and temperature. The minimum solubility temperature, respectively the Krafft temperature, was sufficient for irone extraction using fatty acid salts and further heating could be dispensed. Thus, extracting at room temperature was feasible for most of the investigated sodium fatty acid salts, except sodium myristate. In this case, moderate heating up to 45 °C was needed. Nevertheless, this represents a significant advantage compared to high temperature effects induced by hydro distillation or solvent extraction. Moreover, by changing the counterion, a further drop of Krafft temperature was possible. Consequently, extractions with potassium myristate could be performed at 30 °C. Already after 30 min, an almost complete irone extraction was achieved, whereas a maximum could be found around 2 h.

All in all, an irone yield of 285 ± 16 mg/kg can be obtained by extracting iris rhizomes for 30 min at 45 °C with an aqueous sodium myristate solution of 0.12 mol/L and a solid to liquid ratio of 1/30. For comparison, the total irone content in the rhizomes is 290 ± 5 mg/kg. Thus, applying these optimal extraction parameters, a complete extraction of irones (98 %) from iris rhizomes is possible. Furthermore, other fatty acid salts, which also occur in the rhizomes, are as well suitable as micellar extraction medium. By adding shorter-chained fatty acids salts to longer-chained fatty acid salts, also the selectivity of the extraction can be adapted. Thus, a tailor-made extraction of iris rhizomes is possible.

3.3.8. Isolation methods

Once the optimal parameters for the extraction of irones were determined, the attention was turned towards the isolation of the desired molecules from the extraction solution. In most micelle-mediated extractions described in literature, this step is even omitted[40, 41] or carried out using toxic solvents like methanol, hexane or chloroform.[42, 43] Therefore,

different isolation methods including the neutralization of the myristic acid, the precipitation of poorly soluble lime soaps, breaking the micelles by adding ethanol as well as the combination with hydro distillation were investigated and are discussed in the following.

3.3.8.1. Re-extraction with an organic solvent

The easiest way to isolate the irones from the extraction medium is to use an organic solvent, which is immiscible with the extraction medium but dissolves the fragrances. This re-extraction method was applied in all experiments mentioned above due to its simplicity and quantitative extraction efficiency. Once the rhizomes were removed by centrifugation, the supernatant was liquid/liquid extracted in a separating funnel. To this purpose, solvents can be used, which are commonly applied for plant extractions. Indeed, they must be immiscible with the aqueous extraction solution and completely removable after the extraction. For example, diethyl ether is a suitable solvent. It is highly volatile and can be removed easily afterwards without any residue using rotary evaporation. Alternatively, the environmentally friendly 2-methyltetrahydrofuran can be used as a re-extracting agent. A very clear phase separation can be achieved with this non water-soluble solvent. This solvent is derived from renewable resources and can also be abiotically degraded by sunlight or air.[58] It evaporates almost as quickly as diethyl ether and allows a green extraction of the desired compounds from the extraction medium. Nevertheless, it has to be mentioned that vapors of 2-methyltetrahydrofuran, when mixed with air, are flammable when they are exposed to ignition sources.[59] Diethyl ether and also 2-methyltetrahydrofuran are sensitive to light and air, tending to form explosive peroxides.

The re-extraction method with an organic solvent enables not only the isolation of the fragrances from the aqueous media, but also the recovery of the surfactant solution. This solution can be directly used for additional extractions without further purification. Only after a third repetition, the yield of irones was slightly decreased as can be looked up in a previous work.[28] A similar recycling process was also shown in literature from Ressmann *et al.* (2013) investigating a micellar extraction of piperine from black pepper.[60]

Apart from the fact that an extract prepared by solvent extraction requires considerably higher amounts of solvent, an extract obtained in the manner described above differs significantly from a common solvent extract. In the soap extract, the desired irones have been enriched, and further compounds are not or hardly co-extracted by the organic solvent from the extraction medium. The HPLC-UV chromatograms of a soap extract and a Soxhlet extract can be seen in Figure 38 (see experimental section p. 125 for the HPLC-UV method).

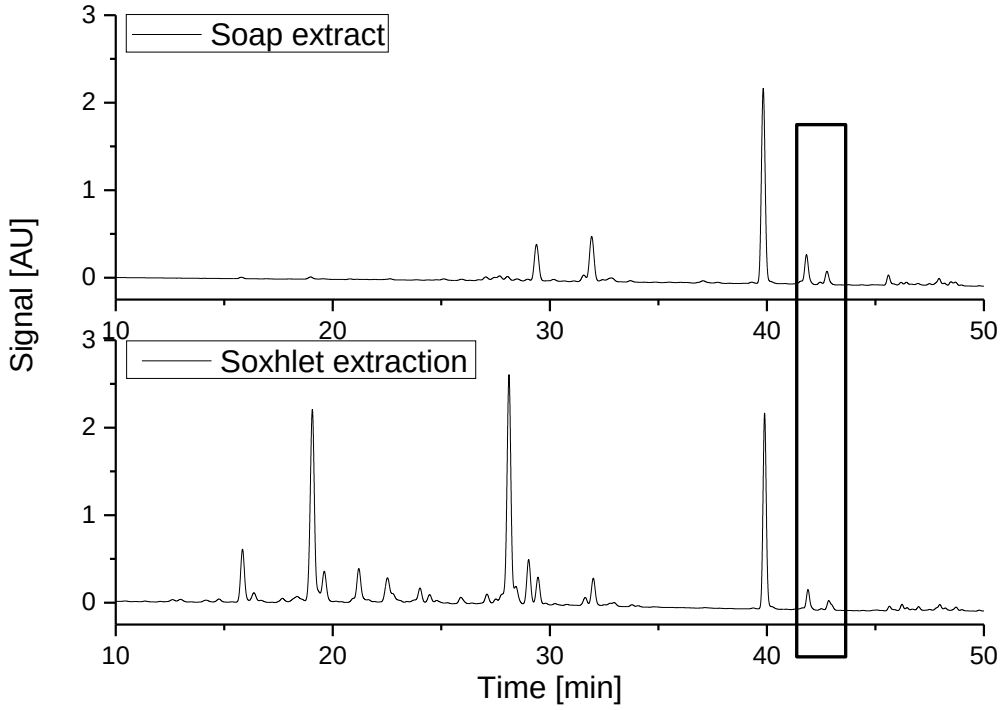


Figure 38: HPLC-UV chromatograms of a soap extract (NaC14) re-extracted with diethyl ether (above) in comparison to a Soxhlet extract with ethanol (below) (see description of extraction and HPLC-UV method in Experimental p. 117, p. 118 and p. 125, respectively). The irone isomer peaks are within the added frame and the peak around 40 min refers to the internal standard α-ionone.

The chromatogram below illustrates that many polar compounds are extracted by Soxhlet extraction with ethanol. The peaks between 20 – 35 min refer to flavonoids.[29] These secondary metabolites are not or hardly present in the soap extract, which was re-extracted with diethyl ether (Figure 38 below). There, mainly non-polar compounds, such

as the irones (~ 42 min), are extracted. Extracting with an aqueous sodium myristate solution followed by re-extraction with small amounts of organic solvent, thus, results in a higher selectivity of irones and saves a lot of solvent compared to pure solvent extraction.

3.3.8.2. Precipitation of myristic acid

Another possibility to isolate the desired fragrances from the aqueous soap solution is to lower the pH value. By adding an acid to the aqueous medium without the rhizomes at moderate temperatures, a silky white precipitate of myristic acid with creamy consistency occurs. A suitable acid for the neutralization reaction is for example hydrochloric acid, but also organic acids like citric acid can be used. As soon as the pH value of the medium is decreased, the micelles break up, because the carboxylic group of the fatty acid is protonated. The neutralized myristic acid is not water-soluble and thus, phase separation occurs. Water-soluble parts and hydrophilic molecules stay in the aqueous phase, whereas all hydrophobic compounds migrate to the fatty acid phase (see Figure 39).

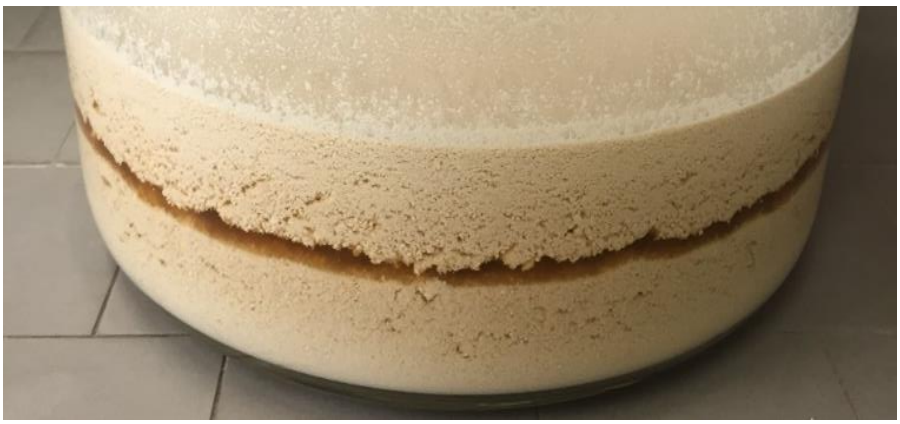


Figure 39: Aqueous extraction solution after the addition of hydrochloric acid (compare Experimental p. 120). The white precipitate contains myristic acid and the desired hydrophobic compounds.

In particular, the most valuable compounds of the extract, the hydrophobic irones, migrate to the fatty acid phase. This behavior could already be observed in the recovery experiment of irones in 3.3.5. The myristic acid precipitate containing the desired irones

can be separated from the aqueous solution by filtration or preferable centrifugation. Furthermore, it was shown that the slower the acid was added under stirring, the smaller was the amount of retained water in the myristic acid precipitate after filtration. This finding is of importance to achieve an almost water-free extract.

The extract obtained as described above can also contain undesirable compounds like suspended solids, which remained in the aqueous soap solution after filtration or centrifugation. Moreover, residual amounts of water remain in the precipitate. Problems with storing and stability may occur, and therefore a further purification step is necessary. As it is well known in the field of perfumery, in particular in the “enfleurage” (compare chapter 5), it is possible to enrich the fragrance molecules, respectively, to isolate the fragrances from the fat matrix by washing or soaking with organic solvent.[61] Accordingly, the fatty acid precipitate was dissolved in ethanol under stirring at moderate temperatures. Only the ethanol-soluble parts got dissolved and the non-soluble parts could be separated by filtration. The residue was washed with ethanol several times (see description of isolation in Experimental p. 120). The dried amount of the separated plant material residue was calculated to be around 5 – 10 % of the rhizome mass initially applied.

This remaining plant material residue was investigated further. By using the iodine test, it was feasible to verify the presence of starch.[62] When treating the remaining plant material residue with a solution of elemental iodine and potassium iodine in water, the contained starch complexes the triiodide anion and results in an intense purple color. Starch is a significant part of iris rhizomes[7], but undesirable in the extract. However, starch is soluble at increased temperatures in water and is therefore firstly dissolved and then precipitated together with the fatty acid at room temperature. Nonetheless, as described above, it is feasible to separate the remaining plant material residue from the fatty acid precipitate with an additional washing and filtration step. Subsequently, the solvent can be removed using rotary evaporation. Thereby, water and ethanol build an azeotrope. Using this effect, it was possible to remove the residual moisture of the extract by distillation. An azeotropic ratio of 95.5 % ethanol and 4.5 % water was determined by using an alcoholmeter.

If the water content of the extract is very high, there is another possibility to get the extract free of water. The purified precipitate without remaining plant material is first melted in centrifugation tubes at 55 °C. After complete melting, the mixture is centrifuged for 10 min at 55 °C with 4500 rpm. Phase separation of water and myristic acid with the contained hydrophobic molecules occurs. After solidifying of the myristic acid phase at room temperature, the water can be removed. However, this procedure is only possible with a purified extract. The direct separation of remaining plant material and water from the extract by melting is not feasible due to a poor phase separation. In the following illustration, the procedure is summarized schematically (Figure 40, compare also Experimental p. 120).

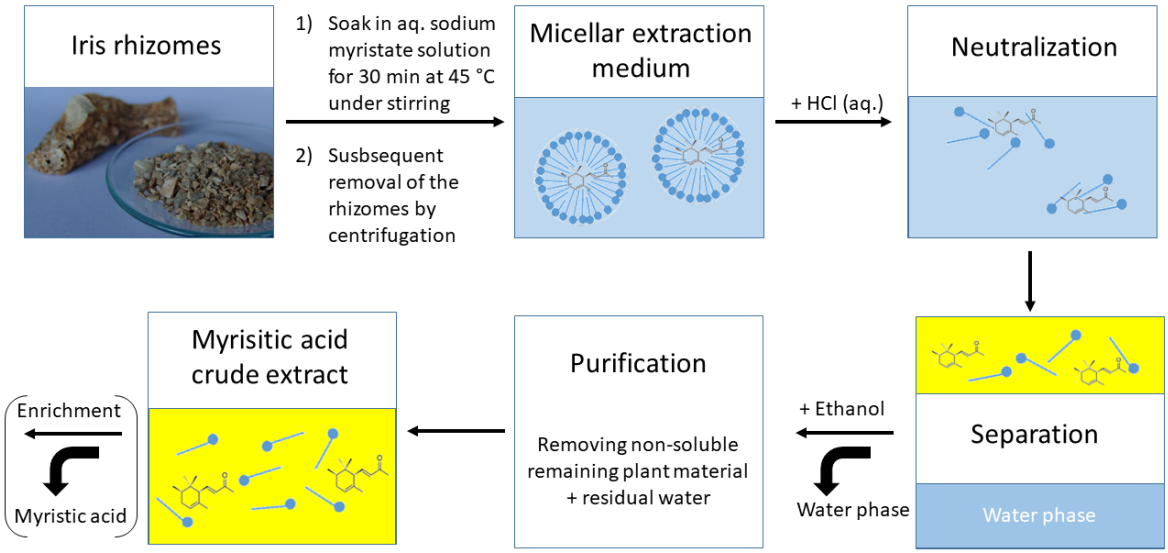


Figure 40: Schematic illustration of the irone isolation by precipitating myristic acid.

The method to isolate the irones by precipitating myristic acid was tested and optimized several times. The following results are based on two independent experiments (n = 2), which show complete reproducibility. *Iris germanica* L. rhizomes with a complete irone content of 290 ± 5 mg/kg were used for the extraction and isolation experiments. Every single step was analyzed by GC-FID/MS (method see Experimental p. 124). The remaining plant material was dried and re-extracted with diethyl ether to check any traces of irones. No irones were detectable. Also, the aqueous phase gained after filtration of the myristic

acid precipitate was analyzed. Thereby, a small amount of irones up to 24 mg/kg was detected. This represents only 8 % of the original irone content and thus can be neglected. The myristic acid crude extract obtained as described above comprises 269.5 ± 0.2 mg/kg irones. As a consequence, by decreasing the pH value of the soap solution, it is possible to isolate 93 % of the total irones determined in iris rhizomes. An additional rinsing of the rhizomes after the extraction process, which is often necessary using common extraction methods like maceration, is not required.[61] The rhizomes have been soaked again in an aqueous soap solution after the first extraction, but no remaining irones could be determined. Thus, the irones were completely transferred from the rhizomes into the extraction medium.

By using soap compounds, which are naturally occurring in the rhizomes (primarily myristic acid), the obtained extract is only composed of the intrinsic components resulting in a harmonic and natural flavor. The crude extract contains 0.4 mg irones per gram extract. As myristic acid is already a frequent ingredient of perfumes and cosmetics, the final product with its remaining fatty acid represents an ideal basic raw material. The weak acid acts as a fixative in perfumes, which is used to equalize the vapor pressures and thus to slow down the rate of evaporation of the volatile fragrances.[63] Furthermore, also in formulations like crèmes and lotions, myristic acid as well as its salts and esters have been reported as opacifier, cleansing and emulsifying agent for instance.[64] It is furthermore possible to enrich the fragrance molecules by separating myristic acid. This procedure is described later in chapter 3.3.9.

3.3.8.3. Breaking the micelles by adding ethanol

For completeness, it should be mentioned that a further attempt to break up the micelles and to release the irones was done. The experiments were carried out as part of the bachelor thesis of Meike Bauer.[65] In this thesis, it was investigated whether it is possible to isolate the irones after breaking the sodium myristate micelles by adding ethanol. The alcohol seems to be able to destroy micellar structures, as it is indicated in literature.[66, 67]

The simplest way to show changes in the mesophase behavior is to employ ternary phase diagrams.[68] Therefore, a ternary phase diagram of sodium myristate/ethanol/water was established at 60 °C (see Figure 41). Just a few samples were prepared due to the high cost of pure sodium myristate (see Experimental p 121).

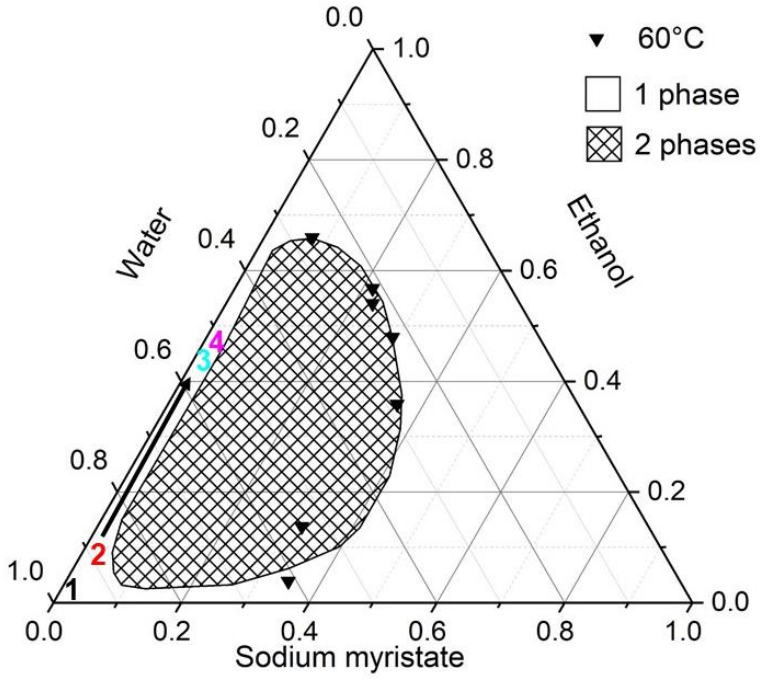


Figure 41: Ternary phase diagram of sodium myristate/ethanol/water at 60 °C. Measuring points are marked with "▼" and the position of the DLS samples with numbers 1-4 (see description in Experimental p. 121).

Sodium myristate is readily soluble in water and slightly soluble in ethanol at 60 °C. In addition, small portions of the salt are soluble in ethanol and water mixtures. The miscibility ends up in an oval area, which is marked in the diagram as a two-phase region. In the one-phase region on the left side of the miscibility gap, dynamic light scattering (DLS) experiments were performed. The positions of the samples are marked with numbers 1-4 in Figure 41 and the fitted DLS curves are shown in Figure 42. DLS is a commonly used method for analyzing systems wherein any kinds of aggregates are expected. It is possible to make qualitative predictions about the presence and the size of

structures. For more detailed information, the reader is referred to literature by Zemb *et al.* ,for instance.[69]

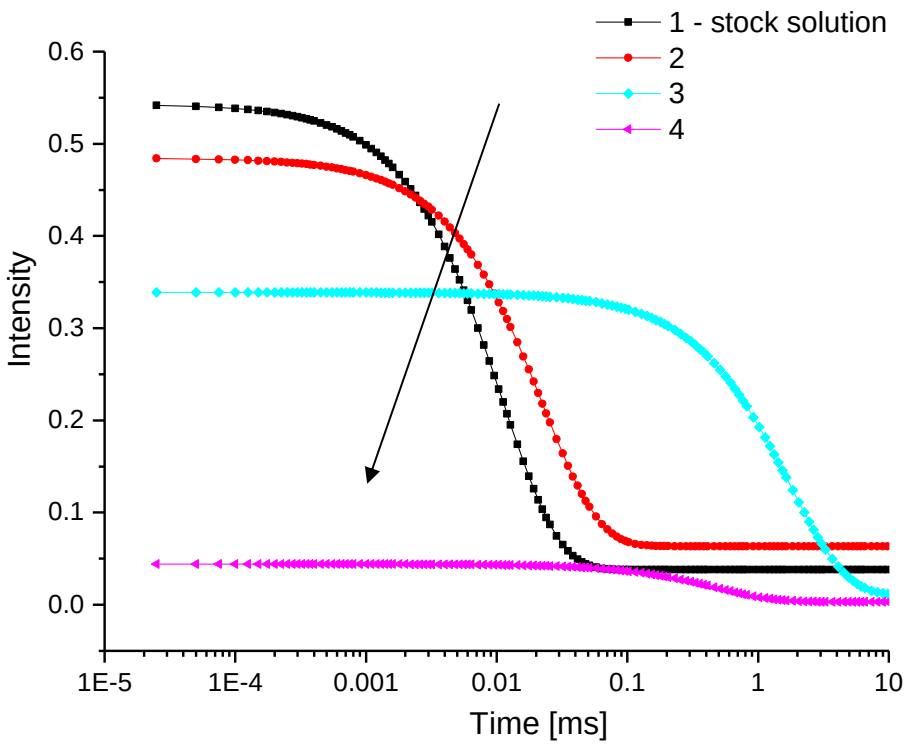


Figure 42: Fitted DLS curves of the samples indicated in the phase diagram above (see description in Experimental p. 121).

A stock solution of 2.5 wt. % sodium myristate in water, which is the same concentration as in the previous experiments, was prepared and measured via DLS. Regarding the scattering of sample 1, where no ethanol was added, it can be seen that micelles are present in the solution owing to the high intensity. Increasing the amount of ethanol leads to an increase of the structure sizes in solution (compare Figure 42). It can be assumed that ethanol acts as a co-surfactant in the beginning. Co-surfactants are molecules which are insufficiently hydrophilic to form micelles with water by themselves, but can influence the structures when mixed with surfactants.[68] Depending on the strength of the polar group hydration, there is a greater or smaller incorporation of the co-surfactant into the micelles. In the case of ethanol here, it can be assumed that the ethanol molecules are

incorporated in the micelle-water-interface. By increasing the mass ratio of ethanol, the micelles swell until the interactions between the soap molecules get weaker. If a critical concentration of ethanol is reached, in this case nearly 50 %, the micelles are so much inflated that the intermolecular interactions get lost and the structure is destroyed. The DLS scattering of sample 4 shows that there are no more micelles present in the solution. Thus, it can be assumed that it is feasible to destroy the micelles by the addition of ethanol, as it is already reported in literature for the anionic surfactant sodium dodecyl sulfate by Javadian *et al.* (2007).[67] However, also the co-solvent effect has to be considered. The more ethanol is added to the aqueous solution of sodium myristate, the more hydrophobic the solvent mixture becomes. As a result, the driving force of the micelle monomers to build aggregates is diminished at a high ethanol content. As it can be seen in the DLS scattering of sample 4, the surfactant molecules remain free in solution.

In any case, whether by a co-surfactant or a co-solvent effect, the micellar structure of sodium myristate can be destroyed by the addition of ethanol, releasing the enclosed irone molecules. Nevertheless, the isolation of the desired irones from the solution is difficult applying this approach. Due to the increasing hydrophobic character, the irones get also dissolved in the mixture, which complicates the isolation. No further trials were carried out to solve this difficulty, since the aim of this thesis was also to avoid the consumption of organic solvents.

3.3.8.4. Precipitation of poorly soluble lime soaps

Another approach to isolate the fragrance molecules from the extraction medium was done by precipitating the myristic acid as lime soap by adding divalent alkaline earth salts. As it is known, magnesium or calcium salts of fatty acids are insoluble or hardly soluble in water.[68] By adding a stoichiometric quantity of calcium or magnesium salts to the aqueous sodium myristate solution, the sodium ion is replaced and poorly soluble lime soaps are formed and precipitate in water. This effect is also known from laundry detergency. Thereby, the drawback of soaps regarding their very low tolerance against polyvalent electrolytes is often reported.[70] Hard water also contains significant amounts of calcium

and magnesium ions. As a result, the insoluble lime soaps precipitate during the washing procedure. This behavior lowers the efficiency of the washing machine and leads to yellowish clothes due to the incorporation of the lime soaps into fabrics.[71] This, in the case of washing, disadvantageous effect, should be used here to isolate the desired irones from the extraction medium.

Precipitating the insoluble lime soaps by adding magnesium salts led to a strongly exothermic reaction. Therefore, calcium chloride was preferably added to isolate the fragrance compounds and to avoid heating during this step. After a short period of agitation, the precipitate was separated by filtration. The irones are not soluble in water and therefore remained in the lime soap. This precipitate of granular structure was nearly free of water and could be handled without further purification. With ethanol, it was possible to re-extract the desired fragrances from the calcium soap. Calcium myristate is not soluble in ethanol and was separated by filtration again. After evaporation of the solvent, the isolated fragrances were obtained (compare description in experimental section p. 101).

Isolating the irones by precipitation of the insoluble lime soaps achieved an extraction yield of 196 ± 1 mg/kg determined by GC-FID/MS (n = 2, description of the GC-FID/MS method see Experimental p. 124). This represents 68 % of the total amount of irones contained in *Iris germanica* L. rhizomes. However, the extract contains 2.0 – 2.2 mg irones/g extract. Compared to only 0.4 mg irones per gram extract by precipitating myristic acid via neutralization, this method achieves an enriched extract of irones. The aqueous solution was re-extracted with diethyl ether, but almost no irones could be found in the waste water (only 12 ± 6 mg/kg compared to the total 290 ± 5 mg/kg contained in the rhizomes, ≙ 4 %). As a consequence, the residual amount of irones must remain in the calcium myristate precipitate. However, it was not feasible to dissolve the irones completely from this precipitate with solvent. Even ultrasound irradiation does not lead to an improved extraction yield. A further disadvantage of this isolation method is that calcium myristate cannot be reused. Therefore, a recycling of the extraction medium is not possible compared to the isolation process by precipitating myristic acid solely as discussed in 3.3.9.

3.3.8.5. Combination of micellar extraction with hydro distillation

Another idea to isolate the irones from the micellar solution was to combine the extraction method with a common hydro distillation. Before hydro distillation, the rhizomes are usually soaked in water or even acidic water for several hours to swell the plant material and to make the desired compounds more accessible.[4, 26] After hours until days of distillation, the essential oil can be separated. The aim of this study was to combine the soaking of the rhizomes in an aqueous micellar solution with a followed short-time hydro distillation. With this combination, shorter distillation times, lower energy costs and also the reduction of the risk of thermal degradation of the fragrance molecules should be possible.

A relatively short time is sufficient to extract the irones from iris rhizomes by soaking them in an aqueous soap solution, as it is described in chapter 3.3.6.3. Maceration for 30 min at a slightly increased temperature led already to an almost complete irone extraction. As the irones are dissolved in the micelles, the mixture can be distilled with or without rhizomes. However, due to the strong foaming property of aqueous soap solutions, an intermediate step to prevent foaming was necessary. Foaming is a well-known problem in industry. Foams prevent efficient filling of containers or cause defects on surface coatings, but also interfere during the washing procedure of laundry or dishes. However, foaming can be prevented by changing the surface potential. Calcium soaps as well as silicone oils, for example, are practically insoluble in water and have a pronounced ability to spread over a surface.[72] Thus, foam formation can be regulated by adding silicone oils or by precipitating the insoluble calcium soaps. Moreover, the attempt to neutralize the fatty acids salts was done to prevent foaming during distillation.

For the experiments, iris rhizomes of unknown origin were used. To check the irone content, a reference hydro distillation using a Clevenger apparatus was carried out. To this purpose, the rhizomes were soaked in water for 90 min at 50 °C. Afterwards, this mixture was distilled for 150 min. The experiments were carried out three times (n = 3, see Experimental p. 122) and an irone yield of 337 ± 8 mg per kg rhizomes was achieved. This value was assigned as reference and was set to 100 %. For comparison, distilling the rhizomes for 6 hours without previous soaking the rhizomes led to an irone yield of

348 ± 4 mg/kg. A longer extraction time does not inevitably lead to a higher yield of irones, but to a higher total yield relative to the mass. In particular, the amount of fatty acids contained in iris rhizomes increases with distillation time.

➔ **Precipitation of myristic acid**

Analogous to the reference experiment, the rhizomes were soaked for 90 min at 50 °C before hydro distillation. In this case, an aqueous sodium myristate solution was used instead of water. To prevent foaming during hydro distillation, the micelles were destroyed by adding hydrochloric acid. One experiment was carried out where the rhizomes were previously separated by centrifugation (n = 1). Another experiment was performed including the rhizomes (n = 1). The subsequent hydro distillation using a special Clevenger apparatus lasted 150 min. The aim was only to isolate the already extracted irones from the aqueous extraction medium, not the extraction itself. Table 14 shows the obtained irone yields isolated with, respectively, without rhizomes.

Table 14: Irone content of the extract and irone yield of the combination of precipitating myristic acid and hydro distillation with and without rhizomes (n = 1, see description of isolation p. 122), determined by GC-FID/MS according to the method described in Experimental p. 124.

With rhizomes	Without rhizomes
20 mg/g extract	25 mg/g extract
68.5 mg/kg rhizomes	80 mg/kg rhizomes
≙ 20 %	≙ 24 %

Combining the micellar extraction procedure with hydro distillation leads to an extract with a low content of fatty acid. Myristic acid is hardly distillable under these conditions. Thus, the fragrance molecules are much more concentrated compared to the neutralization isolation method described in 3.3.8.2. Thereby, solely 0.4 mg irones per gram extract were obtained. With a yield between 20 mg/g and 25 mg/g, this isolation method shows a clear advantage. However, only 20 – 24 % of the irones compared to the reference experiment could be achieved by this combination. During distillation, myristic

acid is liquid and forms a thick film on the top of the water phase. Consequently, the fragrances are hindered to be distilled. Besides, no difference is obtained when the rhizomes were separated before distillation. Maybe the mass transfer during distillation is a little bit improved without rhizomes. Due to the same recovery rate, it can be noted again that the irones are definitely extracted by the micellar extraction medium. Indeed, the isolation of the fragrances failed with this procedure. Prolongation of the distillation duration presumably leads only to an increased yield of myristic acid, but not in irone recovery.

➔ **Precipitation of lime soaps**

Another possibility to reduce the strong foaming of the soap solution during distillation is to precipitate the lime soaps by adding a divalent salt in a stoichiometric amount. Usually, the formation of lime soaps during the washing process caused through hard water is, among other things, unwanted as also foaming is strongly reduced.[73] Here, this effect is used as advantage. The precipitation was carried out after soaking the rhizomes for 90 min at 50 °C in an aqueous sodium myristate solution, as described in the experimental section p. 122). As applied above, this precipitation was done once with and once without rhizomes (in each case n = 1). Afterwards, the mixture was distilled for 150 min. The lime soaps were still insoluble at the applied high temperature. The mixture with the containing rhizomes was very viscous and pappy. Thus, some water had to be added. After 90 min, the distillation was stopped due to the strong swelling and foaming of the rhizome particles. The distillation without rhizomes lasted 150 min, as well as in the other experiments. The results are summarized in Table 15.

Although the distillation including the rhizomes was stopped after 90 min, the irone content of the extract was very high (185 mg/g). The yield concerning the irone content per kg rhizomes of both distillation experiments was comparable. It can be presumed that the isolation of the desired fragrances is possible within shorter distillation time, comparing the irone content of the extract of 185 mg/g with rhizomes for 90 min and 95 mg/g without rhizomes for 150 min. Only the amount of fats and waxes is increasing with time, but the total irone content regarding the yield in mg/kg dried rhizomes stays

constant. An extract with an irone content of 95 mg/g was achieved by distilling 150 min, as in the reference distillation. Thereby, 100 mg irones per gram extract were obtained. But in the former case, the irones were already in solution and the rhizomes have been separated before. In this case, hydro distillation serves as the function of isolating, not extracting the fragrances. Separating the rhizomes before the precipitation of the lime soaps facilitates the handling of the subsequent distillation enormously. With the improved mass transfer, even the irone yield can be increased (compare Table 15).

Table 15: Irone content of the extract and irone yield of the combination of precipitating the lime soap and hydro distillation with and without rhizomes (n = 1, see description of isolation p. 122), determined by GC-FID/MS according to the method described in Experimental p. 124.

With rhizomes	Without rhizomes
185 mg/g extract	95 mg/g extract
256 mg/kg rhizomes	264 mg/kg rhizomes
≙ 76 %	≙ 78 %

To sum up, with an extraction efficiency of around 78 %, the isolation method of combining the precipitation of the lime soaps with followed hydro distillation shows acceptable results. An excessive temperature influence as it is the case applying common hydro distillation, can be avoided using this combination. Only a short distillation time is required. This period can presumably be further reduced and thus thermal degradation of the sensitive fragrances is hindered.

➔ **Anti-foaming agent**

The easiest way to compensate foaming is to add an anti-foaming agent. A variety of defoamers are available to reduce and hinder the formation of foams. The general principle of anti-foaming agents is based on their insolubility in water and the facility to spread rapidly on surfaces due to their insolubility in the foaming medium. The surface active property destabilizes the foam structure.[72] Polydimethyl siloxane is a widely used additive and also applied here. Silicone oil-based anti-foaming agents are polymers

consisting of hydrophobic silica dispersed in silicone oil. This emulsion led to a fast and well spreading in the foaming media. 0.1 g of the anti-foaming agent was sufficient to inhibit foaming of 1 L extraction medium. By adding a small amount of this additive, it was possible to carry out hydro distillation of the soap solution directly. No intermediate step to overcome the foam problem, as described above, was necessary. The GC-FID chromatograms (according to the method described in Experimental p. 124) of the reference hydro distillation and the micellar hydro distillation with the anti-foaming agent after 150 min hydro distillation are shown in Figure 43.

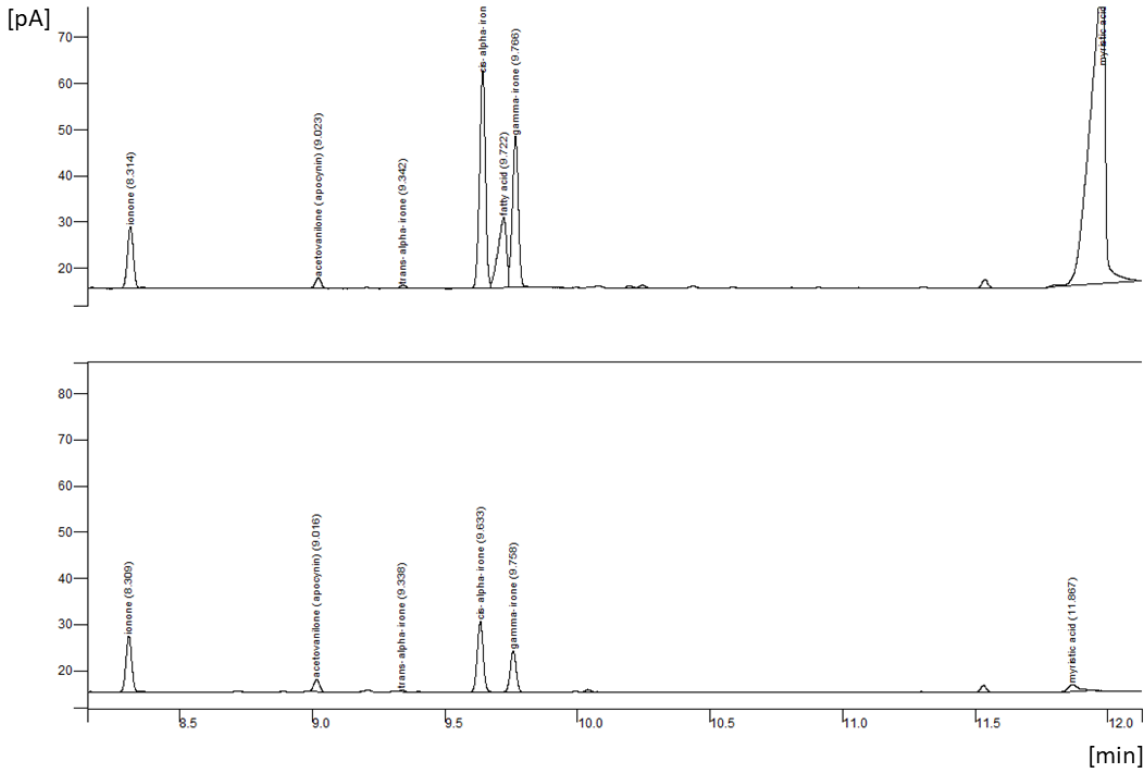


Figure 43: GC-FID chromatogram (according to the method described in Experimental p. 124) of the reference hydro distillation (above) and the hydro distillation combined with the anti-foaming agent (below) (see description of isolation in Experimental p. 122).

The chromatogram of the reference hydro distillation (Figure 43, above) shows a high proportion of fatty acids in the extract. Especially myristic acid at 12.3 min is present in high amounts. In contrast, the micellar extraction combined with hydro distillation and anti-foaming agent shows nearly no fatty acid peaks in the extract (Figure 43, below). At

the high pH-value of the micellar solution, all free fatty acids contained in iris rhizomes are saponified and therefore cannot be distilled, not even in traces. Consequently, this approach would be a good possibility to separate fatty acids from the fragrance molecules. But despite the clearly visible irone peaks at a retention time around 10 min, the combination of micellar extraction and hydro distillation with an anti-foaming agent was not satisfactory. A very bad odor and a bad extraction efficiency of only 52 % led to this conclusion. Likewise, the reduced mass transfer due to the surface-spreading agent shows that this combination is no alternative to isolate the desired fragrances from the micellar media.

To sum up, combining micellar extraction of iris rhizomes and subsequent hydro distillation to isolate the irones showed interesting results, but no decisive advantage compared to hydro distillation solely could be achieved.

3.3.9. Enrichment of the irones in the extract

For some applications, it is desirable to have an extract with a high content of irones. However, the irone content per extract obtained by precipitating the fatty acid, which is the best method to isolate the irones (see 3.3.8.2), is just 0.4 mg/g. To enrich the amount of irones, myristic acid which is the main part of the extract has to be removed. Myristic acid is soluble in ethanol at room temperature, but not at – 20 °C. Thus, the fatty acid precipitates and crystallizes in ethanol under cold conditions. The desired irones remain dissolved, as it is also written in literature.[10]

This effect was utilized to separate myristic acid and to enrich the fragrance compounds. To this purpose, the crude extract was dissolved in warm ethanol. A ratio of 1 g myristic acid crude extract to 14 mL solvent was determined to be best. If less solvent is used, the myristic acid crystals can hardly be filtered, whereas too much solvent is a waste. Also, if the volume of solvent is too high, the risk that parts of myristic acid are still dissolved and remain in solution is high. By cooling the mixture over night at – 20 °C, myristic acid was precipitated and could be removed by filtration. To ensure a complete crystallization of the fatty acid, it was necessary to wait several hours. Subsequent evaporation of the

solvent led to the isolation of the enriched irones. These enriched irones are called "concentrate" in the following. However, a complete separation of myristic acid was not possible by one filtration step. 3.60 g white crystals (HMyr recycled) were isolated from 4.55 g of the crude extract, as it can be seen in Table 16. This represents a recovery of myristic acid around 80 %. A part of the fatty acid (≙ 18 %) remained in the concentrate, and 2 % of the amount originally used were lost during filtration (see description in Experimental p. 122).

Table 16: Enrichment of the irones by precipitating myristic acid at – 20 °C according to the method described in Experimental p. 122 (n = 1), determined by GC-FID/MS (see Experimental p. 124).

	Total mass	Irone yield/g extract	Irone recovery
Reference	4.55 g	95.7 mg/g	-
Concentrate	0.84 g	373.6 mg/g	72 %
HMyr recycled	3.60 g	18.0 mg/g	15 %
Total	97.5 %	-	87 %

In the reference sample, in total 435.4 mg irones were determined by GC-FID/MS and this value was set to 100 %. Thus, an irone recovery of 72 % was possible within this enrichment step, but around 28 % irones were lost. In the dried crystals of myristic acid 15 % of the missing irones were found. The other 13 % were probably left on the filter paper or were lost during solvent evaporation. To increase the irone recovery, the myristic acid precipitate has to be washed more intensively in spite of the danger that a part of the myristic acid gets dissolved again. In this case, a second crystallization and filtration step can be added. Regarding the irone recovery of only 72 %, it can be seen that the handling can certainly be adapted. The experiment was carried out only once (n = 1). However, applying this irone concentration step does not only provide the enrichment of the irones, but also the recycling of myristic acid. The recycled myristic acid can be re-used for saponification and subsequent micellar extraction of iris rhizomes. The irones, which have been lost during the enrichment process, presumably accumulate in the circuit again.

A further suited method to enrich fragrance molecules is to apply molecular distillation. Molecular distillation operates under low pressures and relatively low temperatures. Thus, it also allows the separation, respectively, the purification of thermal sensitive molecules. As another advantage, this process takes place without toxic or flammable solvents, which are indispensable using other purification techniques.[74] The applicability of the molecular distillation process to separate fragrances from fatty acids is confirmed later in chapter 5: modern enfleurage. It has been demonstrated that a direct and gentle separation of fragrances from fatty acids in a genuine composition is possible without losses, including a subsequent reuse of the extraction medium.

3.3.10. Reusability of the extraction medium

As just explained, myristic acid can be reused after the separation of the valuable irones. No further purification step is necessary and the fatty acid can be directly saponified by adding aqueous sodium hydroxide in an equimolar quantity. With this newly generated extraction medium, subsequent micellar extraction of iris rhizomes is feasible and only fresh water and NaOH pellets are needed.

Another possibility to reuse the extraction medium directly was examined. To this purpose, the same aqueous sodium myristate solution was used several times for the extraction of iris rhizomes. Consequently, after soaking the rhizomes in the aqueous soap solution, the plant material was separated by centrifugation. This solution was directly used for further two extraction cycles of iris rhizomes without isolating the rhizomes before. Three independent experiments were carried out (n = 3) and the irone yield was analyzed using GC-FID/MS (see Experimental p. 124). With a very low standard deviation it was shown that only 189 ± 3 mg/kg irone can be extracted by reusing the extraction medium three times. For comparison, 268 ± 15 mg/kg irones are extracted by using the extraction medium only once under the same conditions (compare 3.3.6.4 and 3.3.6.5). Thus, a loss of 29.5 % irone is obtained by reusing the extraction medium several times. It can be assumed that this is due to the deteriorated mass transfer as a result of the increased viscosity. Not only the loss of extraction medium by removing the extracted rhizomes, but also the increased viscosity by dissolving starch and other compounds from

the rhizomes causes this deterioration. After each step, it would be necessary to adjust the volume of the extraction medium. Consequently, the advantage of reusing the extraction medium is limited and almost the same amount of solvent is necessary. Thus, the direct reusability of the extraction medium is not appropriate, if a high irone yield is desired.

Nonetheless, as discussed in a previous work, reusing the extraction medium is possible after re-extracting the irones with an organic solvent.[28] This re-extracted micellar solution can be directly used for additional runs without purification. Only after a third repetition, the yield of irones was slightly decreased. A disadvantage, however, is the need of an organic solvent, which is undesirable regarding the principles of Green Chemistry.

Therefore, the best manner to be in accordance with Green Chemistry is to saponify the separated myristic acid and to start the extraction process from the beginning. Using molecular distillation, this recycling is easily possible. Additionally, this method works without the need of toxic and flammable solvents, and long extraction times as well as high temperatures are minimized.

3.3.11. Scale-up

In cooperation with the company Phytotagante S.A.S. in Toulouges (France), a scale-up of the micellar extraction procedure of iris rhizomes was tested. Phytotagante is specialized in the production of plant extracts for perfumery, natural cosmetics, and aromatherapy. Essential oils, aromatic hydrosols, concretes, resinoids, absolutes, rare vegetable oils and various extracts are produced there by solvent extraction or distillation in big scale.[75] During a 10-day stay in November 2015, a large-scale extraction was carried out to check potential problems and difficulties by changing the procedure from laboratory scale to commercial production.

10 kg of dried and powdered *Iris germanica* L. rhizomes (3 years old) were soaked in 250 L of an aqueous potassium myristate solution (c = 0.1 mol/L) for 60 min at 45 – 50 °C under stirring. For the isolation of the desired irones from the extraction medium, it was necessary to remove the extracted rhizomes first, which turned out to be a great

challenge. Filtration using filter papers with various pore diameters required several hours. The soaked rhizomes were swollen such strongly that it was impossible to filtrate the extraction medium completely. Furthermore, it was necessary to heat the solution slightly during the long-lasting filtration process because of the solubility temperature of potassium myristate (30 °C). Also, the vacuum filtration technique using a large Buchner funnel was not suited for the separation process due to the strong foaming of the micellar solution under vacuum. The method of choice in laboratory scale was centrifugation. Thereby, the rhizomes were removed completely without any loss of extraction medium. Since no centrifuges were available at the company Phytotagante to centrifuge 250 L, and also the filtration process failed, the rhizomes were allowed to settle for 3 hours at slightly increased temperatures. After sedimentation of the plant material, the supernatant was transferred by pumping. The powdered iris rhizomes were of different particle size, as it was already mentioned in 3.3.6.1. This different granulation degree led to variations in sedimentation. A few particles remained in the extraction solution after decantation. Consequently, also the fatty acid precipitate, which was received after neutralization with hydrochloric acid, contained traces of rhizomes. The amount was calculated to be around 10 % of the initially used quantity of rhizomes. The fatty acid precipitate containing the desired irones could be separated by vacuum filtration. In this case, vacuum filtration was possible, because the neutralization was carried out in excess to destroy the foaming character of the soap solution. Heating during this filtration step was no longer required. On the contrary, cooling improved the separation process of the fatty acid precipitate from the aqueous phase. The separated precipitate containing the desired irones was mixed with ethanol in a next step to remove remaining plant material, such as starch as it is described in 3.3.8.2. However, the precipitate still contained remaining iris rhizome particles. Thus, it could not be avoided that during the purification step, ethanol also extracts the remaining iris rhizomes. As a consequence, the composition of the extract obtained this way differs from the extract processed with ethanol without rhizomes.

To conclude, micellar extraction of iris rhizomes in large scale is not possible either, as in laboratory scale, without the use of a centrifuge. To scale-up the green extraction method, even a continuous centrifuge is necessary. Thereby, larger volumes of plant material can be separated within saving of time. Centrifugation is also advantageous for the separation

of the fatty acid precipitate. Working with potassium myristate enables extraction at room temperature, respectively 30 °C. Thus, it is even possible to centrifuge without additional heating due to the short contact time. However, to separate remaining water from the fatty acid phase containing the desired irones, it has been found that melting the mixture and subsequent centrifugation is the method of choice. As a consequence, a continuous centrifuge with an additional heating system would be best for the irone extraction using micellar media.

3.4. Conclusion

In this chapter, an alternative and green approach in laboratory and industrial scale was examined to extract the fragrance compounds from iris rhizomes. Commonly, the valuable irones are obtained by long-lasting hydro distillation or by solvent extraction of the iris rhizomes. Both methods have disadvantages like high energy consumption or the use of flammable, volatile and often toxic solvents.

Extraction with biodegradable, non-toxic and non-flammable aqueous soap solutions represents a green and alternative method for plant extraction.[76, 77] With the already patented method, nonpolar components can be extracted by aqueous solutions due to the amphiphilic character of soaps. The results of the investigated experiments are shortly summarized in the following.

First of all, the optimum parameters to extract the irones from iris rhizomes were examined using an aqueous sodium myristate solution. The extracts respectively the irone yields were analyzed by GC-FID/MS. The total irone content in *Iris germanica* L. rhizomes was determined to be 290 ± 5 mg/kg. Soaking the rhizomes in a soap solution of 0.12 mol/L for a short time (30 – 60 min) at a moderate temperature (45 °C), led to an almost complete extraction of the desired irones. 285 ± 16 mg/kg irones (≙ 98 %) could be extracted with a solid (rhizomes) to liquid (micellar solution) ratio of 1/30. Moreover, it was possible to carry out the extractions at room temperature by using potassium myristate instead of sodium myristate without any loss of fragrance compounds.

Also varying the chain length of the soap surfactant and mixing soap surfactants with different carboxylate chains was investigated, since various fatty acids are naturally occurring in the rhizomes. Adding shorter-chained fatty acid salts to the sodium myristate solution led to an adjustment of the extraction selectivity due to the increase of the polar character of the micellar medium. Consequently, a tailor-made extraction was possible by using different fatty acid salts. Thus, this extraction method uses an intrinsic plant substance as highly efficient extraction medium and solubilizer.

Not only the extraction itself but also the isolation of the irones from the extraction medium was part of this work. To this purpose, several attempts were investigated to find

the best and most efficient isolation method. The easiest and most economical way to recover the desired irones was to lower the pH value of the aqueous soap solution by the addition of an acid. The free fatty acids are formed, and thus, the micelles break up causing the release of the nonpolar irones. These valuable compounds migrate to the likewise insoluble fatty acid phase and can be separated together by filtration or centrifugation. As myristic acid is already a frequent ingredient of formulations like crèmes and lotions or perfumes, the final product with its remaining fatty acid represents an ideal basic raw material. However, the irone concentration in it is very low. In addition, excess myristic acid can be recovered by crystallization in cold ethanol or preferably by molecular distillation. This separation process allows not only the enrichment of the irones but also the recovery of myristic acid, which afterwards can be saponified again and reused for subsequent extraction cycles.

3.5. Experimental

3.5.1. Reagents

The following chemicals were used without further purification: sodium butanoate (Merck, for synthesis), sodium hexanoate (Sigma-Aldrich, 99 – 100 %), sodium octanoate (Sigma-Aldrich, purity ≥ 99 %), sodium decanoate (Sigma-Aldrich, purity ≥ 98 %), sodium dodecanoate (Sigma-Aldrich, purity 99-100 %), sodium myristate (Sigma-Aldrich, purity ≥ 99 %), sodium palmitate (Sigma-Aldrich, purity ≥ 98.5 %), sodium oleate (Sigma-Aldrich, purity ≥ 82 %), potassium myristate (Stéarinerie Dubois, surfactants cleansing agents), myristic acid (Sigma-Aldrich, purity ≥ 99 %), formic acid (Sigma-Aldrich, for synthesis), acetic acid (Sigma-Aldrich, purity ≥ 96 %), sulfuric acid (Merck, purity ≥ 98%), anisaldehyde (Merck, purity ≥ 98 %), calcium chloride (Merck, anhydrous powder Reag. Ph Eur), magnesium chloride (Sigma-Aldrich, anhydrous ≥ 98 %), ethanol (Sigma-Aldrich, purity 99 %), diethyl ether (Merck, for analysis), toluene (Merck, for analysis), ethyl acetate (Carl Roth, purity≥ 99 %), 2-methyltetrahydrofuran (Pennakem, purity ≥ 99 %), hydrochloric acid (Sigma Aldrich, reagent grade 37 %), hydrogen chloride solution (Merck, (1N) Reag. Ph Eur, Reag. USP), sodium hydroxide (Merck, pellets for analysis), sodium hydroxide solution (Sigma-Aldrich, volumetric 1.0 M) and silicone anti-foaming emulsion (Roth, 30 % polydimethyl siloxane). HPLC grade methanol from Merck was used during the analysis. Standard irone, a technical mixture of isomers (purity ≥ 90 %, GC) and α-ionone (purity ≥ 90 %) were purchased from Sigma-Aldrich as well as trimethylsulfonium hydroxide solution (0.25 M in methanol, for GC derivatization). A reference iris butter received by steam distillation was obtained from Phytotagante. Deionized water was used throughout the experiments.

3.5.2. Iris rhizomes

The dried rhizomes of *Iris germanica* L. and *Iris pallida* Lam. were obtained from Phytotagante in Toulouges, France. The rhizomes were cultivated in the south of Marrakech, Morocco, and were harvested approximately three years after planting. *Iris germanica* L. rhizomes were dried for three years and delivered ground. *Iris pallida* Lam.

rhizomes were stored for nine years in pieces and crushed with a kitchen device to obtain a powder before using.

To determine the particle size distribution, the rhizomes were sieved by means of standardized sieves according to DIN. The test was carried out in triplicate with an average sample of *Iris germanica* L. rhizomes.

3.5.3. Soxhlet extractions

Solid-liquid extractions were performed by using a Soxhlet apparatus. About 8 g rhizomes were extracted for 6 h with 50 mL of methanol. This corresponded to approximately 70 extraction cycles. Every experiment was repeated three times (n = 3).

For HPLC-UV analysis, the Soxhlet extracts were adjusted at room temperature with methanol to a final volume of 50 mL. Using α-ionone as internal standard (0.5 mg/mL), the concentration of irones was determined by performing a triplicate analysis with internal calibration (see 3.5.11.2).

The procedure for GC-FID/MS measurements had to be adapted to prevent a contamination of the non-volatile compounds in the column. Therefore, the solvent of the Soxhlet extracts was evaporated using rotary evaporation. After the addition of the internal standard α-ionone, the extract was purified by dissolving in hexane. To separate the non-soluble waxes, the mixture was filtered through 0.2 µm PTFE syringe filters. The concentration of the irones was determined by performing a triplicate analysis with internal calibration (see 3.5.11.1).

3.5.4. Recovery experiment

To 100 mL aqueous sodium myristate solution (0.1 mol/L), 150 µL irone (Sigma-Aldrich, technical mixture of isomers) were added and stirred for 30 min at 50 °C. By the addition of hydrochloric acid until a pH value of 5 was reached (pH paper), the precipitation of myristic acid occurs. This precipitate was filtered under vacuum and dried at atmospheric

conditions. 50 mg of the dried precipitate were dissolved in 1 mL of internal standard solution (0.5 mg α-ionone/mL methanol). The analysis was carried out by GC-FID/MS (see 3.5.11.1) performing three independent measurements. The reference system was prepared by merging the same amount of resulting myristic acid (2.33 g) with the equal quantity of irones (150 µL). Every experiment was carried out in triplicate (n = 3).

3.5.5. pH stability

Ten aqueous samples, representing all pH values from 1 till 10, were prepared using hydrochloric acid and sodium hydroxide (0.1 mol/L respectively). The pH value was adjusted using a *pHenomenal pH 1000 L* pH meter from VWR. To 10 mL of each aliquot, 15 µL of α-ionone were added. The samples were stirred at 55 °C for three hours and afterwards cooled in a refrigerator for the same time. By adding 5 mL diethyl ether, α-ionone was extracted from the aqueous phase. All organic phases including a reference sample (pure α-ionone in the same concentration) were dotted to a TLC plate (classical TLC Silica Gel 60 F_{254} plate) with a concentration of 5 µL as described in 3.5.11.3.

The extractions of iris rhizomes with water, NaOH (0.1 mol/L) and NaC14 (0.1 mol/L), respectively, were carried out as described in the following.

3.5.6. Extraction procedure to optimize the extraction parameters

The soap solutions were prepared by weighing in the fatty acid soaps in snap cover glasses (sodium octanoate, sodium decanoate, sodium dodecanoate, sodium myristate, sodium oleate, sodium palmitate, potassium myristate, respectively), adding a certain amount of water and stirring at slightly increased temperature until a clear solution was obtained (45 °C, except for sodium palmitate 60 °C). Generally, approaches of 10 mL were carried out. To these solutions, a certain amount of rhizomes were added. The extraction of irones was performed by employing various extraction conditions, including different soap solutions and concentrations, various extraction times, solid to liquid ratios and extraction temperatures. The processing was performed the same way with all samples

and every experiment was carried out in triplicate (n = 3). After soaking, the rhizomes were removed via centrifugation at 3700 rpm for 10 min at 50 °C using a *Sigma 3-18KHS centrifuge*. To re-extract the irones out of the aqueous solution, a small amount of diethyl ether was added and mixed directly in lockable centrifuge tubes (volumetric capacity 15 mL). The mixture was centrifuged again at 3700 rpm for 5 min at 20 °C to get a clear phase separation between aqueous and organic phase. The solvent layer was transferred, and the re-extracting step was repeated two times. For the isolation of the fragrances, the collected solvent layers were evaporated to dryness. The obtained residue was dissolved in a solution of internal standard (0.5 mg α-ionone/mL methanol), mixed in an ultrasonic bath at room temperature to enhance the solubility, filtered through a 0.2 µm PTFE syringe filters and measured immediately via GC-FID/MS (see 3.5.11.1), alternatively with HPLC-UV (see 3.5.11.2).

Ultrasound-assisted extractions were carried out at room temperature by placing the samples in an ultrasonic bath for several minutes. Microwave-assisted extractions were performed using a *SP Discover (CEM) Microwave Synthesizer*. Extractions were realized at 100 W for the same time to evaluate the different extraction methods.

For the percolation experiments, a heatable column was purchased from the glass blowing workshop of the University of Regensburg. With this individual design, it was possible to percolate iris rhizomes with an aqueous sodium myristate solution (0.1 mol/L) at 50 °C. The ground rhizomes were mixed with sea sand and filled into the column. The extraction medium was added dropwise. To accelerate the percolation process, the column was evacuated repeatedly. To determine the extracted irone yield, the obtained and directly filtered solution was re-extracted with diethyl ether. After evaporation of the solvent to dryness, the extract containing the fragrance compounds was dissolved in internal standard solution (0.5 mg α-ionone/mL methanol) and measured immediately via HPLC-UV (see 3.5.11.2).

3.5.7. Isolation methods

3.5.7.1. Re-extraction with an organic solvent

The isolation of the irones from the extraction medium by re-extraction with an organic solvent was done like mentioned in 3.5.6. As organic solvent, diethyl ether and 2-methyltetrahydrofuran were used.

For larger batches (> 100 mL), separating funnels were applied. To gain a better phase separation, waiting over night or additional centrifugation was implemented. This re-extraction step was repeated three times, respectively. Afterwards, the collected solvent layers were evaporated to dryness. The determination of the irone yield was carried out using GC-FID/MS (see 3.5.11.1) using α-ionone as internal standard (0.5 mg α-ionone/mL methanol).

3.5.7.2. Precipitation of myristic acid

Analogously, after soaking the rhizomes in an aqueous sodium myristate solution, the rhizomes were removed by centrifugation at 50 °C. By equimolar addition of hydrochloric acid (c = 1 mol/L) (+ 10 % excess) until a pH of 5 was reached, the fatty acid salts were neutralized. The fatty acid precipitate containing the desired irones was settled over night before filtration (4 °C). Due to the still high content of remaining plant material and residual water, the precipitate was dissolved in warm ethanol und filtered again. After solvent evaporation, the fatty acid crystals were further dried by melting and centrifuging at 55 °C. After cooling, the crystallized myristic acid phase could be separated from the water phase. The extract containing the fragrance compounds was dissolved in internal standard solution (0.5 mg α-ionone/mL methanol) and measured immediately via GC-FID/MS (see 3.5.11.1). The experiment was carried out two times (n = 2).

3.5.7.3. Breaking the micelles by adding ethanol

The ternary phase diagram of sodium myristate/ethanol/water was measured at 60 °C in a water bath. Mixtures of sodium myristate and ethanol with determined weight ratios were prepared in test tubes with a magnetic stir bar and a starting weight of 1.5 g. The samples were mixed by vortexing for 30 seconds and afterwards stirring in a tempered water bath. Water was added until the mixtures get clear and the mass of the added water was determined. Afterwards, ethanol was added until the solutions get cloudy again. The mass of additional ethanol was added to the mass of ethanol added first to get further weight ratios. This was done to economize the expensive sodium myristate salt. The weight percent of each compound was plotted to a ternary phase diagram.

DLS measurements were performed using an ALV/CGS-3 goniometer system equipped with a vertical polarized HeNe laser and an ALV/LSE-5004 correlator under thermostatic control at 60 °C. All one-phase samples nearby the phase boundary were measured. To remove dust, the samples were filtered using a 0.2 µm PTFE membrane filter. Data have been collected in various periods from at least 60 seconds to 180 seconds while samples are tempered at 60 °C. The wavelength of the laser was set to 632.8 nm and the angle was 90 °.

3.5.7.4. Precipitation of poorly soluble lime soaps

To isolate the irones from the aqueous sodium myristate solution, a stoichiometric amount of calcium chloride (+ 10 % excess) was added under stirring at room temperature to precipitate the insoluble lime soaps (n = 2). Afterwards, the precipitate was filtered under vacuum using a Buchner funnel. The filtered aqueous phase was re-extracted with diethyl ether in a separating funnel to determine potentially remaining irones. The irones contained in the precipitate were dissolved in ethanol under stirring and ultrasound sonication. The insoluble calcium myristate precipitate was filtered under vacuum again and was washed several times with fresh ethanol. After evaporation of the solvent, the obtained fragrances were dissolved in internal standard solution (0.5 mg α-ionone/mL methanol) and measured via GC-FID/MS immediately (see 3.5.11.1).

3.5.7.5. Combination with hydro distillation

For the reference hydro distillation, 100 g *Iris germanica* L. rhizomes of unknown origin were soaked in 2 L water for 90 min at 50°C. Subsequently, hydro distillation was carried out for 150 min using a Clevenger apparatus (n = 3). The temperature of the condenser was set to 8 °C. The obtained iris butter was dissolved in methanol and adjusted to a final volume of 10 mL. Using α-ionone as internal standard (0.5 mg/mL), the concentration of irones was determined by GC-FID/MS (see 3.5.11.1).

In the other cases, 100 g rhizomes were soaked in 2 L aqueous sodium myristate solution (c = 0.12 mol/L) for 90 min at 50 °C. The combination with hydro distillation was carried out once with and once without rhizomes (in each case n = 1). To remove the rhizomes, centrifugation was implemented for 10 min at 50 °C and 4500 rpm. Precipitation of myristic acid was done by slowly addition of hydrochloric acid (c = 1 mol/L) under stirring until a pH of 4 was reached. Precipitation of the lime soaps was done by adding a stoichiometric amount of calcium chloride (+ 10 % excess) under stirring. Immediately after the precipitation, respectively, hydro distillation was performed for 150 min.

The anti-foaming agent was directly added after soaking the rhizomes in an aqueous sodium myristate solution (c = 0.12 mol/L) for 90 min at 50 °C. The rhizomes were not removed before, and subsequent hydro distillation was carried out for 150 min as well (n = 1).

The obtained extracts were dissolved in methanol and adjusted to a final volume of 10 mL, respectively. In addition to the reference sample, TMSH as methylation reagent was added to the solution, as it is described in 3.5.11.1. Using α-ionone as internal standard (0.5 mg/mL), the concentration of irones was determined by GC-FID/MS (see 3.5.11.1).

3.5.8. Enrichment of the irones in the extract

For the experiments to enrich the irones in the extract, a reference extract with a known amount of irones was prepared. To this purpose, 4.55 g of an extract, which consists of 4.25 g myristic acid and 0.30 g irone (technical mixture, Sigma-Aldrich), was artificially

produced by melting and homogenizing. This artificially extract was dissolved in 70 mL ethanol at slightly increased temperature under stirring (n = 1). The mixture was allowed to crystallize overnight at - 20 °C. The subsequent filtration of the crystallized myristic acid particles was done at the same temperature under vacuum. The precipitate was washed with cold ethanol and dried at room temperature afterwards. The mass of recovered myristic acid was determined by weighing. To isolate the irones, the solvent was evaporated using rotary evaporation. Using α-ionone as internal standard (0.5 mg/mL), the concentration of irones was determined by GC-FID/MS (see 3.5.11.1).

3.5.9. Reusability of the extraction medium

Micellar extractions of *Iris germanica* L. rhizomes to reuse the extraction medium were carried out as described in 3.5.6 using an aqueous sodium myristate solution (c = 0.12 mol/L) with the following parameters: solid to liquid ratio 1/30, extraction time 30 min and extraction temperature 55 °C. After removing the soaked rhizomes by centrifugation, the same amount of rhizomes was added to the extraction medium again. This step was repeated twice so that the extraction medium was reused three times. This experiment was carried out three times (n = 3). The further procedure was handled as described above and measurements were carried out via GC-FID/MS (see 3.5.11.1).

3.5.10. Scale-up

At the company Phytotagante S.A.S. in Toulouges (France), a scale-up of the micellar extraction of iris rhizomes was carried out. 10 kg of dried, powdered *Iris germanica* L. rhizomes (3 years old) were soaked in 250 L of an aqueous potassium myristate solution (c = 0.1 mol/L) for 60 min at 45 – 50 °C under stirring. Then, the rhizomes were allowed to settle for 3 hours before the aqueous solution was removed by pumping. To approximately 200 L solution (largely free of rhizomes), hydrochloric acid was added under stirring until a pH of 3 was reached. This mixture was stored over night at 5 °C before the myristic acid precipitate was filtered under vacuum using a Buchner funnel.

7 kg of the precipitate were mixed with around 25 L ethanol (96 %) and stored without further processing.

3.5.11. Analysis and quantification

3.5.11.1. Gas chromatography

The analysis was performed on a GC-FID system (Agilent 7890A) consisting of a 7693 Autosampler, and a FID-Detector coupled with a 220 Ion Trap GC/MS. Separations were achieved on a VF-5ms column (30 m x 250 µm x 0.25 µm) with helium as carrier gas with a constant flow of 1 mL/min. The following temperature program was used (compare Figure 44): 100 °C to 250 °C at 10 °C/min and then to 300 °C with 50 °C/min. The samples (1 µL) were injected with a split/split less injector (split 1/50). The injector temperature was set to 250 °C and the temperature of the flame ionization detector was set to 300 °C. Qualitative and quantitative analysis was performed by analyzing retention times and peak areas. Irones were quantified by internal calibration.

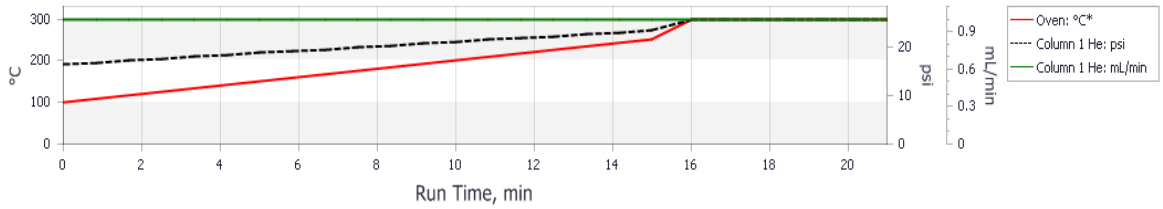


Figure 44: GC method to quantify irones.

Internal calibration was carried out using α-ionone as internal standard. To this purpose, a stock solution, of 10 mg/mL irones (Sigma-Aldrich) in methanol was prepared. From this stock solution dilutions of 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL were produced. Another stock solution of α-ionone in methanol (10 mg/mL) was prepared and diluted to 1 mg/mL. Each time, 1 mL of the diluted internal standard solution was added to 1 mL of the various analyte dilutions. After mixing for a few seconds in an ultrasonic bath, the mixtures were filtered using 0.2 µm PTFE syringe filters and

measured by GC-FID/MS. Every sample was prepared in triplicate and also the measurements were carried out three times.

In case of overlapping of the fatty acid peak with the irone isomers (described in 3.3.1.3), methylation with TMSH was carried out. To 100 µL sample solution 50 µL TMSH reagent (0.2 M in methanol) was added. After mixing for a few seconds in an ultrasonic bath, the mixture was filtered using a 0.2 µm PTFE syringe filter and measured via GC-FID/MS.

3.5.11.2. High-Pressure Liquid chromatography

The analysis was performed on a Waters HPLC system consisting of two Waters 515 HPLC Pumps, Waters 717 plus Autosampler, Waters UV/VIS-Detector and Waters Empower 3 Software. Separations were achieved on a Knauer Eurosphere C18-column (100 Å, 250 x 4.6 mm, 3 µm). The mobile phase consisted of: (A) 0.1 % aqueous formic acid and (B) methanol (HPLC grade, Merck). With a flow of 0.7 mL/min, the gradient was increasing from 30 % B to 100 % B in 35 min and this composition was held for 15 min. The equilibration time before and after a measurement was 7 min. Further conditions were set as following: injection volume 10 µL, maximum absorption wavelength 230 nm and column temperature 40 °C. All measurements were repeated twice. The peak assignment was carried out using standard irones from Sigma-Aldrich and the iris butter purchased from Phytotagante. Irones were quantified by internal calibration with α-ionone as described above.

3.5.11.3. Thin-layer chromatography

Thin-layer chromatography was performed by applying a small spot of solution (usually 5 µL, 1 mg/mL) to a classical silica TLC plate (Silica Gel 60 F_{254} plate, Merck). After sample preparation, the plate was placed in a separation chamber with toluene: ethyl acetate (97/3, v/v) as the mobile phase. After staining with an anisaldehyde/sulfuric acid solution and heating to 105 °C, sharp spots of irones were visible in a dark violet color. The staining solution consisted of 0.5 mL anisaldehyde (98 %, Merck), 10 mL acetic acid (96 %, Merck), 85 mL methanol (HPLC grade, Merck) and 5 mL sulfuric acid (98 %, Merck).

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4. Micellar extraction of roses

4.1. Introduction

In a first study and as outlined in the last chapter, it could be demonstrated that micellar extraction with biocompatible and biodegradable aqueous soap solutions can be applied to extract and isolate the valuable nonpolar irones from iris rhizomes. By using a myristate solution, an almost complete extraction was possible within a short time and at moderate temperatures. This gentle method not only enables a reduction in extraction time and energy consumption, but also prevents the degradation and volatilization of the fragrances at high temperatures, which are disadvantages of conventional extraction methods.

The high temperature is also a known problem by distilling the essential oil of roses.[1] Rose fragrances are key ingredients in perfume and cosmetic industry. Citronellol, geraniol, nerol, and 2-phenylethanol are characteristic and valuable ingredients among others. Because of its versatility and harmonizing effect on body and soul, the Damask Rose was selected by NHV Theophrastus as medicinal plant 2013. The medicinal plant of the year is proclaimed annually in Germany since 1990 by an association for the promotion of natural healing.[2, 3] In contrast to distillation, the majority of the constituents contained in rose blossoms remain unaltered throughout solvent extraction, since the oil is not subjected to heat. Thus, thermal degradation and destructive oxidations are avoided. Moreover, rose oil obtained by distillation contains no or only a very low concentration of water-soluble substances. Especially, 2-phenylethanol, which is a very characteristic fragrance molecule of roses, is only present in minor amounts due to its water solubility.[4] Thus, for perfume industry, the rose absolute, which is produced by solvent extraction, is of high interest. However, not only the risk of handling large quantities of flammable and often toxic solvents, but also the danger of potential residues in the extract led to the fact that solvent extraction, especially with the most commonly used *n*-hexane, is increasingly questioned.[5]

In the human body, hexane can be bio-transformed to 2,5-hexanedione, which leads to nerve damage. As a consequence, in the medically orientated aromatherapy and also

partly in the food industry rose absolute is rarely used, because toxic residues of hexane are possible.[3, 6, 7] In the production or fractionation of fats and oils and the production of cocoa butter, only hexane residues of 1 mg/kg are admitted, for instance.[8] In addition, hexane is included in the list of the European Parliament among the substances, which are prohibited in cosmetic products.[9] This regulation (EG 1223/2009) harmonizes legislation on cosmetic products in the European Community to ensure a high level of protection of human health. The regulation has been applicable in all Member States of the European Union since 2013. Furthermore, in 2012, the impact of *n*-hexane on the human health and the environment has been investigated within the framework of the substance assessment under REACH (European Union regulation concerning the Registration, Evaluation, Authorization and Restriction of Chemicals). The evaluation has not yet been finished, but it is not excluded that hexane will be completely forbidden as extraction medium in the future.[10]

Owing to the numerous drawbacks of the conventional rose extraction methods as well as the possible necessity of replacing hexane, it was investigated in a second study if the micellar extraction method tested for iris rhizomes can be transferred to roses. In collaboration with a major international perfume company, the micellar extraction method using an aqueous sodium myristate solution was applied on rose blossoms and is described in the following. Furthermore, an approach to isolate also the water-soluble fragrance compounds from the aqueous extraction medium by adding inorganic salts was examined.

4.2. Fundamentals

4.2.1. Rose plant

Roses are perennial flowering plants. The genus rosa comprises 100 to 150 species and belongs to the family *Rosaceae*. Roses are erect, climbing, or trailing shrubs. The stems are usually copiously armed with prickles, called thrones. The flowers vary in size and shape and the color is ranging from white to multi-color. Roses are cultivated for their beautiful flowers and their delightful fragrance, which varies according to the variety and climatic conditions. Most rose species are native to Asia, North America, Europe, and northwest Africa. Today, roses are cultivated practically in all countries where the climate is suited. Roses are best known as ornamental plants and have minor medicinal uses. Moreover, they are widely used for perfumery. Especially the flowers of *Rosa x damascena* Mill. and *Rosa x centifolia* L. are cultivated owing to their contained fragrances (see Figure 45).

Figure 45: Field of *Rosa x centifolia* L. in Pégomas, France.

The most important cultivation areas are France, Morocco, Italy, Bulgaria, Egypt, India, Persia, and Turkey. In December and January, rows of ditches (50 cm in depth and 50 cm in width) are prepared, in which the rose twigs cut at the soil are planted. It takes around

three years for a rose plant to attain maturity. Then, normally 5 tons of fresh roses can be harvested per hectare. The rose harvest is limited to approximately 30 days in May/June. The harvest has to start early in the morning hours, preferably before sunrise, as the oil content of the flowers decreases rapidly as the temperature rises during the day. [3, 4, 11-13]

4.2.2. Ingredients of the rose petals

The most important ingredient of rose petals is the essential oil, which is present in a very low concentration of less than one percent. In *Rosa x centifolia* L. only 0.02 – 0.073 % and in *Rosa x damascena* Mill. only 0.02 – 0.05 % essential oil is contained. However, all flower parts are more or less oily. Thus, the whole flower heads are used for the extraction. The composition of the essential oil depends on the variety and, above all, on the extraction process. It can be distinguished between rose oil obtained by distillation (see 4.2.3) and rose absolute obtained by solvent extraction (see 4.2.4).

Furthermore, 0.4 % flavonoids such as quercetin, campherol, and their galacto- and glycosides are present in rose blossoms. Triterpenes such as β-amyrin, β-sitosterol, stigmasterol, ursolic acid and 2-hydroxy-ursolic acid as well as the diterpene callitrisin acid and benzoic acid are also components of the plant and have beneficial effects on the human health.[3, 4] Several pharmacological properties including anti-HIV, antibacterial, antioxidant, antitussive, hypnotic, antidiabetic, and relaxant effects on tracheal chains have been reported for this plant.[14]

4.2.3. Rose oil and rose water

Rose oil is produced by steam distillation of the rose blossoms. Particularly suitable are blossoms of *Rosa x damascena* Mill. due to their high content of essential oil. In industrial production, generally 400 – 500 kg flowers are distilled with the fourfold amount of water steam. The distillation is carried out for 1.5 – 2 hours. Thereby, the condenser temperature is kept at 35 °C to avoid the crystallization of the contained waxes. The distillate is collected in special Florentine flasks. The so-called “first oil” can be decanted

and the distillation water, which still contains rose oil, is further distilled. The second distillation achieves around 2/3 of the complete yield and differs from the odor and the physicochemical properties of the first oil (1/3). Due to the influence of high temperature decomposition products, such as H_2S, NH_3, CH_3OH, CH_3COOH, CH_3COCH_3, and CH_3CHO, are formed. Nevertheless, the first and second oils are mixed in the natural ratio afterwards to gain the final rose oil. The water, left from the distillation, is sold as rose water and finds application in many kitchens, for example, to flavor food.[1, 4, 13, 15, 16]

Rose oil is a yellow partly crystallized liquid at room temperature with a very characteristic odor of rose blossoms. It finds application in skin care, medicine aromatherapy, and is a key ingredient in fine fragrances used by leading perfume companies like Kenzo, Chanel, Dior, Fendi and many others. The world annual consumption of rose oil comprises 3000 - 4500 kg. Bulgaria and Turkey are the main producers of rose oil supplying 80 – 90 %. The rest of the production is supplied by Morocco, Iran, Mexico, France, Italy, Lebanon, India, Russia, and China. Since approximately 3 tons of blossoms are needed to prepare 1 kg of oil, rose oil is very expensive. In fact, one kilogram rose oil was sold between 5750 and 6000 US$ in 2005. Generally, the price of organically produced rose oil is approximately 20 % higher compared to the oil produced with regular agricultural practices.[2, 15, 17, 18]

The Bulgarian rose oil (*Rosa x damascena* Mill.) contains around 400 substances. The characteristic components are citronellol (20 – 38 %), geraniol (14 %), non-fragrant waxes (16 %), nerol (7 %), 2-phenylethanol (2.8 %), methyl eugenol (2.4 %), linalool (1.4 %), eugenol (1.2 %), farnesol (1.2 %), ethanol (1.2 %), geranyl acetate (0.7 %), α-terpineol (0.7 %), 1-citronellyl acetate (0.5 %), *cis*-rose oxide (0.46 %), hexanol (0.2 %), *trans*-nerolidol (0.2 %), *trans*-geranium acid (0.18 %), *trans*-rose oxide (0.17 %), and phenyl ethyl acetate (0.05 %). Other important components which are only contained in trace amounts are nerol oxide, rosefuran, β-ionone, and especially β-damascenone and β-damascone. Even though these compounds are present only in traces, they contribute more than 90 % to the odor and are blamed for the typical smell of the oil. These rose ketones are derived from carotenoid degradation and only arise during the distillation process. Also, the sulfur compounds contribute to the smell of rose oil.[3, 4, 17, 18] The chemical structure of some important ingredients is given in Figure 46.

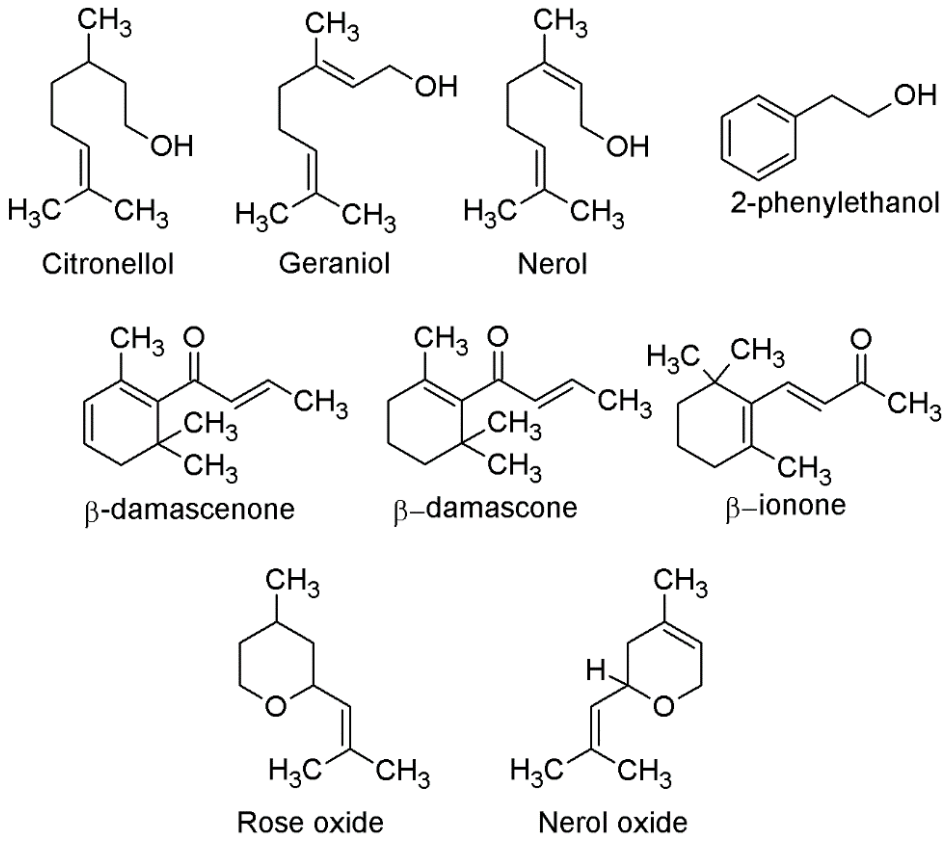

Figure 46: Overview of some important rose oil ingredients.

In view of the multitude of odoriferous compounds, rose oil is one of the most complex essential oils. However, as a result, adulterations are often difficult to detect. Because of the high price, rose oils are sometimes diluted to extend the fragrance compounds. Typically, palmarosa oil, geranium oil and especially the synthetic geraniol, citronellol, and 2-phenylethanol are used to dilute the rose oil.[4]

4.2.4. Rose absolute

Another possibility to gain the valuable ingredients of rose blossoms is solvent extraction. The centifolia species are particularly suited for solvent extraction, because they possess a lower oil content as *Rosa x damascena* Mill., which are commonly treated with water steam to produce the rose oil.[3, 4] Traditionally, the rose absolute was obtained by soaking the petals in animal fat (see enfleurage, chapter 5). Nowadays, this elaborate process has been completely replaced by petrochemical solvents. It can be distinguished between the

static and the rotary process. In the static procedure, the roses are fed on perforated tablets and the extractant (commonly *n*-hexane) is flowed through in the countercurrent process. This procedure is still applied, as it can be seen Figure 47.

Figure 47: Solvent extraction of rose blossoms in the South of France, May 2016.

The rotary process, in which the solvent consumption can be minimized, is typically carried out in 3000 L extraction vessels equipped with stirrers. 600 – 750 kg rose flowers are extracted with around 1500 L *n*-hexane for 20 min at 60 – 65 °C. After removing the extraction solution, the process is repeated with fresh solvent at least once. In both processes, the solvent is evaporated under vacuum afterward and the so-called rose concrete is achieved. It is a waxy, semi-solid material with a pink-red color and represents the true fragrance of rose blossoms. Approximately 400 kg fresh blossoms are needed to receive 1 kg rose concrete. This represents the 10-fold yield compared with rose oil distillation. In 2005, the price of 1 kg rose concrete was between 600 and 650 US$.[15]

However, because of its limited solubility, the concrete cannot be used directly in the perfume industry. Therefore, the concrete has to be extracted again. In this extraction process, 1 kg concrete is dissolved in 8 – 10 L hot ethanol. Afterwards, the mixture is

cooled to freezing temperatures to precipitate waxes and fatty acids. The cooled ethanolic extract is filtered and solvent evaporation under vacuum yields the rose absolute with a yield around 65 %. The absolute is a reddish liquid and its scent is described as rich, sweet, rose spicy, and honey. Rose absolute mainly consists of 2-phenylethanol, citronellol, geraniol, nerol, eugenol, methyl eugenol, geranyl acetate, benzyl alcohol, nonadecane, nonadecene, and farnesol. Although the rose absolute contains the typical essential oil components, it differs strongly from the rose oil obtained by distillation (see Table 17). The primary difference is the content of 2-phenylethanol. In rose absolute, the content amounts to 60 – 75 % and thus, represents the main constituent. Since 2-phenylethanol is soluble in water, its content in rose oil is with 1 -3 % generally low. Since the fragrance compounds are not subjected to such high temperatures as compared with distillation, most of the ingredients remain unchanged in the rose absolute.[4, 15, 18-20]

Table 17: Chemical composition of rose oil and rose absolute.[4, 21]

	Rose oil (distillation)	Rose absolute (extraction)
Geraniol and citronellol	50 – 70 %	30 – 34 %
Nerol	5 – 10 %	5 – 10 %
2-phenylethanol	1 – 3 %	60 – 75%
Eugenol	1 %	1 %
Linalool and citral	Traces	Traces

Rose absolute, as well as rose oil, find mainly application in the perfume and cosmetic industry. In contrast to rose oil, rose absolute is rarely used in the medically orientated aromatherapy and in the food industry because toxic residues of hexane are possible.[3]

One alternative extraction method for roses is already described in the literature. Rose oils which are extracted with supercritical CO_2 are characterized as pure and very close to the natural scent of roses. In addition, they are completely free of toxic residues.[15, 16] However, the investment costs of an industrial plant are very high and the method development is very time-consuming.[22] In the following, the micellar extraction method using soap surfactants is examined as alternative method to gain the desired compounds.

4.3. Results and discussion

4.3.1. Analytics

Before starting the extraction experiments to examine whether it is possible to transfer the micellar extraction procedure to rose blossoms, it was necessary to establish an appropriate analysis method to determine the yield of the recovered rose fragrances. Due to the fact that geraniol is a key compound in rose oil and present in a high and known concentration, the analysis was focused on this molecule. For a fast screening, thin-layer chromatography was applied. For visualizing the spots, a staining reagent based on p-anisaldehyde was sprayed onto the plate. Geraniol became visible in a dark violet color. This method was applied to make first predictions about the presence of geraniol or other similar compounds in the extracts.

A more detailed analysis was achieved using GC-FID/MS (see method in Experimental p. 158). Due to the coupling of a MS detector and the linkage with the NIST databank, the analysis and the verification of several important fragrance compounds was possible.

An artificial rose scent (obtained from an industrial partner) was used for various extraction experiments due to the lack of fresh rose petals with a high content of essential oil. In this case, the extraction efficiency of the experiments was determined by comparing the obtained peak areas with the peak areas of a reference sample. The artificial rose scent consisted of 50 % 2-phenylethanol, 32 % citronellol, and 18 % geraniol.

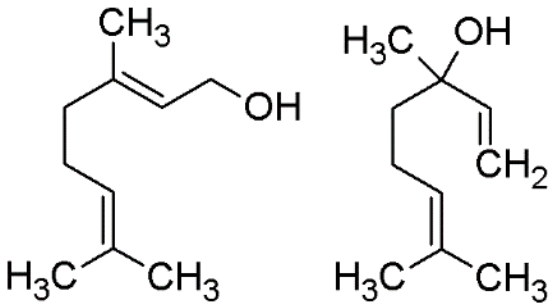

Figure 48: Chemical structure of geraniol (left) and the corresponding internal standard linalool (right).

For a quantitative prediction, the content of geraniol was determined via internal standard calibration. To this purpose, linalool was used as internal standard, because of its isomerism and chemical similarity to geraniol (see Figure 48). Although linalool is present in minor amounts in roses, it is absent in the rose scent from our industrial partner, which was used during most of the experiments. The response factor was determined by performing a multipoint calibration, which can be seen Figure 49. In the course of this, the ratio of the geraniol peak area and the linalool area against the ratio of geraniol concentration and linalool concentration was plotted. The reciprocal slope of this linear function represents the response factor, which was calculated to be K = 0.96. For the analysis, linalool was added to every sample in a known concentration, and the amount of geraniol was estimated according to the equation indicated in 3.3.1.2.

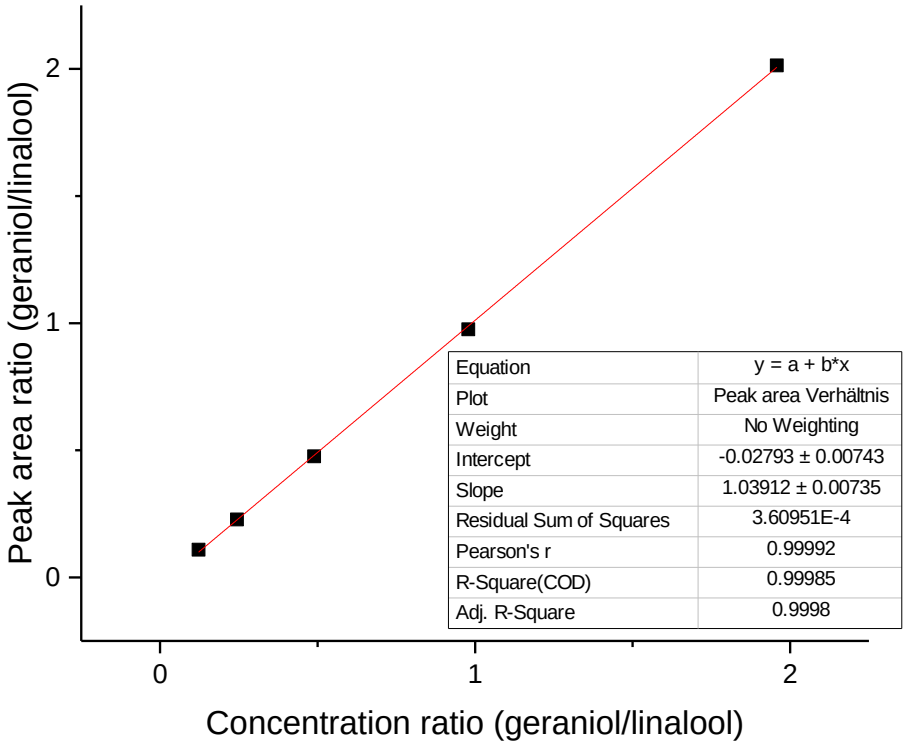

Figure 49: Determination of the response factor for internal standard calibration using GC-FID/MS (as described in Experimental p. 158).

4.3.2. pH stability of geraniol

Throughout the micellar extraction procedure, the desired fragrances pass a wide range of different pH values. As established with the extraction of iris rhizomes, first of all, the plant material is soaked in an aqueous myristate solution with a pH value of around 10 for a certain time. After removing the plant material, the soap solution is neutralized to isolate the fragrance molecules by adding hydrochloric acid until a pH value of 4 – 5 is reached. Before, it is necessary to consider the behavior of the rose fragrances under extreme conditions to ensure that no decomposition occurs during micellar extractions. A reduced yield and an adulterate fragrance composition would be the consequence.

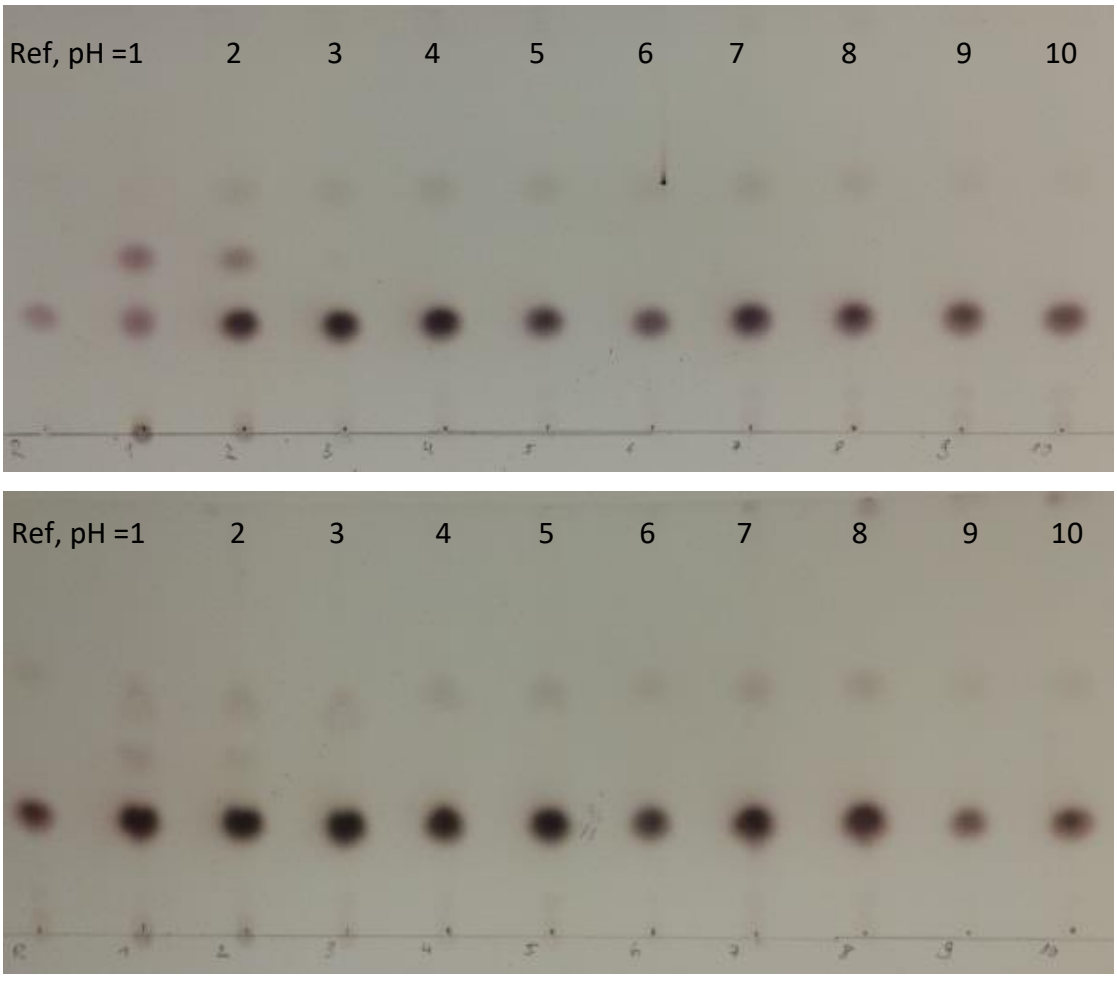

Figure 50: TLC of the stability test of geraniol at different pH values, above: without myristate matrix, below: with myristate matrix (as described in Experimental p. 158).

The stability tests were carried out with geraniol as representative for the rose scent. To this purpose, samples of geraniol with different pH values were stirred at 55 °C for 3 h and cooled in a refrigerator to simulate the conditions during micellar extraction. Thereby, not only the influence of the pH value solely, but also the influence of the extraction medium was examined.

In Figure 50 above, the TLC of the pH stability of geraniol in the pure pH solutions without myristate matrix is shown. For better comparison, a spot of the reference solution (geraniol in diethyl ether of the same concentration) is placed in the beginning of the TLC. The sharp violet spot refers to geraniol. However, also traces of impurities can be seen slightly above the violet spot. Also, at the start line a small residue remains (purity of geraniol ≥ 97 %, purchased from Sigma-Aldrich). At high pH values, geraniol does not show any decomposition as the appearance of the spots is similar to the reference sample. Only at low pH values, clear differences can be seen. At very acidic pH values, including pH = 1 and pH = 2, an additional intense spot appears. Furthermore, the residue at the start line is more colored than in the reference sample. This indicates that geraniol is partly decomposed at low pH values.

The same pH stability experiments were carried out with aqueous sodium myristate as matrix (c = 0.1 mol/L). The obtained TLCs are presented in Figure 50 below. Thereby, the same conclusions can be drawn. At high pH values, no decomposition of geraniol occurs, whereas at pH = 1 and pH = 2, chemical degradation can be noticed. In contrast to the samples without myristate matrix, here the decomposition is less pronounced, as rough quantitative predictions can be made by the intensity of the spots. As a result, it can be assumed that the myristate matrix protects the fragrance compounds from decomposition. To be more precise, the fatty acid produced at low pH values presumably encloses the geraniol molecules, thus preventing them from acid attack.

For a more detailed analysis, the samples with pH = 1 (with and without myristate matrix) were analyzed qualitatively by GC-FID/MS, as it is described in Experimental p. 158. The chromatograms are plotted in Figure 51. The peak of geraniol is marked at a retention time of 5.9 min. In the chromatogram of the sample without myristate matrix (below), it can be seen that geraniol is only present in low concentration recognizing by a small peak

height. In contrast, a lot of decomposition products are apparent. Especially linalool (4.8 min), α-terpineol (5.6 min), and citronellol (5.8 min) were found in high concentrations. Moreover, α-pinene (4.4 min), β-*cis*-ocimene (4.5 min), ocimenol (5.3 min), 1,8-terpin (6.5 min), and isopelugol (6.9 min) were assigned by matching with the NIST database. These findings confirm the results already achieved by TLC.

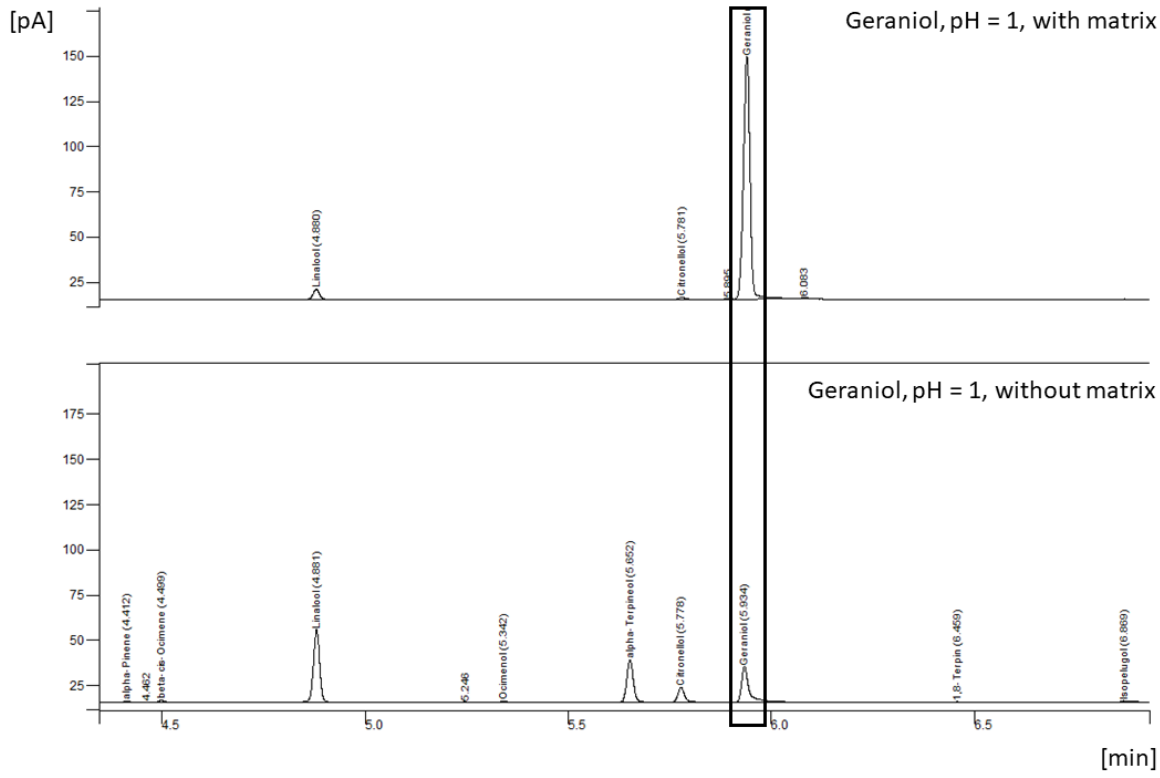

Figure 51: GC-FID/MS chromatogram of geraniol at pH = 1, with myristate matrix (above) and without myristate matrix (below). The geraniol peak is within the added frame (see description of GC-FID/MS method in Experimental p. 158).

The chromatogram of the sample with sodium myristate matrix (Figure 51 above) is much clearer. Only a few decomposition products can be outlined. These are linalool at 4.9 min and citronellol at 5.8 min with very low concentrations. Thus, geraniol is only slightly decomposed in the sample of pH = 1 including myristic acid, as it can be seen regarding the still high peak height.

To sum up, geraniol is stable throughout a wide range of different pH values. Solely at very acidic pH values, decomposition of geraniol occurs. However, the myristic acid contained in the aqueous soap solution protects the fragrance compounds from decomposition. Even a very acidic environment for a short time does not lead to an alteration of the fragrance composition or a loss in extraction yield. Moreover, it could be proven that the alkaline extraction medium has no influence on the stability of geraniol. Thus, the extraction as well as the isolation of rose fragrances is possible applying micellar extraction.

4.3.3. Micellar extraction of roses

In collaboration with a leading perfume company, the micellar extraction procedure, successfully examined for iris rhizomes, was transferred to roses. Since the rose blossoms are harvested very early in the morning and have to be processed fast, the experiments were carried out directly in the laboratories in the South of France. Experiments with *Rosa x centifolia* L. were done during the rose harvest in May 2016.

Fresh roses were added to a solution of sodium myristate (0.1 mol/L) with a solid to liquid ratio of 13.3/1 and stirred for 30 min at 55 °C. The reduced solid to liquid ratio was chosen due to the fact that roses are much more fragile compared to the woody iris rhizomes. Already after one minute, the color of the transparent solution changed strongly to dark green and after 30 min the blossoms were almost completely decomposed, which can be seen in Figure 52 (left). The remaining rose blossoms were filtered through a stainless steel filter and washed with hot water. In contrast to separating the swollen iris rhizomes, filtering of the partly decomposed rose blossoms did not constitute any problem. In order to precipitate myristic acid together with the fragrance compounds, hydrochloric acid was added slowly at room temperature, until a pH of 3 – 4 was reached (see Figure 52 (right)). Because the plant dyes were also protonated, the mixture turned into a pink color. The solution settled for around one hour, allowing the precipitate to mature. Afterwards, the precipitate was filtered under vacuum using a Buchner funnel. The precipitate consists of myristic acid, the fragrances, but also of remaining plant material as known from the extraction of iris rhizomes. To separate remaining cellulose fibers and other plant parts,

the precipitate was dissolved in warm ethanol and filtered again. Approximately 2.4 % of the plant material originally used for the extraction could be found in the precipitate and separated in this way. The residual amount of water was separated by removing ethanol by rotary evaporation additionally.

In a second experiment, this purification step was omitted. To get the myristic acid precipitate containing the desired fragrance compounds completely free of water, the mixture was melted and centrifuged. Due to the low amount of remaining plant material, the separation of water and remaining plant material could be done simultaneously in one step. Compared to the laborious processing of iris rhizomes, this represents an immense saving in time, energy and solvent. Moreover, the risk of losing valuable fragrance molecules is minimized. Since most of the dyes are hydrophilic, the extract is only slightly colored with a magnificent smell of roses. It is completely soluble in ethanol.

Figure 52: Micellar extraction of rose blossoms in an aqueous sodium myristate solution: soaking the blossoms (left) and precipitating the fatty acid after the separation of the roses (right).

In Table 18, an overview of the mass balance of the micellar extraction of roses can be seen. In total, 46.6 g of myristic acid were found in the aqueous sodium myristate solution. After the micellar extraction and precipitation of myristic acid together with the fragrance compounds, the yield of the extract amounted to 41.8 g. That means that 89.7 % of the originally used quantity of myristic acid was recovered. The missing 10 % of myristic acid got presumably lost during filtration.

Table 18: Mass balance of the micellar extraction experiment with roses.

Sample	Yield	
Fresh roses	150 g	
Micellar extraction medium	2.0 L	
Myristic acid (HMyr)	46.6 g	
contained in the extraction medium		
Remaining plant material (dry)	3.6 g	Brown powder, odorless
Maceration with hexane	1.2 g	Contains a lot of HMyr
Washing solution (l/l extraction)	0.1 g	Yellowish oil with odor
Extract	41.8 g	Yellowish crystals with odor

According to the information of our industrial partner, 700 mg fragrance compounds must be present in the extract after the maceration of 150 g rose blossoms. Assuming that the extraction was exhaustive, this corresponds only to 0.017 mg fragrance compounds per gram extract. As the extract should contain a very large number of different fragrances such as geraniol, citronellol, nerol etc., it was impossible to analyze quantitatively the extraction efficiency via GC-FID/MS (see method in Experimental p. 158).

Instead, the fragrance compounds which maybe still remained in the rose blossoms were analyzed. To this purpose, the extracted roses were macerated with hexane over night at room temperature. The yield of the macerate accounted 1.2 g, mainly consisting of waxes and remaining myristic acid. Perfume molecules could not be determined. In the remaining plant material residue also no fragrances could be analyzed. For further analysis, the aqueous solution after the separation of myristic acid was re-extracted with

hexane three times. Approximately 0.1 g of a yellowish oil were recovered. A GC-FID/MS analysis of this oil showed that several fragrance compounds were still present in the washing solution (compare Experimental p. 158). The water-soluble 2-phenylethanol was found in the aqueous phase, but also traces of citronellol, farnesol, geraniol, eugenol, and methyl eugenol. Only qualitative statements were possible.

4.3.4. Recovery of geraniol

To investigate why the desired fragrances are partly dissolved in the aqueous phase and not in the myristic acid phase, recovery experiments with geraniol as key compound were carried out. To this purpose, a known amount of geraniol was added to an aqueous myristate solution and stirred for 60 min at 50 °C. Afterwards, the micelles were destroyed by neutralization with hydrochloric acid and the myristic acid precipitate containing the analyte was analyzed by GC-FID/MS (see method in Experimental p. 158). The peak area of geraniol was compared before and after the recovery experiment, as also performed in 3.3.4. Only 75 % of the initially amount of geraniol were recovered in the fatty acid phase. Compared to the complete recovery of the irones in the previous experiments, this represents an unsatisfactory result. However, this outcome explains why geraniol was found in the aqueous solution of the micellar extraction of roses (see 4.3.3).

With a solubility of 686 mg/L at 20 °C, geraniol is hardly soluble in water.[23] But compared to the very low concentration of geraniol in the rose blossoms and consequently, the low content in the aqueous extraction medium, geraniol is dissolved at slightly increased temperatures in a non-negligible amount. The addition of a kosmotropic inorganic salt can reduce the solubility of hydrophobic compounds in water, whereas the partitioning in the organic layer increases.[24] The only requirement for this salting-out effect, which is described in 2.5, is that the salt is soluble in the water phase and not in the organic phase.

Using sodium chloride, this behavior was investigated in regard to the recovery of geraniol in an aqueous sodium myristate solution. On a trial basis, sodium chloride was added to the neutralized micellar solution. The analysis of the precipitate was carried out

analogously to the experiment without salt addition. 88 % of geraniol could be recovered this way. This implements an increase in yield of 13 %. Consequently, by adding an inorganic salt to the aqueous solution, it is possible to enhance the recovery of geraniol.

4.3.5. Salting-out of 2-phenylethanol

The fact that desired molecules get lost by means of solubility is also known for the very characteristic 2-phenylethanol in industrial preparation of rose fragrances. During hydro distillation, the 2-phenylethanol contained in roses gets lost because of its miscibility with water (compare 4.2.4).[4, 15, 20] Over two-thirds of the 2-phenylethanol is retained during distillation and only one-third of the amount can be recovered within the distillate.[25, 26] This results in a different smell of the rose oil compared to the true rose scent. According to literature, 22 g/L 2-phenylethanol are soluble at room temperature in water.[27] In rose water obtained from our industrial partner and Phytotagante, significantly amounts of 2-phenylethanol were found, as analyzed by GC-FID/MS (described in Experimental p. 158). But, also citronellol and geraniol were found.

The same observation was made by extracting rose blossoms with micellar media. Therefore, the addition of various salts was examined in regard to enhance the extraction efficiency of 2-phenylethanol. This molecule was chosen because of its significance, high water-solubility and high concentration in rose blossoms. It serves as a key substance. If the micellar extraction of 2-phenylethanol with the addition of salts is possible, salting-out is definitely feasible with less water-soluble substances such as geraniol.

The salting-out efficiency of six different salts was tested: sodium chloride (NaCl), potassium pyrophosphate ($K_4P_2O_7$), phytic acid sodium salt ($C_6H_{18}O_{24}P_6 \bullet xNa^+ \bullet yH_2O$, PA), magnesium sulfate ($MgSO_4$), ammonium sulfate ($(NH_4)_2SO_4$), and potassium carbonate (K_2CO_3). The experiments were performed by weighing the desired salt masses (5 – 40 wt. %) into 100 mL graduated tubes and adding water and 2-phenylethanol (19.4 g/L). The concentration of 19.4 g 2-phenylethanol per one liter water was determined as the maximum amount that can be dissolved at 25 °C. The salting-out

efficiency was ascertained visually. The experiments were carried out in triplicate (n = 3) as part of the bachelor thesis of Chantal Walser.[28]

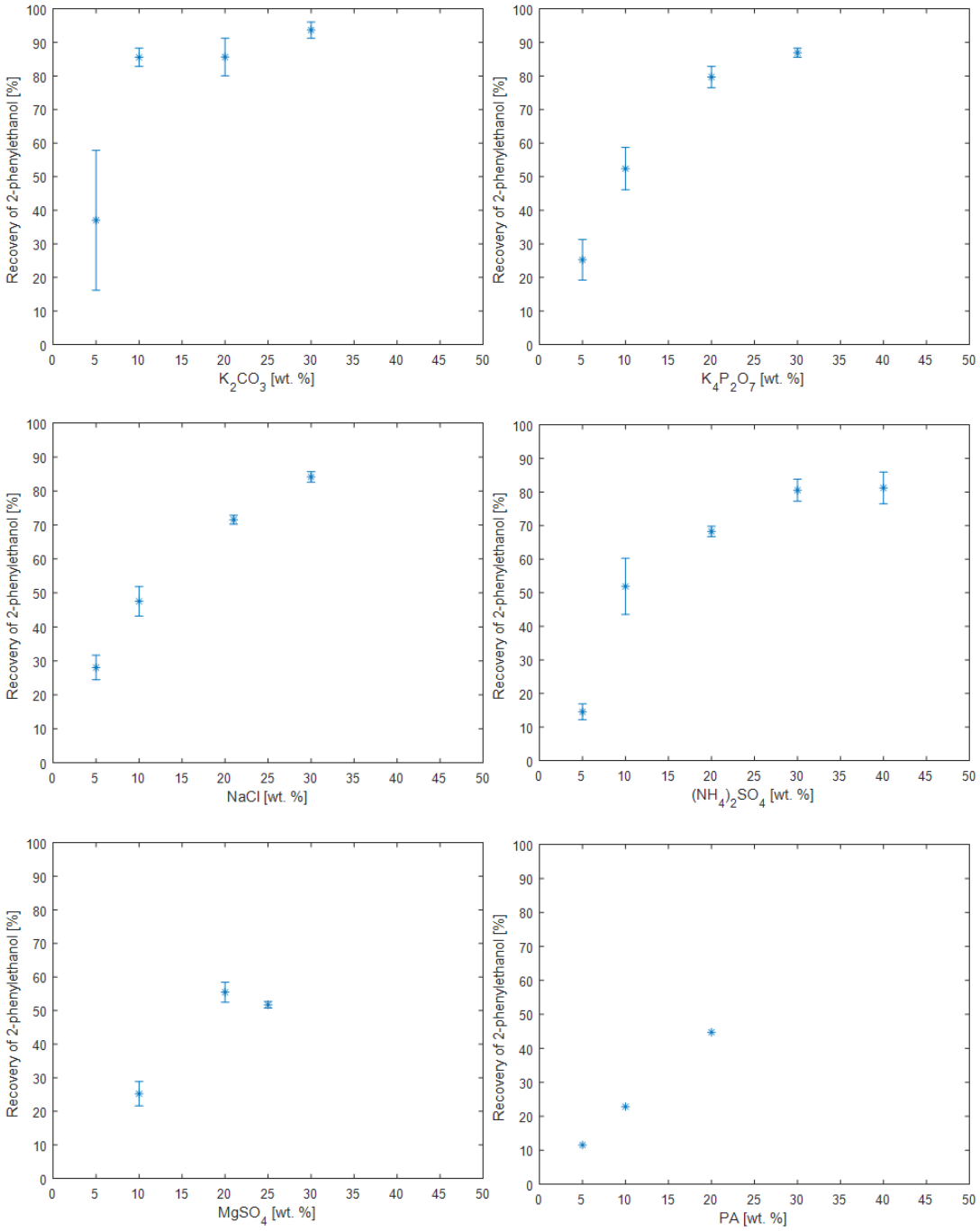

Figure 53: Potassium carbonate (K_2CO_3), potassium pyrophosphate ($K_4P_2O_7$), sodium chloride (NaCl), ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate ($MgSO_4$), and phytic acid sodium salt ($C_6H_{18}O_{24}P_6 \bullet xNa^+ \bullet yH_2O$) addition in wt. % in regard to the salting-out efficiency of 2-phenylethanol, based on three independent experiments (n = 3) and determined by visual observations.

Figure 53 represents the yield of recovered 2-phenylethanol after adding various salts in different concentrations to the aqueous solution of 2-phenylethanol (19.4 g/L). At low concentrations, the salting-out efficiency of the respective salts is not completed. On the one hand, this can be seen at the low yields of 2-phenylethanol expressed in percentages. On the other hand, the incomplete recovery becomes apparent regarding the high error bars at low salt concentrations. Especially for K_2CO_3, a high standard deviation in the beginning can be determined. This implements that adding only minor amounts of the salt leads to an incomplete salting-out of 2-phenylethanol. Increasing the salt concentration further, an almost complete salting-out of 2-phenylethanol occurred. Extracting with 10 wt. % K_2CO_3 achieved almost the identical yield (85.6 ± 2.7 %) compared with the addition of 20 wt. % (85.7 ± 5.6 %). Only the addition of 30 wt. % K_2CO_3 to the aqueous 2-phenylethanol solution increased the yield further. 93.7 ± 2.4 % of the dissolved 2-phenylethanol in water could be separated from the water phase.

All the other tested salts show similar behavior regarding the salting-out efficiency. With increasing salt concentration, also the yield of recovered 2-phenylethanol increases until a certain saturation or rather an almost complete separation is reached. Further increasing of the salt concentration is not possible due to the limited solubility of the salts in water, respectively. Salting out with PA and $MgSO_4$ only achieved a 2-phenylethanol yield approximately around 50 %. Thereby, a salt concentration of 20 wt. % was used. Increasing the concentration of $MgSO_4$ was not feasible due to its solubility limit of 25 wt. % in water. An increase of PA was avoided considering the high price of the salt. As a consequence, none of these two salts were suited for the separation of 2-phenylethanol from water.

$K_4P_2O_7$ is the second efficient additive for the separation of 2-phenylethanol from water, after K_2CO_3. 86.9 ± 1.4 % 2-phenylethanol can be recovered by adding 30 wt. % of $K_4P_2O_7$ to the solution. Salting-out with NaCl achieved a yield of 84.1 ± 1.5 % at the same concentration, whereas salting-out with $(NH_4)_2SO_4$ recovered 80.5 ± 3.2 % of 2-phenylethanol. Thus, the separation of 2-phenylethanol from the aqueous phase by the addition of $K_4P_2O_7$, NaCl and $(NH_4)_2SO_4$ is also possible. But, a high concentration of the respective salt is required and the separation is not exhaustive. Salting-out with sodium

chloride is, however, a good compromise as it is also formed during the neutralization step in micellar extraction.

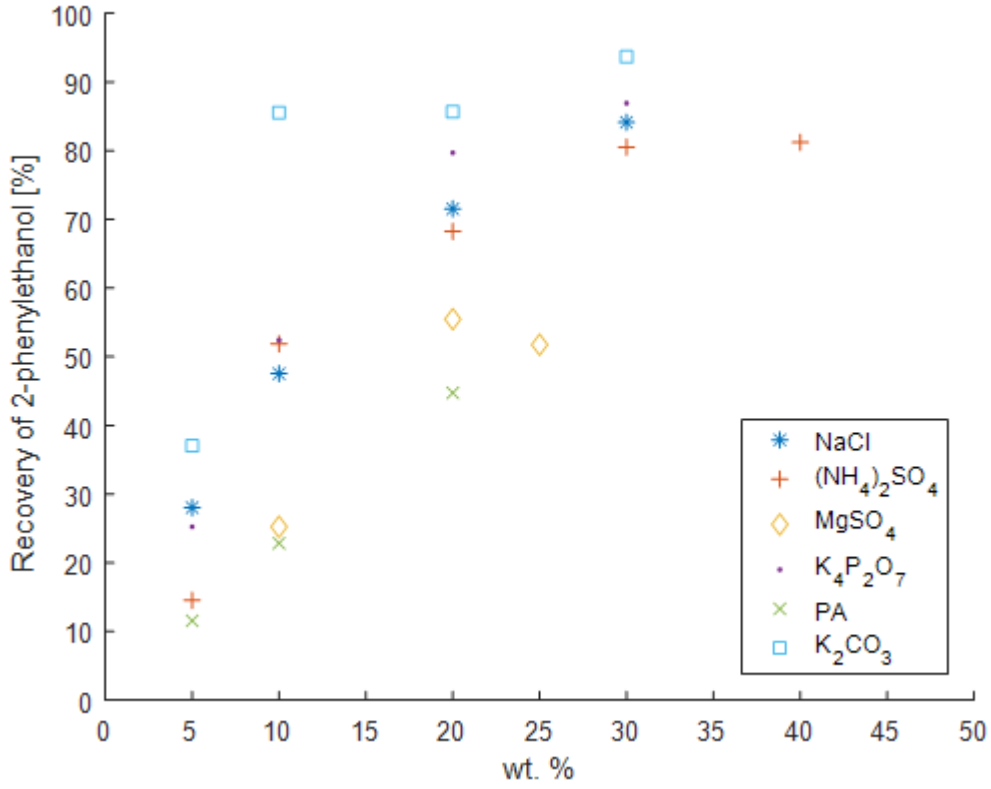

Figure 54: Comparison of the salting out efficiency of 2-phenylethanol of all salts as a function of their concentration (wt. %) determined by visual observations.

In Figure 54, all tested salts are plotted together in one graph. Ammonium sulfate and sodium chloride were found to be good salting-out salts, but higher concentrations were needed to achieve a similar salting-out performance, compared with potassium carbonate and potassium pyrophosphate. At only 10 wt. % K_2CO_3, a salting-out efficiency of 2-phenylethanol of 85.6 ± 2.7 % was practicable. Not only the water solubility of 2-phenylethanol, but also of less water-soluble compounds such as geraniol, citronellol and further desired fragrance compounds can be decreased by adding an inorganic salt. This results in an enhanced yield of odoriferous substances in the micellar extraction procedure, as well as an improved scent similar to the pure roses, only by adding a harmless inorganic salt.

As already discussed, during the micellar extraction and isolation of fragrance compounds, the pH value covers a wide range from alkaline to acidic. Therefore, it is important to consider the influence of the pH value on the salting-out efficiency of 2-phenylethanol. Daneshfar *et al.* (2016) developed a microextraction procedure based on dispersive liquid-liquid extraction for the analysis of 2-phenylethanol in rose water.[29] This method employs carbon tetrachloride as extractant and ethanol as disperser. Thereby, it is also confirmed that the solubility of 2-phenylethanol in the aqueous solution decreases by adding sodium chloride. Moreover, in this study, the effect of varying the pH value was examined. It is shown that protonation or deprotonation of 2-phenylethanol significantly influences the solubility in water. At a pH value of 6, the salting-out efficiency was best. Consequently, neutralization of the micellar solution may only be done in slight excess to guarantee the neutral form of 2-phenylethanol and thus, an efficient partitioning between water and the fatty acid phase.

Owing to the lack of fresh rose blossoms containing a high content of essential oil, such as *Rosa x centifolia* L., and the fact that a full quantification of the characteristic fragrances would go beyond the scope of this work, the salting-out experiments were only examined theoretically on the simulated aqueous system with 2-phenylethanol. Salting-out has a lot of industrial applications as well. It is applied for large-scale purification of chemicals and pharmaceuticals, for instance.[30] As a result, it is assumed that adding a salt to the micellar solution during the extraction of roses not only increases the yield significantly, but also can be applied industrially.

4.4. Conclusion

In this chapter, it was investigated whether the micellar extraction procedure optimized for iris rhizomes can be transferred to rose blossoms. The fragrances of roses are used as key ingredients for perfumes and are commonly extracted with hexane. Some of the most important fragrance molecules are citronellol, geraniol, and 2-phenylethanol. Not only the risk of handling large quantities of the toxic solvent for humans and the environment, but also the danger of potential residues in the extract led to the fact that Green Extraction methods gain more and more importance.

In collaboration with a major international perfume company, the micellar extraction of roses using an aqueous sodium myristate solution was examined. Compared to iris rhizomes, it was observed that the experimental procedure is much less complicated due to the nature of the plant material. The extracted rose blossoms can be easily separated by filtration and no further purification step is necessary to remove remaining plant material. Thus, the rose fragrances can be directly isolated from the extraction medium by the addition of HCl and the subsequent separation of the myristic acid phase. The odoriferous extract is completely soluble in ethanol and with its remaining fatty acid, it represents an ideal basic raw material for cosmetics or perfumes.

But apart from the simpler practicability, also the extraction efficiency has to be taken into account. Due to the high content of myristic acid, it was not possible to quantify the fragrance compounds in the odoriferous extract. However, the aqueous solution after the separation of the myristic acid precipitate was analyzed by GC-FID/MS. Thereby, important fragrance compounds such as 2-phenylethanol, geraniol, and citronellol were found. That means that during the micellar extraction, valuable odoriferous substances get lost. The observation that geraniol partly remains in the aqueous phase was also confirmed by a recovery experiment. Only 75 % of the initially used amount of geraniol could be recovered in the fatty acid phase.

The problem that partially water-soluble compounds get lost is also known in industry when extracting the essential oil of roses by steam distillation. Especially, 2-phenylethanol is an important perfume molecule and due to its water-solubility absent in the distillate.

Sodium chloride, potassium pyrophosphate, phytic acid sodium salt, magnesium sulfate, ammonium sulfate, and potassium carbonate to an aqueous solution of 2-phenylethanol was added in order to decrease its solubility in water. With this approach, the water-soluble typical rose fragrance molecule could be salted out either with 30 wt. % $K_4P_2O_7$ (recovery of 87 % 2-phenylethanol) or with 30 wt. % K_2CO_3 (94 % recovery). Also by adding sodium chloride, the solubility of 2-phenylethanol in water could be decreased significantly. As a consequence, 2-phenylethanol but also other hydrophobic molecules such as geraniol and citronellol migrate to the fatty acid phase by the addition of a salt.

To sum up, micellar extraction can be applied onto rose blossoms by adapting the procedure slightly. Adding a harmless inorganic salt is still in accordance with the principles of Green Chemistry. With this method, it is possible to recover the water-soluble 2-phenylethanol using an aqueous extraction medium. Not only the scent of the extract is similar to the pure rose blossoms, but also no toxic residues remain. 2-phenylethanol could so far only be extracted using flammable and often toxic solvents.

4.5. Experimental

4.5.1. Reagents

The following chemicals were used without further purification: sodium chloride (Sigma-Aldrich, ≥ 99.5 %), potassium pyrophosphate (Sigma-Aldrich, 97 %), phytic acid sodium salt (Sigma-Aldrich, ≥ 98 %), potassium carbonate (Merck, p.A.), potassium pyrophosphate (Fluka, purity ≥ 98 %), sodium sulfate (Fisher Scientific, purity ≥ 98 %), myristic acid (Sigma-Aldrich, purity ≥ 99 %), hydrochloric acid (Sigma Aldrich, reagent grade 37 %), hydrogen chloride solution (Merck, (1N) Reag. Ph Eur, Reag. USP), sodium hydroxide (Merck, pellets for analysis), sodium hydroxide solution (Sigma-Aldrich, volumetric 1.0 M), ethanol (Sigma-Aldrich, purity 99 %), diethyl ether (Merck, for analysis), toluene (Fisher Scientific, purity 99.99 %), ethyl acetate (Fisher Scientific, purity 99.98 %), p-anisaldehyde (Merck, purity ≥ 98 %), glacial acetic acid (Sigma-Aldrich, purity ≥ 99.8 %) and concentrated sulfuric acid (Merck, purity 95-97 %). HPLC grade methanol from Merck was used during the analysis. Linalool (Sigma-Aldrich, purity ≥ 98 %), geraniol (Sigma-Aldrich, kosher, ≥ 97 %), citronellol (Sigma-Aldrich, kosher, ≥ 97 %) and 2-phenylethanol (Sigma-Aldrich, purity ≥ 99 %) were purchased for the quantification. Rose water was obtained from Phytotagante and an industrial partner. Deionized water was used throughout the experiments.

4.5.2. pH stability of geraniol

4.5.2.1. With myristate matrix

An aqueous sodium myristate solution (c = 0.1 mol/L) was prepared and divided into fractions of 10 mL. To each sample, 15 µL geraniol were added and stirred for 10 min at 55 °C. Using sodium hydroxide and hydrochloric acid, the pH value of the aliquots was adjusted to represent all pH values ranging from 1 – 10. The pH value was monitored using a *pHenomenal pH 1000 L* pH meter from VWR. The samples were stirred at 55 °C for 3 h in a water bath. Afterwards, the samples were cooled in a refrigerator for one hour. To get a TLC with high resolution, geraniol was re-extracted with 5 mL diethyl ether from the

aqueous phase. The TLC was prepared as described in 4.5.6.1. In addition, the organic phase of the sample with pH = 1 was analyzed by GC-FID/MS directly (see 4.5.6.2).

4.5.2.2. Without myristate matrix

Using sodium hydroxide and hydrochloric acid, 10 mL samples with pH values ranging from 1 – 10 were prepared using a *pHenomenal pH 1000 L* pH meter from VWR. To each sample, 15 µL geraniol were added and stirred for 3 h at 55 °C. Afterwards, the samples were cooled in a refrigerator for one hour. Geraniol was re-extracted with 5 mL diethyl ether and TLC was carried out (as described in 4.5.6.1). In addition, the organic phase of the sample with pH = 1 was analyzed by GC-FID/MS directly (see 4.5.6.2).

4.5.3. Micellar extraction of roses

2 L of an aqueous sodium myristate solution (c = 0.1 mol/L) was prepared under stirring at 55 °C by mixing sodium hydroxide pellets and myristic acid in a stoichiometric amount. 150 g of fresh *Rosa x centifolia* L. blossoms were added to the solution and stirred for 30 min at 55 °C. Afterwards, the rose petals were removed by filtration through a stainless steel filter and washed with hot water. The solution was neutralized by adding hydrochloric acid (V = 200 mL, c = 1 mol/L) slowly (1 h) at room temperature. This mixture was stored for one hour at 4 °C, before the myristic acid precipitate was filtered under vacuum using a Buchner funnel. In one experiment, the precipitate was dissolved in warm ethanol (96 %) to remove remaining plant material. In another experiment, the remaining plant material and water was directly separated by melting and centrifuging the mixture. For analysis, 50 mg of the extract were dissolved in 1 mL methanol and measured immediately via GC-FID/MS applying internal calibration (see 4.5.6.2).

4.5.4. Recovery of geraniol

150 µL geraniol were added to 100 mL of an aqueous sodium myristate solution (0.1 mol/L) and stirred for 60 min at 50 °C. To precipitate the myristic acid afterwards, hydrochloric acid was added until a pH value of 4 – 5 was reached (pH paper). After stirring the mixture for 1.5 h, this precipitate was filtered under vacuum and dried at atmospheric conditions. 50 mg of the dried precipitate were dissolved in 1 mL of internal standard solution (0.5 mg linalool/mL methanol). The analysis was carried out via GC-FID/MS (see 4.5.6.2). The reference system was prepared by merging the same amount of resulting myristic acid (2.33 g) with the equal quantity of geraniol (150 µL).

The same experiment was repeated, but with the addition of sodium chloride. After precipitating myristic acid, 5.8 g sodium chloride were added to the aqueous solution. The mixture was stirred for 1.5 h. Subsequently, this precipitate was filtered under vacuum and dried at atmospheric conditions. 50 mg of the dried precipitate were dissolved in 1 mL of internal standard solution (0.5 mg linalool/mL methanol) and measured via GC-FID/MS (see 4.5.6.2).

4.5.5. Salting-out experiments

The samples were prepared in 100 mL graduated flasks with a volumetric scale by weighing the various salt masses (5 – 40 wt. %) into the graduated flasks and adding water and 2-phenylethanol (19.4 g/L), respectively. Six different salts were tested: sodium chloride (5, 10, 20 and 30 wt. %), ammonium sulfate (5, 10, 20, 30 and 40 wt. %), magnesium sulfate (10, 20 and 25 wt. %), potassium pyrophosphate (5, 10, 20 and 30 wt. %), phytic acid sodium salt (5, 10 and 20 wt. %) and potassium carbonate (5, 10, 20 and 30 wt. %). The mixture was shaken and then put in a water bath at 25 °C ± 1. The recovered 2-phenylethanol phase was ascertained visually after one night. All experiments were carried out three times (n = 3).

4.5.6. Analysis

4.5.6.1. Thin-layer chromatography

A mixture of toluene and ethyl acetate (88/12, v/v) was used as eluent for thin-layer chromatography. Small spots of solution (usually 5 µL with a concentration of 1 mg/mL) were applied to a classical silica TLC plate (Silica Gel 60 F_{254} plate, Merck) by using micro capillaries. For visualizing the spots, a staining reagent was sprayed onto the plate and developed by heating to 105 °C. The reagent was composed of 0.5 mL p-anisaldehyde, 10 mL glacial acetic acid, 85 mL methanol and 5 mL concentrated sulfuric acid.

4.5.6.2. Gas chromatography

GC analysis was realized using an Agilent Technologies 7890A Chromatography System. The system implies a FID Detector coupled with a 220 Ion Trap GC/MS. The samples were injected automatically by an Agilent 7693 Autosampler. Separations were achieved on a VF-5ms column (30 m x 250 µm x 0.25 µm) with helium as carrier gas with a constant flow of 1 mL/min. The samples (1 µL) were injected with a split/split less injector (split 1/50). The following temperature program was used (see Figure 55): holding 80 °C for 0.5 min, ramping up to 300 °C at 20 °C/min and then holding 300 °C for 5 min. The injector temperature was set to 250 °C and the temperature of the flame ionization detector was set to 300 °C. Qualitative and quantitative analyses were performed by analyzing retention times and peak areas. Geraniol was quantified by internal standard calibration.

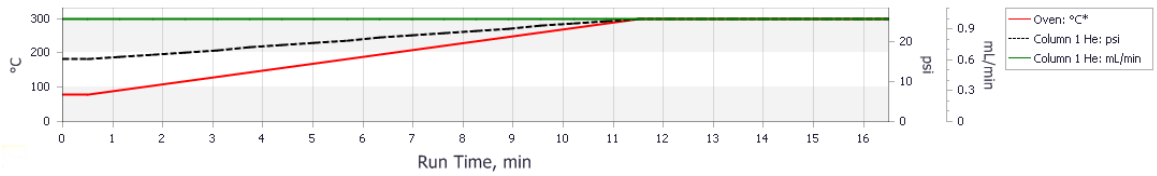

Figure 55: GC method to quantify geraniol.

Internal standard calibration was carried out using linalool as internal standard. To this purpose, a stock solution of 10 mg/mL geraniol in methanol was prepared. This stock solution was used to prepare dilutions of 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL. Another stock solution of linalool in methanol (10 mg/mL) was prepared and diluted to 1 mg/mL. Each time, 1 mL of the diluted internal standard solution was added to 1 mL of the various analyte dilutions. After mixing for a few seconds in an ultrasonic bath, the mixtures were filtered using 0.2 µm PTFE syringe filters and measured by GC-FID/MS, immediately. Every sample was prepared in triplicate and also the measurements were carried out three times.

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5. Modern enfleurage

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5.1. Introduction

So far, it has been shown that by using an extraction medium, which is naturally occurring in various plants, the isolation of valuable odoriferous compounds from plant material is possible. Within 30 min at moderate temperatures, the nonpolar irones from iris rhizomes can be extracted using an aqueous sodium myristate solution. Compared with steam distillation or solvent extraction, this so-called micellar extraction is an efficient, mild, gentle and green extraction method without high energy consumption or the risk of handling flammable and often toxic solvents.

Nonetheless, when transferring the micellar extraction method to rose blossoms, it turned out that a lot of the valuable water-soluble ingredients, such as 2-phenylethanol, remained in the aqueous phase and could not be co-precipitated with the fatty acid. The addition of large quantities of an inorganic salt was necessary to constrain the perfume molecules into the fatty acid phase.

In roses, the fragrance molecules are located on the surface of the petals inside fragile glandular trichomes and are thus easily accessible to the extraction medium.[1] Therefore, by extracting roses, the use of lipophilic fatty acids without water should be more advantageous than the complex micellar extraction procedure described above.

The idea of the approach “modern enfleurage” is based on a traditional but no longer applied extraction method, the so-called enfleurage. Due to the high expenditure on manual work, this method is no longer competitive and is hardly used, although in this way excellent fragrances can be obtained. Typically, animal fats, which are solid at room temperature, were used to capture the odoriferous compounds from the plant material, especially from petals or whole flowers.

In this study, the advantages of classical enfleurage and solvent extraction were combined, whereby odorless molten free fatty acids were used as liquid extraction medium. By completely embedding the plant material, the extraction medium is better able to penetrate into the plant tissue and the mass transport of the analytes is increased. Also, the challenging isolation of the fragrance compounds from the fatty acid mixture was investigated. To this purpose, a molecular distillation, also known as short-path distillation, was examined since a gentle thermal separation process is ideally suited for isolating the fragrance compounds. Usually, certain constituents of the flower oil, especially the most volatile ones, are lost during solvent distillation.[2] In contrast, by applying molecular distillation, only the desired perfume compounds are distilled, but not the extraction medium. The result of molecular distillation is not only a nearly residue-free extraction of the fragrances, but also an efficient recycling of the extraction medium, which can be used for further extraction cycles.

5.2. Fundamentals

5.2.1. Principles and history of enfleurage

Most essential oils are isolated from the plant material by steam or hydro distillation. However, the flowers of certain plants, such as jasmine, tuberose, violet, narcissus, mimosa, hyacinth and a few others yield no oil at all, when they are distilled. The oil is partially destroyed by the boiling water, which affects the decomposition of the sensitive plant constituents. But in part, also high-boiling constituents or compounds which are soluble in water are not able to be distilled. As a consequence, a distilled oil does not always represent the natural scent of the originally plant. Furthermore, flowers like jasmine and tuberose, continue their plant physiological activities after picking and maintain emitting small quantities of perfume molecules.[2, 3] However, distillation destroys the vital functions of the plant instantly.

Hence, a method had to be established to isolate the fragrances from flowers of this type. In the 19th century, extraction with cold fat, also known as enfleurage à froid, was developed and carried out in southern France.[4] In doing so, a glass plate was coated with odorless fat and loaded with freshly picked flowers. The volatile components released by the flowers were absorbed from the fat over a long period of time. The glass plates were stored on top of each other in an air-tight compartment, called chassis. After 24 h, the flowers were removed and the chassis was recharged with fresh flowers.[5] In the case of jasmine, this process was repeated 70 times. Consequently, the entire period of enfleurage lasted 8 – 10 weeks.[2] But this high effort was rewarding since the cold fat did not destroy the vital functions of the plant and thus a much greater yield of oil was obtained. These flower oils, which were subsequently extracted from the fat with alcohol (see 5.2.3), are the finest and most delicate fragrances for perfumery representing the authentic scent as exhaled by the flowers.[2]

It can be distinguished between cold and hot enfleurage depending on the temperature of the fat. In hot enfleurage, also known as enfleurage à chaud, the flowers were drawn into previously heated fat. The process is similar to the maceration process described in 2.3.2.1, with the fundamental difference that hot fat is employed. It was especially used for flowers, which stop their physiological activities after picking, for instance roses,

orange blossoms, and mimosa. Since no further oil is developed in these flowers, the time-consuming and elaborate method of cold enfleurage was dispensable. To this purpose, several batches of fresh flowers were treated with the same batch of hot fat until the fat became saturated with the fragrance molecules. Usually, every extraction step lasted about half an hour and the temperature was set at 80 °C. Afterwards, the mixture was cooled for one hour and reheated to filter the extracted plant material. This procedure was repeated about ten times, until the fat was saturated with perfume molecules.[2]

Despite the advantages, enfleurage has been replaced by extraction with volatile solvents almost completely, since petroleum-based solvents were introduced in the 20th century. Nonetheless, the original scent of flowers can only be isolated by expression, which is limited to plants or plant parts with a high oil content and cold enfleurage.[5] However, the procedure of enfleurage is time-consuming, elaborate, tricky, and requires much experience.[2]

5.2.2. Preparation of the fat base

The quality of the fat base was of high importance, since it largely affected the success of the enfleurage. The fat had to be odorless and of adequate consistency to wet the flowers sufficiently. If the fat was too hard, absorption was incomplete due to the reduced contact area between the flowers and the fat base. In contrast, if the fat was too soft, it was difficult to remove the adhering fat from the flowers. As a consequence, the yield of the recovered flower oil was reduced and also a loss of the fat base was involved. Thus, the fat base had to have a semisolid consistency at the temperature at which enfleurage was carried out.

Years of empirical experience had shown that a mixture of one part tallow and two parts lard was best suited. The fats were cleaned and carefully purified by hand. Afterwards, the fat base had a uniform consistency and was free of water and impurities. To prevent that the base turned rancid during the hot summer months, benzoin was added as preservative.[2, 5]

This fat base possessed a high power of absorption of the perfume compounds. However, the production of highly-purified animal fat was time-consuming, cumbersome and very expensive. Since the fat spoiled and became rancid quickly, it only could be used once. Various experiments with mixtures of vegetable oils had been investigated as alternative for the old-fashioned mixture of lard and tallow. Despite the fact that especially the hardened vegetable fats do not turn rancid easily, the result was a variety of interesting qualities and widely different yields, but the highest quality was achieved using the animal fat base, described above. Also, mineral oils were used as base for the cold extraction of flowers. Not only the power of absorption was lower compared to the animal fats, but also the isolation of the fragrances from the mineral oil afterwards was more cumbersome. Moreover, esters of polyhydric aliphatic alcohol (ester of glycol, glycerol, mannitol, hexitol) were tested and patented as base for enfleurage. However, none of them found their way into commercial applications due to simpler but questionable solvent extraction.[2, 6] A literature research with the help of SciFinder, Google Scholar and Google Patents revealed that saturated fatty acids, as envisaged in this study, have not yet been tested as base for enfleurage.

5.2.3. Isolation of the perfume compounds from the fat base

In the early days of perfumery, the fat containing the fragrance molecules was used directly and designated as pomade. Later, these pomades were extracted with highly purified alcohol several times to separate the perfume molecules from the fat. The alcoholic washings were called extrait.[2, 4]

Since no heat was applied during these washing steps, the extrait contained the fragrances as exhaled by the living flowers. Sometimes fatty by-notes contributed to the smell. But, by cooling the extrait in a refrigerator, they could be eliminated to a certain content. The crystallized exhausted fat was separated by filtration. Since it was useless for a new extraction cycle of flowers, but not completely odorless, it was used for the manufacture of soaps. The extraits were concentrated by distilling off the alcohol. To do this, a vacuum was applied to remove the alcohol at low temperatures, producing the so-called absolute of enfleurage. It is a semisolid flower oil with a small quantity of alcohol

soluble fat (about 1 %). However, this fatty by-note can also have a positive impact on the overall impression of the perfume, as it also imparts a certain roundness and fixation value.[2, 5]

5.3. Results and discussion

5.3.1. Melting point reduction of myristic acid

As the salt of myristic acid was already successfully used as a solubilizer in the previously discussed micellar extractions, the fatty acid serves as the basis of the here examined approach: modern enfleurage. The aim of this study was to use myristic acid solely without water as extraction medium for the easily accessible fragrances on the surface of rose petals. Thereby, the advantages of classical enfleurage can be combined with those of solvent extraction. As a consequence, the extraction medium should be liquid to facilitate the mass transfer of the perfume molecules, but should also be gentle to the plant material.

Myristic acid is a common saturated fatty acid, which occurs naturally in most vegetable and animal fats, such as nutmeg, palm kernel oil or coconut oil for instance. Saturated fatty acids are chemically stable, biodegradable, widely available in high quality, inexpensive, and safe for humans, animals, and the environment. Thus, they represent the ideal extraction medium in regard to the requirements and principles of Green Extraction.[7] However, the melting point (mp) of myristic acid of 54.4 °C is quite high.[8] To increase the viability of the plants during the extraction procedure, several different approaches were carried out to lower the melting point of myristic acid, as part of a research work of Manuel Rothe.[9]

5.3.1.1. Binary mixture of myristic acid and lauric acid

Lauric acid is also a saturated fatty acid with two carbon atoms less than myristic acid. It is a medium chain fatty acid with a slight soapy odor. Its melting point is still relatively high (44.2 °C).[8] However, by mixing myristic acid and lauric acid it was noticed that a system is formed, where the melting point is lower than of the pure components. By means of optical melting point determination (n = 3), a eutectic point was determined at 34.9 °C. A eutectic point is defined as the lowest possible melting point of a mixture.[10] The eutectic mixture was composed of 70 wt. % lauric acid and 30 wt. % myristic acid. Thus, by mixing lauric acid and myristic acid the melting point can be decreased by 20.3 °C,

compared to pure myristic acid. In the molten state, the eutectic mixture is a transparent homogenous solution with a low viscosity and almost no odor. The detailed phase diagram is plotted in Figure 56.

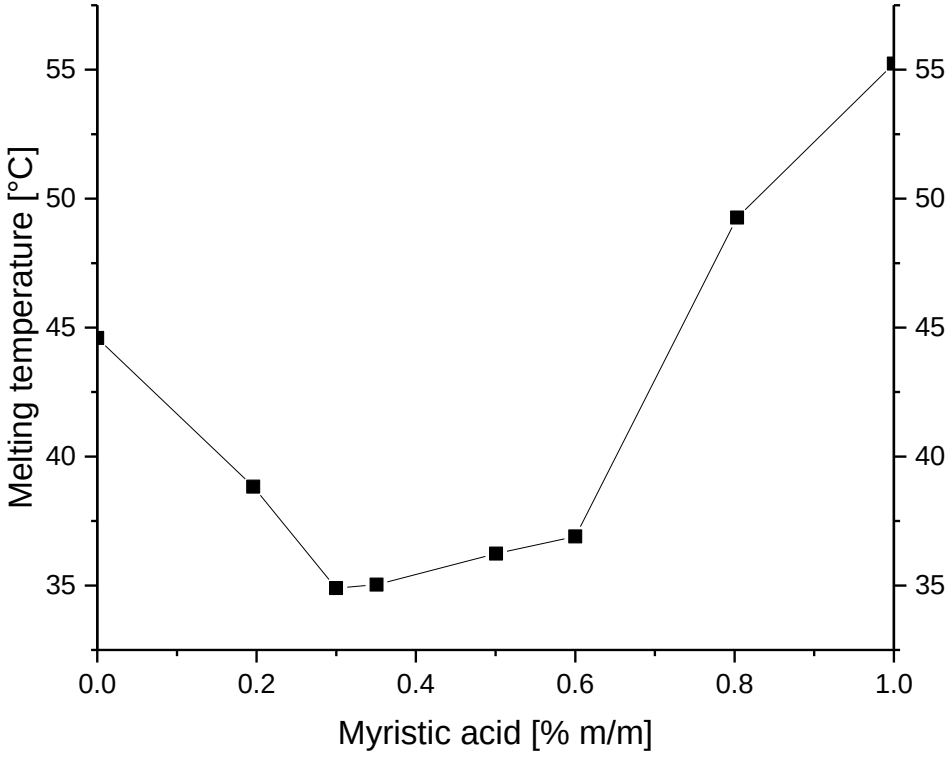

Figure 56: Melting point diagram of the binary mixture myristic acid/ lauric acid with a eutectic point at 34.9 °C, determined by optical melting point determination (n = 3, see Experimental p. 188).

In literature, a eutectic point of 34.2 °C is reported for a mixture of 66 wt. % lauric acid and 34 wt. % myristic acid determined by differential scanning calorimetry.[11] This mixture has superior thermal properties and thermal reliability for solar heating applications. Due to its melting congruency, good chemical stability, and non-toxicity, binary fatty acid eutectics are used as phase change materials (PCM). In regard to the shortage of fuel sources, fatty acids represent a continuous security of supply since they are derived from vegetable and animal oils.[11, 12]

5.3.1.2. Ternary mixtures of myristic acid, lauric acid and palmitic acid

Fatty acids are generally known to form ternary or even quaternary eutectics.[13] Due to the strong smell of short-chained fatty acids, only the addition of longer-chained fatty acids was investigated in order to prevent an unpleasant smell of the extraction medium. Thus, palmitic acid (C16 chain length, mp 62.9 °C)[8] was added to a varying mixture of lauric acid and myristic acid. The melting points were determined using a melting point apparatus (n = 3).

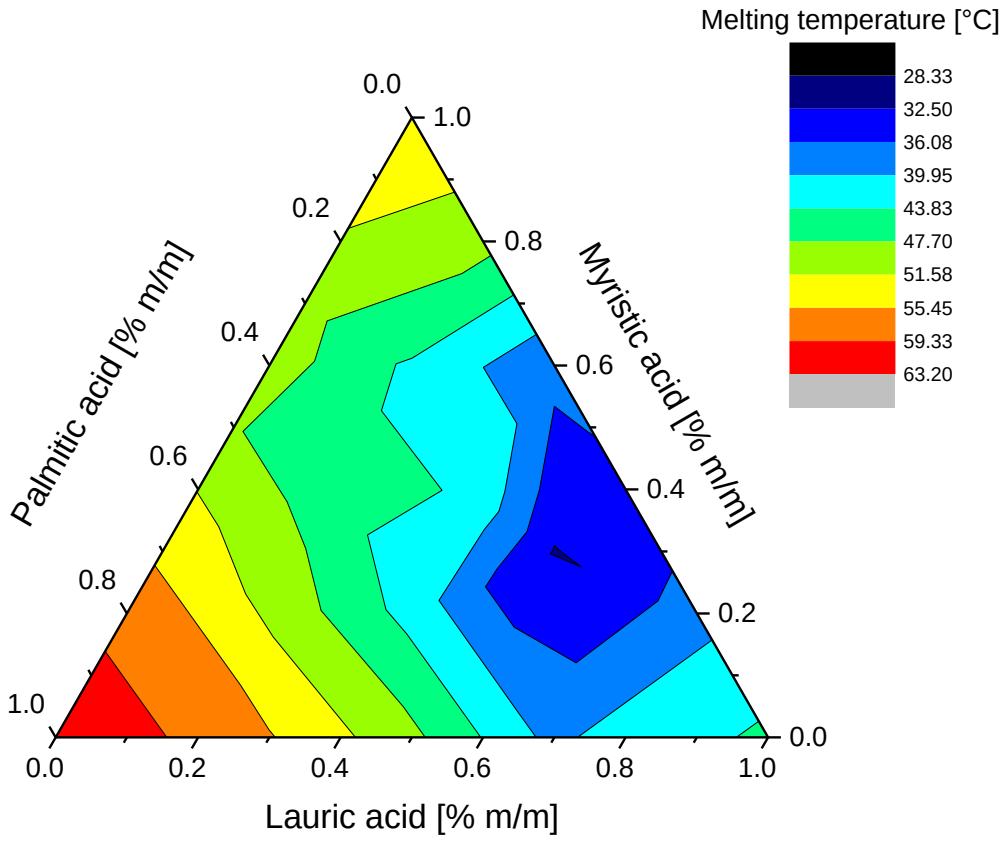

Figure 57: Melting point diagram of the ternary system lauric acid, myristic acid and palmitic acid, determined by optical melting point determination (n = 3, see Experimental p. 188).

As also described in literature, a eutectic point lower than of a binary mixture can be obtained by mixing several fatty acids.[14] The eutectic mixture of lauric acid (55 wt. %), myristic acid (30 wt. %) and palmitic acid (15 wt. %) allowed a further melting point

lowering, compared to the binary mixture of lauric acid and myristic acid (34.9 °C, compare 5.3.1.1). A eutectic point of 32.3 °C was determined for the ternary fatty acid mixture (see Figure 57). Thus, by adding a third fatty acid to the mixture of lauric acid and myristic acid, a further decrease in melting temperature is possible.

In the work of Zhao *et al.* (2014), an experimental eutectic point (29.35 °C) was calculated for a ternary mixture of lauric acid/myristic acid/palmitic acid = 60:30:10.[14]

5.3.1.3. Quaternary fatty acid mixture

Moreover, the addition of a fourth component, stearic acid, to the mixture of lauric acid, myristic acid and palmitic acid was tested. Stearic acid is a saturated fatty acid with a C18 chain length and one of the most common fatty acid found in animal and vegetable fat. Stearic acid is odorless and has a melting point of 69.9 °C. Randomly, a few mixing ratios of the quaternary mixture were selected and tested in regard to decrease the melting point of the mixture further (n = 3).

Table 19: Melting points of the quaternary mixture of lauric acid, myristic acid, palmitic acid and stearic acid, determined by optical melting point determination (n = 3, see Experimental p. 188).

Lauric acid [wt. %]	Myristic acid [wt. %]	Palmitic acid [wt. %]	Stearic acid [wt. %]	Melting point [°C]
63	21	10	6	**31.1**
55	25	13	7	**31.1**
66	19	9	5	**29.9**
57	28	9	5	**30.3**
57	19	18	5	**31.4**
57	19	9	15	**32.9**

The lowest melting temperature was achieved by mixing 66 wt. % lauric acid, 19 wt. % myristic acid, 9 wt. % palmitic acid and 5 wt. % stearic acid. As it can be seen in Table 19, a melting point of 29.9 °C was obtained. The results indicate that the melting point of a

quaternary mixture is even lower compared to the melting points of the corresponding ternary systems. Thus, by adding a fourth component to the mixture, a further reduction of the melting point (2.4 °C) is feasible, even though stearic acid has a very high melting point. Adding longer chained fatty acids additionally, for instance arachidic acid or behenic acid, might result in a further decrease of the melting temperature. However, the influence of these long-chained fatty acids is expected to be relatively small, as the results above show a decreasing effectiveness, when adding more compounds to the mixture.

As a consequence, a ternary mixture of 55 wt. % lauric acid, 30 wt. % myristic acid and 15 wt. % palmitic acid was used as an optimum extraction medium. In the following, this fatty acid mixture was applied to extract the fragrances contained in rose blossoms. In addition, it is also conceivable that this medium can be used for the extraction of jasmine or tuberose flowers. At an extraction temperature of 32.3 °C, the physiological activity of the flowers can continue, in contrast to the high temperature applied in hydro distillation or solvent extraction. Furthermore, significant amounts of oxygen can be dissolved in fatty acids.[15] Consequently, the flowers can form and deliver their fragrances to the extraction medium for a long time.

5.3.2. Extraction of rose petals with a ternary fatty acid mixture

To investigate the extraction power of free fatty acids, the ternary eutectic mixture (55 wt. % lauric acid, 30 wt. % myristic acid and 15 wt. % palmitic acid) was used to extract rose petals. With this mixture, only heating up to 32.3 °C was required and therefore, a gentle extraction of the sensitive rose fragrances was possible. Due to the lack of fresh scented roses, roses of unknown origin without smell were artificially scented. Thus, a rose oil provided by our industrial partner was individually applied on the petals with a rose oil to rose petal ratio of 4 g/kg. The artificially scented flowers were macerated with a solid to liquid ratio of 1/12. Occasionally, the mixture was stirred carefully. In Figure 58, the permeation progress of the fatty acid mixture into the rose petals is shown. Already after a short time (Figure 58 left), the mixture started to permeate the petals. After 1 h (Figure 58 middle), a large part of the rose petals was already penetrated by the fatty acids, whereas the extraction seemed to be completed after 3 h (Figure 58 right).

Figure 58: Rose petals after 5 min (left), after 1h (middle) and 3 h (right) of maceration in a ternary mixture of lauric acid, myristic acid, and palmitic acid.

Compared to the *Rosa x centifolia* L. blossoms used during the micellar extraction experiments (compare 4.3.3), this rose of unknown origin was very rigid. Thus, the maceration time presumably can be reduced further by extracting the delicate variety.

After soaking overnight, the rose petals were filtered through a stainless steel filter. The low melting point of the fatty acid mixture and the fact that the mixture takes several hours to crystallize, allowed us to work without additional heating. The extraction process was further repeated three times using the same extraction medium. Theoretically, the flowers should be extracted until the fatty acid mixture is saturated with perfume molecules.

The collected roses of all four batches were washed with a small quantity of fresh fatty acid mixture. To remove the remaining adhering fatty acids, the collected batches were squeezed and washed with hot water in the filter sieve. After cooling down the fatty acid mixture, the water was easily separated from the hardened extraction media. The aqueous phase was rich in dye, but contained no odoriferous compounds, as it was tested via GC-FID/MS (described in Experimental p. 191). For the analytics of rose fragrances, it is referred to chapter 4.3.1: micellar extraction of roses. Due to the high proportion of fatty acid, a direct quantitative determination of the fragrances in the extraction medium by GC-FID/MS measurements was not possible. In the following, different strategies to isolate the fragrance compounds from the fatty acid mixture are presented.

5.3.3. Vacuum distillation for the separation of a compound mixture

A possibility to isolate the fragrance compounds from the ternary fatty acid mixture is to re-extract the extraction medium with an appropriate solvent. As it was also investigated by isolating the irones from the myristic acid phase, see 3.3.9, fatty acids precipitate and crystallize in ethanol under cold conditions. However, several crystallization and filtration steps were necessary to enrich the fragrance compounds. Only 72 % of the irones could be recovered this way. The evaporation of the solvent afterward is a crucial step, since a decreased pressure and elevated temperature is applied. Due to the large amount of ethanol, which is required to remove the desired compounds from the extraction medium, the fragrances are exposed to this influence a long time.

Saturated fatty acids are chemically very stable and withstand several thousand thermal cycles, as it was reported by Sari (2005).[11] Therefore, thermal separation of the perfume compounds was examined by carrying out various distillation experiments. In this course, not the extraction medium, but the desired fragrances are distilled. The perfume compounds are exposed to elevated temperatures and decreased pressures only for a short time. Moreover, the use of solvents can be completely avoided this way, which is a main target of Green Extraction.[7] An overview of the respective boiling points of the investigated system at atmospheric conditions is given in Table 20. It can be seen that the difference between the boiling points of the fatty acids and the boiling points of the perfume compounds is large enough to achieve separation by distillation.

Table 20: Boiling points at atmospheric conditions of the investigated system obtained from the open GESTIS Substance Database, IFA.

Compound	Boling temperature
Lauric acid	298 °C
Myristic acid	326 °C
Palmitic acid	351 °C
2-phenylethanol	220 °C
Citronellol	225 °C
Geraniol	230°C

5.3.3.1. Simple vacuum distillation

First of all, a simple vacuum distillation was carried out. To this purpose, the ternary fatty acid mixture consisting of lauric acid (55 wt. %), myristic acid (30 wt. %), and palmitic acid (15 wt. %), as optimized in 5.3.1, was mixed with 5 % of the rose oil obtained from our industrial partner at elevated temperature. The rose oil consists of 50.2 % 2-phenylethanol, 31.5 % citronellol, 18.3 % geraniol, as it was determined by GC-FID/MS.

The distillation using a common Claisen bridge was carried out until no more dripping of the distillate occurred at 100 °C and 2.0 • 10^{-2} mbar. In this process, a temperature of the vapor phase between 38 – 48 °C was measured. The distillates were analyzed by GC-FID/MS (see Experimental p. 191). The results in comparison to the reference rose scent are shown in Table 21.

Table 21: Composition of the rose oil separated by vacuum distillation from the fatty acid mixture in comparison to the reference, determined by GC-FID/MS (see Experimental p. 191).

Composition	1. Experiment	2. Experiment	3. Experiment	Reference
2-Phenylethanol	57.9 %	80.6 %	80.9 %	50.2 %
Citronellol	27.7 %	13.6 %	13.9 %	31.5 %
Geraniol	14.4 %	5.8 %	5.2 %	18.3 %
Total yield	**77 %**	**37 %**	**47 %**	**-**

Although the temperature of the bottom phase was very high, no decomposition products of 2-phenylethanol, citronellol or geraniol became apparent, considering the GC-FID/MS chromatograms. However, using a common Claisen bridge, a complete separation of the fragrance compounds from the fatty acid mixture was not possible. The volume of the vapor phase was too low to keep the apparatus at temperature. Moreover, the oily residues in the distillation apparatus were very different each time. Thus, it was not possible to determine a meaningful total yield of the distillate. In addition, by calculating the chemical composition of the distillate, it was demonstrated that the proportion of 2-phenylethanol, citronellol and geraniol differs widely (compare Table 21). It seems that the first experiment was most likely successful in regard to the total yield and the chemical

composition of the rose oil. In the second and third experiment, presumably an equilibrium has not been attained.

Therefore, a vacuum distillation with fractionation was carried out to evaluate the establishment of the equilibrium between the fragrance compounds during the distillation process. A special glassware (distillation spider) was adapted to the Claisen bridge to allow the direct fractionation into individual round-bottomed flasks. The conditions for the distillation were the same as applied above. A fatty acid mixture with 5 % rose scent was distilled at $2.0 \bullet 10^{-3}$ mbar and 100 °C. The temperature of the vapor phase was measured continuously. The first fraction was collected at a temperature T(head) of 45 °C. With increasing time, the temperature T(head) increased to 48 °C (second fraction) before it dropped again to T(head) = 42 °C (third fraction). Finally, the temperature of the vapor phase was determined to be 38 °C. This fraction was collected until no more dropping occurred.

The temperature has to be increased to 122.5 °C until the fatty acids, mainly lauric acid, start to distillate. As a result, the difference of the boiling points of the fragrances and the fatty acids of 22.5 °C is high enough to achieve a separation. However, as also observed performing the simple vacuum experiments without fractionation, it was not possible to separate the fragrances completely. In total, only 67 % of the rose scent was recovered in this experiment with fractionation. The other 33 % remained in the fatty acid mixture. The composition of the individual fractions, measured by GC-FID/MS (see Experimental p. 191), is given in Table 22.

Table 22: Composition of the rose oil fractions separated by vacuum distillation from the fatty acid mixture in comparison to the reference, determined by GC-FID/MS (see Experimental p. 191).

Composition	1. Fraction	2. Fraction	3. Fraction	4. Fraction	Reference
2-phenylethanol	76.9 %	67.9 %	61.3 %	56.2 %	50.2 %
Citronellol	16.0 %	22.1 %	26.2 %	29.8 %	31.5 %
Geraniol	7.1 %	10.0 %	12.5 %	14.0 %	18.3 %

In all collected fractions, only the molecules contained in the rose scent, 2-phenylethanol, citronellol and geraniol, were detected by GC-FID/MS (as described in Experimental p. 191). No decomposition products and also no traces of fatty acids were determined. In the first fraction, mainly 2-phenylethanol was present (76.9 %). This content decreased by increasing distillation time. In contrast, the content of citronellol and geraniol was low in the beginning (16.0 % and 7.1 %) and increased with time. Thus, 2-phenylethanol is more volatile than citronellol and geraniol, as it can also be compared with Table 19. In the course of time, an equilibrium was established. The composition of fraction 4 showed a relatively similar composition to that of the reference sample. 56.2 % 2-phenylethanol, 29.8 % citronellol, and 14.8 % geraniol were found in comparison to 50.2 % 2-phenylethanol, 31.5 % citronellol and 18.3 % geraniol in the reference. As a result, a better equilibration is needed to achieve a high distillation efficiency.

5.3.3.2. Vacuum distillation with rectification

In general, the distillation efficiency can be increased by distillation with rectification, since the vapor is in countercurrent contact with the liquid several times. If the contact time is long enough, an equilibrium can be established. The rectification process represents an extension of the simple vacuum distillation. The theoretical background is explained in chapter 2.6.2.

The vacuum distillation experiments with rectification were examined at the Clausthal University of Technology at the Institute for Separation and Process Technology headed by Prof. Dr.-Ing. Jochen Strube (Germany). The construction of the distillation process can be seen in Figure 59. A Vigreux column of 40 cm length was installed for an improved separation of the fragrance compounds from the fatty acid mixture. In order to increase the efficiency of the distillation column further, the system was coupled with a backflow valve. Only a part of the condensate was discharged. The other part flows back as reflux into the column and moves downwards as a liquid counterphase to increase the contact between vapor and liquid phase.

A mixture of the ternary fatty acid system (55 wt. % lauric acid, 30 wt. % myristic acid and 15 wt. % palmitic acid) containing 5 % rose oil (50.2 % 2-phenylethanol, 31.5 % citronellol, 18.3 % geraniol) was used for the distillation experiment. The condenser temperature was set to 5 °C, whereas the temperature of the bottom was set at 130 °C. This increased temperature in comparison to the previous implemented experiments was necessary, as here only a vacuum of 3 mbars could be applied. After 15 mins equilibration time, the backflow was set to 90 %.

Figure 59: Construction of a distillation with rectification.

During the distillation process, the temperature of the vapor phase was 80 °C. A light yellowish color of the fatty acid mixture in the bottom was observed. To get sure that the distillation was fully completed, the temperature of the bottom was slowly increased to 145 °C. The temperature of the vapor phase was first constant and then dropped to 70 °C. Presumably, the low-boiling 2-phenylethanol was distilled first. Afterwards, geraniol and

citronellol, which are the higher-boiling compounds of the fragrance mixture, built an azeotrope with 2-phenylethanol. A further temperature increase did not lead to a change of the vapor phase. Only at 175 °C, the temperature of the vapor phase increased strongly. In the course of this, the evaporation of the low-boiling lauric acid started. The distillation was terminated immediately.

The distillate was liquid at room temperature and also the GC-FID/MS analysis (as it is described in Experimental p. 191) confirmed that no fatty acids were contained. Despite the high temperature, no decomposition products were found. However, only 30 % of the initial amount of rose oil could be recovered this way. The system is not suitable for compounds with such low concentrations, since a large part remained in the Vigreux column.

On its way to the head of the column, the vapor mixture exchanges with the liquid phase, continuously. The less volatile components of the vapor phase condense and enrich the liquid phase. At the same time, the released heat of the condensation ensures the evaporation of the more volatile compounds of the liquid phase. Due to these processes in the column, the content of the more volatile components increases in the vapor phase from the bottom to the top of the column. Consequently, the amount of the low-boiling 2-phenylethanol was the highest in the distillate. 76.5 % 2-phenylethanol were determined by GC-FID/MS, whereas only 17.0 % citronellol and 6.5 % geraniol were present in the distillate (the original composition of the rose oil contained in the fatty acid mixture was 50.2 % 2-phenylethanol, 31.5 % citronellol and 18.3 % geraniol).

Overall, by distilling under rectification, only 30 % of the contained rose oil could be separated from the fatty acid mixture and this with a completely different composition. Mainly the low-boiling component was separated as intended by this distillation method. The volume of the vapor phase was simply too low to be distilled completely in a batch process. Even a long equilibration time did not result in a consistent fragrance composition. Therefore, a continuous distillation process has to be applied.

5.3.3.3. Molecular distillation

A molecular distillation, also known as short-path distillation, is a continuous process. The mixture to be distilled flows along the wall of the evaporator as a thin liquid film, while being agitated and distributed constantly. The vapor stream reaches the condenser, which is in the middle of the evaporator chamber, within a “short path”. Thus, the volatile substances are transported away continuously within a short time and with minimal thermal stress. A schematic presentation is given in chapter 2.6.3. (Figure 9).

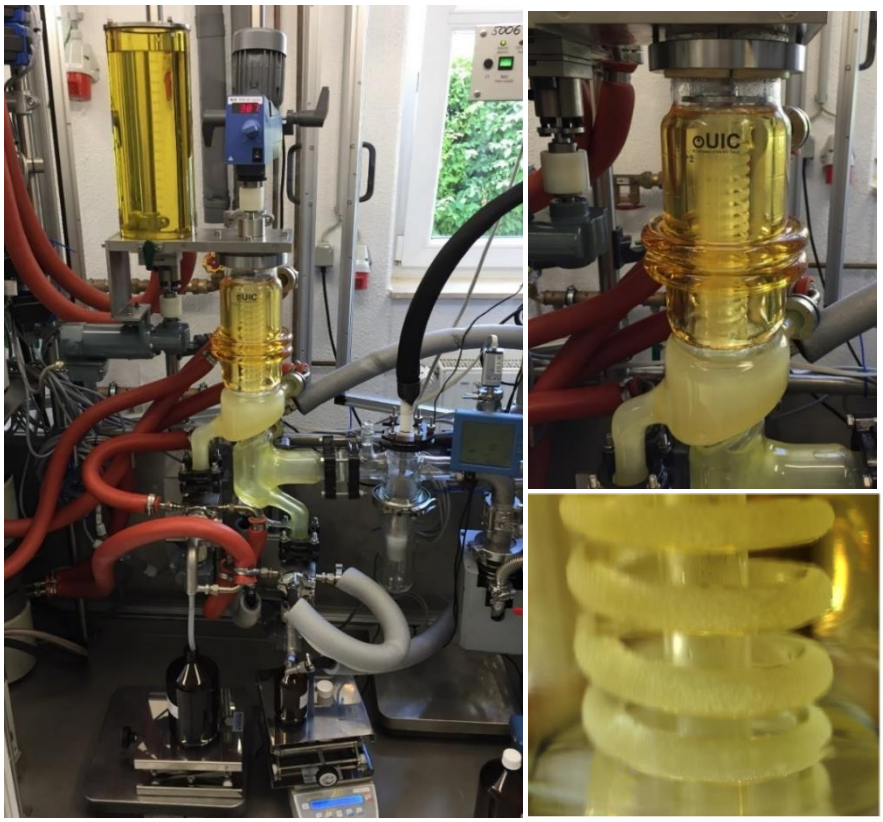

Figure 60: Apparatus of the KDL5 short-path distillation of UIC GmbH used for the experiments.

In the Process Development Center at UIC GmbH in Alzenau-Hörstein (Germany) experiments with a molecular distillation device KDL5 were carried out (see Figure 60). The aim was to separate the fragrance compounds from the ternary fatty acid mixture with a consistent fragrance composition, as it was not possible applying a simple batch distillation as described above. For this purpose, a ternary fatty acid mixture of 55 wt. %

lauric acid, 30 wt. % myristic acid and 15 wt. % palmitic acid with 5 % rose oil (in this case 50 % 2-phenylethanol, 30 % citronellol and 20 % geraniol) was provided in the feed vessel. Nine tests were performed to reach the targeted amount of around 5 % of the distillate (corresponding to the oil quantity contained in the fatty acid mixture). At 0.5 mbars, 90 °C evaporator temperature, 40 °C condenser temperature and a feed quantity of around 600 g/h, the experiments were started and the evaporator temperature was changed until approximately 5 % distillate was reached. Furthermore, the evaporator temperature, the feed quantity and the pressure of the vacuum were varied during the experiments, as it can be seen in the distillation protocol in Table 23.

Table 23: Distillation protocol of the examined experiments with the quantity of distillate and residue and the content of fragrances in the distillate measured by GC-FID/MS (as described in Experimental p. 191).

Test #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Evaporator [°C]	90	88	86	86	88	88	90	88	96
Internal Condenser [°C]	40	40	40	40	40	20	40	40	40
Vacuum [mbars]	0.5	0.5	0.5	0.5	0.5	0.5	2.0	2.0	5.0
Feed rate [g/h]	667	632	658	493	487	472	491	490	482
Distillate [wt. %]	6.0	5.1	4.4	4.1	5.2	4.7	5.3	4.5	3.0
Residue [wt. %]	94.0	94.9	95.6	95.9	94.8	95.3	94.7	95.5	97.0
Content of fragrances in the distillate [%]	64.9	70.1	74.5	68.7	65.5	68.9	74.6	78.1	83.4

From all experiments, samples of the distillate and the residue were taken. The respective mass fractions in wt. % of the distillate and the residue are given in the distillation protocol. The targeted distillation amount of the contained 5 % was realized largely during the various tests. To achieve this yield, the condenser temperature should be in the order of about 40 °C. If the temperature is set lower, for instance 20 °C as performed in experiment 6, the condensing fatty acids freeze, and hinders the fragrance molecules to be drained (compare Figure 60 on the bottom right).

The composition of the distillate was analyzed by GC-FID/MS (see Experimental p. 191). The content of the fragrance compounds in the distillate is given in percentage and indicated in the distillation protocol (see Table 23). For example, in the first experiment, the content of rose oil in the distillate was calculated to be 64.9 %. The other 35.1 % refer to the fatty acids (mainly lauric acid) in the distillate. By decreasing the evaporator temperature, see test 2 and 3, the selectivity of the distillation increases, as it can be seen by an enhanced content of fragrance compounds in the distillate, up to 74.5 %. In the experiment 4 and 5, the feed rate was decreased from approximately 600 g/h to 500 g/h. Since the time spent on the evaporator wall is extended, an increased distillation efficiency and thus, an increased content of the fragrance content in the distillate was expected. However, the reduction of the feed rate had only little influence. In the experiments 7, 8 and 9, the difference between evaporator and inner condenser temperature was varied. The higher this temperature difference, the better was the separation of the fragrance compounds from the fatty acid mixture. 83.4 % rose fragrances were contained in the distillate of test 9, when the vacuum was set to 5 mbars, the temperature of the condenser to 40 °C and the temperature of the evaporator to 96 °C. To sum up, in a first test series, the rose oil content of 5 % in the starting mixture was increased up to 83.4 % in the final distillate. The distillates of the last three tests were liquid at room temperature indicating a high content of rose scent and only minor amounts of fatty acids.

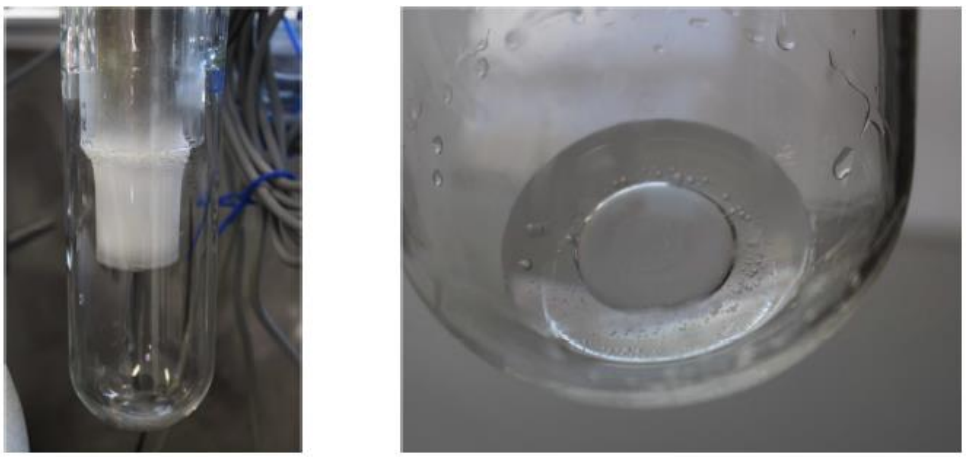

Figure 61: Cold trap at the end of the experiments (left) and melted (right).

During the whole experiments, 7 g (≙ approximately 1 g/h) of a water/oil solution were collected in the cold trap, see Figure 61. The solution consisted mainly of 2-phenylethanol (80 %). This collected condensate can be presumably further reduced by operating with a vacuum around 5 mbars. At 0.5 mbars, the boiling temperature of 2-phenylethanol (45 °C, calculated by UIC GmbH) is too close to the condenser temperature of 40 °C. At 5 mbars, the boiling temperature is 83 °C (calculated by UIC GmbH) and thus, a sufficient difference in temperature is ensured.

Not only an almost complete separation of the fragrance compounds from the fatty acid mixture was possible applying molecular distillation, but also the composition of the rose oil remained constant. In Figure 62, the GC-FID/MS chromatogram (obtained as described in Experimental p. 191) of a test distillation (blue) in comparison to the reference rose oil (red) of the same concentration is shown. The distillate (here test 3) has the same rose oil composition as in the starting mixture, as it can be confirmed with Table 24. For all other experiments, similar results were obtained. Only the lauric acid peak varies slightly in height. Thus, with the continuous distillation process, it is possible to isolate the rose fragrances in an authentic composition from the fatty acid mixture, which was not feasible with a conventional vacuum distillation.

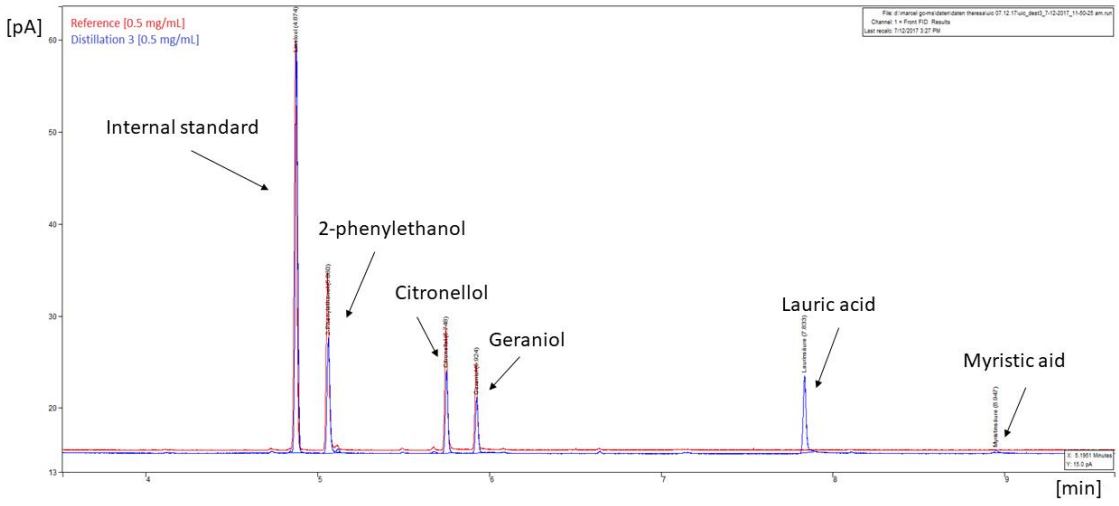

Figure 62: Comparison of a GC-FID/MS chromatogram (see Experimental p. 191). of the reference sample (red) and a test distillation (blue).

Table 24: Rose oil composition of the reference in comparison to a test distillation, determined by GC-FID/MS (as described in Experimental p. 191).

Rose oil composition	Reference	Test distillation
2-phenylethanol	49.05 %	48.63 %
Citronellol	30.64 %	30.49 %
Geraniol	20.31 %	20.88 %

It should be possible to optimize the distillation parameters further to achieve a complete isolation of the fragrance compounds from the ternary fatty acid mixture. However, in this study, it was proven that applying molecular distillation enables the isolation without a change in the composition of the fragrance mixture. More than 83 % of the rose oil could be recovered in a first test series under gentle conditions. Presumably, it is necessary to repeat the distillation procedure twice for a complete separation. Nonetheless, fatty acids are part of cosmetic or perfumery formulations and thus, they are added to the final product in any case. In comparison to other isolation techniques using solvents, molecular distillation has a lot of advantages. In this case, not the extraction medium, but the desired fragrances are distilled resulting in a product free of toxic residues. As with solvent extraction, the separated extraction medium can be directly reused for further extraction steps, since the fatty acids are chemically and thermally very stable. Due to the short exposure of the fragrance compounds to high temperatures and to a high vacuum, also sensitive compounds can be separated without decomposition.

In the work of Kurkcuoglu *et al.* (2003), molecular distillation was investigated for the production of rose absolute from rose concrete.[16] A lower yield was reported compared to dissolving the concrete in ethanol and subsequent freezing and filtration. However, the obtained distillate showed similar composition to commercial absolutes. Moreover, Ren *et al.* (2005) studied the purification of rose oil by molecular distillation.[17] A product of high purity could be obtained with a total recovery of more than 60 %. These results confirm the molecular distillation as ideal separation process for fragrance compounds, as thereby no decomposition products and a composition similar to the starting material are reported. In addition, the yield can be controlled by the distillation parameters.

5.4. Conclusion

In this chapter, the extraction of rose blossoms with lipophilic fatty acid mixtures without water was investigated. This “modern enfleurage” seemed to be more advantageous for the extraction of roses than the complex micellar extraction procedure (see chapter 4). The fragrance molecules are located on the surface of the petals inside fragile glandular trichomes and are thus easily accessible to the extraction medium. Using the free fatty acids, also the extraction of the water-soluble 2-phenylethanol should be possible, which is not feasible applying the micellar extraction procedure without the addition of inorganic salts.

Since the advantages of classical enfleurage and solvent extraction should be combined, a liquid extraction medium was targeted. Myristic acid, which already showed good solubilizing properties during micellar extraction, has a melting point of 54.4 °C. This temperature is too high to ensure that the plants respectively plant parts continue their physiological activities during extraction. Therefore, eutectic mixtures of various fatty acids were examined. With a ternary mixture of 55 wt. % lauric acid, 30 wt. % myristic acid and 15 wt. % palmitic acid, it was possible to lower the melting point to 32.3 °C. Thus, only a slight temperature increase was necessary to liquefy the extraction solution. In this mixture, fresh roses were macerated experimentally. The handling of the extraction process as well as the filtration of the rose petals and removal of remaining water was possible without major problems.

Moreover, this study was focused on the isolation of the fragrance compounds from this ternary fatty acid mixture. To this purpose, molecular distillation experiments were carried out in the Process Development Center at UIC GmbH (Alzenau-Hörstein, Germany). The distillate showed the same composition of the rose fragrances as in the starting mixture. Thus, with this continuous distillation procedure, it was possible to isolate the artificial rose fragrances in authentic composition, which was not feasible applying simple vacuum distillations. The original content of rose fragrances in the fatty acid mixture was 5 %. In a first series of experiments, it was possible to increase this content up to 83 %. That means that the fatty acid content from 95 % could be decreased up to 17 %. Not the extraction medium, but the desired fragrances were isolated without

decomposition, since the exposure to increased temperature at decreased pressure is very short during molecular distillation. As a result, a product free of toxic residues is obtained, in contrast to conventional separation techniques of perfume compounds using solvents. The extraction medium can be directly reused for further extraction steps due to the chemical and thermal stability of fatty acids.

Many parameters are significant to ensure the qualitative and quantitative extraction of fragrances from plant material. So far, a proof of concept has been successfully performed. However, many questions are still open primarily regarding the extraction power of the fatty acid mixture with varying extraction parameters. The aim is to clarify these questions in future. In view of the potential prohibition of hexane as extraction medium and the generally increasing trend of Green Chemistry, finding a sustainable and green extraction method has strategic importance. A solvent-free, completely non-toxic and sustainable extraction process for fragrances from plant material without thermal degradation of sensitive compounds could be possible by applying “modern enfleurage”.

5.5. Experimental

5.5.1. Reagents

The following chemicals were used without further purification: lauric acid (Sigma-Aldrich, purity ≥ 98 %), myristic acid (Sigma-Aldrich, purity ≥ 99 %), palmitic acid (Sigma-Aldrich, purity ≥ 97 %), stearic acid (Sigma-Aldrich, purity ≥ 98 %), ethanol (Sigma-Aldrich, purity 99 %) and diethyl ether (Merck, for analysis). HPLC grade methanol from Merck was used during the analysis. Linalool (Sigma-Aldrich, purity ≥ 98 %), geraniol (Sigma-Aldrich, kosher, ≥ 97 %), citronellol (Sigma-Aldrich, kosher, ≥ 97 %) and 2-phenylethanol (Sigma-Aldrich, purity ≥ 99 %) were purchased for the quantification. Rose oil consisting of 2-phenylethanol, citronellol and geraniol was provided by a major international perfume company. Deionized water was used throughout the experiments.

5.5.2. Melting point determination

The melting points of the various fatty acid mixtures were examined by mixing lauric acid, myristic acid, palmitic acid and stearic acid in the respective weight ratios. The mixtures were first homogenized by melting and then transferred as powder to melting point capillaries. The capillary was then submerged in a thermostatic oil bath and the melting end point was determined with a digital thermometer (GHM Messtechnik GmbH, Germany, precision < 1 %). The results are based on three independent measurements (n = 3).

5.5.3. Extraction of rose petals with a ternary fatty acid mixture

1 kg extraction medium was prepared by mixing 550 g lauric acid, 300 g myristic acid and 150 g palmitic at 50 °C. As no inherently odoriferous roses were available at that time, odorless rose petals were artificially scented with the rose oil provided by our international partner (c.f. 4.3.1). To this purpose, a rose oil to rose petal ratio of 4 g/kg was used. The scented roses (83.3 g) were added to the fatty acid mixture with a solid to liquid ratio of 1/12. The rose petals were soaked in the mixture over night at 35 °C.

Afterwards, the rose petals were removed by filtration through a stainless steel filter to reuse the extraction medium again. The extraction of scented rose petals was repeated three times with this solution. The extracted and separated petals were collected and stored at 4 °C. Finally, the combined petals were washed with 0.5 kg of fresh fatty acid mixture in the stainless steel filter. Additionally, the roses were squeezed out and washed with hot water to remove adhering fatty acids from the petals. After solidifying at room temperature, the small amount of water was separated by decantation. The fatty acid mixture containing the desired fragrances as well as the aqueous washing solution was stored in the fridge and analyzed by GC-FID/MS (see 5.5.5).

5.5.4. Vacuum distillation

5.5.4.1. Simple vacuum distillation

For the simple vacuum distillation experiments, the following experimental setup was assembled. A distilling bridge with Liebig condenser and vacuum stopper was installed on a 1000 mL round-bottomed flask. The condenser temperature was set to 5 °C. The temperature of the round-bottomed flask was regulated with a heating plate using an oil bath and adjusted to 100 °C. The distillation mixture was constantly agitated with a magnetic stirrer. The vacuum was set to $2.0 \bullet 10^{-2}$ mbars. A thermometer was installed at the Claisen bridge, which allowed us to check the temperature of the vapor phase. For fractionated distillation, a distillation spider was adapted to allow the direct fractionation into individual round-bottomed flasks.

Respectively, 142.5 g fatty acid mixture (55 % lauric acid, 30 % myristic acid and 15 % palmitic acid) were mixed with 7.5 g rose oil (50.2 % 2-phenylethanol, 31.5 % citronellol and 18.3 % geraniol). The distillation was continued until no more dripping occurred. The separated rose oil was diluted with methanol (5 mg/mL) and analyzed by GC-FID/MS (see 5.5.5). The pure rose oil (5 mg/mL methanol) was used as a reference value for comparison.

5.5.4.2. Vacuum distillation with rectification

The experiments were carried out at the Clausthal University of Technology at the Institute for Separation and Process Technology headed by Prof. Dr.-Ing. Jochen Strube, Germany. The rectification system consisted of a 40 cm Vigreux column combined with a Backflow controller. The temperature of the condenser was set to 5 °C and the vacuum was adjusted to 3 mbars. 300 g of the fatty acid mixture containing 5 % rose oil (composition see 5.5.4.1, respectively) was heated up to 145 °C in a round-bottom flask and stirred continuously. After 15 min equilibration time, the rose oil was distilled with a backflow of 90 %. The distillation was terminated after 2 hours. The obtained distillate was analyzed by GC-FID/MS (5 mg/mL methanol, see 5.5.5).

A second experiment was carried out using the same conditions, but without backflow.

5.5.4.3. Molecular distillation

The experiments were performed at the Process Development Center at UIC GmbH in Alzenau-Hörstein, Germany. A partly automated short-path distillation plant (KDL5) with an evaporator area of 0.05 m^2 and a throughput of up to 1.5 kg/h was used. The feed and also the discharge of distillate and residue was carried out by heated gear pumps.

Nine tests were investigated to reach the separation of the rose oil (in total 5 %, composition 49.1 % 2-phenylethanol, 30.6 % citronellol, 20.3 % geraniol) from the fatty acid mixture (55 % lauric acid, 30 % myristic acid and 20 % palmitic acid). Around 5 kg of this mixture were stored at 40 °C in the feed vessel. The tests were started at 0.5 mbar and the evaporator temperature was increased until 5 % of distillate had been distilled. Subsequently, the evaporator temperature, the feed quantity and the vacuum were varied. The temperature of the condenser was adjusted in experiment 6. At all settings, samples of the distillate and residue were taken and analyzed by GC-FID/MS with linalool as internal standard. 1 mg of the distillate was dissolved in 1 mL methanol. This solution was mixed with 1 mL of internal standard solution (2 mg linalool/mL methanol) and analyzed immediately (see 5.5.5).

5.5.5. Gas chromatography

GC analysis was realized using an Agilent Technologies 7890A Chromatography System. The system implies a FID Detector coupled with a 220 Ion Trap GC/MS. The samples were injected automatically by an Agilent 7693 Autosampler. Separations were achieved on a VF-5ms column (30 m x 250 µm x 0.25 µm) with helium as carrier gas with a constant flow of 1 mL/min. The samples (1 µL) were injected with a split/split less injector (split 1/50). The following temperature program was used (see Figure 63): holding 80 °C for 0.5 min, ramping up to 300 °C at 20 °C/min and then holding 300 °C for 5 min. The injector temperature was set to 250 °C and the temperature of the flame ionization detector was set to 300 °C. Analysis was performed by analyzing retention times and peak areas in comparison to the internal standard linalool.

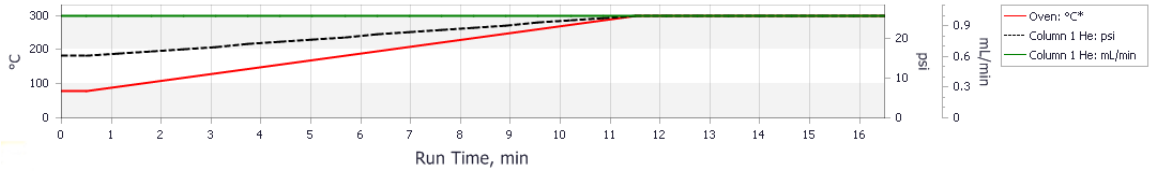

Figure 63: GC method for the analysis of rose scent.

5.6. References

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6. General conclusion and outlook

Considering the drawbacks which are linked to the conventional extraction methods distillation and solvent extraction, the need for finding new alternative green extraction methods is very high. In regard to the principles of Green Extraction, two non-toxic, non-flammable, and very mild extraction methods were invented for the isolation of fragrance compounds from plant material: micellar extraction with aqueous soap solutions and modern enfleurage with free fatty acids.

Based on iris rhizomes, a simple, efficient and gentle extraction method for fragrance compounds with natural, biocompatible and biodegradable soap solutions was established. Generally, the iris butter is obtained from rhizomes of *Iris germanica* L. or *Iris pallida* Lam. by steam or hydro distillation. During this long-lasting process, not only a high amount of energy is required, but also the thermal degradation and volatilization of the fragrance compounds are induced. By using aqueous soap solutions, especially a sodium myristate solution (0.12 mol/L), an almost complete extraction of the valuable non-polar irones is possible within 30 – 60 min at 45 °C due to the amphiphilic character of soaps. Various fatty acids, particularly myristic acid, are naturally occurring in the rhizomes. Thus, this gentle extraction method uses an intrinsic plant substance as a highly efficient extraction medium. By using a mixture of fatty acids with different chain lengths, a tailor-made extraction is possible. Adding shorter-chained fatty acids to the sodium myristate solution leads to an adjustment of the selectivity due to the increase of the polar character of the extraction solution. Not only the extraction, but also the isolation of the irones is possible by applying micellar extraction. The free fatty acids are formed by lowering the pH value of the soap solution and thus, the micelles break up causing the release of the irones. These nonpolar compounds migrate to the insoluble myristic acid phase and can be separated together. As myristic acid is already a frequent ingredient of formulations like crèmes and lotions or perfumes, the final product with its remaining fatty acid represents an ideal basic raw material. However, the irone concentration in it is very low. In addition, excess myristic acid can be recovered by crystallization in cold ethanol or preferably by molecular distillation. This separation process allows not only the enrichment of the irones in the extract, but also the recovery of myristic acid. The fatty

acid can be saponified again and reused for subsequent extraction cycles. This newly invented, alternative and green extraction method had been already protected by a European patent (EP 3 130 655 A1., “Process for Isolation of Odoriferous Agents”, Theresa Höß, Marcel Flemming, Didier Touraud, Werner Kunz, University of Regensburg, 2017). For a scale-up to industrial application, however, some difficulties still have to be overcome.

Moreover, the micellar extraction procedure was investigated for the isolation of fragrance compounds such as citronellol, geraniol, and 2-phenylethanol from rose blossoms. These fragrances are key ingredients for perfumes and cosmetics and are commonly extracted with solvents like *n*-hexane. Thereby, the majority of the constituents contained in the rose blossoms remain unaltered. In contrast, rose oil, produced by steam or hydro distillation, contains no or only a very low concentration of water-soluble substances. Especially the characteristic 2-phenylethanol is only present in minor amounts. Moreover, due to the high temperature during the distillation process, thermal degradation of sensitive compounds often occurs. Thus, rose absolute, which is produced by solvent extraction, is mainly used despite the risk of handling large quantities of flammable and toxic solvents and the danger of potential residues in the extract. In collaboration with a major international perfume company, it was found that the experimental procedure of the micellar extraction of *Rosa x centifolia* L. is less complicated, compared to iris rhizomes, due to the different nature of the plant material. The odoriferous extract is completely soluble in ethanol and represents with its remaining fatty acid an ideal basic raw material. However, in contrast to the extraction of the water-insoluble irones, the addition of an inorganic salt is necessary to decrease the solubility of partially water-soluble substances, such as 2-phenylethanol and geraniol. By adding potassium carbonate or sodium chloride, for instance, around 90 % of the amount of 2-phenylethanol in water can be recovered. As a consequence, by using the salting-out effect, also the isolation of water-soluble compounds is possible. Not only the scent of the extract is similar to the pure rose blossoms, but also no toxic residues remain.

Furthermore, in collaboration with the Ph. D. student A. Wollinger, it could be proven that the micellar extraction procedure using aqueous soap solutions is suited as well for other plants, respectively plant material. The extraction of antioxidants from rosemary leaves

was successfully demonstrated and has already been protected by an International patent application (WO 2017/194629 A1 “Process for Extraction of Antioxidants from Plant Material”, Alexander Wollinger, Theresa Höß, Didier Touraud, Werner Kunz, 2017, University of Regensburg). The main antioxidants in rosemary are rosmarinic acid (hydrophilic) and carnosic acid (hydrophobic). Antioxidants of natural sources are of high importance due to the fact that artificial antioxidants are partially prohibited as food additives. With micellar extraction, the total amount of rosmarinic acid and carnosic acid contained in *Rosmarinus officinalis* L. can be extracted within few minutes at moderate temperatures. Compared to conventional extraction methods, this represents a significant saving in time and energy. In addition, it is the first process reported to obtain carnosic acid by extraction of rosemary leaves without using toxic and flammable solvents.

The second invented green extraction method, “modern enfleurage”, is a simple alternative to the relatively complex procedure of the micellar extraction. Since the fragrances are located on the surface of the rose petals inside fragile glandular trichomes, they are easily accessible to the extraction medium. In modern enfleurage, the advantages of classical enfleurage and solvent extraction are combined. Odorless molten free fatty acids without water are used as liquid extraction medium. By completely embedding the plant material compared to classical enfleurage, the lipophilic extraction medium is better able to penetrate into the plant tissue and the mass transport of the analytes is increased. Moreover, also the extraction of the water-soluble 2-phenylethanol should be possible without the addition of additives. Since a eutectic mixture of lauric acid, myristic acid and palmitic acid with a melting point around 30 °C was chosen as liquid extraction medium, also the extraction of plants respectively plant parts, which continue their physiological activities after picking, is conceivable. This study was mainly focused on the isolation of the fragrance compounds from this ternary fatty acid mixture. Therefore, molecular distillation experiments were carried out in the Process Development Center at UIC GmbH (Alzenau-Hörstein, Germany). In contrast to solvent extraction, by applying molecular distillation, not the extraction medium but the fragrances are separated by distillation. Molecular distillation is a continuous thermal separation process with short exposure to increased temperature and decreased pressure

and thus, ideally suited for the purification and separation of sensitive compounds. As a result, a product free of toxic residues with a fragrance composition similar to the genuine character is obtained, which was not feasible applying simple vacuum distillation. The original content of rose fragrances in the fatty acid mixture was 5 %. In a first series of experiments, it was possible to increase this content up to 83 %. That means that the fatty acid content from 95 % could be decreased up to 17 %. In addition, the separated extraction medium can be reused for further extraction cycles directly. The solution does not have to be purified or saponified as in the micellar extraction procedure. So far, a proof of concept regarding the isolation of the fragrance composition was accomplished. A funding proposal to the “Bayerische Forschungsstiftung” in Munich, Germany, was submitted (AZ-1311-17) in order to examine the modern enfleurage further. In view of the potential prohibition of *n*-hexane as extraction medium and the generally increasing trend of Green Chemistry, finding a sustainable and green extraction method has strategic importance. With this approach, a solvent-free, completely non-toxic and sustainable extraction of fragrances from plant material is possible without thermal degradation of sensitive compounds.

For the extraction of plant material, a high extraction power of the medium is essential. In future, the already gained knowledge of micellar extraction should be combined with the approach “modern enfleurage”. In micellar extraction, amphiphilic molecules form aggregates, which enable lipophilic molecules to be dissolved in water. Moreover, aqueous surfactant solutions have the property to penetrate the plant matrix and thus, to accelerate the extraction of fragrances, for instance. By adding sodium myristate with a small amount of water to the mixture of fatty acids, the extraction power of pure fatty acids can be probably further increased. Also, the extraction of less fragile plant material, like iris rhizomes, is conceivable. Subsequently, the small amount of sodium myristate can be neutralized and the fragrance compounds can be separated from the fatty acid mixture by molecular distillation. Thus, the natural scent of plants, for instance also of orange blossoms, jasmine, tuberose or narcissus, can be gently recovered without toxic solvents. Moreover, this extraction method represents a circuit with the recycling and reusing of the extraction medium. The separated extract is free of toxic residues. Only traces of edible fatty acids may be present.

To sum up, both of the two newly invented extraction methods are in accordance with the principles of Green Extraction (compare chapter 2.2): bio-based solvents are used for the extraction of plant material; energy consumption is reduced compared to conventional extraction methods since extraction occurs at moderate temperatures and within short extraction time; no co-products respectively waste is produced since the extraction medium can be recycled; the risk of handling large quantities of flammable and toxic solvent is limited completely; and the extract is biodegradable and free of toxic residues compared to conventional solvent extraction. As a consequence, high valuable products can be obtained using these green and sustainable extraction processes. Thus, the high demand for less toxic, renewable und biodegradable solvents for the extraction of plant material can be satisfied.

As with conventional extraction methods, the values of the extraction parameters are not universal and have to be adjusted and optimized to the nature of the plant material, respectively to the compounds to be isolated. In particular, the isolation of the fragrance compounds from the extraction medium can be challenging.

7. Appendix

7.1. Table of Figures

7.2. Index of Tables

7.3. Table of Symbols

Symbol	Name
a	Peak area
a_0	Cross sectional area
b_1	Slope
c	Concentration
ChC_4	Choline butanoate
ChC_6	Choline hexaonate
ChC_8	Choline octanoate
CMC	Critical micelle concentration
CPE	Cloud point extraction
DLS	Dynamic light scattering
EPA	Environmental Protection Agency
EtOH	Ethanol
FDA	Food and drug administration
FID	Flame Ionisation Detector
GC	Gas chromatography
HMyr	Myristic acid
HPLC	High-performance liquid chromatography
ILs	Ionic liquids
K	Response factor
KC14	Potassium myristate
l	length
m	Mass
MAE	Microwave-assisted extraction
MeOH	Methanol
MHC	Minimal hydrotrope concentration
m/m %	Mass fraction
mp	Melting point
MS	Mass spectrometry
n	Number of repetitions

NaC4	Sodium butanoate
NaC6	Sodium hexanoate
NaC8	Sodium octanoate
NaC10	Sodium decanoate
NaC12	Sodium dodecanoate
NaC14	Sodium myristate
NaC16	Sodium palmitate
NIST	National Institute of Standards and Technology
NaOleate	Sodium oleate
N_s	Packing parameter
p	pressure
p-value	Statistical significance
PA	Phytic acid sodium salt
PCM	Phase change material
PTFE	Polytetrafluoroethylene
SDS	Sodium dodecylsulfate
SFE	Supercritical fluid extraction
s/l	Solid to liquid ratio
T(head)	Temperature of the vapor phase during distillation
T_{Krafft}	Krafft temperature
TMSH	Trimethylsulfonium hydroxide
TLC	Thin-layer chromatography
UAE	Ultrasound-assisted extraction
UV/VIS	Ultraviolet/visible
v	Volume of a surfactant molecule
V	Volume of a solvent
VOCs	Volatile organic compounds
wt. %	weight percent
x_i	Mole fraction
y_i	Activity coefficient

7.4. List of Publications

(1) Theresa Höß, Marcel Flemming, Didier Touraud, Werner Kunz, Process for Isolation of Odoriferous Agents, University of Regensburg, 2017, EP 3 130 655 A1.

(2) Alexander Wollinger, Theresa Höß, Didier Touraud, Werner Kunz, Process for Extraction of Antioxidants from Plant Material, University of Regensburg, 2017, WO 2017/194629 A1.

7.5. List of Presentations

10/2014 **7th Green Solvents Conference**, Dresden (Germany)

"Extraction of *Iris pallida* Lam. with choline-based ionic liquids" (Poster)

02/2015 **Jahrestreffen der Fachgruppe für Phytoextrakte**, Bonn (Germany)

"Extraction of *Iris pallida Lam*. with choline-based ionic liquids" (Poster)

06/2015 **International Workshop on "Alternative Solvents for Extraction, Purification and Formulation"**, Avignon (France)

"Extraction of *Iris pallida* Lam. with choline-based ionic liquids" (Poster)

05/2016 **Green Extraction of Natural Products Conference**, Turin (Italy)

"Micellar Extraction of iris rhizomes using green soap solutions" (Poster)

07/2016 **Formula VIII Conference**, Barcelona (Spain)

"Micellar Extraction of iris rhizomes using green soap solutions" (Poster)

03/2017 **Jahrestreffen der Fachgruppe für Phytoextrakte,** Köln (Germany)

"Micellar Extraction of iris rhizomes using green soap solutions" (Poster)

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Regensburg, 31.01.2018 _____

(Höß Theresa)