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The Investigation of the Formation of Volatile Compounds in Unrefined Vegetable Oils and their Misleading Association with Residues from Solvent Extraction

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Abstract

Vegetable oils are an essential part of a healthy and balanced diet and have major economic significance. The kind of production, whether performed by extraction with solvents, or by mechanical techniques, significantly influences the quality of the oil and its potential field of use. Occasionally, volatile, organic substances, which are considered indicative for an oil that has been produced by solvent extraction, are detected in unrefined vegetable oils. Being accused of producing vegetable oils by solvent extraction can have major negative impact on high-quality oil mills and their reputation. The goal of the study was to analyse unrefined oils of different raw materials for such compounds. For this task a robust and sensitive method using headspace gas chromatography coupled with a mass selective detection system was developed and validated. Another aim was to determine the origin of any elegit solvent residues in the vegetable oils and to find ways to avoid them. Complementary measurements by solid phase micro extraction gas chromatography were conducted to compare the results of the measurements to an orthogonal methodology. Multivariate data analysis was used to find correlations between the spectrum of substances in the oil and other parameters like the producer of the oil or the pattern of fatty acids.

Zusammenfassung

Pflanzliche Öle sind ein essentieller Bestandteil einer gesunden, ausgewogenen Ernährung und haben große wirtschaftliche Bedeutung. Pflanzliche Öle werden meist entweder durch die Extraktion mit Lösemitteln oder durch Pressen des Rohmaterials hergestellt, wobei die Wahl des Herstellungsverfahrens großen Einfluss auf die Qualität und die möglichen Anwendungsbereiche des Produkts nimmt. Gelegentlich werden flüchtige, organische Substanzen, die als Indikatoren für die Anwendung eines Extraktionsverfahrens mit Lösemitteln dienen können, in unraffinierten Pflanzenölen detektiert. Ölmühlen die sich auf die Produktion hochwertiger und naturbelassener Öle spezialisiert haben und mit dem Vorwurf konfrontiert sind Extraktionsverfahren eingesetzt zu haben, müssen mit einer starken Schädigung ihrer geschäftlichen Basis und ihres Images rechnen. Das Ziel dieser Studie war es unraffinierte Öle aus unterschiedlichen Rohstoffen auf flüchtige, organische Verbindungen zu untersuchen. Zu diesem Zweck wurde eine robuste und empfindliche Methode auf Basis der Headspace-Gaschromatographie gekoppelt mit massenselektiver Detektion entwickelt und validiert. Des Weiteren sollte ermittelt werden wie die mutmaßlichen Kontaminationen in die Öle gelangten und wie diese in Zukunft vermieden werden könnten. Komplementärmessungen, die mit Festphasenmikroextraktion und Gaschromatographie durchgeführt wurden, dienten dazu die Ergebnisse mit einer orthogonalen Technologie vergleichen zu können. Multivariate Datenanalyse ermöglichte die Korrelierung der Messergebnisse mit Parametern wie dem Fettsäuremuster oder dem Produzenten und die Ermittlung von Zusammenhängen zwischen dem Substanzspektrum der Proben und externen Einflussgrößen.

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1 Introduction

The production of virgin, unrefined vegetable oils has a long tradition in southern Styria. The Styrian pumpkin seed oil is well known as a local specialty and has economical relevance for the region as well. It has been registered as a "Protected Geographical Indication" item (PGI) in the European Union's (EU) Database Of Origin & Registration (DOOR) in 1996 (The European Commission, 1996). However, other high-quality oils from different oil seeds are produced in the region as well, like linseed oil, hazelnut oil and walnut oil. In contrast to animal fats, vegetable oils have a high content of unsaturated fatty acids and other essential nutritious components, like fat-soluble vitamins and antioxidants. Due to these valuable ingrediencies, they play an important role in a balanced healthy diet. (Timmermann, 1990). The choice of the oil production technique is a first indication of the quality of the final product. Oils can be produced either by mechanical techniques or by extraction with organic solvents (Krist, et al., 2009). The pressing of oilseeds is the traditional technique in Styria. The produced oil is used for high-quality foods or direct consumption. Most of the flavouring substances and other ingredients are preserved and a valuable product can be achieved regarding both, its nutritional as well as its sensory parameters. The roasting of the crushed oil seeds prior to the pressing process enables the separation of the oil from other parts of the plant, as well as the development of substances, which are essential for the typical flavour (Siegmund & Murkovic, 2004). In contrast to mechanical processes, extraction with solvents is a more cost-effective technique, and is therefore used in the production of oils which are produced in high volume for the food industry as well as other industrial applications. The complete removal of these solvents from the produced oil can be a challenge. Even small traces of solvents can be detected with modern analytic techniques. (Kumar & Gow, 1994). Investigating oil samples for volatile, organic substances is therefore a common method for determining whether an oil was extracted or produced by mechanical means. (Michulec & Wardencki, 2004) Oil mills, which are confronted with the accusation of producing their oil via extraction of the oilseeds, face substantial economical and reputational damage. Several studies have already investigated the influence of the roasting process of oil seeds, on the formation of volatile substances. However, the focus is usually put on flavouring substances instead of compounds which are also used as solvents. Short-chain alkanes (n-pentane, n-hexane, n-heptane) are still common in the food industry today, whereas aromatics (benzene, toluene, xylene) had been used in the past (Ozel, et al., 2014) (Zhang, et al., 2016) (Gracka, et al., 2016). Especially in Europe, the topic of contamination of edible oils with mineral oils and solvent residues is one which has significantly gained in importance over recent years. Since a variety of hydrocarbons can be found naturally in unrefined vegetable oils, it is often not easy to distinguish between natural occurrence and contamination (Moreda, et al., 2001). In addition to technical

challenges, producers are faced with the consumer's wish of obtaining a product which is without impurities, but also has been produced under natural and organic conditions. The oil's tendency to absorb compounds from the environment further complicates the matter. Existing EU law regulates contaminants (e.g. polycyclic aromatic hydrocarbons, PAH) in edible oils as well as residues of certain solvents which are generally used for extractions. But not all occurring compounds are regulated within EU law. Moreover, limits for solvent residues apply only for oils which have been declared to be extracted with solvents. There are no legal limits for solvents in virgin oils since no residues are supposed to be found. Some legislations allow residues which cannot be technically avoided without further defining limits (The European Parliament and the Council of the European Union, 2009), (The European Commission, 2017). With analytical instrumentation and methodology becoming increasingly sensitive even the smallest amounts of volatiles and other substances can be detected in the oil which leads to unclear situations, especially if the legal requirements are not explicitly defined. Producers who are faced with analytical reports which claim the presence of minor residues of solvents in their virgin oils have no legislative leeway if food traders reject their products. Choosing a sensible and careful approach to this important issue, is critical for producers of virgin oils and food traders, as well as for analytical chemists. Lawmakers must be aware that there is need of addressing these problems and avoiding legislative uncertainty concerning the occurrence of solvent residues in unrefined vegetable oils.

The project, which was the basis for this master thesis was largely conducted at Institut Dr. Wagner Lebensmittel Analytik GmbH in Lebring, Austria. Its aim was to analyse oils which have demonstrably been produced by pressing of the oilseeds, for volatile, organic substances, which could be associated with solvent extraction. The analysis of oils from different producers and various oilseeds, was conducted at the Institut Dr. Wagner using static headspace gas chromatography coupled with a mass selective detection system (HS-GC/MS) technology. However, some comparative measurements were done at the Institute of Analytical Chemistry and Food Chemistry at the University of Technology in Graz. Selected samples were treated with a thermal stress test, which would provide information about the influence of heat on the development of these volatile substances. The focus was therefore put on volatile substances, which are specifically used as solvents, and not on the whole spectrum of volatile compounds.

1.1 Vegetable oils

Vegetable oils and fats consist of a variety of triacylglycerides which are tri-esters of glycerol and fatty acids. Most of the fatty acids are linear and even-numbered in the range of 4 to 26 carbon atoms. In contrast to animal fat which in most cases is solid, most vegetable oils are liquid. One exception is palm oil which has a fatty acid spectrum which is quite similar to animal fats. The state of matter depends on the length of the fatty acid chains in the triacylglyceride as well as their degree of saturation. The shorter the fatty acid chains and the higher the number of double bonds, the lower the melting point. Since oleic acid, the most common fatty acid in plant oils, is monounsaturated, most plant oils are liquid. Whether a mixture of triacylglycerides is called an oil or a fat, depends on the aggregate state and therefore on the composition of the fatty acids. (Krist, et al., 2009) With 59 million tons of annual global production, palm oil is the most produced vegetable oil, followed by soybean oil (45 million tons), rapeseed oil (27 million tons) and sunflower oil (16 million tons). Other oils, with an annual production of less than 10 million tons, are palm kernel oil, cotton oil, peanut oil, and coconut oil. High quality oils, like virgin olive oil and linseed oil which have lower annual production volumes, are popular especially in Europe (OVID 2015, 2014). Fats have the highest energy density of all foods and deliver around 40% of the daily energy intake of a normal diet. Fats play an especially important role for the overall health since they are a major building block for the synthesis of phospholipids in the human body. These compounds are essential for the formation of a stable and healthy cell membrane. Fats also serve as a means of storing energy. Plant oils are physiologically healthier due to their much higher share of unsaturated acids. Several of the polyunsaturated fats which cannot be obtained by eating animal fats are essential for the human organism. They cannot be synthesised in the body and therefore must be introduced externally through the diet. The share of saturated fatty acids like stearic acid, which is the most common fatty acid in animal fats, should not exceed 10% of the total energy delivered by fats and oils. Most plant oils fulfil this condition and should therefore be an integral part in a healthy and balanced diet. However, not all vegetable oil can unrestrictedly be recommended for the daily consumption. The composition of the oils is not only determined by the plant which it originated from, but also by the specific production technique. Further treatment of the oil also significantly influences its dietary value (Krist, 2013) (Chow, 2008).

1.1.1 Production of vegetable oils

Vegetable oils have been part of the common diet of humans for thousands of years with the oldest findings of oil production equipment dating back to the stone age. There is archaeological evidence that vegetable oils have already regularly been produced and used on a large scale 6000 years ago, especially in Babylon and Egypt (Roth & Kormann, 2000). The oil was not only produced for dietary use

but for medical applications as well. Around the Mediterranean basin, olive oil became extremely popular with the rise of developed cultures like the Persians, the Greeks and later the Romans. Historic oil production relied heavily on gravity to extract the oil from the fruit. The necessary force was exerted either by the olives' own weight or by stones which were put on top of the fruits. Only inventions like the screw thread which was not known until approximately 50 BC, allowed the construction of presses which enabled the large-scale production of oils made from oil seeds as well. Today two major production techniques for vegetable oils can be distinguished: Pressing by mechanical means and extraction using organic solvents. (Krist, 2013)

1.1.1.1 Solvent extraction

Solvent extraction is either employed initially or used to increase the yield after a mechanical production technique has been used. To produce cheap oil in large quantities, the former usually applies and extraction techniques as well as mechanical methods are used in succession. When solvent extraction was first employed at the end of the 19th century, the most common solvents were benzene, tetrachloromethane, and trichloroethene. (Krist, 2013) Due to the toxic and sometimes carcinogenic nature of these solvents, they are no longer used. The most common solvents in use today are hexane and methylethylketone. (Baltes & Matissek, 2011) The advantage of solvent extraction over any mechanical production technique is the superior yield and therefore its efficiency. To guarantee sufficient access of the solvent into the plant matter, the oil seeds must be ground first. To further increase the yield, the extraction is often conducted under heating. There are two extraction techniques which are employed most commonly. In the percolation process the crushed oil seeds are constantly in contact with the solvent which is flowing in the opposite direction. A conveyor belt transports the seeds through the counter-stream of the solvent. This guarantees high extraction efficiency since fresh solvent is constantly delivered to the seeds. Also, the mechanical stress on the seed material is low which decreases the number of small particles in the oily solution. The second technique is the immersion process. It is required when the extracted matrix is especially dense or not easily accessible by the solvent due to high fibre content. In this process, the whole batch of crushed seeds is immersed into the solvent. Since the solvent is not in movement, it becomes saturated with oil after some time. To help with this issue the solvent is agitated, and fresh solvent can access the seed material. This is mechanically demanding for the oil seeds which leads to more floating particles in the oil-solvent mixture. These solid impurities must later be removed by filtering (Gustav Heess GmbH, 2010). Regardless of the extraction process which is employed to separate the oil from the residual plant material, the solvent must be removed, and the oil must be refined before it can be used. This is not a single process but consists of a cascade of individual steps, which are carried out in different vessels and reactors. Its goal is the removal of undesired odours, flavours, and colours. (Krist, 2013) The first step is the degumming of the oil in which salt solutions or acids are added to remove

lecithin. This residue is purified and used in the production of margarine. The next step is the deacidification which removes free fatty acids from the oil which are not esterified with glycerol. This is done by adding strong alkalic substances which saponify the fatty acids (Baltes & Matissek, 2011). The addition of glycerol can also bind the acids by esterification. Then the oil is bleached by adding adsorbing material like aluminium silicate or charcoal. (Krist, 2013) In a last step the oil undergoes deodorisation which removes undesired odours and other volatile contaminants like light PAH or pesticides. This is usually done by a steam distillation at 240 – 270 °C and under reduced pressure. Oils which are intended for use in the food industry usually undergo a winterisation process which precipitates waxes and prevents the oil from becoming cloudy. Only if the extracted vegetable oil is put through the whole process it is safe for consumption. If desired, additional ingrediencies like antioxidants and vitamins can be added to the oil after the refining process. (Baltes & Matissek, 2011)

1.1.1.2 Pressing

Mechanical technologies like pressing of the oil fruits or seeds is historically the far older method and has been employed since the invention of screw threads enabled the construction of tools which could exert enough force on the plant material. Before that the oil had been produced by making use of the weight of stones and the oil plants themselves which had crushed the lowest plants and had separated the oil. The pressing process is the technology which is most frequently used if the quality of the oil and the integrity of its original ingredients are prioritised over the production quantity. Usually several steps of pressing the oil seeds are conducted which produce different qualities of oil, depending on the production conditions. (Krist, 2013) The Austrian federal ministry of health and consumer protection enforces strict regulation on which treatments a vegetable oil can be subject to if certain quality parameters should be obtained. Pressed vegetable oils are usually not refined and therefore can only be purified by mechanical and physical means like washing, precipitations of floating matter, filtration, or centrifugation. If only a mechanical pre-treatment had been done, the first fraction of the pressing process, which was produced without any heating can be called “virgin”. The fraction of the next higher quality is called “cold pressed” and certain pre-treatments like a roasting process are allowed if they are specifically declared. Heating to 140 °C under vacuum as a purification and cleaning process is allowed only for a short period and no additives can be introduced into the oil. If the pressing process is conducted under heating, the yield is usually higher because the viscosity of the oil decreases. Certain quality designations like “virgin” or “cold pressed” however cannot be used then. (Österreichisches Bundesministerium für Gesundheit, 2012) For the pressing process itself a variety of technologies are employed. They can be divided into open presses, closed discontinuous presses, and closed continuous presses. Open presses are only used to produce olive oil and are a discontinuous process. In this technique, layers of olive pulp separated by sheets of filter material are pressed hydraulically. Closed discontinuous presses are mainly used for the production of high-quality oil in

low quantities. The crushed oil seeds are filled into a cylinder and pressed with a hydraulic piston. The oil flows through a set of filters and is collected. To increase the yield the pulp is often layered with metal sheets separating each layer of oil seeds. Closed continuous oils are usually screw presses in which the oil seeds are conveyed through the press by a screw. Since the diameter of the cylinder around the screw is continuously decreased, the pressure on the oil seeds increases and the oil is separated from the plant material. Due to the high pressures, the temperature can rise which has to be taken into account for the production of virgin oils (Krist, 2013). The last two processes, the close discontinuous and the closed continuous presses, are the most common ones for the local production of Styrian vegetable oil. Although the production quantity of pressed and unrefined oils ranges far behind warm pressed oils or even extracted ones, there are significant advantages of not extensively using heat to increase the oil output. Most of the native, nutritiously extremely valuable substances like vitamins and antioxidants stay in the oil. The oil is not chemically treated, and no artificial addition of antioxidants is required (Parker, et al., 2003). Since these oils are not refined they keep many of the healthy ingredients. However, substances which are introduced into the oil via contamination or which form unintentionally, cannot be removed after the production of the oil. Special care must therefore be taken to select only raw material of the highest quality and use only proven production techniques. Unrefined vegetable oils are not well suited for being heated if they have a high content of unsaturated fats and other thermally labile components. Potentially unhealthy and toxic substances like trans-fatty acids and PAH can form in the oil upon heating. Compared to refined vegetable oils, the sensitivity towards sunlight is much higher with these oils and therefore they should be stored in a dark environment.

1.1.2 Fat degradation

As a natural product, virgin vegetable oils are prone to degradation over time. The chemical structure of fatty acids allows for various mechanisms of degradation due to different factors like heat, light, or enzymatic reactions. The main influences on the reactivity towards degradation are the fatty acid spectrum of the oil, the physical storing conditions as well as the presence of antioxidants in the oil. However, also the way in which the individual fatty acids are incorporated into the triacylglycerides has a significant influence onto the degradation pattern. The most common degradation mechanism is the lipid autoxidation. This process will happen in every fat or oil over time. However, the higher the share of unsaturated fatty acids in the triacylglycerides, the higher the rate of autoxidation. This reaction usually triggers multiple follow-up reactions which yield secondary and tertiary products and metabolites. The process changes the flavour and the smell of the oil and is commonly known under

the term “rancidity”. The overall process is very complex and consists of various reactions which result in the formation of a wide spectrum of substances (Belitz, et al., 2008).

1.1.2.1 Hydrolysis of triacylglycerides

Triacylglycerides consist of fatty acids which are linked to glycerol via ester moieties. The carbonyl groups are preferred reaction sites for acidic and basic reagents as well as enzymes. The resulting hydrolysis reaction will lead to the formation of mono- and diacylglycerides, glycerol, and free fatty acids which can be seen in Figure 1 (Frankel, 2012).

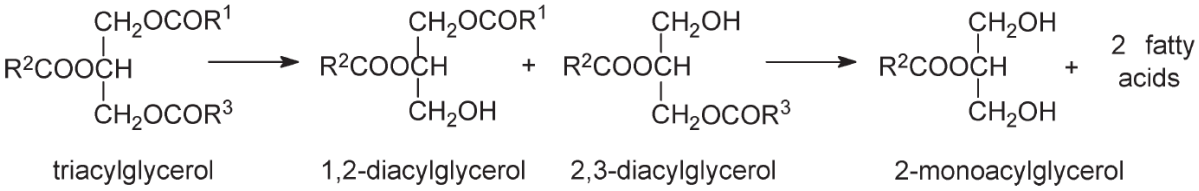

Figure 1: Reaction scheme of the hydrolysis of triacylglycerides to mono- and diacylglycerides and free fatty acids (Frankel, 2012)

The presence of moisture, traces of heavy metals and oxides as well as the influence of heat can significantly increase the rate of the hydrolysis reaction. A critical factor are enzymes like lipases which can be found in every plant and animal organism. They help the growing plants to break down the fats and access the stored energy. During the oil production some enzymes will get into the oil and initiate the decomposition. The free fatty acids which are formed during the hydrolysis reaction significantly influence the taste of the vegetable oil and are responsible for the typical impression of rancidity. Besides organoleptic sensations, the free fatty acids enable further reaction pathways which result in the formation of secondary and tertiary metabolites. The extend of the hydrolysis of the triacylglycerides in the vegetable oil and therefore the amount of free fatty acids is usually determined via titration and reported using the acid value. This value is given in the mass of potassium hydroxide (KOH) in milligram which would be needed to neutralise the free fatty acids in 1 g of fat. Refined fats must have a significantly lower acid value (<0.6 mg KOH g^{-1}) than non-refined oils (<4.0 mg KOH g^{-1}) (Österreichisches Bundesministerium für Gesundheit, 2012).

1.1.2.2 Autoxidation and formation of hydroperoxides

Free fatty acids which have been cleaved off the triacylglycerides are the basic substrate for further autoxidation processes which lead to the formation of hydroperoxides. The overall mechanism of this process can be split into three distinct reaction steps: initiation, propagation, and termination. An overview of the three reaction steps is depicted in Figure 2 (Afaf, 2003).

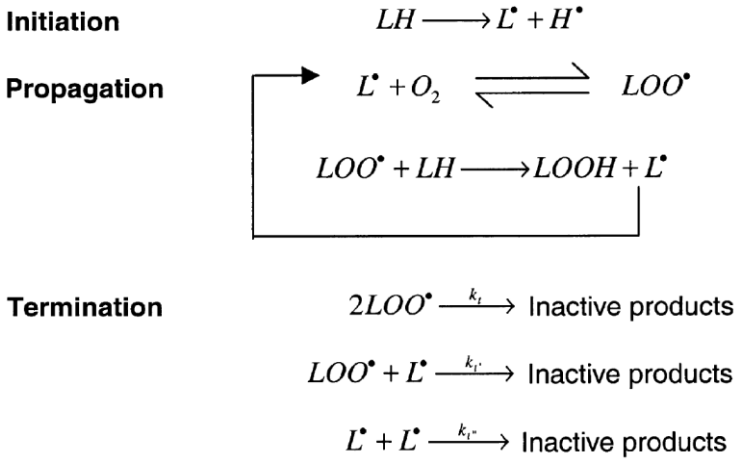

Figure 2: The three reaction steps of the autoxidation process of fatty acids (Afaf, 2003)

The reaction is initiated by the abstraction of a hydrogen atom at a carbon atom adjacent to a double bond in the fatty acid chain. The abstraction is influenced by different parameters like the presence of oxygen, antioxidants and prooxidants as well as the temperature, light exposure, and the degree of unsaturation of the fatty acids. These parameters also influence the duration of the so-called lag or induction phase that precedes the initiation and in which no reaction products can yet be observed. The free radicals which are generated in the initiation reaction then can react with other fatty acids which in turn yield a peroxide moiety and a new free radical. At this point the process enters the second reaction phase, the propagation. Termination usually occurs when two radicals react with each other to form a stable molecule. This only happens when most of the substrate, usually unsaturated fatty acids, have already been oxidised. As already mentioned, the reactivity of the fatty acids on the triacylglycerides heavily depends on their degree of unsaturation. The presence of double bonds significantly increase the sensitivity to oxidation with oleic acid (18:1) being a hundred times more reactive than stearic acid (18:0). Linoleic acid (18:2) is 1200 times more reactive than stearic acid and linolenic acid (18:3) even 2500 times more sensitive to oxidation (Chow, 2008). This is due to the better distribution of the charge over multiple double bonds after the hydrogen atom has been abstracted (Frankel, 2012). Since polyunsaturated fatty acids have multiple hydrogen atoms which are prone to abstraction by a radical, the complexity of the reaction and the number of possible products increases. While there are only four possible arrangements of hydroperoxides after the degradation of oleic acid, linolenic acid offers six different possibilities Figure 3 (deMan, 1999).

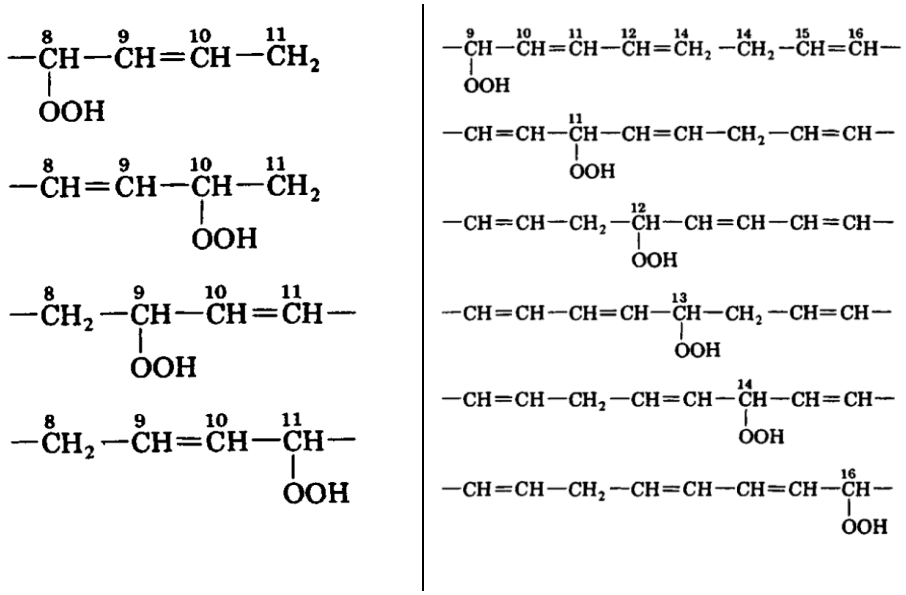

Figure 3: Possible hydroperoxides formed during the oxidation of oleic acid (left) and linolenic acid (right) (deMan, 1999)

The hydroperoxides and radicals which were formed during the propagation phase of the reaction react further to secondary reaction products with the most important ones being carbonyls like aldehydes and ketones. The peroxide number which is frequently determined in vegetable oils provides reliable information about the extent of the oxidation reactions which had already occurred. Aldehydes and alcohols which are some of the secondary metabolites, as well as tertiary metabolites like other fatty acids (not to be confused with the initial substrate) are responsible for the typical rancid flavours and smells which develop in a vegetable oil over time. An overview of various reactions and their resulting metabolites can be seen in Figure 4 (Afaf, 2003).

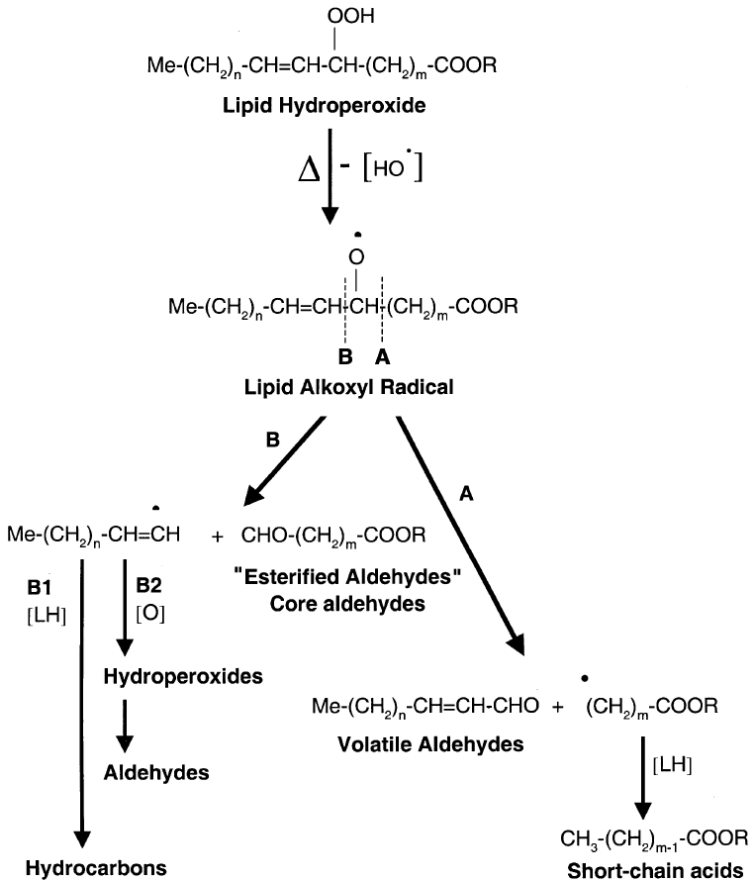

Figure 4: Overview of possible secondary reactions after the formation of lipid hydroperoxides (Afaf, 2003)

This autoxidation process is the most important reason, most virgin vegetable oils are always pressed fresh and based on the demands of the market. If the oils were produced in high volume too early, the oxidation processes would have already significantly degraded the oils when they are consumed. Proper storage after the pressing process is nevertheless critical since any influence like light and impurities might increase the reaction rate.

1.1.2.3 Thermal decomposition

Many vegetable oils are exposed to high temperatures at some point during their production or later when they are used in the kitchen. The conditions of these processes vary, nevertheless they will influence the substance spectrum in the oils. Since non-refined vegetable oils undergo only limited cleaning, they still contain many impurities which derive from the raw material. This makes them especially valuable regarding their nutritional quality, however, the presence of these substances can also increase their sensitivity to heat. The raw materials of the oils, like pumpkin seeds, often undergo a roasting process prior to pressing (chapter 1.1.3). During this production phase the crushed seeds and the oil which is already separating from the plant material is exposed to temperatures of up to 130 °C. These conditions are maintained for approximately 60 minutes with a high surface area and under the presence of moisture and oxygen. During the roasting process many of the typical flavouring

substances are formed in the oil with heat playing an important role in these reactions (Poehlmann & Schieberle, 2013). However, the coinciding degradation of the ingrediencies could also lead to the formation of undesired substances, many of which are volatile These include free acids, aldehydes, ketones, but also alkanes, olefins, and aromatic compounds (Kubátová, et al., 2011). The presence of water and oxygen during the roasting process accelerates the degradation reactions (Nawar, 1969). Possible pathways of thermal degradation can be seen in Figure 5 (Chang & Wan, 1947).

Decomposition of the glyceride

$$\begin{array}{l} CH_2OCOR' \\ | \\ CHOCOCH_2R'' \\ | \\ CH_2OCOR''' \end{array} \longrightarrow \begin{array}{l} CH2 \\ | \\ CH \\ | \\ CHO \end{array} + R'COOH + R'''COOH + R''CH=CO$$

Decomposition of fatty acids

$$RCOOH \longrightarrow CO_2 + RH$$
$$2RCOOH \longrightarrow CO_2 + H_2O + RCOR$$

Decomposition of ketenes and acrolein

$$2R''CH=CO \longrightarrow 2CO + RHC=CHR$$
$$CH_2=CHCHO \longrightarrow CO + C_2H_4$$
$$RCOCH_2R \longrightarrow R{-}R + CH_2CO$$
$$2RCOCH_2R \longrightarrow 2R_2 + CO + C_2H_4$$

Decomposition into elements

$$C_nH_{2n+2} \longrightarrow nC + (n+1)H_2$$

Dehydrogenation of paraffins

$$C_nH_{2n+2} \longrightarrow C_nH_{2n} + H_2$$

Splitting decomposition of paraffins

$$C_nH_{2n+2} \longrightarrow C_{n-m}H_{2n-2m+2} + C_mH_{2m}$$

Alkylation of paraffins, the reverse of the splitting decomposition of paraffins

Isomerization of paraffins

$$N - C_nH_{2n+2} \longrightarrow iso - C_2H_{2n+2}$$

Aromatic cyclization of paraffins

$$C_{(2n+6)}H_{(2n+14)} \longrightarrow C_nH_{2n+1} + 4H_2$$

Polymerization of olefins

$$2C_nH_{2n} \longrightarrow C_{2n}H_{4n}$$
$$C_nH_{2n} + C_mH_{2m} \longrightarrow C_{(n+m)}H_{2(n+m)}$$

Depolymerization of olefins, reverse of "Polymerization of olefins"

Decomposition of olefins to diolefins

Decomposition of olefins to acetylenic hydrocarbons

Aromatization or cyclization of olefins

Hydrogenation of olefins

$$C_nH_{2n} + H_2 \longrightarrow C_nH_{2n+2}$$

Isomerization of olefins

$$n - C_nH_{2n} \longrightarrow iso - C_nH_{2n}$$

Figure 5: Decomposition of triacylglycerides (Chang & Wan, 1947)

While the formation of oxygen-containing substances like methyl ketones and esters are frequently discussed, the appearance of hydrocarbons like short chained alkanes and volatile aromatic compounds are less often found in literature about roasting processes of oil seeds (Hoell, et al., 2012), (Ozel, et al., 2014), (Gracka, et al., 2016). The formation of these hydrocarbons plays a more relevant role in the production of fuels from vegetable oil. A possible reaction which results in the formation of short-chained alkanes (n-pentane, n-hexane, n-heptane), as well as aromatic compounds (benzene, toluene, xylene) is shown in Figure 6 (Schwab, et al., 1988).

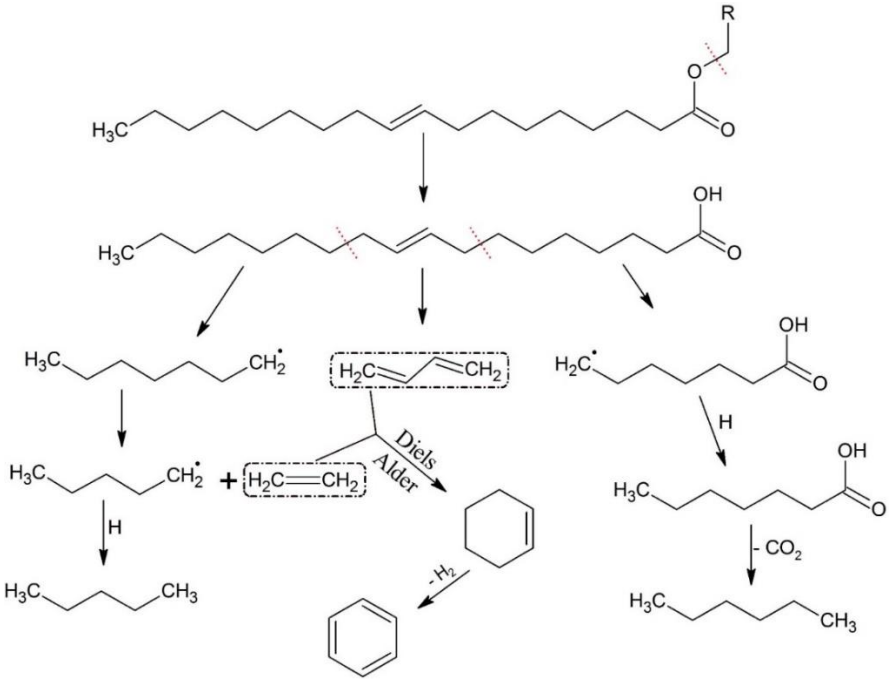

Figure 6: Mechanistic pathway of the degradation of oleic acid, which results in various volatile linear alkanes and aromatic compounds, that were found in the analysed oil samples (Schwab, et al., 1988)

These aromatic or aliphatic compounds generally are formed through a free-radical or a carbenium ion mechanism (Schwab, et al., 1988). Thermally cleaved free fatty acids react to form aliphatic or olefinic radicals through decarboxylation. Unsaturated hydrocarbons often are cracked further into short-chained homologs. The olefins undergo Diels-Alder-cyclisation reactions to produce cyclic substances, which can yield aromatics after dehydrogenation. Remaining radicals react to stable hydrocarbons through termination reactions (Kubátová, et al., 2011). Compounds with antioxidative effect, like β-carotene or tocopherol, can retard the degradation of triacylglycerides and therefore increase the thermal stability of oils (Taghvaei & Jafari, 2015). Mechanical load, as it is used during the pressing of the oil, can also lead to these reactions and increase the temperature of the oil as well. All these processes lead to a complex mixture of substance which influence the character of the vegetable oil.

While studies about the roasting process usually focus on temperatures below 100 °C, experiments regarding the production of biofuels most often employ heating beyond 200 °C. The conditions which

are typical for the roasting process of pumpkin seeds are found in between these two temperatures. This and the focus of many studies on flavouring compounds leads to a lack in literature material regarding the formation of volatile hydrocarbons in vegetable oils. Although the formation of volatile hydrocarbons in vegetable oils is acknowledged, the amount and expected concentrations are not investigated.

1.1.3 Styrian pumpkin seed oil

The production of virgin vegetable oils in Europe has a history and tradition which goes back thousands of years. The most prominent example of this is olive oil which is still produced mainly around the Mediterranean basin. It is of so much significance for both, culture and nutrition, that multiple legislative pieces have been put into place by the European Union to regulate its production and distribution (The European Commission, 2016) (The European Commission, 2012). Styria and other parts of Austria also have a long tradition with the production of vegetable oils for nutritious use. The most important source for the production of unrefined Styrian vegetable oils are locally grown and harvested pumpkin seeds. The variety which is grown in Styria for the exclusive use of producing oil is the *Cucurbita pepo styrica*. This special mutation has lost its seed shell, while it was cultivated for oil production. This made the whole oil production process easier and was the initiator for the steep increase in production volume. Initially it was mainly produced individually by farmers for their own use and was therefore considered food for poor people. Meanwhile this image is long gone, as it is well known and liked as a high-quality gourmet oil. In 1996 the Styrian pumpkin seed oil was added as a PGI-item to the EU's Database of Origin and Registration (DOOR). Therefore, only specifically declared fields are used for growing pumpkins and no imports of seeds are allowed for the production of this special oil. (Gemeinschaft Steirisches Kürbioskernöl g.g.A., 2018) The Styrian oil pumpkin can be grown on many kinds of soil since its demands on the ground are low. However, it is quite temperature sensitive and requires a comparably warm environment without frost periods and plenty of precipitation. After harvesting the pumpkins, the seeds must be separated from the pulp which until the last decade had mostly been done by hand. Meanwhile specialised machinery is employed for the task of harvesting the fruits and extracting the seeds. The seeds are dried and then stored in special silos until just before the oil is produced. Before the seeds are pressed they are finely ground and mixed with salt and water to a doughy paste (Gemeinschaft Steirisches Kürbioskernöl g.g.A., 2018). This mixture is then roasted under stirring in large pans for approximately 60 minutes at 130 °C (Poehlmann & Schieberle, 2013). Due to the Maillard reaction at elevated temperatures many of the typical aromas and flavours of the oil are developed. Typical volatile substances in the pumpkin seed oil which are relevant for its smell and taste are listed in Table 1 (Matsui, et al., 1998).

Table 1: Volatile substances typical for flavour and smell of pumpkin seed oil after roasting (Matsui, et al., 1998)

Ethyl-2-methylbutyrate	3-Methylbutanal
2-ethyl-3,5-dimethylpyrazine	2-Nonenal
2,3-Diethyl-5-methylpyrazine	Phenylacetaldehyde
2,4-Nonadienal	2,4-Decadienal
2-Methoxy-4-vinylphenol	β-Damascenone
Hexanal	2-Acetyl-1-pyrroline
2-Ethyl-5-methylpyrazine	Dimethyl trisulfide
3-Methylbutyric acid	3-Ethyl-2,5-dimethylpyrazine
4,5-Epoxy-2-decanal	2-Ethenyl-3,5-dimethylpyrazine
4-Hydroxy-2,5-dimethyl-3-furanone	Benzaldehyde
4-Ethyl-2-methoxyphenol	2-Methyl-6-isopropylpyrazine

However, the oil contains many other flavour carrying substances which all contribute significantly to the special taste and smell of the oil (Poehlmann & Schieberle, 2013). The roasting process is finished when all the water in the dough has been evaporated. The residual mixture, which is still at elevated temperature, is then pressed at a pressure of 30 to 35 MPa. Steel discs which are inserted between layers of the pumpkin seed pulp guarantee an even distribution of the pressure and increase the yield. (Krist, 2013) Usually 2.5 to 3 kg of pumpkin seeds are needed to produce 1 l of pumpkin seed oil. After the pressing process, the fresh oil is kept still in special containers which allows the precipitation of small particles and other impurities. No further treatments or clean-up procedures are done. The pressing process usually can extract 90% of the oil in the seeds. The residual 10% stay in the press cake, which contains other valuable ingredients like protein and fibre. This makes it a valuable source of animal feed. Another way of utilising the press cake is the production of so-called salad oil which are blends of oils that contain more than 2% of an oil other than the original one. The press cake is ground once more, mixed with other vegetable oils like rapeseed oil and pressed again. Alternatively, the oils are blended directly. The resulting oil mixture is a cheap alternative to pumpkin seed oil and is often used in the gastronomy sector. The percentage of the mixed oils must be declared, and it must clearly be differentiated from pure oil (Österreichisches Bundesministerium für Gesundheit, 2012). Even though the colour of salad oil can be similar to pure pumpkin seed oil, other sensory parameters like the smell and the flavour are completely different. (Gemeinschaft Steirisches Kürbioskernöl g.g.A., 2018)

1.2 The project

The project which this master thesis is built around was not from the beginning intended to be used for a master thesis. It was only thought about using it as a basis for this thesis after most of the initially planned goals of the project had already been completed. This is also the reason some development steps regarding the instrumental method and other objectives of the project were not approached with the same attitude and scientific carefulness as if it had been planned as a master thesis from the beginning. Original considerations and goals changed with the project over time and some decisions regarding the method development and the experimental design would have been different in hindsight. The nature of the project also transmitted a sense of urgency which led to taking shortcuts at some points in the execution. Although the clear majority of the obtained results and conclusions are still valid, optimisations would have been possible. The conclusions which were drawn and possible optimisations for future projects on the topic are discussed in chapter 4.

The idea for the project was triggered by the accusation of a food trading company of a locally produced pumpkin seed oil being contaminated with residues of solvents. The oil was intended to be sold in the grocery shop chain of that company. By chance it was selected for undergoing a suite of analytic investigations which had not all been part of the routine analysis for proofing marketability. Besides a pesticide screening and the analysis for PAH (polycyclic aromatic hydrocarbons), MOSH (mineral oil saturated hydrocarbons), POSH (polyolefinic oligomeric saturated hydrocarbons) and MOAH (mineral oil aromatic hydrocarbons), the oils were also analysed for solvent residues. This is unusual since the oil was, as all Styrian pumpkin seed oil, produced by pressing the oil and not by solvent extraction. Usually only oils which have been produced by solvent extraction instead of pressing are analysed for solvent residues. These extracted oils must undergo a refining process which removes any residues of the solvents to make them safe for consumption and other applications in the food industry. Existing EU law regulates the residues of solvents which can legally be used as extraction solvents for the vegetable oil production. (The European Commission, 2017) (The European Parliament and the Council of the European Union, 2009) However, these only apply for oils which have been produced by extraction. There is no specific regulation regarding solvent residues in oils which have been produced by pressing. These oils do not come in contact with any solvents at any point of the production process and no solvent residues are expected to be found. There is a passage in the regulation which states that no solvents can be found except residues which cannot technically be avoided. But there are no details given on what technically unavoidable residues would mean. The analysis report for this oil however made notice of traces of several volatile compounds like acetone, 2-butanone, styrene, toluene, and xylene. Due to these results the oil was rejected by the company and a statement from the producer was demanded. The oil had been produced locally under strict

quality controls and with techniques which have been employed for multiple decades and had never caused any problems. After a meeting with other local oil mills it became clear that most of the producers were faced with accusations of having various solvent contaminations in their high-quality vegetable oils. A statement of one of the food safety laboratories which had conducted some of the analysis stated that the solvents clearly were contaminations which had to be avoided. These developments occurred within a short period of time and caused substantial uncertainty among the oil producers. They had to expect their products to be from now on frequently rejected by grocery store chains. A solution to the problem had to be found as soon as possible.

The Institut Dr. Wagner Lebensmittel Analytik GmbH, a food safety laboratory in southern Styria, is well connected within the local food production industry. Therefore, the company has a lot of experience in conducting chemical food analysis as well as providing consulting services and planning audits. The producers asked for help regarding the elegit contaminations of their vegetable oils. Confronted with this information the Institut Dr. Wagner suggested to initiate a project which would aim at working on the following goals:

1) Process analysis of the current production techniques and tools
2) Analysis of samples for solvent residues to see whether they are present in the samples
3) Research on the cause of the contaminations and solutions to the problem

In addition to analysing the solvent residues in the vegetable oils, MOSH, POSH, and MOAH were investigated as well, but the analyses were performed by an external laboratory. Therefore, the results of the MOSH, POSH, and MOAH investigation are not part of this master thesis.

Since the Institut Dr. Wagner almost exclusively analyses high quality vegetable oils which are pressed, there was no readily available method for analysing solvent residues in vegetable oils. So, this method had to be developed, proven reliable, and validated. The next part of the project was to measure 69 project samples. These were mostly pumpkin seed oils but also oils made from other oil seeds, like hemp seed, linseed, poppy seed, hazelnut, and walnut. These results would either confirm or contradict the initial reports which had led to the food trader refusing the oil. If any residues were found in the samples, the third part of the project was to conduct some research why these substances occur in oils. The sample set of these 69 samples was split into two sampling sessions which were six months apart to see whether any changes in the oils would occur over time. The whole situation and the uncertainty which accompanied it led to a sense of urgency for finding the origins of these residues and a possible solution on how to avoid them. It cannot be emphasised enough that the task which was communicated by the oil producers was to find contaminations in the oils. The statements from both, grocery store chains as well as accredited food safety laboratories only spoke of avoidable

contaminations. A scientific report by the European Food Safety Authority (efsa) specifically discussed the appearance of mineral oil hydrocarbons in food and identified contamination during harvesting or processing of the raw material as the main source. Most of these contaminations would be longer chained hydrocarbons with 10 carbon atoms and more however, also shorter chained alkanes are expected alongside the others (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2013). With this overwhelming amount of information and the emphasis on trying to confine the apparently extensive contamination of the oils with solvent residues and mineral oils, the perception was centred around contamination. Other possible causes for the substances in the samples were initially not thought off. This master thesis therefore aims to point out that although many signs lead into one direction, all possible scenarios should always be considered.

2 Materials and methods

2.1 Analytes and standard material

The scope of the method was the reliable detection and quantitation of the following analytes: Trichloromethane, trichloroethene, tetrachloroethene, trans-1,2-dichloroethene, 1,1-dichloroethane, cis-1,2-dichloroethene, 1,2-dichloroethane, dichloromethane, 1,1,1-trichloroethane, tetrachloromethane, 1,1,2-trichloroethane, 1,1,1,2-tetrachloroethane, dibromochloromethane, bromodichloromethane, bromoform, benzene, toluene, ethylbenzene, m-xylene, p-xylene, o-xylene, styrene, 2-butanone, ethyl acetate, n-pentane, n-hexane, n-heptane, and acetone. Standards of all the analytes were purchased from Lactan Chemikalien und Laborgeräte Vertriebsgesellschaft m.b.H & Co KG (Graz, Austria), and used for the development of the GC/MS-method, as well as for the preparation of the calibration standards. Benzene D6, Acetone D6 and Chloroform D1, which were used as internal standards, were also bought from Lactan. The pure standard substances were stored at -20 °C. As a standard material for the calibrations and the method validation, highly refined olive oil (Sigma-Aldrich Handels Gmbh; Vienna, Austria) was used. A list of all the analytes with their purities and densities can be seen in Table 2. This information is especially important since the standard solutions were prepared volumetrically using capillaries with fixed volumes. The actual concentrations used in the calibrations and validation was therefore individually calculated for each analyte.

Table 2: Analytic scope of the method, the standard's purities, and the analyte's densities

Substance	Purity [%vol]	Density [g cm^{-3}]
n-Pentane	99.0	0.63
Acetone	99.0	0.79
Acetone-D6	99.5	0.87
Dichloromethane	99.0	1.33
trans-1,2-Dichloroethene	98.0	1.25
n-Hexane	95.0	0.66
1,1-Dichloroethane	98.0	1.17
cis-1,2-Dichloroethene	99.0	1.28
2-Butanone	99.5	0.80
Ethyl acetate	99.0	0.89

Trichloromethane	99.0	1.48
Trichloromethane-D1	99.8	1.50
1,1,1-Trichloroethane	96.6 (5000 µg ml^{-1})	1.34
Tetrachloromethane	99.9	1.59
Benzene	99.0	0.88
Benzene-D6	99.8	0.95
1,2-Dichloroethane	99.5	1.25
n-Heptane	99.0	0.68
Trichloroethene	99.5	1.46
Bromodichloromethane	98.0	1.98
Toluene	99.0	0.87
1,1,2-Trichloroethane	98.0	1.44
Tetrachloroethene	99.5	1.62
Dibromochloromethane	98.0	2.42
1,1,1,2-Tetrachloroethane	99.0	1.56
Ethyl benzene	99.0	0.87
m-Xylene	99.0	0.86
p-Xylene	99.0	0.86
o-Xylene	99.0	0.88
Styrene	99.5	0.91
Tribromomethane	99.0	2.89

2.2 Analytic system

The instrument assembly consisted of an HP 6890 gas chromatograph coupled with an Agilent Technologies 5973 network MSD mass spectrometer. The ionisation of the analytes in the mass spectrometer was performed by electron impact ionisation. The sampling was done with an HP G1290A headspace autoinjector. The capillary column used for all analyses was an Agilent Technologies DB-624, 30 m x 0.25 mm, 1.40 µm film. The following parameters were not modified over the course of the method development and optimisation and therefore remained the same also in the final method. Helium 5.0 was used as carrier gas and exerted a constant column pressure of 10 psi (68.95 kPa), while the sample inlet was held at 250 °C. The average flow velocity was 40 cm sec^{-1}. The transfer line from the gas chromatograph to the mass spectrometer was heated to 290 °C, the MS ion source to 230 °C and the MS quadrupole to 150 °C.

2.3 Software and computer programs

A variety of different software tools were used over the course of the project to conduct all the measurements and data evaluations. The data acquisition on the GC/MS was performed using the software GC MSD Acquisition G1707EA, Version 02.02. Since the data was recorded using Agilent ChemStation software, the data files had to be converted into the appropriate format to enable the more comfortable quantitative evaluation on the Agilent MassHunter software. For the file conversion the program GC/MS Translator B.06.01 was used. During the method development phase, data quality was also checked with the software Agilent MSD ChemStation E.02.02.1431, which could handle the data without any conversion. For quantitative as well as qualitative data evaluation after converting the files, the programs Agilent MassHunter Quant B.07.01 and Agilent MassHunter Qual B.07.00 were used. The evaluation of the analytic data which was obtained for the SPME-GC/MS measurements was done with the software tool GCMSsolutions version 4.11 by Shimadzu Corporation. The method validation and other evaluations were calculated in Microsoft Office Excel 2013 and 2016, while all multivariate data analysis was performed using Agilent MassProfiler Professional Version 14.5.

2.4 Method development

The analytical method used for the analysis of the samples was developed independently although studies regarding methods for solvent residue analysis have already been published. These publications were used to some extent as guides for certain steps in the method development like choosing the right headspace parameters (Kumar & Gow, 1994), (Michulec & Wardencki, 2004), (Michulec & Wardencki, 2005). From an early point in the development it was decided to aim for the

accreditation of the method according to ÖVE/ÖNORM ISO/IEC 17025. Therefore, the development of the method was oriented at EN ISO 16035:2005 (Österreichisches Normungsinstitut, 2005). The method was modified regarding to the analysed compounds which originally were only halogenated hydrocarbons. The detection system which was used in the norm was an electron capture detector which wouldn't work on non-halogenated compounds. A mass selective detector was used instead to be able to detect all the substances. Further changes were the use of an autosampler instead of the manual injection which was performed in the original method and slight changes in the GC's temperature program. This was done to sufficiently separate the additional analytes. As is explained in chapter 2.4.3.1 it was significantly less time spent on the optimisation of the headspace method compared to the chromatographic and mass spectrometric parameters. The parameters which were ultimately chosen were mostly based on other frequently used methods at the Institut Dr. Wagner like the detection of CS_2 in fruits and vegetables and measuring aromatic compounds in water. Due to the inherent urgency of the project, the focus was put on enabling fast sample preparation and measurement at the expense of sensitivity.

2.4.1 Compound acquisition

According to EN ISO 16035 the standard solutions and mixes thereof were prepared volumetrically (Österreichisches Normungsinstitut, 2005). Volumetric glass capillaries were used to introduce the pure analytes into the solvent which was put into a volumetric flask beforehand. Since the densities of the individual analytes vary significantly the actual concentrations in mass of analyte per mass of sample material would also have been very different with each analyte. The purity of the analytes also influenced the overall concentration. Therefore, a system of nominal concentration was employed which would state a general concentration for the analytes assuming a density of 1 g cm^{-3} and a purity of 100%. Every analyte was now associated with a factor which would correct for the actual densities and purities. The actual concentration of trichloromethane would now be higher than the concentration of n-pentane if the same volume was added due to the higher density of trichloromethane. The relationship of actual concentrations to given nominal concentrations can be seen in Table 6 on page 30.

The first phase of the method development was obtaining chromatographic as well as mass spectrometric data for every analyte. A drop of an individual analytical standard was filled into a GC-vial, closed with a crimp cap, and heated at 100 °C for 5 minutes. A GC syringe was then used to manually inject 50 µl of the gas phase in the vial into the GC inlet. The GC oven's initial temperature at injection was 70 °C which was then steadily increased to 150 °C within 15 minutes. The single quadrupole mass selective detector was acquiring data in scan mode with a range of m/z 20 – 200.

Using this approach, the most prominent m/z signals as well as the elution order of all the analytes could be determined. First qualitative evaluation methods could be generated which would simplify the data evaluation during the further development of the method.

2.4.2 Optimising the instrumental parameters

After the individual analytes were proven to be reliably detectable on the GC/MS system, the retention times and thus the distribution of the analytes over the available run time was optimised. The initial temperature of the GC oven was decreased from 70 °C to 50 °C to increase the separation of early eluting compounds. The linear temperature ramp was split into a flatter part with a heating rate of 8 °C min^{-1} and a steeper ramp after most of the analytes were already eluted, with a rate of 20 °C min^{-1}. In the final program, the GC oven was held at 50 °C for four minutes (i), then heated at 8 °C min^{-1} to 100 °C, which was held for five minutes (ii) and lastly heated at 20 °C min^{-1} to 200 °C, which was held for one minute (iii). This resulted into a total runtime of 21 minutes and 15 seconds. This temperature program enabled satisfactory separation compared to the original experimental program, especially in the first half of the chromatogram. A chromatogram which shows an overlay of all the individual SIM signals is shown in Figure 7.

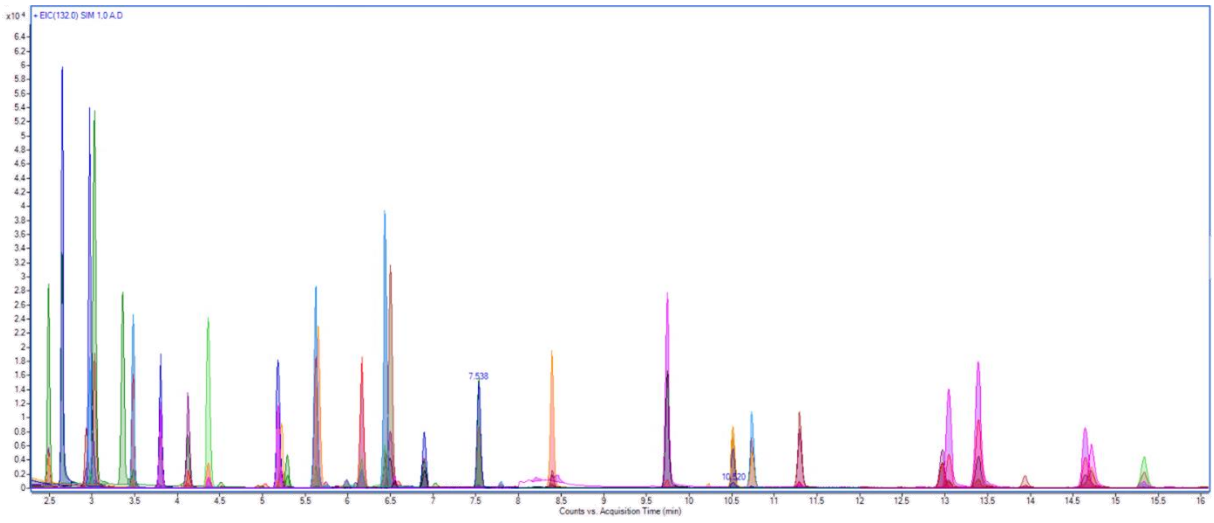

Figure 7: A chromatogram which shows the individual SIM signals (concentration equivalent to approx. 1 mg kg^{-1})

Table 3 shows the substances which were measured with the method, the retention times as well as the m/z values of each substance. For each analyte three m/z values were selected to be measured for a sufficiently unequivocal identification. The only exemptions are acetone and acetone-d6 since only two relevant fragments could be detected. The m/z values written in bold font were intended to be used as quantifiers in later quantitative evaluation methods due to either the good signal height, or the high signal to noise ratio. M-xylene and p-xylene could not be separated using this instrumental

setup. These two isomeric substances were too similar for a chromatographic separation. They also gave the same signal intensity when detected. Therefore, they were expressed as one single substance two simplify further analysis.

Table 3: Chromatographic and mass spectrometric method parameters

Substance	RT [min]	m/z 1	m/z 2	m/z 3
n-Pentane	2.355	**57**	43	72
Acetone-D6	2.788	**64**	46	
Acetone	2.835	**43**	58	
Dichloromethane	3.268	**84**	86	88
trans-1,2-Dichloroethene	3.575	**96**	98	63
n-Hexane	3.883	**86**	57	71
1,1-Dichloroethane	4.103	**63**	98	83
cis-1,2-Dichloroethene	4.907	**96**	98	63
2-Butanone	4.938	**72**	57	43
Ethyl acetate	5.007	**88**	70	73
Trichloromethane-D1	5.338	**84**	86	119
Trichloromethane	5.368	**85**	83	118
1,1,1-Trichloroethane	5.633	**97**	119	99
Tetrachloromethane	5.872	**119**	84	117
Benzene-D6	6.136	**56**	82	81
Benzene	6.200	**78**	77	52
1,2-Dichloroethane	6.248	**98**	100	62
n-Heptane	6.600	**100**	71	70
Trichloroethene	7.231	**130**	132	95
Bromodichloromethane	8.078	**83**	127	129
Toluene	9.418	**91**	92	93
1,1,2-Trichloroethane	10.188	**97**	99	132

Tetrachloroethene	10.389	**166**	168	129
Dibromochloromethane	10.920	**127**	210	129
1,1,1,2-Tetrachloroethane	12.508	**117**	131	119
Ethyl benzene	12.580	**106**	91	65
m-, p-Xylene	12.919	**106**	105	91
o-Xylene	14.095	**91**	105	106
Styrene	14.166	**104**	103	78
Tribromomethane	14.743	**173**	252	171

With the completion of the optimisation of the gas chromatographic parameters, the next step was to optimise the mass spectrometric method. The goal was to further increase the sensitivity and the data rate of the mass spectrometer which meant that the detection mode hat to be changed from the scan mode, which had been needed to obtain the m/z values, to the selected ion monitoring (SIM) mode. Relevant ions were only recorded over a certain period, depending on the analyte's elution pattern. The temperature program was therefore also optimised to leave big enough gaps between the peaks to be able to switch from one time segment to the next, without any of the analytes eluting over two segments. In the past, this had caused problems with the calculation of the peak areas and therefore was to be avoided. Besides the m/z values and the time of each time segment, two additional parameters could be modified: The dwell time which was given in milliseconds and the cycle rate in cycles per second. These two parameters were reversely dependent on each other. The higher the dwell time, the more significant was a detected signal at a single point in time. However, it decreased the cycle rate which determined how many times the signal could be acquired in order to generate a mass spectrum. As a rule of thumb, there should be 12 acquisition points throughout a single peak to get a meaningful result. This also guaranteed reliable measurements over multiple runs, e.g. for a calibration (Prest & Peterson, 2001). The average peak range in the chromatogram shown in Figure 7 was 7.5 seconds. Therefore, the dwell time was modified in way, that the acquisition rate would always be higher than 2 cycles per second. Table 4 shows the arrangement of the m/z which were distributed over five time segments. With the equipment used for the experiments, the number of acquisition points per second increased over the course of the measurements. n-Pentane which is had a retention time of 2.355 had exactly 12 acquisition points at its base peak width, whereas later eluting substances like toluene had between 15 and 20. All the acquisition rates were kept above 2 cycles per second, however this was more important for the early eluting substances than for the later ones.

Table 4: The time segment arrangement of the selected ion monitoring (SIM) acquisition method

Time segment no.	1	2	3	4	5
Acquisition time [min]	2.0 - 4.8	4.8 - 5.8	5.8 - 8.0	8.0 - 12.0	12.0 - end
m/z	43	47	52	83	65
	46	57	56	91	78
	57	70	70	92	91
	58	72	71	93	103
	63	83	77	97	104
	64	84	78	99	105
	72	85	82	127	106
	83	86	84	129	117
	84	88	97	132	119
	86	96	98	166	131
	88	97	100	168	171
	96	98	119	210	173
	98		130		252
			132		
Dwell time [ms]	34	36	32	36	34
Acquisition rate [cycles s^{-1}]	2.12	2.16	2.08	2.16	2.11

2.4.3 Development of the sample preparation procedure

While the development of the instrumental method required only small amounts of the pure substances, for the development of the sample preparation, a designated stock solution containing all the analytes was needed. According to the EN ISO 16035:2005 (Österreichisches Normungsinstitut, 2005) the stock solution was to be prepared in isooctane. However, measuring diluted stock solutions without any matrix material would in no way be comparable to real samples. Therefore, it was decided early on, that an oil which did not contain any solvents was to be used as matrix material for the calibration. An olive oil with no detectable traces of solvents was spiked with 100 µl of a solution in isooctane which resulted in 1 mg kg^{-1} of each of the analytes in the oil. The analytes were added

volumetrically into the solution and the amount is therefore an approximation. The real concentrations of the analytes were dependent on the density as well as the purity of the standard material. The actual concentrations and the preparation of the solutions are discussed in more detail in chapter 2.6.1. Compared to the chromatograms which were obtained from measurements of the gas phase of mixed pure standards without isooctane, the chromatographic resolution as well as the peak shapes were very poor. Additionally, large signals appeared in the chromatogram which had not been observed previously. Repeated measurements which were compared with previous ones led to the assumption that the isooctane was the reason for the worsening of the chromatographic quality. A different solvent for the analytes had to be found. A commercially available hydrocarbon standard mix which contained some of the analytes was diluted with methanol (LGC Standards). Since methanol was not in the method's scope it was decided to try it as the solvent for the stock solution. Another approach would have been high-boiling point solvents like triacetin, however, methanol was readily available and worked well with the analytes. A preliminary experiment, similar to the measurement with the isooctane solution, showed significant improvements of the chromatographic quality regarding the resolution as well as the peak shapes. A comparison between the measurement using isooctane and the later measurement using methanol as solvents of the standard solution can be seen in Figure 8. The peak height in the measurement of the standard prepared with isooctane was slightly higher due to the higher concentration of the analytes in this particular standard.

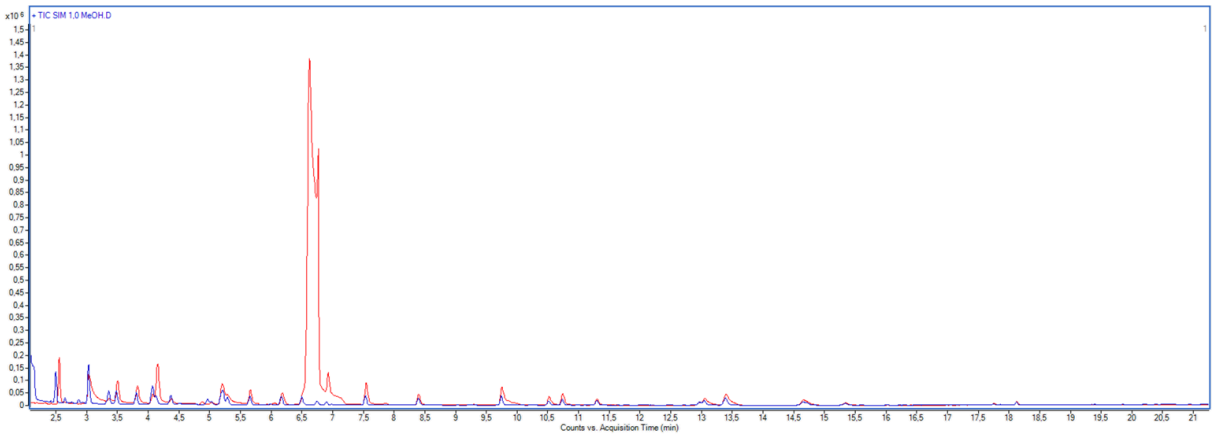

Figure 8: Comparison of the chromatograms of measurements of all analytes diluted in isooctane (red) and methanol (blue) with olive oil as matrix material.

The large signal which was mentioned earlier, and which was most certainly caused by the isooctane in the oil can be seen between 6.5 minutes and 7.0 minutes. The signal disappears with the use of methanol as the solvent for the standard solution. Not only did every single peak shape improve

significantly, but also the baseline stabilised and decreased in height. This can be seen especially well in Figure 9 which shows an enlarged view of the chromatogram between 7.4 minutes and 14.8 minutes.

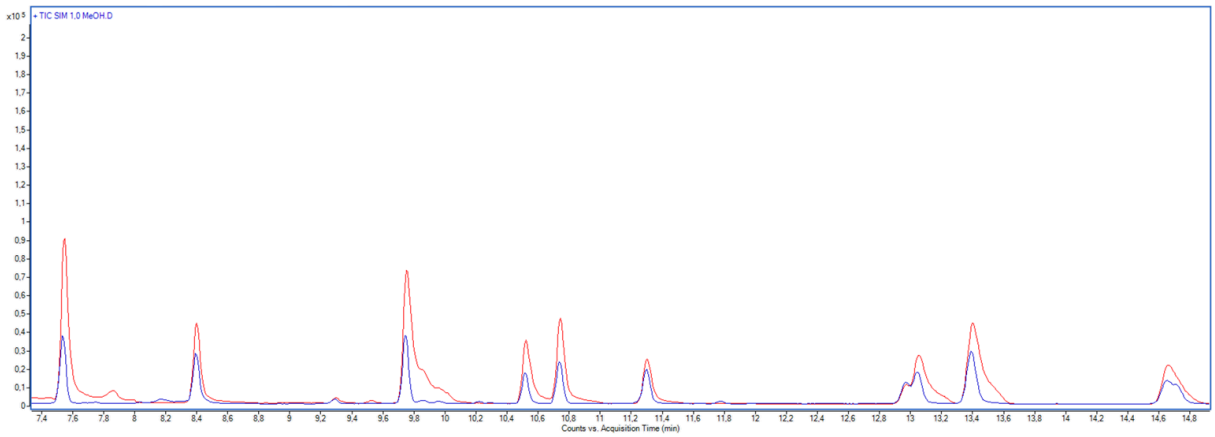

Figure 9: Enlarged view of the chromatogram of the comparison between a standard solution prepared in isooctane (red) and a standard solution in methanol (blue) with olive oil as matrix material

Because of these results it was decided to prepare the stock solution in methanol instead of isooctane. In these first experiments the nominal concentration of all the analytes was the same, except for 1,1,1-Trichloroethane, which already was a diluted solution (5000 µg ml^{-1}). Therefore, a validation had to be prepared and measured which would be used to calculate the limit of detection (LOD) and the limit of quantitation (LOQ) of each analyte. This data would later be used to prepare a stock solution in which the concentrations of the analytes would be oriented according to the respective LODs. The amount of methanol solution added to each sample should be 100 µl independent of the concentration of the analytes. 10 g of the oil for each sample were decided to be the right sample amount for the 20 ml Headspace vials. After adding the methanol, the vials were shaken briefly by hand to properly mix the solution with the oil. After the addition of the methanol, which would contain the analytes and internal standards, the vials were closed with crimp caps. It was taken care, that the sample preparation and weighing would be done outside of the chemical laboratory room to prevent contamination with traces of solvent fumes in the air. Multiple blank measurements were performed with each sequence of analyses. The closed vials were put into the HP G1290A headspace autoinjector which completed the last steps of sample preparation like heating the samples.

2.4.3.1 Headspace autoinjector conditions

The instrumental method was based on the norm EN ISO 16035:2005 (Österreichisches Normungsinstitut, 2005) and it was tried to follow it as closely as possible. However, it was decided from the start that the conditioning time should not exceed 15 minutes due to optimising the analysis duration and improving the overall sample throughput performance. At the time of the method development, it was not yet fully grasped to which extent temperature would affect the oil. The focus

was put on extracting the analytes from the matrix as efficiently as possible. The recommended time for the sample conditioning was 60 minutes. Shortening the equilibration time without sacrificing too much sensitivity could only be accomplished if the temperature of the headspace oven was increased. The norm specifically suggested raising the temperature from the originally recommended 80 °C to achieve higher sensitivity. Additionally, a higher equilibration temperature in the headspace oven can help to minimise matrix effects (Cao, et al., 2013). A temperature had to be determined, which would enable the highest possible signal for most of the analytes but would not interfere with the samples. However due to the initially quite tight timeframe and the urgency of the matter it was decided that considering published results and experience on working on gas chromatographic methods with headspace sampling techniques would be more efficient than conducting prolonged experiments. M. Michulec and W. Wardencki have conducted research on the optimisation of headspace parameters for the robust and reliable analysis of hexane, benzene, toluene and five chlorinated hydrocarbons (Michulec & Wardencki, 2005). All these analytes were also in the scope of this method. Therefore, their results were used to select parameters for the headspace oven temperature, the equilibration time, as well as the sample volume. Their conclusions for the best headspace parameters depended on whether the analyte was halogenated. Reports from various food analysis laboratories which were provided by project partners explicitly showed, that it was non-halogenated compounds which caused concern. No halogenated compounds were ever detected, which led to the decision to optimise the headspace conditions for the detection of the non-halogenated compounds. In the unlikely case that any halogenated methods would be detected later, the method would have been modified to enable reliable quantification of theses analytes. Their experiment on the oven temperature showed that the signal intensity steadily rises the higher the sample is heated although some saturation effect could be seen at around 160 °C. The oven temperature which they settled on for non-halogenated substances was 120 °C which was therefore selected for this method. The equilibration time is especially crucial to make sure that method delivers the same results with every measurement. Michulec and Wardencki chose 20 minutes as the equilibration time for the non-halogenated substances in their method. However, their research data showed that there was no observable difference in the signal intensity after 15 minutes of equilibration. Due to the time restrictions in the project it was decided to equilibrate for 15 minutes. As a last experiment they tried different sample amounts starting from 2 ml up to 12 ml in 22 ml headspace vials. The best volume for halogenated substance appeared to be 4 ml. However, the best results for non-halogenated substances was at 10 ml. Therefore 10 g were decided as the sample amount which would be used throughout the project. Other headspace parameters are mostly independent of the sample type and kind of method. Therefore, these parameters were taken over from various other sources (Kumar & Gow, 1994), (Michulec & Wardencki, 2005). The conditions

which were used to prepare the samples in the autoinjector for the gas chromatographic measurement can be seen in Table 5.

Table 5: The settings which were used for the headspace autoinjector for all measurements

Parameter	Description	Value
Oven temperature	Temperature of the oven the samples are equilibrated in before injection.	120 °C
Sample loop temperature	Temperature of the sample loop which is filled with the headspace gas to obtain a constant volume for each measurement.	160 °C
Transfer line temperature	Temperature of the heated transfer line between the headspace autoinjector and the GC inlet.	160 °C
GC cycle time	Time which counts down after each injection until the autosampler prepares the next sample. Since the sampler can only send information to the GC but not receive any, the runtime should be set with a margin. Otherwise the next injection will occur before the GC is ready.	28 min
Vial equilibration time	Duration for which the sample is heated in the oven of the autoinjector.	15 min
Pressurisation time	Duration for which the sample is pressurised with helium through the needle.	0.2 min
Loop fill time	Duration for which the overpressure which has built up in the sample can force headspace gas into the sample loop.	0.15 min
Equilibration time	Duration for which the pressure in the sample loop is equilibrated after filling the loop.	0.05 min
Injection time	Duration for which the carrier gas flow can flow through the sample loop to flush the sample gas over the transfer line into the GC inlet.	1 min
Shake intensity	Intensity of the shaking motion of the sample rack in the oven. There are three possible settings: no shaking, low and high.	High
Vial pressure	The pressure which the helium flow can build up in the vial. A manual valve is used to change the pressure.	20 psi (137.9 kPa)

2.5 Method validation

Before the samples were measured systematically, an extensive method validation was performed to confirm the reliability as well as the sensitivity of the method and to calculate critical method parameters. Highly refined olive oil (Sigma-Aldrich Handels Gmbh; Vienna, Austria), was used as the matrix material for the measurements. Six concentration levels as well as a set of blank samples with four parallel measurements each were measured to obtain the required data. For each analyte the same amount was added volumetrically into the solutions which were used to spike the samples. Since the actual concentrations were influenced by the density as well as the purity of the standards, it varied for each individual analyte. The purities and densities of the analytes are shown in Table 2 on page 18 and must be considered for each calibration. The HS-GC/MS system was more sensitive for some analytes than for others. The lowest nominal concentration level had to be chosen in a way that accommodated both effects. It had to enable a meaningful calculation of the LOD for high-density substances with high detection sensitivity as well as for analytes with lower density and low detector response. The nominal concentrations of the analytes, which apply for a density of 1 kg dm^{-3} and a purity of 100%, were: 0.005, 0.01, 0.25, 0.1, 0.25, and 1.0 mg kg^{-1} (amount of analyte per amount of oil). The concentration values of 1,1,1-Trichloroethane were especially low, because there was no pure standard available but only a solution. The calculated actual concentrations of each level considering the purity as well as the density of the analytes can be seen in Table 6.

Table 6: The calculated concentrations of each level of all the compounds in the validation experiment considering the density and the purity of the analytes

Substance	Calculated conc. of each level with given nominal conc. (with ρ=1 g cm^{-3}) [µg kg^{-1}]					
	5.000	**10.00**	**25.00**	**100.0**	**250.0**	**1000**
n-Pentane	3.118	6.237	15.59	62.37	155.9	623.7
Acetone	3.911	7.821	19.55	78.21	195.5	782.1
Dichloromethane	6.583	13.17	32.92	131.7	329.2	1317
trans-1,2-Dichloroethene	6.135	12.27	30.68	122.7	306.8	1227
n-Hexane	3.135	6.27	15.675	62.7	156.75	627
1,1-Dichloroethane	5.558	11.12	27.79	111.2	277.9	1112
cis-1,2-Dichloroethene	6.356	12.712	31.78	127.12	317.8	1271
2-Butanone	3.980	7.960	19.90	79.60	1990	7960

Ethyl acetate	4.426	8.851	22.13	88.51	221.3	885.1
Trichloromethane	7.326	14.65	36.63	146.5	366.3	1465
1,1,1-Trichloroethane	0.02450	0.04900	0.1225	0.4900	1.225	4.900
Tetrachloromethane	8.075	16.15	40.37	161.5	403.7	1615
Benzene	4.356	8.712	21.78	87.12	217.8	871.2
1,2-Dichloroethane	6.219	12.44	31.10	124.4	311.0	1244
n-Heptane	3.366	6.732	16.83	67.32	168.3	673.2
Trichloroethene	7.264	14.53	36.32	145.3	363.2	1453
Bromodichloromethane	9.717	19.43	48.58	194.3	485.8	1943
Toluene	4.307	8.613	21.53	86.13	215.3	861.3
1,1,2-Trichloroethane	7.056	14.11	35.28	141.1	352.8	1411
Tetrachloroethene	8.075	16.15	40.37	161.5	403.7	1615
Dibromochloromethane	11.86	23.72	59.29	237.2	592.9	2372
1,1,1,2-Tetrachloroethane	7.722	15.44	38.61	154.4	386.1	1544
Ethyl benzene	4.307	8.613	21.53	86.1	215.3	861.3
m-, p-Xylene	8.500	17.00	42.50	170.0	425.0	1700
o-Xylene	4.356	8.712	21.78	87.12	217.8	871.2
Styrene	4.508	9.015	22.54	90.15	225.4	901.5
Tribromomethane	14.30	28.61	71.53	286.1	715.3	2861

The measured data was used to determine critical method parameters like the coefficient of determination, the relative deviation as well as limit of detection (LOD) and limit of quantitation (LOQ). The LODs were calculated according to the calibration method using Equation 1. The LOQ of each analyte was set to be three times the LOD.

$$x_{LOD} = \frac{1}{m} \cdot s_{res} \cdot P_t(\alpha, n-2) \cdot \sqrt{\frac{1}{n} + \frac{1}{l} + \frac{\bar{x}^2}{\sum(x_i - \bar{x})^2}}$$ **Equation 1**

m…slope
S_{res}…Residual standard deviation
P_t…t-distribution one sided T-test
α…uncertainty
n…number of measurements
l…number of parallel measurements
x…concentration values

The method parameters determined by the data of the method validation is shown in Table 7.

Table 7: List of analysed compounds and their calculated LOD and LOQ (base on the calibration method with seven concentration levels and four repetitions each, P < 0.05)

Substance	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	RSD%	Coefficient of determination
n-Pentane	0.1	0.3	4%	0.9996
Acetone	0.3	1	10%	0.9878
Dichloromethane	0.03	0.09	9%	0.9984
trans-1,2-Dichloroethene	0.003	0.01	5%	0.9998
n-Hexane	0.01	0.03	8%	0.9997
1,1-Dichloroethane	0.003	0.01	4%	0.9998
cis-1,2-Dichloroethene	0.003	0.01	3%	0.9998
2-Butanone	0.03	0.1	2%	0.9995
Ethyl acetate	0.1	0.3	2%	0.9997
Trichloromethane	0.01	0.03	5%	0.9987
1,1,1-Trichloroethane	0.001	0.003	4%	0.9986
Tetrachloromethane	0.003	0.01	4%	0.9999
Benzene	0.003	0.01	3%	0.9999
1,2-Dichloroethane	0.003	0.01	4%	0.9979
n-Heptane	0.01	0.03	3%	0.9941
Trichloroethene	0.003	0.01	2%	0.9979

Bromodichloromethane	0.003	0.01	2%	0.9997
Toluene	0.003	0.01	2%	0.9999
1,1,2-Trichloroethane	0.003	0.01	2%	0.9997
Tetrachloroethene	0.003	0.01	2%	0.9995
Dibromochloromethane	0.003	0.01	2%	0.9994
1,1,1,2-Tetrachloroethane	0.003	0.01	2%	0.9989
Ethyl benzene	0.003	0.01	3%	0.9999
m-, p-Xylene	0.003	0.01	4%	0.9998
o-Xylene	0.003	0.01	3%	0.9976
Styrene	0.003	0.01	2%	0.9996
Tribromomethane	0.003	0.01	2%	0.9999

The substances in Table 7 are ordered according to their elution sequence and therefore their retention times. It can be observed that the relative deviation tends to get smaller the later the analytes elute. The substances were more focused by the column and there was a higher chance for interferences to be separated from the signal of the analyte. The required data was in every respect more than satisfactory and proved that the method worked reliably. For any future calibrations however, it made no sense to use the same concentrations for each analyte. This had been done to gather comparable information for each compound and to collect unbiased data. It was evident that some substances could be detected more sensitively than others. For some substances like acetone and dichloromethane it blank signals were detected despite special care to avoid contamination by the laboratory environment. The blank signals were considered in the calculations of the LOD and LOQ. With these considerations, it made sense to individualise the concentrations of the substances in future calibrations.

2.6 Analysis preparation and measurements

Before the routine analysis of the samples could be done, all the procedures regarding standard solutions, calibration, and sample preparation had to be developed and tested.

2.6.1 Preparation of the stock solutions and calibrations

The first stock solutions which were prepared, either in isooctane or methanol, contained the same volume of each analyte. This made it easier to compare the results of the individual compounds during the method development phase. However, after the successful validation of the method the obtained information could be used to prepare a much more suitable stock solution for future calibrations. For preparing the stock solution, the pure substances were diluted in methanol. As described in chapter 2.4.3, using methanol led to multiple improvements over using isooctane as the solvent. The added volumes were dependent on the LODs, which are listed in Table 7, and on the expected concentration of the analytes in most of the samples. These expected concentrations were based on preliminary results. The resulting customised stock solution was prepared in a 2 ml-volumetric flask according to Table 8. Volumetric glass capillaries were used to add the pure substances to some methanol which was already in the volumetric flask and it was filled to the line with methanol.

Table 8: Scheme of the preparation of the stock solution which took into account LOD of the analytes as well as their occurrence in typical samples

Substance	added volume pure standard [µl]	calculated actual conc. of lowest cal. level [mg kg^{-1}]	nominal conc. of lowest cal. level (ρ=1 g cm^{-3}) [mg kg^{-1}]
n-Pentane	200	0.062	0.10
Acetone	200	0.078	0.10
Dichloromethane	100	0.066	0.050
trans-1,2-Dichloroethene	20	0.012	0.010
n-Hexane	100	0.031	0.050
1,1-Dichloroethane	20	0.011	0.010
cis-1,2-Dichloroethene	20	0.013	0.010
2-Butanone	100	0.040	0.050
Ethyl acetate	200	0.089	0.10

Trichloromethane	50	0.037	0.025
1,1,1-Trichloroethane	200	0.00049	0.0010
Tetrachloromethane	10	0.0081	0.0050
Benzene	20	0.0087	0.010
1,2-Dichloroethane	20	0.012	0.010
n-Heptane	50	0.017	0.025
Trichloroethene	10	0.0073	0.0050
Bromodichloromethane	10	0.0097	0.0050
Toluene	20	0.0086	0.010
1,1,2-Trichloroethane	10	0.0071	0.0050
Tetrachloroethene	10	0.0081	0.0050
Dibromochloromethane	10	0.0012	0.0050
1,1,1,2-Tetrachloroethane	10	0.0077	0.0050
Ethyl benzene	20	0.0086	0.010
m-, p-Xylene	20	0.0085	0.010
o-Xylene	20	0.0085	0.010
Styrene	20	0.0090	0.010
Tribromomethane	10	0.014	0.0050

This stock solution was then used to prepare five individual calibration solutions with increasing concentrations. The concentration pattern of the calibration can be seen in Table 9.

Table 9: Calibration levels according to the concentration of the lowest standard and the dilution pattern

Calibration level	Conc. Factor compared to lowest calibration level	concentration (nominal lowest conc.: 0.01 mg kg^{-1})
1	x 1	0.010
2	x 2.5	0.025
3	x 10	0.10
4	x 25	0.25
5	x 100	1.0

The internal standard solution was added into each individual calibration solution in a way that the concentration of the internal standards would have a nominal concentration of 1 mg kg^{-1}. For the actual concentrations, Table 2 on page 18 can be consulted. Highly refined olive oil, purchased at Sigma-Aldrich Handels Gmbh (Vienna, Austria), was used as a matrix material for the preparation of the calibration standards, because it contained negligible amounts of volatile substances (<LOD). For every calibration standard, 10.00 g of this oil were spiked with the corresponding calibration solution to achieve the goal of adding 100 µl of methanol solution to every sample. A usual calibration consisted of five levels with an additional blank. The calibrated range can be derived from Table 8 and Table 9. For each measurement, a new calibration was prepared and measured, because repeated measurements of headspace samples could lead to deviating results.

2.6.2 Sample preparation

The sample preparation was strictly performed in a laboratory environment in which no solvents were ever used for other analyses. This would prevent contamination from the air which could potentially be a major problem. 10.00 g of every sample were weighed into a 20 ml headspace vial and 100 µl of a methanol solution were added. This solution contained either only the internal standards for standard samples and blanks, or the respective calibration solution for calibration samples. The vial was closed with an aluminium crimp cap which was equipped with a Teflon-coated butyl rubber septum. As standard material for blanks and calibrations, the highly refined olive oil was used. The prepared samples were then transferred into the analytic system. 69 individual oil samples from nine different oil mills were analysed in two sample sets. There was a period of six months between the measurement of the two sample sets. Each sample was measured three times in three distinct worklists to determine the measurement uncertainty. To prevent any bias, the sequence in which the samples were prepared and measured was altered every time.

2.7 Thermal stress tests

The aim of the project was not only to develop an analytic method for the reliable and robust detection of volatile substances but also to find out why these substances occurred in the oils. First results of measured samples had already shown that the volatile substances occurred in all the analysed samples. These preliminary results were confirmed by results of a third party, another analysis laboratory which was tasked with the analysis. All these parameters led to the conclusion that the substances might form in the oil at some point of the production. The results of a preliminary experiment which is discussed in more detail in chapter 3.2.1 seemed to confirm these suspicions. Therefore, a series of experiments were designed to prove that some volatile compounds which could be mistaken for solvent residues in the oil, formed intrinsically. It was suspected that high temperature could have an influence on the formation of these substances. Especially pumpkin seed oils are subject to elevated temperatures during the roasting process prior to the pressing of the seeds. To investigate the influence of temperature on the oilseeds and the oil during the roasting process, samples of vegetable oils from different seeds and a batch of crushed pumpkin seeds were heated to 120 °C for 24 hours. After 2 hours, 4 hours, 8 hours, 16 hours, and 24 hours samples were drawn and measured with the same analysis method as the other project samples. Additionally, blank samples which should represent the content of substances without any heating were measured as well. The whole experiment was carried out three times to confirm the repeatability of the thermal treatment of the samples and the measurements. The results of these measurements are shown and further discussed in chapter 3.2.2.

2.7.1 Headspace condition reconsiderations

The results of the thermal stress test experiments, which are shown in chapter 3.2.2, raised second thoughts about the headspace sample method. For each measurement, the samples were shaken in the headspace oven for 15 minutes at a temperature of 120 °C. The same temperature had been used in the thermal conditioning experiment and had caused significant increase in many volatile substances which could erroneously be interpreted as solvent residues. The first sample was drawn after 2 hours, so there was no information about the behaviour of the oil in the time before that. It was possible that some of the detected substances in the project samples were formed during the sample heating period in the headspace oven. To investigate these suspicions further an experiment was designed. As sample material for the experiment, a Styrian pumpkin seed oil which contained very low concentrations of the analytes, was used. It did contain small amounts of n-pentane, acetone, 2-butane, and toluene. 10.00 g of the oil were weighed into vials fourteen times. Seven of these samples were spiked with 100 µl a solution containing all the analytes and the internal standard in methanol. The nominal

concentration of the analytes was 0.25 mg kg^{-1} (assuming a density of 1 g cm^{-3} and a purity of 100% for each compound). The nominal concentration of the internal standards was 1.0 mg kg^{-1}. For the actual concentrations, Table 6 on page 30 can be consulted. Both, the spiked and the non-spiked samples were measured with oven temperatures, ranging from 80 °C to 140 °C using intervals of 10 °C. The signal areas of the analytes were recorded and compared to determine the temperature which was best suited for the task. The results of the experiments can be seen in chapter 3.2.3.

2.8 Comparative measurements using solid phase micro extraction GC/MS

The results of the HS-GC/MS method which was developed for this project, were compared to the measurements of a complementary system – Solid Phase Micro Extraction (SPME) GC/MS. The instrument was also equipped with a cryogenic cooling system for the GC oven. SPME injection systems make use of fine fibres on which the analytes adsorb. The fibre is then inserted into the injection system of the GC and heated to desorb the analytes and transport them into the separation column. This technique allows for the analysis of thermally labile substances which would possibly be destroyed in the harsher conditions of static headspace samplers. The adsorption of the analytes on the fibre also leads to a concentration effect which increases the sensitivity and eliminates a lot of the influence of the sample matrix. The cryogenic conditions of the GC oven allow much lower initial temperatures which can be well below 0 °C. Highly volatile substances can therefore be separated much better. The aim of the comparative analyses was to verify the results using a different sample injection system, which would impose less thermal stress on the samples. For the measurements, 1 g of pumpkin seed oil was weighed into a 20 ml-SPME vial, a glass coated stirring bar was added, and the vial closed using a magnetic crimp cap with a Teflon-coated rubber septum. A rudimentary two-point calibration was prepared with samples containing a nominal concentration of 0.1 mg kg^{-1} and 0.25 mg kg^{-1} respectively. Table 8 (page 34) and Table 9 (page 36) can be used to determine the actual concentration for each analyte at these two nominal concentration levels. In addition to confirm the results of the HS-GC/MS method the results of the SPME measurements were used to estimate the LOD and LOQ of this method. While the sample preparation for this experiment was conducted at the Institut Dr. Wagner Lebensmittel Analytik GmbH, the measurements were done at the Institute of Analytical Chemistry and Food Chemistry at the University of Technology in Graz.

3 Results and discussion

3.1 Samples

The 69 samples from nine different oil mills were delivered and measured in two sets with a period of six months between the sets. This was done to guarantee consistency in the results as well as to see whether any substantial changes could be observed between the sample sets. Each sample was measured three times to confirm the repeatability of the method. All samples of one sample set were measured within one worklist overnight. However, the sequence in which the samples were prepared and analysed, was altered with every worklist to prevent any sequential bias. The results of the measurements confirmed the initial reports which had led to initiating the project. In every single one of the 69 analysed oils, at least small residues of volatile substances were found, all of them being non-halogenated compounds. All these substances are commonly used solvents in chemical laboratories and for other industrial applications. The substances which occurred most frequently and in the highest concentrations were acetone, 2-butanone, n-pentane (0.11 – 1.9 mg kg^{-1}), as well as toluene, n-heptane, ethyl acetate and styrene (0.014 – 0.40 mg kg^{-1}). Individual oils also contained traces of ethyl acetate and aromatic analytes like m-xylene, p-xylene, and ethyl benzene. However, in most cases these compounds were well below the LOD. The relative deviation of all the samples over the three repetitions and over all substances was below 20%. Table 10 and Table 11 show the detailed results of the measurements of both sample series which were drawn and measured with a six-months period in between.

Table 10: Results of the measurements of the first series of samples and their deviations (three repetitions)

sample	n-pentane [mg kg^{-1}]	acetone [mg kg^{-1}]	2-butanone [mg kg^{-1}]	n-heptane [mg kg^{-1}]	toluene [mg kg^{-1}]	m-/p-xylene [mg kg^{-1}]	Styrene [mg kg^{-1}]
1 poppy	<0.30	n.d.	<0.10	n.d.	0.013 ± 0.001	<0.010	n.d.
2 pumpkin	0.34 ± 0.02	1.6 ± 0.2	0.96 ± 0.03	n.d.	0.061 ± 0.004	0.025 ± 0.002	0.014 ± 0.001
3 pumpkin	0.84 ± 0.02	2.2 ± 0.1	1.3 ± 0.1	n.d.	0.055 ± 0.001	0.015 ± 0.001	0.014 ± 0.001
4 walnut	1.1 ± 0.1	<1.0	0.24 ± 0.01	<0.03	0.023 ± 0.001	<0.010	0.022 ± 0.001
5 linseed	n.d.	<1.0	n.d.	n.d.	n.d.	n.d.	n.d.
6 pumpkin	n.d.	1.4 ± 0.2	0.44 ± 0.02	n.d.	0.028 ± 0.001	n.d.	n.d.
7 pumpkin	<0.30	1.8 ± 0.2	0.85 ± 0.03	n.d.	0.055 ± 0.001	n.d.	n.d.
8 pumpkin	<0.30	1.4 ± 0.1	0.68 ± 0.03	n.d.	0.031 ± 0.001	n.d.	n.d.
9 pumpkin	<0.30	1.8 ± 0.1	0.83 ± 0.03	n.d.	0.033 ± 0.001	n.d.	n.d.

10 pumpkin	0.72 ± 0.04	1.4 ± 0.1	0.64 ± 0.04	n.d.	0.080 ± 0.007	0.035 ± 0.006	<0.010
11 pumpkin	0.54 ± 0.05	1.4 ± 0.1	0.69 ± 0.02	0.04 ± 0.01	0.056 ± 0.001	<0.010	<0.010
12 pumpkin	0.33 ± 0.01	1.4 ± 0.1	0.65 ± 0.02	n.d.	0.054 ± 0.001	n.d.	n.d.
13 pumpkin	0.33 ± 0.01	1.4 ± 0.1	0.67 ± 0.02	n.d.	0.061 ± 0.001	n.d.	n.d.
14 pumpkin	0.46 ± 0.02	1.8 ± 0.1	1.1 ± 0.1	n.d.	0.059 ± 0.002	n.d.	<0.010
15 pumpkin	0.70 ± 0.03	1.9 ± 0.1	0.81 ± 0.03	<0.03	0.050 ± 0.001	n.d.	<0.010
16 pumpkin	0.32 ± 0.02	1.6 ± 0.1	0.79 ± 0.02	n.d.	0.040 ± 0.001	n.d.	<0.010
17 pumpkin	n.d.	<1.0	0.20 ± 0.01	n.d.	0.021 ± 0.001	n.d.	n.d.
18 pumpkin	0.34 ± 0.02	1.2 ± 0.1	0.48 ± 0.02	n.d.	0.049 ± 0.001	n.d.	<0.010
19 pumpkin	n.d.	1.5 ± 0.1	0.55 ± 0.02	n.d.	0.029 ± 0.001	n.d.	<0.010
20 pumpkin	n.d.	1.4 ± 0.1	0.24 ± 0.01	n.d.	0.033 ± 0.001	n.d.	n.d.
21 pumpkin	<0.30	<1.0	0.19 ± 0.01	n.d.	0.015 ± 0.001	n.d.	n.d.
22 pumpkin	n.d.	<1.0	0.36 ± 0.01	n.d.	0.017 ± 0.001	n.d.	n.d.
23 pumpkin	n.d.	<1.0	0.48 ± 0.02	n.d.	0.020 ± 0.001	<0.010	<0.010
24 pumpkin	n.d.	<1.0	0.21 ± 0.01	n.d.	0.021 ± 0.001	n.d.	n.d.
25 pumpkin	<0.30	<1.0	0.29 ± 0.01	n.d.	0.021 ± 0.001	n.d.	n.d.
26 pumpkin	<0.30	<1.0	0.26 ± 0.01	n.d.	0.019 ± 0.001	n.d.	n.d.
27 pumpkin	<0.30	<1.0	0.29 ± 0.02	n.d.	0.022 ± 0.001	n.d.	n.d.
28 pumpkin	n.d.	<1.0	0.25 ± 0.01	n.d.	0.025 ± 0.001	n.d.	n.d.
29 pumpkin	0.50 ± 0.04	1.3 ± 0.1	0.62 ± 0.04	n.d.	0.041 ± 0.001	n.d.	<0.010
30 pumpkin	0.62 ± 0.05	1.2 ± 0.1	0.46 ± 0.03	n.d.	0.041 ± 0.002	n.d.	n.d.
31 pumpkin	0.50 ± 0.03	1.2 ± 0.1	0.53 ± 0.03	n.d.	0.037 ± 0.001	n.d.	<0.010
32 pumpkin	n.d.	<1.0	0.15 ± 0.01	n.d.	0.020 ± 0.001	n.d.	n.d.

Table 11: Results of the measurements of the second series of samples and their deviations which were obtained six months after the first sample set (three repetitions)

sample	n-pentane	acetone	2-butanone	ethyl acetate	n-heptane	toluene	ethyl benzene	m-/p-xylene	styrene
	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]
33 pumpkin	0.59 ± 0.05	1.2 ± 0.08	0.45 ± 0.03	n.d.	<0.03	0.051 ± 0.002	0.017 ± 0.001	0.074 ± 0.004	<0.01
34 pumpkin	0.37 ± 0.02	1.2 ± 0.09	0.51 ± 0.04	n.d.	n.d.	0.037 ± 0.001	n.d.	<0.01	<0.01
35 pumpkin	0.69 ± 0.06	1.5 ± 0.01	0.69 ± 0.05	n.d.	<0.03	0.068 ± 0.003	n.d.	<0.01	<0.01
36 pumpkin	1.0 ± 0.05	1.5 ± 0.02	0.57 ± 0.02	n.d.	<0.03	0.041 ± 0.002	n.d.	n.d.	<0.01
37 pumpkin	0.56 ± 0.05	1.4 ± 0.01	0.6 ± 0.01	n.d.	<0.03	0.043 ± 0.001	n.d.	n.d.	<0.01
38 pumpkin	0.48 ± 0.02	1.4 ± 0.01	0.64 ± 0.04	n.d.	n.d.	0.062 ± 0.002	n.d.	<0.01	<0.01
39 pumpkin	0.60 ± 0.03	1.3 ± 0.01	0.46 ± 0.02	n.d.	<0.03	0.041 ± 0.001	n.d.	n.d.	<0.01
40 poppy	0.44 ± 0.02	<1.0	<0.1	n.d.	<0.03	<0.01	n.d.	n.d.	n.d.
41 walnut	1.3 ± 0.09	<1.0	0.25 ± 0.03	n.d.	<0.03	0.021 ± 0.001	n.d.	<0.01	0.016 ± 0.001
42 pumpkin	n.d.	<1.0	n.d.	0.40 ± 0.02	n.d.	<0.01	n.d.	n.d.	n.d.
43 linseed	n.d.	<1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
44 pumpkin	1.3 ± 0.1	1.3 ± 0.08	0.74 ± 0.05	n.d.	0.047 ± 0.009	0.065 ± 0.004	n.d.	0.023 ± 0.002	0.015 ± 0.001
45 pumpkin	1.6 ± 0.1	1.2 ± 0.09	0.70 ± 0.05	n.d.	<0.03	0.058 ± 0.003	n.d.	0.011 ± 0.001	0.014 ± 0.001
46 pumpkin	<0.3	1.9 ± 0.2	0.81 ± 0.02	n.d.	n.d.	0.029 ± 0.001	n.d.	n.d.	<0.01
47 pumpkin	<0.3	1.6 ± 0.07	0.58 ± 0.05	n.d.	n.d.	0.029 ± 0.001	n.d.	n.d.	<0.01
48 pumpkin	0.40 ± 0.04	1.6 ± 0.03	0.76 ± 0.07	n.d.	n.d.	0.035 ± 0.001	n.d.	n.d.	<0.01
49 pumpkin	0.35 ± 0.01	1.4 ± 0.01	0.49 ± 0.01	n.d.	n.d.	0.046 ± 0.002	n.d.	n.d.	<0.01
50 hemp	0.56 ± 0.05	<1.0	<0.1	n.d.	n.d.	<0.01	n.d.	n.d.	0.010 ± 0.001
51 pumpkin	0.34 ± 0.01	1.4 ± 0.02	0.61 ± 0.01	n.d.	n.d.	0.049 ± 0.002	n.d.	n.d.	<0.01
52 linseed	0.42 ± 0.03	<1.0	<0.1	n.d.	0.056 ± 0.006	<0.01	n.d.	<0.01	<0.01
53 pumpkin	<0.3	1.0 ± 0.06	0.34 ± 0.02	n.d.	n.d.	0.027 ± 0.001	n.d.	n.d.	<0.01
54 pumpkin	<0.3	<1.0	0.27 ± 0.01	n.d.	n.d.	0.026 ± 0.001	n.d.	n.d.	<0.01
55 pumpkin	<0.3	1.1 ± 0.04	0.47 ± 0.03	n.d.	n.d.	0.033 ± 0.001	n.d.	n.d.	<0.01
56 pumpkin	<0.3	<1.0	0.27 ± 0.02	n.d.	n.d.	0.025 ± 0.001	n.d.	n.d.	<0.01
57 pumpkin	<0.3	<1.0	0.32 ± 0.02	n.d.	n.d.	0.037 ± 0.001	n.d.	<0.01	<0.01
58 pumpkin	0.66 ± 0.06	1.1 ± 0.07	0.49 ± 0.03	n.d.	<0.03	0.051 ± 0.002	n.d.	<0.01	<0.01
59 pumpkin	0.39 ± 0.04	1.0 ± 0.1	0.25 ± 0.02	n.d.	n.d.	0.022 ± 0.001	n.d.	n.d.	<0.01
60 pumpkin	0.39 ± 0.04	1.1 ± 0.08	0.27 ± 0.01	n.d.	n.d.	0.021 ± 0.001	n.d.	n.d.	<0.01

61 pumpkin	<0.3	1.2 ± 0.06	0.69 ± 0.05	n.d.	n.d.	0.044 ± 0.002	n.d.	<0.01	0.012 ± 0.001
62 pumpkin	n.d.	<1.0	n.d.	n.d.	n.d.	0.014 ± 0.001	<0.01	0.023 ± 0.001	n.d.
63 pumpkin	n.d.	1.1 ± 0.02	0.22 ± 0.01	n.d.	n.d.	0.030 ± 0.001	n.d.	0.011 ± 0.001	<0.01
64 pumpkin	n.d.	<1.0	0.16 ± 0.01	n.d.	n.d.	0.025 ± 0.001	n.d.	<0.01	<0.01
65 pumpkin	n.d.	<1.0	0.15 ± 0.01	n.d.	n.d.	0.017 ± 0.001	n.d.	<0.01	<0.01
66 salad oil	<0.3	<1.0	0.27 ± 0.03	n.d.	<0.03	0.034 ± 0.002	n.d.	<0.01	<0.01
67 hazelnut	<0.3	<1.0	0.28 ± 0.03	n.d.	0.070 ± 0.007	<0.01	n.d.	n.d.	0.11 ± 0.01
68 walnut	<0.3	<1.0	n.d.	n.d.	n.d.	<0.01	n.d.	n.d.	n.d.
69 pumpkin	n.d.	<1.0	0.16 ± 0.01	n.d.	0.065 ± 0.007	0.020 ± 0.002	n.d.	<0.01	<0.01

Figure 10 and Figure 11 show the results of the two sample sets with the concentrations of all the detected substances. It must be pointed out that some of the concentrations are below the LOQs which had been determined in chapter 0.. With strictly considering the LOQs in the depiction, a lot of information about the samples would have been lost since most of the samples had traces of n-pentane but many of the concentrations were below the LOQ. The same applied for 2-butanone. The concentrations in Figure 10 and Figure 11 are therefore depicted as if the LOQs would have been zero.

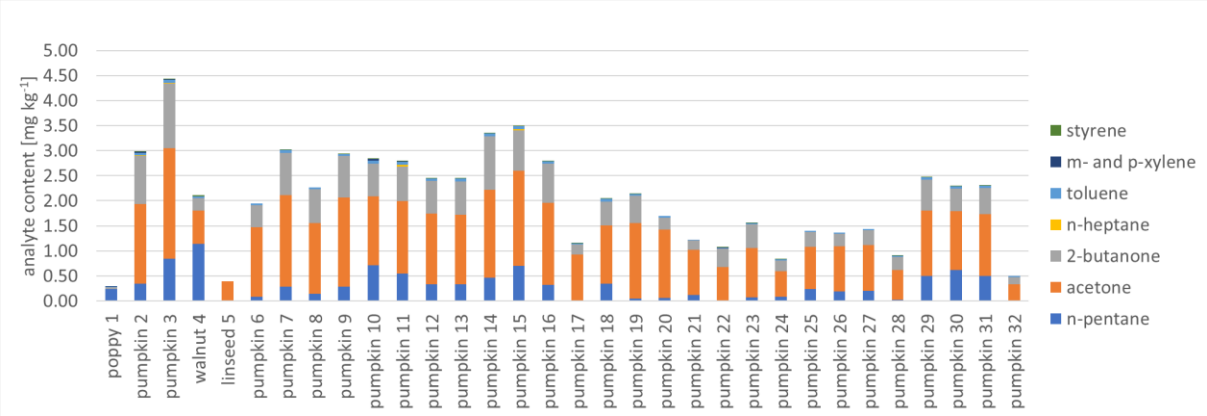

Figure 10: Concentration of the volatile analytes in the first sample set in mg kg^{-1}. (3 repetitions, RSD<20%) Some of the shown concentrations are below the calculated LODs for better comparability of the samples. The seed from which the oil was made is shown below the sample number

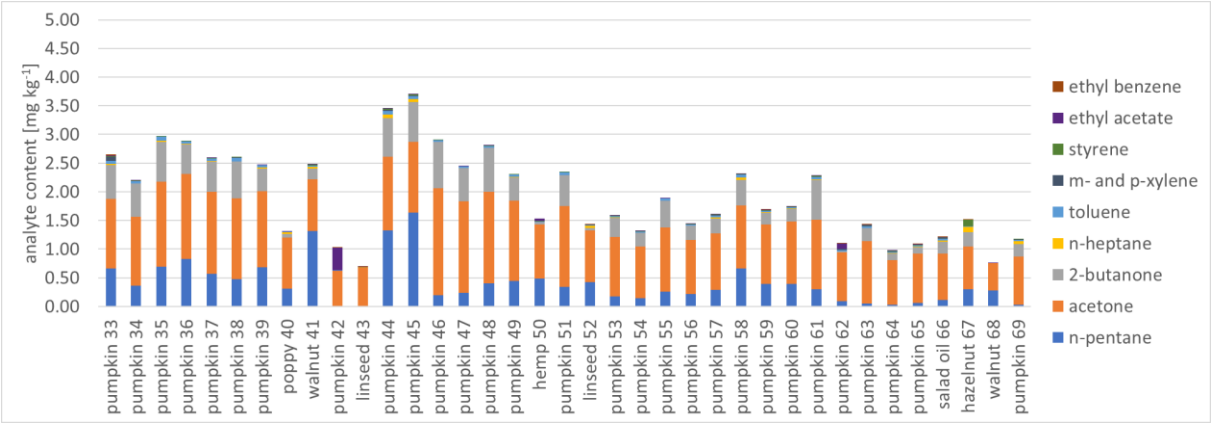

Figure 11: Concentration of the volatile analytes in the second sample set in mg kg^{-1}. (3 repetitions, RSD<20%) Some of the shown concentrations are below the calculated LODs for better comparability of the samples. The seed from which the oil was made is shown below the sample number

The average contamination of the oils with volatile, solvent-like substances was around 2 mg kg^{-1}. This value appeared to be too high for unintentional contamination and the continuity of the contamination in almost all the samples raised suspicion. When individual samples were compared, additional patterns emerged. The samples 17 and 42 were both pumpkin seed oils, however in contrast to almost all the other oils there were no traces of n-pentane. Both oils had been produced by cold pressing without the roasting process prior to pressing. Other typically cold pressed oils like linseed oil (samples 5 and 43) and poppy seed oil (samples 1 and 40) showed quite a different spectrum of volatile substances compared to most of the other oils. The investigation of the origins of these "solvent contaminations" was part of the project and patterns like these provided important indications. Since all the examined oil samples had been produced by pressing and not by solvent extraction, the measured residues must have gotten into the oils in some other way. Especially n-pentane can be used as an indicator for oxidative degradation of vegetable oils (Pinnel & Vandegans, 1996), which lead to the assumption that the other substances also might have had a similar origin.

3.2 Sample conditioning experiments

3.2.1 Preliminary experiment with exposure to sunlight

The results of both measurement series which were discussed in chapter 3.1 have already indicated some correlation between the spectrum of solvents and the type of oil. The production of some of the oils, especially pumpkin seed oil, involves a roasting step. During this process, the mixture of finely ground seeds, water, and salt is heated under stirring in a large pan to temperatures up to 130 °C. This roasting process is conducted until all the water has evaporated which can take 60 minutes or more. (Gemeinschaft Steirisches Kürbioskernöl g.g.A., 2018) During this period the mixture is exposed to high

temperatures, water, oxygen from the air, and light. All these factors could influence the substance spectrum in the oil by decomposition of the ingrediencies. It was not yet clear whether the amounts which had been found in the samples could be formed intrinsically. To find out how the substance spectrum in the oil could be altered by exposing it to light and heat, a small experiment was set up. A pumpkin seed oil (sample number 29) was weighed four times as usual, the internal standards were added, and the vials were closed. One sample was stored in the dark at room temperature, a second one was stored in the refrigerator at 5 °C and two were positioned outside in the warmth of the sun for a day. The next day, the samples were all brought to room temperature and measured on the HS-GC/MS system. The results of this first experiment can be seen in Figure 12 and Figure 13.

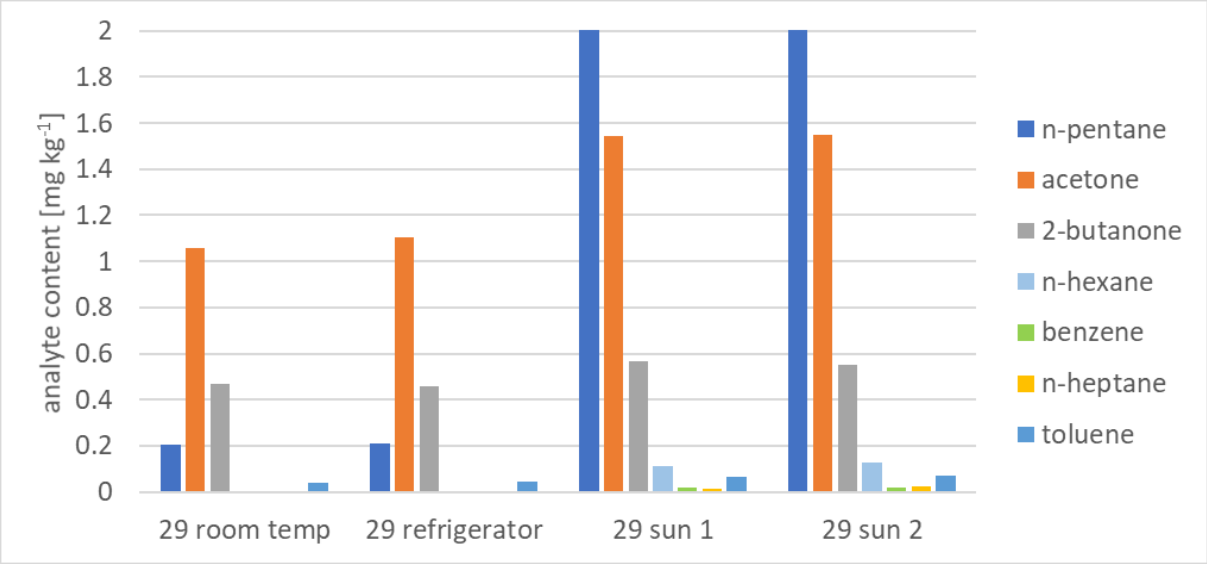

Figure 12: Results of the measurement of the four samples which had been stored under different conditions for a day

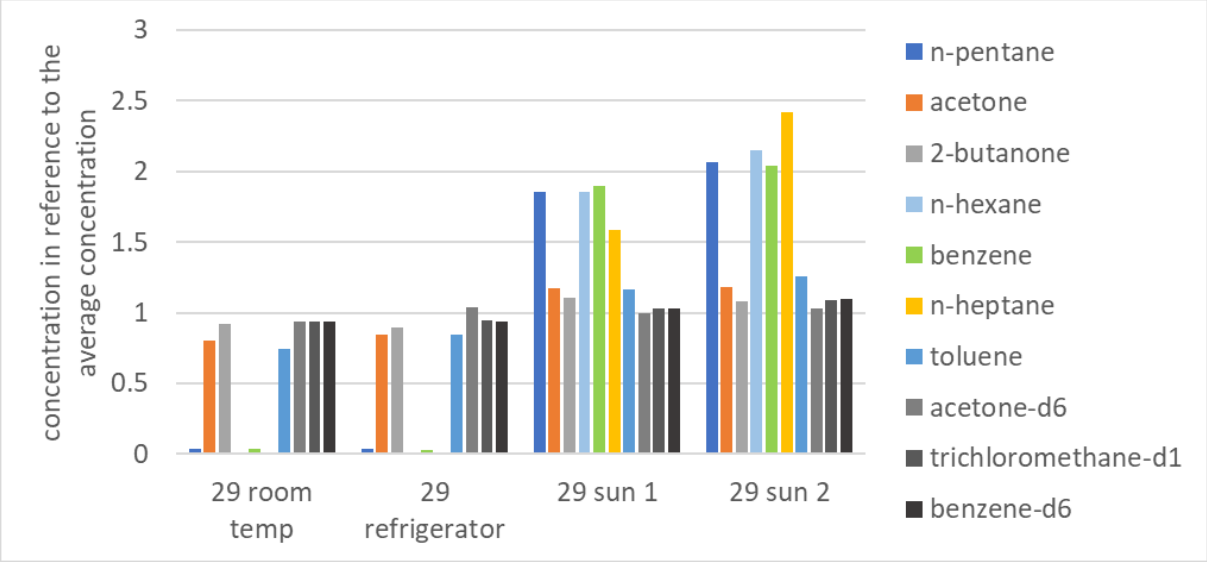

Figure 13: Results of the measurements of the four samples which were stored under different conditions for a day. The analyte contents were divided by the average analyte content over all four samples for better comparability

The results of the measurements showed that all substances which had already been present in the oil, increased in abundance during the exposure to the sunlight (Figure 12). N-pentane increased significantly but so did acetone and 2-butanone. To improve comparability for compounds with lower concentrations, the individual concentrations were divided by their average, which is shown in Figure 13. It could be observed that other alkenes like n-hexane and n-heptane increased in abundance as well. Aromatic compounds like benzene and toluene also increased significantly. The internal standards however showed almost no increase in abundance during the experiment. This was strong evidence that the elegit solvent residues were formed intrinsically during the production process when the sample was exposed to heat and light.

3.2.2 Thermal stress tests

The preliminary experiment (chapter 3.2.1) had shown, that the vegetable oils formed volatile substances which could erroneously be interpreted as solvent residues. The substances were formed during the exposure to sunlight and heat. It was assumed that heating processes during the oil production, for example roasting, could be responsible for the formation of the compounds. Usually the oil production is done indoors and neither the oil nor the raw materials are exposed to sunlight. Therefore, the influence of heat could be the more contributing factor. To investigate this assumption, that the roasting process causes the formation of volatile substances in the oil, which could be mistaken for common organic solvents, a thermal stress test was conducted. The detailed procedure was described in chapter 2.7. The comparison of the TICs (total ion chromatogram of all recorded SIM signals) of the sample measurements after the thermal treatment (Figure 26 on page 49) had already shown that some of the signal intensities grew with prolonged exposure to high temperature. To illustrate the data, the signal areas of the substances were plotted against the duration of the heat conditioning experiment, which can be seen in Figure 14-25. For reasons of comparability and better scaling, some of the graphs show only a selection of oils.

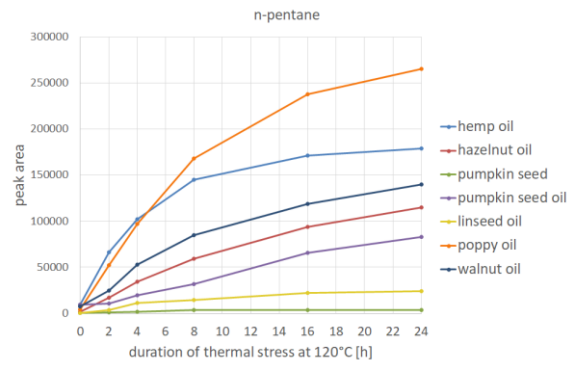

Figure 14: Change of the n-pentane content in various oils

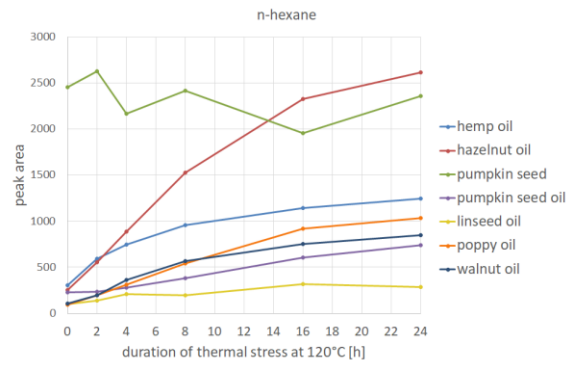

Figure 15: Change of the n-hexane content in various oils

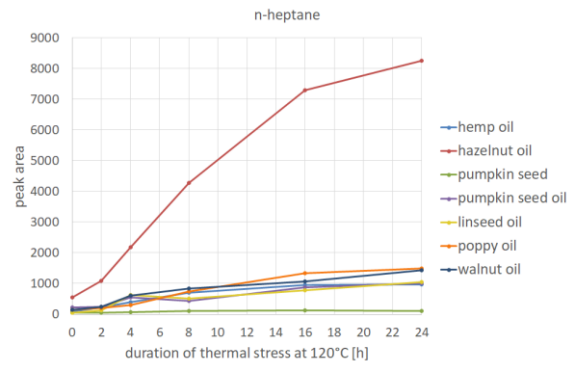

Figure 16: Change of the n-heptane content in various oils

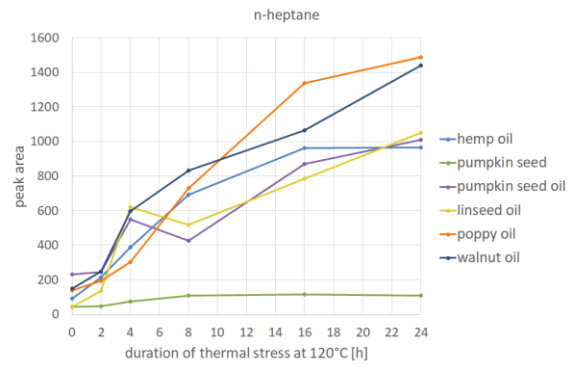

Figure 17: Change of the n-heptane content in various oils (without hazelnut oil)

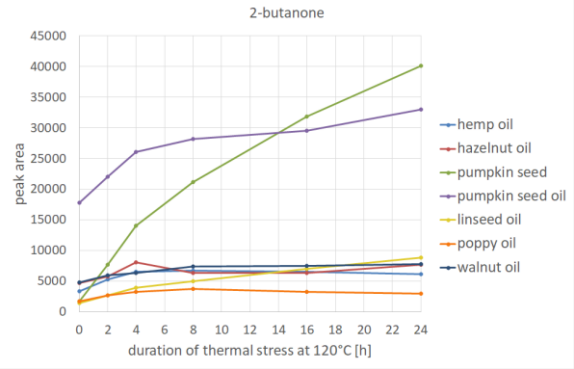

Figure 18: Change of the 2-butanone content in various oils

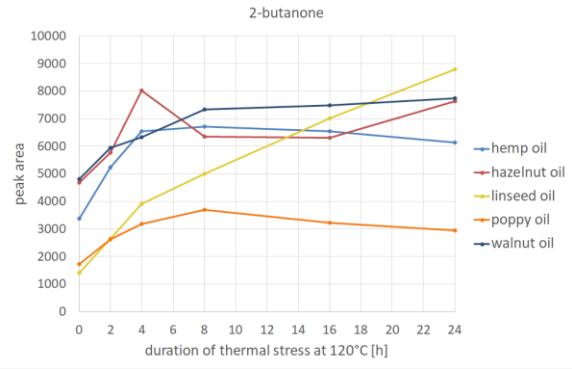

Figure 19: Change of the 2-butanone content in various oils (without pumpkin seed and pumpkin seed oil)

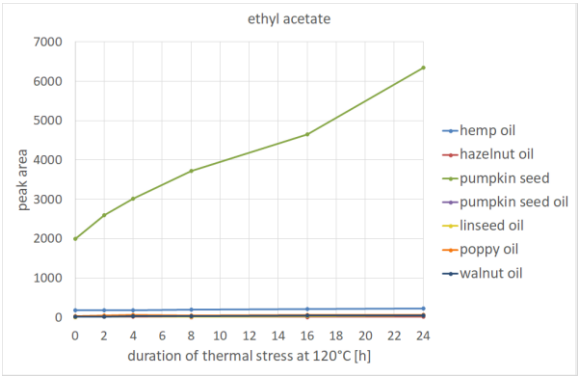

Figure 20: Change of the ethyl acetate content in various oils

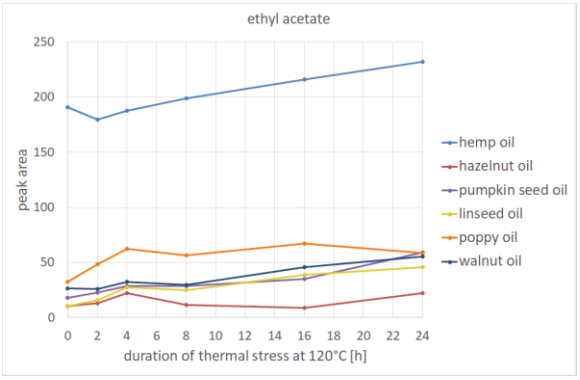

Figure 21: Change of the ethyl acetate content in various oils (without pumpkin seed)

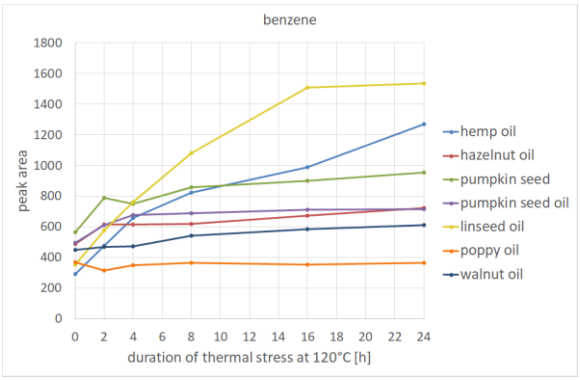

Figure 22: Change of the benzene content in different oil types, over 24 hours of thermal treatment at 120 °C

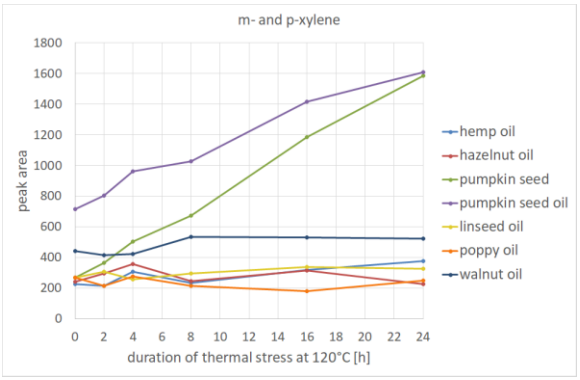

Figure 23: Change of the m- & p-xylene content in various oils

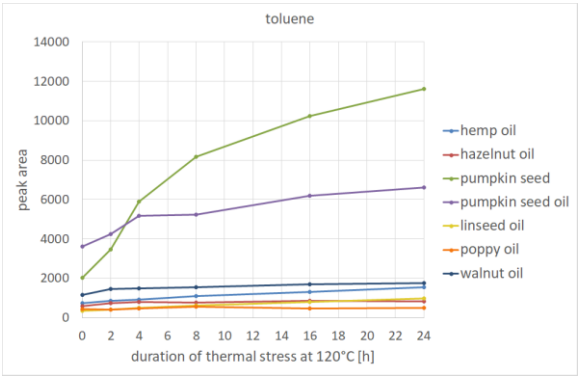

Figure 24: Change of the toluene content in various oils

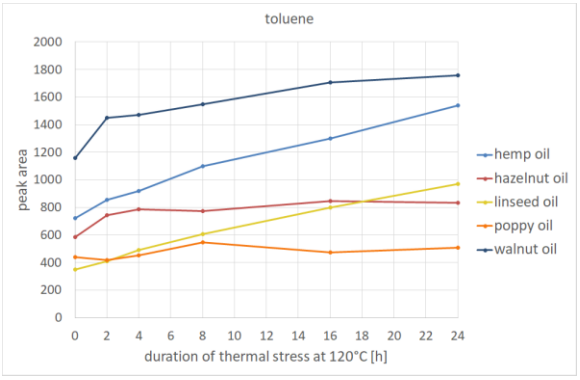

Figure 25: Change of the toluene content in various oils (without pumpkin seed and pumpkin seed oil)

Every oil which was tested in the experiment had been produced from a different oilseed (hemp seed oil, hazelnut oil, pumpkin seed oil, linseed oil, poppy seed oil, walnut oil). Also, a sample of crushed pumpkin seeds was investigated in this experiment. It became evident, that the concentration of the measured substances increased with the duration of the thermal stress. The extent of the increase in concentration, strongly depended on the type of oil. This can be seen in the increased concentrations of aromatic compounds like m-xylene and p-xylene in pumpkin seeds and pumpkin seed oil. Other aromatic substances like benzene and toluene however were formed in all the oils, although the rate of the formation varied in different samples. Linear alkanes like n-pentane, n-hexane and n-pentane increased drastically in all the tested oils, but not at all in the pumpkin seeds. The hazelnut oil stands out with the remarkable increase of the n-heptane concentration. N-heptane was already significantly more abundant in the hazelnut oil before the heat treatment and the concentration increased even further. 2-butanone which is present in almost every analysed pumpkin seed oil increased significantly in the pumpkin seeds, the pumpkin seed oil and in the linseed oil (Figure 18 and Figure 19). In the other types of oil, no increase in methyl ketones could be observed. Ethyl acetate was formed exclusively in the pumpkin seeds and not in any of the oils, including pumpkin seed oil. This behaviour indicates that other ingredients in the plant material which had been separated from the oil during the pressing process could have been responsible for the formation of these oxygen-containing substances. The pumpkin seeds, as well as pumpkin seed oil generally seemed to behave differently under thermal stress than the other tested oil types. This may be due to certain ingredients, which might not be present in the other oils. There is also a significant difference in colour intensity with the pumpkin seed oil being much darker than the other oils. The increase in the signal intensity of volatile substances can be observed in Figure 26. Besides the substances which were in the scope of the method, other substances which had responded to the same SIM signals could be detected in the measurements. Scan-measurements were used to conduct a database search (Wiley) using the fragmentation pattern of the unknown substances.

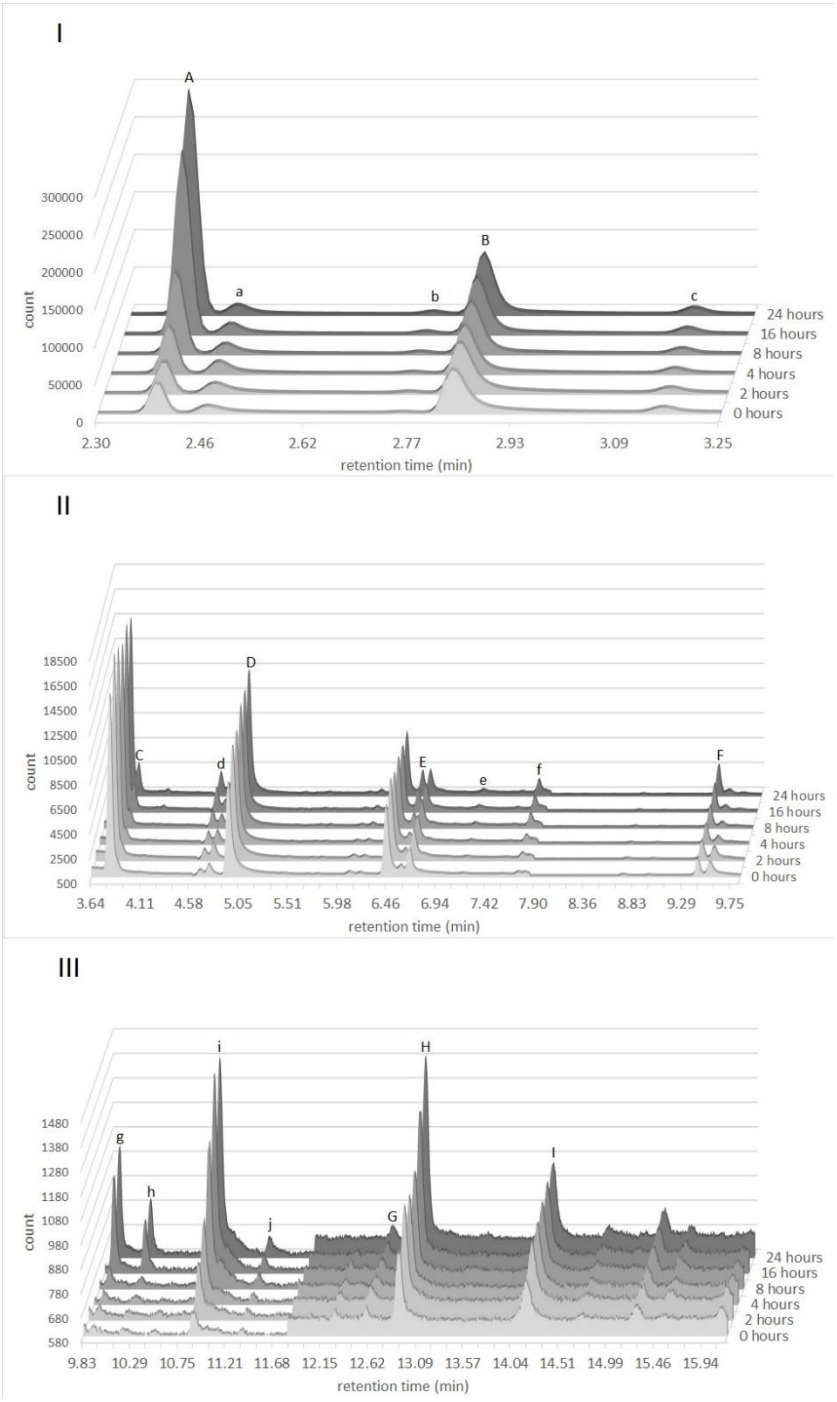

Figure 26: Comparative TIC's of pumpkin seed oil after different periods of thermal treatment at 120 °C. Because of significantly varying signal intensities, the TIC was divided into three parts labelled with I (2.30-3.25 min), II (3.64-9.75) and III (9.83-15.94). Peaks labelled with uppercase letters show compounds which are part of the method's scope, whereas lowercase letters indicate signals which respond to the SIM parameters in the method but were not initially looked for. By doing a mass spectrum database search, some of the signals were identified. Since no analytical standards were available for reliable confirmation, there is no guarantee for correctness. But many of them also show the increase in concentration with prolonged heating. Signals: (A) n-pentane; (B) acetone; (C) n-hexane; (D) 2-butanone; (E) n-heptane; (F) toluene; (G) ethylbenzene; (H) m- and p-xylene; (I) styrene. (a) ethanol; (b) propanol; (d) n-butanal; (g) 2-octene; (h) hexahydro pyridine; (i) n-hexanal.

The compounds which were identified with the highest prediction score (>95%) were ethanol, propanol, n-butanal, 2-octene, hexahydropyridine, and n-hexanal. All these substances are known to naturally occur in vegetable oils and some are responsible for the flavour of the oil (deMan, 1999). The signals of 2-octene and hexahydro pyridine in Figure 26 show interesting behaviour compared to the other substances. While most signals increase steadily over time, the two named substances only seem to appear after 16 hours of thermal treatment. These two substances could be tertiary oxidation products and other reactions were required for these substances to form.

All these results show that the initial claims of food stores as well as analytic laboratories (chapter 1.2), which stated that the solvents in oils were due to contamination or residues of solvent extraction, had most likely been wrong. The substances and the concentrations in which they occurred in the samples, could be explained with intrinsic formation due to heating during the production process.

3.2.3 Headspace oven temperature experiment

As already discussed in chapter 3.2.2 there was a chance of compounds forming in the oil when high temperature was exerted onto the samples for too long. This led to the concern that the selected headspace conditions might have influenced the substance spectrum of the samples. It would be very difficult to determine whether higher concentrations upon heating were due to substances being forced into the gas phase, or due to substances being formed in the sample material because of the heating process. To find out which temperature would work best for the method, an experiment had been designed (chapter 2.7.1). A set of spiked samples had been used to see whether any temperature in the tested range could have already led to a saturation of gas phase. If that would have been the case any further increase in temperature would not have been necessary. A set of pure oils served as a control which indicated if the oil matrix itself started to influence the measurement. For the evaluation, the analytes were divided into four groups: the alkanes, the oxygen-containing compounds, aromatic compounds, and the halogenated compounds. The halogenated compounds were a good indication whether any saturation limits were already reached, since they could not occur naturally in vegetable oils and would not be subject to any degradation. The conditioning period of the samples in the headspace oven had been 15 minutes for all the samples. Figure 27 shows the measured concentration with increased temperature of the headspace oven.

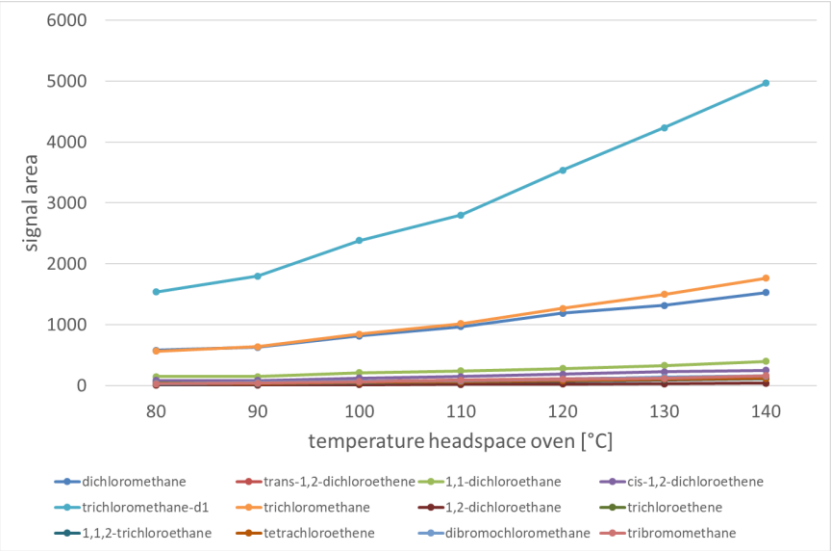

Figure 27: The signal area of halogenated compounds increasing with higher headspace oven temperature in pumpkin seed oil which had been spiked with a nominal concentration of 0.25 mg kg^{-1} of each analyte

Since the concentration of the internal standard (trichloromethane-d1) was higher than the concentration of the other analytes, the corresponding signal was significantly higher as well. But as it can be observed, the increase of the signal area with rising temperature of the headspace oven close to linear with all the halogenated compounds. This meant that the temperature of the HS oven could have been heated even higher, although technical specifications might have limited this. Heating to 140 °C and equilibrating this temperature already took quite long compared to lower temperatures. The next group of analytes which were investigated were aromatic compounds. In contrast to halogenated hydrocarbons, these could occur frequently in natural products. The change in signal area with increasing temperature is shown in Figure 28.

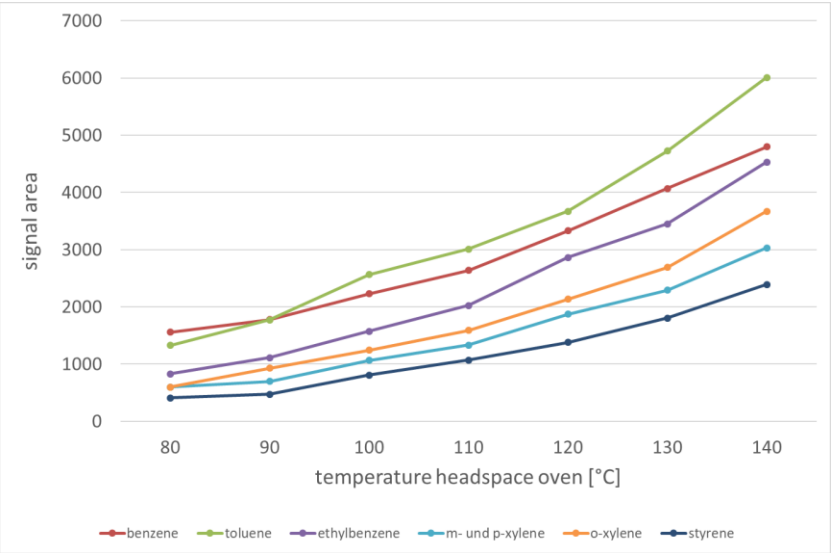

Figure 28: The signal area of aromatic compounds increasing with higher headspace oven temperature in pumpkin seed oil which had been spiked with a nominal concentration of 0.25 mg kg^{-1} of each analyte

As with the halogenated analytes in Figure 27 the signal area increased almost linearly with rising headspace oven temperature. However, the slope of the lines seemed to steepen at temperatures beyond 130 °C. Higher temperatures seemed to be able to force more of the volatile analytes in the samples into the gaseous phase. These results however were not to be given too much weight regarding reaching the equilibration point since all the samples had been conditioned for the same period. Figure 29 shows the development of the signal areas of short alkanes and oxygen-containing analytes.

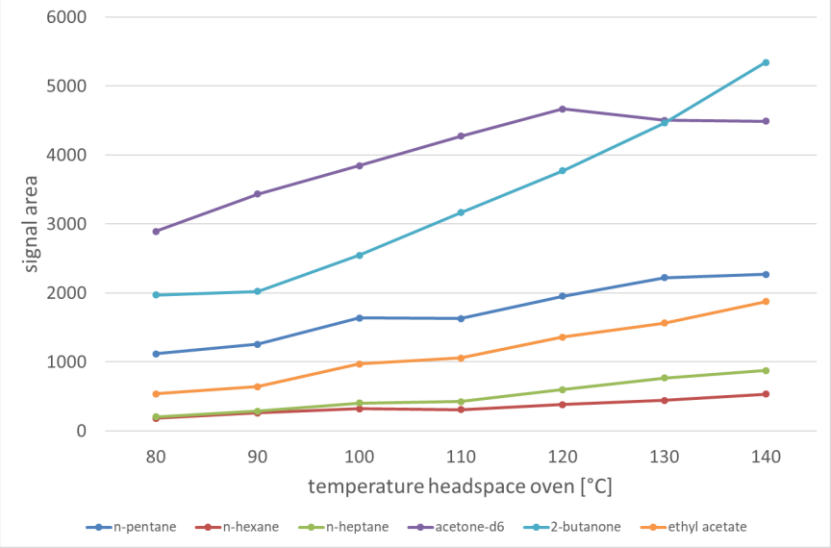

Figure 29: The signal area of short alkanes and oxygen-containing compounds increasing with higher headspace oven temperature in pumpkin seed oil which had been spiked with a nominal concentration of 0.25 mg kg^{-1} of each analyte

The signal area of most compounds seemed to increase steadily with the temperature. An exemption was the deuterated acetone whose signal intensities even decreased above 120 °C. It was difficult to interpret this behaviour but it might have been some sort of saturation effect in the gas phase. The results of the spiked samples indicated that in all cases a higher temperature caused higher signal intensities and therefore better sensitivity To see whether the high temperatures influenced the sample matrix itself, the non-spiked samples were investigated more closely. There were no halogenated compounds detected in any of the samples, with the exemption of dichloromethane. However, this was a contamination caused by the laboratory environment. It had to be accounted for in the quantitative evaluation of the sample measurements by subtracting blank signals from the sample signals. The results of the non-halogenated compounds in the spiked samples, showed no suspicious data. Many of these compounds could occur naturally in oils and other food stuffs. Some of

the analytes were detected in the pure, untreated pumpkin seed oil sample as well. Figure 30 shows the change in signal area for the aromatic compounds in the non-spiked sample matrix.

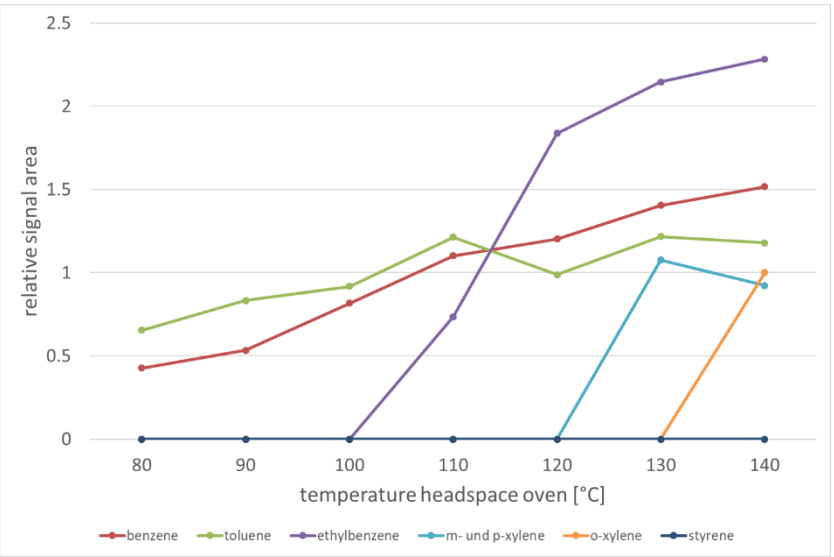

Figure 30: The relative signal area of aromatic compounds increasing with higher headspace oven temperature in pure, non-spiked pumpkin seed oil

The sensitivity as well as the abundance of the individual aromatic analytes in the sample vary significantly. Therefore, the signal area of each temperature level was divided by the average signal over all the measurements for each analyte to make them comparable (Figure 30). However, this also leads to large exaggerations of the signal intensities of some of the compounds, and careful consideration is advised. It shows that toluene, which is quite a common substance in vegetable oils, increases steadily in signal area up to 110 °C. Then the concentration in the head space stabilises. Ethylbenzene which is a frequently occurring substance too is only detectable beginning at 110 °C and the signal increases a lot up to 120 °C. The curve than flattens. At higher temperatures xylenes appear as well although only with very low intensity. The curves in Figure 28 have already shown that xylenes are already detectable with lower headspace oven temperatures. Their appearance at 130 °C and 140 °C therefore seems suspicious and there shouldn't be a need to heat the sample to such temperatures to enable their detection. Although benzene seems to increase steadily, it must be mentioned that the system which was used can detected it at very low concentrations already. The measured signal is far below the calculated LOD and therefore of no concern. This data overall would suggest an oven temperature of approximately 120 °C. This setting would extract a large part of the analytes without altering the sample which might have caused the traces of xylenes at 130 °C and above. Further considerations on this can be made when looking at Figure 31 which shows the behaviour of various analytes in the pure, non-spiked sample with increasing headspace oven temperature.

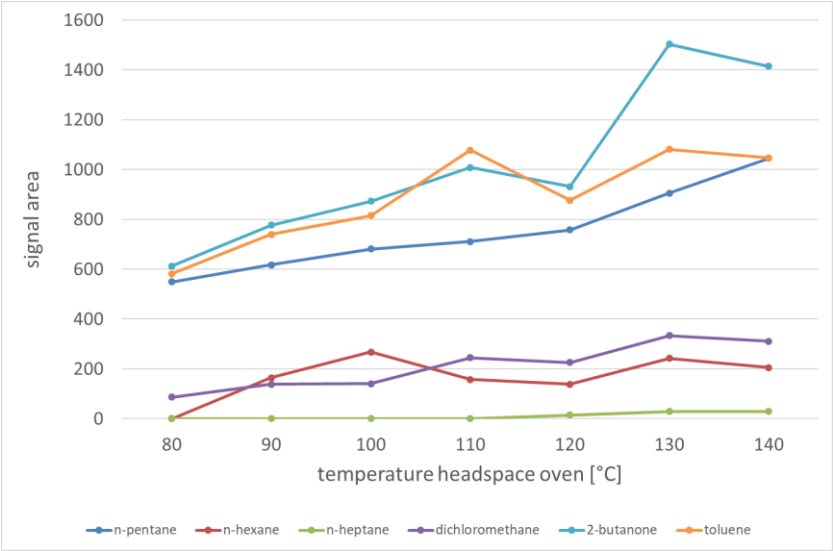

Figure 31: The signal area of various compounds at increasingly higher headspace oven temperature in pure, non-spiked pumpkin seed oil

All the substances depicted in the graph are typical for vegetable oil measurements, although dichloromethane is a contamination which is very common and difficult to avoid in an analytic laboratory environment. However, the fact that it does not occur naturally in the oil made it useful for comparing it to the results of substances which do naturally occur in the oil like n-pentane, toluene, and others. The behaviour of the graphs of dichloromethane, toluene, 2-butanone, and n-hexane with increasing oven temperature looked very similar from 110 °C onwards. Up to that temperature the signals rose steadily and then fluctuated without a certain direction which could have meant the establishing of a saturation effect in the gas phase. The concentration of n-heptane, which was very low, increased up to 130 °C and then stabilised. The signal intensity of n-pentane on the other hand rose steadily up to 120 °C and then the slope steepened again without any signs of stabilisation. This might have indicated that there was some influence of the matrix onto the concentration of that compound. This confirmed the assumption of the previous graphs, which had said that the right temperature of the oven was 120 °C. Over the equilibration time of 15 min enough analyte was driven into the gas phase to enable reliable and sensitive detection but there was no significant influence of the high temperature onto the results yet. Therefore, 120 °C had been chosen as headspace oven temperature for all measurements. Previous measurements regarding the equilibration time at a temperature of 120 °C had already shown that the equilibration point for most of the analytes was reached much earlier, even before 10 minutes. These results fell in line with the headspace autoinjector settings which were suggested by M. Michulec and W. Wardencki (Michulec & Wardencki, 2005). Over the course of the project, calibrations had been made with both, different pumpkin seed oil as well as multiple olive oils and the peak areas as well as the differences between the areas of the various calibration levels had been consistent. This showed that any degradation of the oils was either

similar in extent or did not yet occur at the selected conditions. The latter assumption was supported by the experiments above. Further experiments regarding the sample amount could have been done as well. However, it had not been seen as critical at the beginning of the project since the sensitivity of the method had met the requirements. However, there would have been further potential for optimisation.

3.3 Comparative measurements using SPME-GC/MS

As described in chapter 2.8, a comparative experiment had been designed using SPME-GC/MS technology. The aim had been to confirm the results from the HS-GC/MS measurement and to compare the two methodological approaches. The measurements had been conducted at the Institute of Analytical Chemistry and Food Chemistry at the University of Technology in Graz.

All the analytes which had been spiked into the samples could be identified in the measurements. Due to differences in the stationary phase and the temperature program of the GC method, the retention times and the elution sequence varied compared to the measurements using HS-GC/MS. Figure 32 shows the chromatogram of the extracted SIM signals.

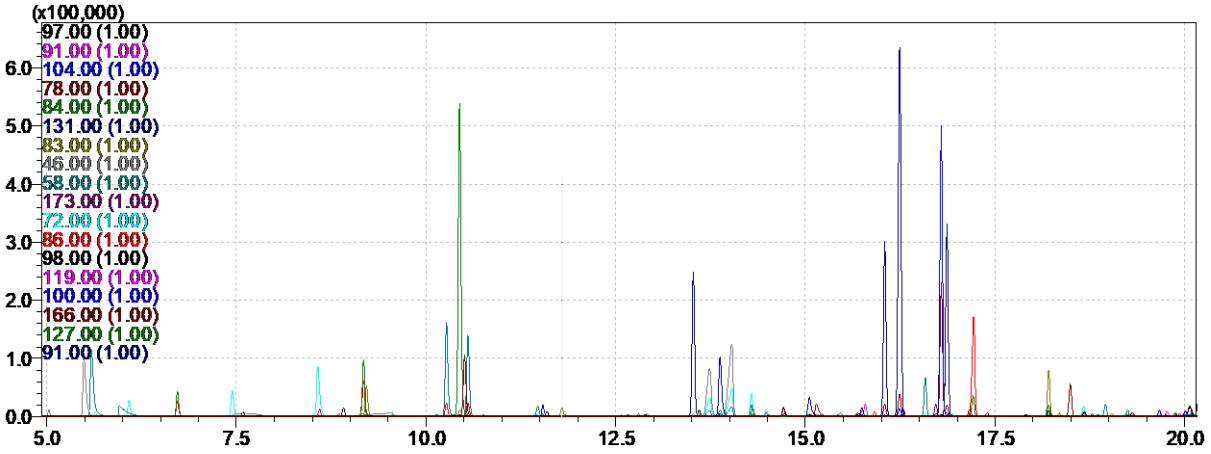

Figure 32: Chromatogram (SIM) of volatile hydrocarbons in a pumpkin seed oil measured with SPME-GC/MS

The chromatographic resolution was more than satisfactory and every signal could easily be identified. The signal to noise ratio (S/N) which was calculated automatically in the GCMSsolutions software was used to extrapolate the LOD and the LOQ, with the assumption of the LOD being three times the S/N and the LOQ being nine times the S/N. This was done for data obtained in the scan mode as well as the SIM mode. However, not all SIM traces for all the substances had been recorded, therefore the LODs calculated from the SIM measurements are incomplete. The real concentrations in the sample which had a nominal concentration of 0.1 mg kg^{-1} were taken into account. The results are listed in Table 12.

Table 12: Calculation of LOD and LOQ based on the S/N values of the SPME-GC/MS measurements of pumpkin seed oil spiked with a nominal concentration of 0.1 mg kg^{-1} of every analyte

			Scan data			SIM data	
Substance	**RT [min]**	**Calc. conc. [µg kg^{-1}]**	**S/N**	**LOD [µg kg^{-1}]**	**LOQ [µg kg^{-1}]**	**S/N**	**LOD [µg kg^{-1}]**
1,1,1,2-Tetrachloroethane	15.744	77.2	239	1	3	1471	0.2
1,1,1-Trichloroethane	10.035	4.83	24	0.6	2	37	0.3
1,1,2-Trichloroethane	13.6	70.6	85	2	7	108	2
1,1-Dichloroethane	7.593	111	395	0.7	3	2826	0.1
1,2-Dichloroethane	10.129	124	123	3	9	**No SIM data**	
2-Butanone	8.578	398	213	6	20	424	3
Acetone	5.599	782	962	2	7	984	2
Acetone-D6	5.495	866	6977	0.3	1	55780	0.05
Benzene	10.508	87.1	1820	0.1	0.4	736	0.3
Benzene-D6	10.443	948	2403	1	4	**No SIM data**	
Bromodichloromethane	11.792	97.2	793	0.3	1	2600	0.1
cis-1,2-Dichloroethene	8.913	127	820	0.3	1	6920	0.06
Dibromochloromethane	14.307	119	903	0.3	1	5376	0.07
Dichloromethane	6.728	658	1734	1	3	625	3
Ethyl acetate	9.151	885	3933	0.7	2	**No SIM data**	
Ethylbenzene	16.045	86.1	11308	0.02	0.07	26665	0.01
m-/p-Xylene	16.241	85.1	2198	0.1	0.3	2453	0.1
n-Heptane	10.132	168	68	6	20	475	1
n-Hexane	8.856	314	168	6	20	542	2
n-Pentane	5.643	624	291	6	20	3061	0.6
o-Xylene	16.864	87.1	8031	0.03	0.1	13378	0.02
Styrene	16.788	90.1	18914	0.01	0.04	15778	0.02

Tetrachloroethene	14.713	80.7	812	0.3	0.9	8629	0.03
Tetrachloromethane	10.529	79.6	264	0.9	3	2626	0.09
Toluene	13.522	86.1	11524	0.02	0.07	32939	0.007
trans-1,2-Dichloroethene	7.591	123	413	0.9	3	2382	0.2
Tribromomethane	16.718	143	912	0.3	1	10996	0.03
Trichloroethene	11.592	72.6	720	0.3	0.9	5959	0.03
Trichloromethane	9.216	36.6	1812	0.06	0.2	9040	0.01
Trichloromethane-D1	9.177	1500	817	0.5	2	68817	0.07

The S/N values of the measurements obtained in the SIM mode were up to 12 times higher than the values from the scan data. This was due to the longer time which the mass spectrometer was able to take for each ion. Since the data from the HS-GC/MS measurements had also been recorded in the SIM mode, a comparison had to be done with these measurements. However, not all S/N values increased with the use of the SIM mode, for example in the cases of benzene, dichloromethane, and styrene. Depending on the substance, the calculated LODs and LOQs of the SPME-GC/MS measurements were approximately 5 to 150 times lower than the ones which had been calculated for the HS-GC/MS method (Table 7 on page 32). This indicated that the SPME-GC/MS method was significantly more sensitive, which could be explained with the SPME sample preparation. The analytes were deposited on the SPME fibre and therefore concentrated. Another factor was the different temperature program of the GC oven. Since the SPME-GC/MS used a cryogenic program, many of the impurities which especially disturbed signals at the beginning of the run could be removed. Additionally, the separation of early eluting substances increased. However, the two methods which were used to calculate the LOD and LOQ were extremely different. The calculation for the HS-GC/MS method was based on the validation measurements using the calibration method. Therefore, the selected concentrations for the standards depended on the concentration range for which the method was intended to be used. If lower standards would have been used, the LOD values could have been lower as well. Additionally, the values which were calculated that way took blank signals into account, which were expected to occur in a normal laboratory environment. These blank signals were also noticed in the SPME-GC/MS experiment, but were not taken into account for the LOD and LOQ calculations based on the S/N. Other studies with the aim of comparing a HS-GC/MS method with a SPME-GC/MS method under comparable conditions came to similar conclusions regarding the sensitivity. The LOQ of the SPME-GC/MS method was lower than the LOQ of the HS-GC/MS method. However, the differences were not as drastic as in this comparison, which might have been due to more comparable GC methods. In

contrast to this study, the experiments by Ligor and Buszewksi had been conducted using flame ionisation detection, which was much more comparable. (Ligor & Buszewski, 2008) In this experiment the GC/MS which were used for the measurement were not only from two different companies (HP and Shimadzu) but also from very different technologic generations. The SPME-GC/MS was the significantly newer model. This difference in development might also have had an influence on the sensitivity of the detection system.

In addition to the calculation of the sensitivity of the SPME-GC/MS method, the results were also used to confirm the spectrum of typical substances found in a pumpkin seed oil. The non-spiked and the two spiked samples were used to calculate the slope and the intercept of the resulting straight-line function. This information was then used to calculate the intercept of the function with the abscissa which corresponded to the value of the concentration of an analyte in the non-spiked pumpkin seed oil. The blank signals which already had been mentioned earlier were subtracted from the signals of the sample measurements. The results can be seen in Table 13.

Table 13: Results of the comparative measurement of pumpkin seed oil with a SPME-GC/MS system based on a standard addition experiment

Substance	Slope	blank area	Intercept	Conc. [mg kg^{-1}]
2-Butanone	203469	0	44281	0.22
Acetone	120684	25355	85312	0.71
Benzene	1127532	24423	8083	0.007
Ethylbenzene	3031537	59	54831	0.018
m-/p-Xylene	7244505	13414	91947	0.013
n-Pentane	6104	1312	7615	1.3
o-Xylene	3371596	4984	44369	0.013
Toluene	2117468	40698	102874	0.049

The results are well within the expected concentrations which were also determined for other pumpkin seed oils that underwent a roasting step prior to the pressing process (chapter 3.1). With the consistency of the results which were determined with two separate methods and two entirely different analytic systems from different providers, the method could be determined to be valid. However, the results obtained by the SPME-GC/MS experiment are only approximate values since standard addition experiment work best if the added concentration are in the in the same concentration range as the concentrations in the sample. Since the concentrations which had been

added to the samples were not always in the optimal concentration range, some deviations must be expected. Repetitions of the measurements of the SPME-GC/MS experiment would have increased the significance of the results. However, the main aim of the experiment, which was the confirmation of the results of the HS-GC/MS results, could still be accomplished. The SPME-GC/MS approach is a viable if also more expensive one. HS-GC/MS is the simpler and more affordable method and the cryogenic conditions of the gas chromatograph add to both, separation quality but also increased financial effort and instrumental complexity.

3.4 Fatty acid profiles and their implications

The results of the vegetable oils which had undergone the heat conditioning experiment which was discussed in chapter 3.2.2 had shown an increase in the concentration of many volatile substances. The content of these substances had grown higher, the longer the oils had been heated. The increase in concentration however had depended strongly on the type of oil, even though all the oils had been treated the same way. This could only be explained with differences in the ingredients of the oils. Therefore, the profile of the fatty acids of all oils, which had undergone the thermal treatment was obtained. The analysis was conducted by an independent laboratory. Figure 33 shows the relative content of the most abundant fatty acids in the different oil samples.

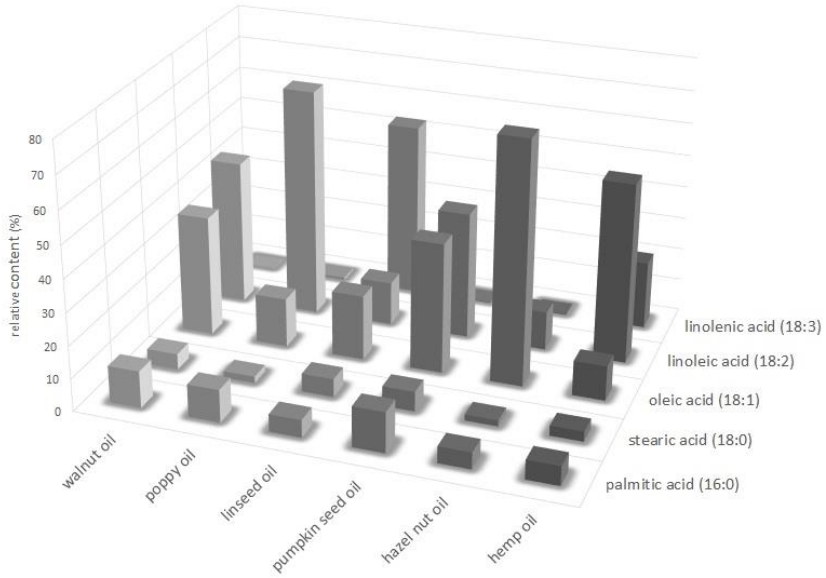

Figure 33: Fatty acid profile of the oils which had been subjected to the thermal treatment at 120 °C which was discussed in chapter 3.2.2

The comparison of the fatty acid distribution with the increase of volatile compounds after the thermal treatment enabled the observation of certain relations. The concentration of n-pentane after 24 hours of thermal treatment correlated with the relative content of linoleic acid, which, compared to other

oils, occurred in high abundance in hemp seed oil, poppy seed oil and walnut oil (Figure 34 and Figure 35). The concentrations of n-hexane and n-heptane, however, increased mainly in oils, which were rich in oleic acid, like the hazelnut oil. The relative content of the individual fatty acids in the oil thus led to a varying increase in volatile compounds, when treated with higher temperatures.

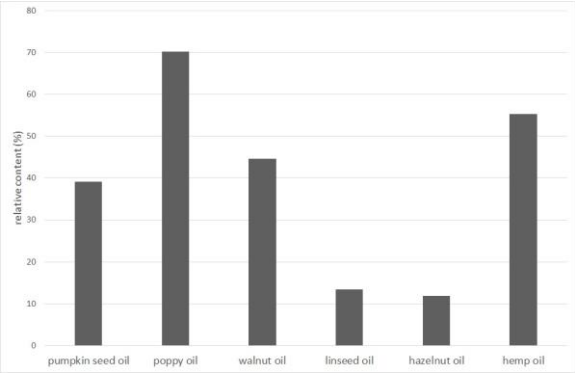

Figure 34: Relative content in linoleic acid in the investigated oils (relative to the total fatty acid content)

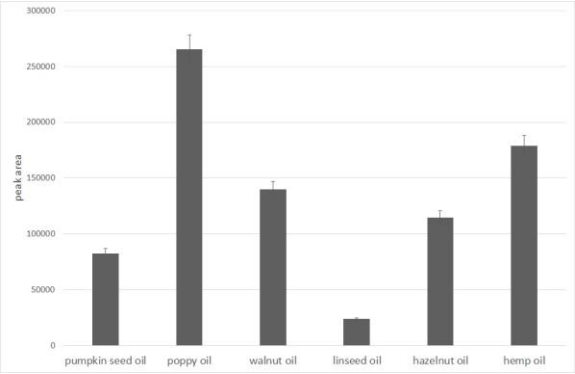

Figure 35: Content of n-pentane in different oil types after 24 hours of thermal treatment. (RSD <10%, n=3)

This phenomenon had also been observed in investigations regarding the pyrolysis of tropical oils, which usually only contain a small share of polyunsaturated fatty acids. The pyrolysis of piqui oil, which has an oleic acid content of around 50%, for example, yields n-heptane as the most abundantly formed, volatile compound (Alencar, et al., 1983). The results of the thermal conditioning experiment, however, could not be explained with the fatty acid profile alone. Other substances like antioxidants, which are present in unrefined vegetable oil, also influence the oil's behaviour. However, not only the overall ratio of fatty acids has an influence, but also the combination of fatty acids on each individual triacylglyceride. Triacylglycerides that carry three linoleic acid moieties (linolein) are significantly less stable, than triacylglycerides which carry only oleic acids (olein), or a mixture of both (Zeb & Murkovic, 2010). This effect could be observed in Figure 14 on page 46 since the concentration of n-pentane in pumpkin seed oil had increased only after several hours of heating, whereas it had raised almost immediately in poppy seed oil. Linseed oil on the other hand, although highly unsaturated, had formed very little n-pentane due to the fact, that it consisted of a low share of linoleic acid. Both, pumpkin seed oil as well as linseed oil, contain exceptionally high amounts of β-carotene which has a stabilising effect on the triacylglycerides (Rafalowski, et al., 2008). The immediate increase of the concentration of linear alkanes in hazelnut oil could be explained either by the ratio of different fatty acid groups carried by the glycerol, which was already discussed above, or by the possible lack of β-carotene or other antioxidants in the oil. Although hemp seed oil does contain large amounts of stabilising antioxidants (Yu, et al., 2004), it has only a small share of oleic acid, and a large one of linoleic acid (Figure 33), which increases the probability that glycerol carries three linoleic acid groups. Because of

their thermal instability and despite the large amount of β-carotene in the oil, they degrade quickly (Montserrat-de la Paz, et al., 2014). This might explain the early increase in volatile substances during heating (Figure 15 and Figure 16 on page 46).

3.5 Substance spectrum and production technology

The results of the thermal conditioning experiment had shown that some relations could be drawn between the formation of volatile substances in the oils and other parameters of the oils like the spectrum of fatty acids (chapter 3.4). For further investigations a statistical software was used - Agilent MassProfiler Professional. The sample files and their results (chapter 3.1) were imported into the software and multivariate data analysis enabled the comparison of the samples. The statistical analysis was based on the content of various volatile substances and their correlation with metadata like the exposure to high temperature over time, content of certain fatty acids, or the oil producer. The substances and corresponding concentrations which were used for the calculations are listed in Table 14.

Table 14: Substances which were used for the multivariate data analysis and the correlation with metadata

acetone	benzene	toluene
ethyl benzene	m- and p-xylene	o-xylene
styrene	2-butanone	ethyl acetate
n-pentane	n-hexane	n-heptane

The data was visualised using principal component analysis (PCA) plots. The PCA plot comparing the results of the temperature conditioning experiment is shown in Figure 36.

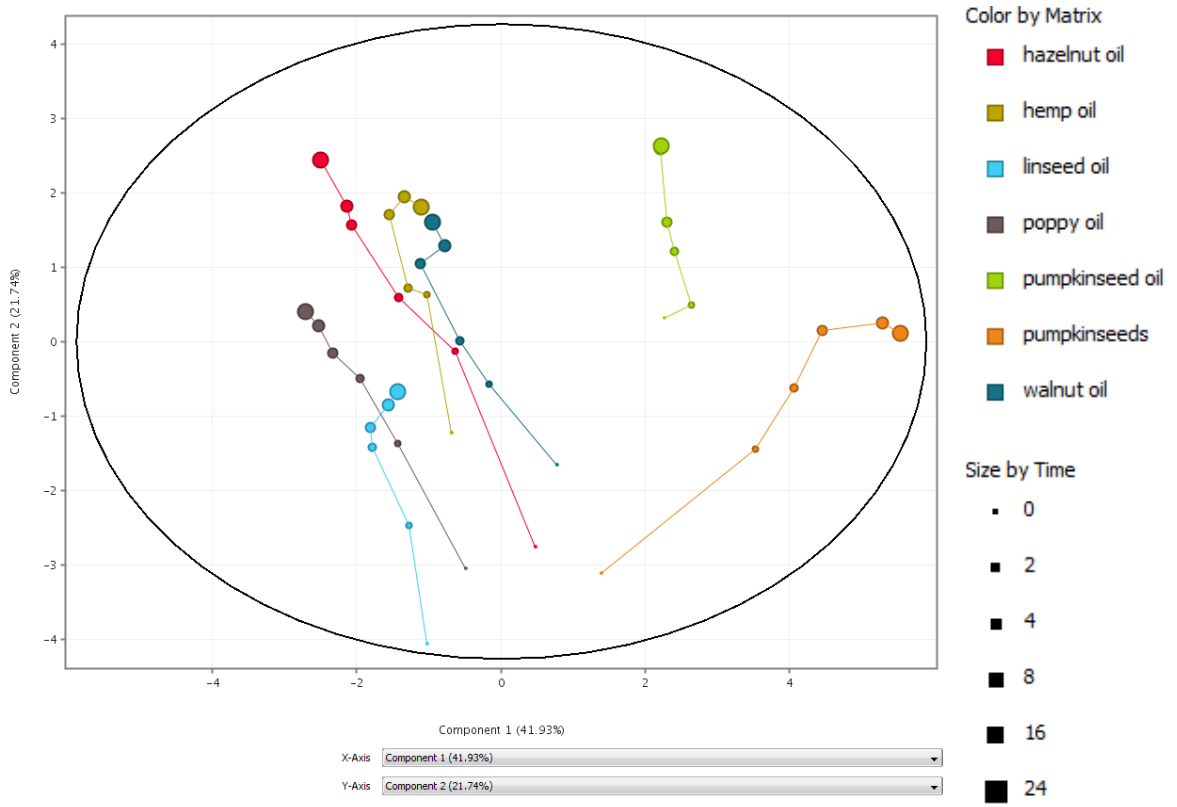

Figure 36: PCA plot comparing the results of the thermal conditioning experiment of different types of oils based on the spectrum of volatile substances and the change over time (from smallest to larges dot: 0, 2, 4, 8, 16, and 24 hours of thermal treatment)

The PCA plot clearly showed, that the oils had a different spectrum of volatile substances which continuously changed over time. The pumpkin seeds developed into a different direction regarding the formation of substances than the other oils. This was most likely due to plant materials in the seeds which had reacted under high temperature. The pumpkin seed oil however appeared to be in between the pumpkin seeds and the other vegetable oils. The composition of pumpkin seed oil is close to the other oils, but it has a darker colour than the other vegetable oils and contains some of the substances which differentiated the pumpkin seeds from the other oils. The pumpkin seed oils generally tended to have higher concentrations of the analysed volatile substances. This was reflected by the fact that the line originated from a different area than the other oils and the pumpkin seeds.

The differences between the pumpkin seed oil and the other oils could be caused by the lack of thermal treatment during the oil production process. The pumpkin seed oil however, had been subject to a roasting step before the pressing. Figure 34 and Figure 35 had already shown the relationship between some of the fatty acids in the vegetable oils and the occurring spectrum of volatile alkanes after heating. Figure 37 shows PCA plots with the results of the samples which had been subject to the

thermal conditioning experiment. They were compared regarding their content in oleic acid and linoleic acid in reference to the concentrations of n-pentane, n-hexane, and heptane.

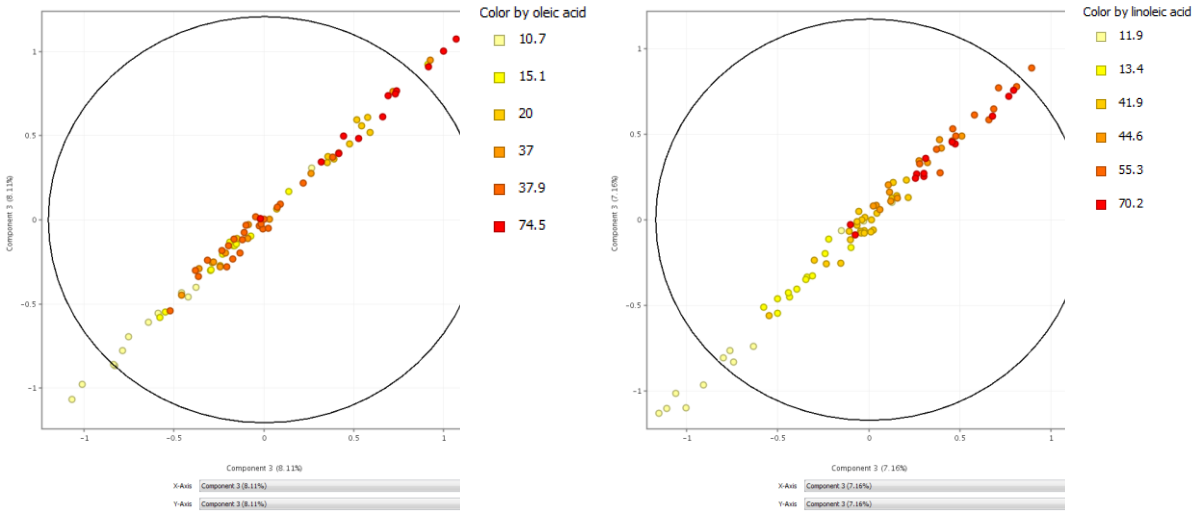

Figure 37: PCA plots of the alkane contents of the samples which were measured for the thermal conditioning experiment in reference to the relative contents of oleic acid (left) and linoleic acid (right)

There was significant correlation between the concentration of volatile alkanes in the oil samples and their respective amounts of linoleic acid (Figure 37, right). Vegetable oils with only a small share of linoleic acid, e.g. hazelnut oil, had lower concentrations of n-pentane, n-hexane, and n-heptane and had formed less of these substances under thermal stress. Oils with a high content in linoleic acid, like poppy seed oil or hemp oil, however showed a very significant increase in volatile alkanes when heated. A direct correlation of the degradation of this fatty acid at elevated temperature and the formation of volatile alkanes could be drawn. This behaviour was less significant when comparing different amounts of oleic acid in the samples (Figure 37, left). However, a trend was still visible with the high oleic acid oils gathering to the upper right of the graph. The linoleic acid which is more reactive than the oleic acid apparently had a higher tendency to form volatile alkanes. However, metadata which could be used to categorise the samples was not restricted to the type of oil or the corresponding shares of the fatty acids. The producers of the vegetable oils were known as well. The conditions which were used to produce the vegetable oils, usually vary from one oil mill to the other and have often been unchanged for many years. The formation of the volatile substances seemed to depend on factors like temperature and the time of exposure to these temperatures. Therefore, it appeared reasonable that the spectrum of volatile substances could be traced back to the producer and the respective production parameters. Again, the substances listed in Table 14 and the corresponding abundances in the samples were used for the comparison of the different producers. Although nine producers handed in samples for the project, only eight of them provided samples for

both sample sets. The corresponding PCA plots of the analysis are shown in Figure 38. Only pumpkin seed oils were used for the comparison, since the other oils were too different regarding their spectrum of substances to be clustered with the pumpkin seed oil.

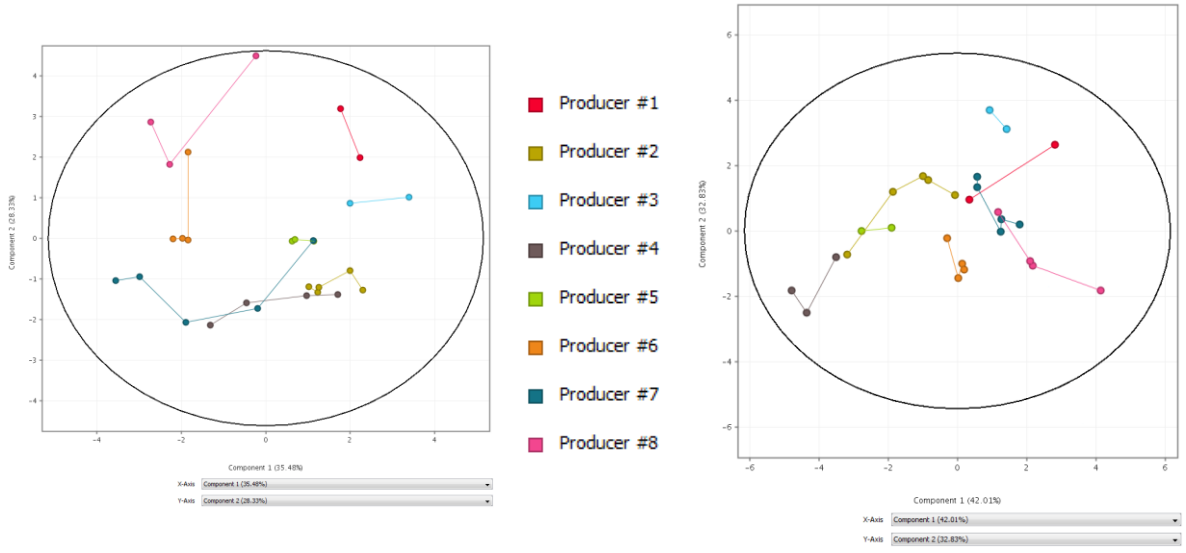

Figure 38: PCA plots of the relation between the spectrum of volatile substances in the samples and the producer of the oil. Only pumpkin seed oils were used from the first sample set (left) and the second sample set (right).

The results from both sample sets show clustering of the samples based on the producer of the oil. The separation between the sample clusters vary in significance, but very clear predictions could still be made. Producer #6, for example, could always be clearly differentiated from producers #2, #3 and #5. This held true for both sample sets although a period of six months had separated the measurements. Limitations regarding the evaluation software prevented the comparison of both sample sets in a single PCA plot, however the results were significant on their own. The measurement of volatile substances which had formed during the production process of the sample could therefore not only enable the differentiation of different oil seeds but also distinguish different oil producers and production technologies. The experiment also proved, that the initial claims, that contaminations had caused the presence of the substances, could not be confirmed.

4 Summary and conclusion

The project which spanned over a year of method development and optimisation, sample measurement and further investigations regarding thermal conditioning, has changed and transformed over this time. The oil producers had initially been suspicious of the results from various food laboratories and had claimed that the solvent residues were artefacts. Statements from these food analysis laboratories had made contamination responsible for the findings and had advised the oil producers to take better care regarding their production conditions and environmental influences. With the development of a sensitive HS-GC/MS method which was capable of reliably detecting the substances in the oil, the existing analysis reports could be confirmed. The first goal of the project could therefore be fulfilled, and the still existing suspicions of false-positive results from the various food laboratories could finally be resolved. However, the inherent urgency of the situation had led to putting the emphasis of the method development on impeccably identifying the analytes to avoid any false-positive results. A lot of effort was therefore put on setting up a reliable mass spectrometric method and to have a good chromatographic separation. Less thought was put into the optimisation of the headspace method and most of the parameters were taken from literature. The method was put through an extensive method validation and proved to be very reliable, robust, and sufficiently sensitive for the aims of the project (chapter 0). Additionally, it had not yet been known at this point that the project would be used as topic for a master thesis and had initially not had scientific intentions. The approach to the method development would have put more emphasis on the optimisation of the headspace method if this would have already been known. However, the experiments which were conducted later regarding the influence of the headspace oven temperature on the sample had shown that the selected headspace conditions were well chosen (chapter 3.2.3). Therefore, the results of both sample sets which had been acquired using the developed method could be regarded as reliable which was confirmed by the low deviation of the three repetitions (chapter 3.1). Additionally, a complementary experiment using an SPME-GC/MS system and an entirely different method and temperature program confirmed the results which were found to be typical for pumpkin seed oil (chapter 3.3). Any doubts that the substances were indeed present in unrefined vegetable oils and pumpkin seed oils were resolved. All analysed samples contained at least traces of volatile hydrocarbons, whereby no halogenated compounds were found. This would have been evidence for the use of solvents for the extraction of the oil because they do not normally occur in nature. Of the substances which were found, acetone, 2-butanone and n-pentane were detected in most of the samples in significant amounts, and toluene was found in many samples as well. The origin of these substances however had still not been entirely clear and since demonstrably no solvents had been used for the production of the oils, an alternate explanation had to be found for the occurrence of

these volatile organic compounds. The visualisation of the substance spectrum in each sample revealed specific patterns. Vegetable oils which had been produced by cold pressing, including truly cold pressed pumpkin seed oil, showed a different spectrum of volatile substances than the pumpkin seed oils which had been subject to roasting before the pressing. This step is usually conducted for approximately 60 minutes and employs temperatures up to 130 °C. This raised the suspicion that the roasting of the pumpkin seeds, which evidently led to the formation of many flavouring substances (Poehlmann & Schieberle, 2013), could also have caused the formation of the volatile compounds which would then have been mistaken for contaminants. A preliminary experiment which compared pumpkin seed oil under different environmental influences (storage in the dark, storage in the refrigerator, and storage in the sunlight) showed that the concentrations of linear, short-chained alkanes (n-pentane, n-hexane, n-heptane) and aromatics (toluene, benzene, xylene) increased over time. Based on these findings a thermal conditioning experiment was designed, for which various types of oils were heated to 120 °C for 24 hours. The periodically drawn samples showed clearly that the content of some of the analytes in the oil increased significantly. The thermal degradation of triacylglycerides, resulted in the formation of many, partly volatile compounds, which were enriched in the oil. This could lead to potentially wrong interpretations regarding the production method of the oil. The amount, as well as the ratio of the resulting volatile substances were dependent on the type of the oil and thus on the fatty acid profile. However, other factors could influence the formation of these substances as well, like the ratio of different fatty acid groups, which are carried by a single glycerol entity. Triacylglycerides that carry three oleic acid groups, have significantly higher thermal stability than triacylglycerides, which carry three linoleic acid groups. The presence of antioxidants, like β-carotene could influence the spectrum of resulting volatile compounds too. Other catalytically active compounds however, could also accelerate the degradation of triacylglycerides. Besides linear alkanes and aromatic compounds, the methyl ketones acetone and 2-butanone were present in the oils as well. Oxygen-containing compounds like acetone and 2-butanone could derive from decarboxylation of β-keto-acids. These are formed either during β-oxidation, or through the oxidation of aliphatic hydrocarbons (Forney & Markovetz, 1971). 2-Butanone had already been found in an earlier study about substances which are formed during the roasting process of pumpkin seeds (Siegmund & Murkovic, 2004) as well as during the roasting of rapeseeds (Gracka, et al., 2016). Although 2-butanone was found in most of the oils, which were investigated in the thermal conditioning experiment, no increase in the 2-butanone-concentration could be observed during the heating in any of the oils. However, the 2-butanonen content did increase in the crushed pumpkin seed sample, which led to the assumption that other compounds in the pumpkin seeds were responsible for the formation of 2-butanone. This applied for other oxygen-containing substances like acetone and ethyl acetate as well. Acetone as well as 2-butanone are also common metabolic products, which often

originate from microorganisms and are found regularly in nature (Forney & Markovetz, 1971). These processes however seemed not to happen in the pumpkin seed oil itself. Acetone and 2-butanonen are the ketones with the largest global annual production and are used in a large variety of cleaning supplies, coatings, and varnishing. They are also used for degreasing metal surfaces and for disinfecting medical tools and instruments (Hoell, et al., 2012). Therefore, some contamination could not be completely excluded as the cause for acetone and 2-butanone in the vegetable oil samples.

The substance spectrum did not only offer information regarding the type of oil but other influences as well. Since many of the substances were likely formed during the production of the oil, some correlations could be drawn to the production methods. The substances are usually formed when the raw materials are heated, either during the roasting process, or due to mechanical stress during the pressing. Multivariate data analysis of the substance spectrum showed that the vegetable oils from one producer could be clustered and therefore distinguished from other producers. This was further evidence for the claim that the production method did influence the formation of these substances. Analysing the spectrum of substances in the oil could be used to trace back oils to their origin and differentiate it from others. For this study, only substances which are industrially used as solvents were analysed. If the scope of the method would be expanded to other volatile substances, many of which are already known, this would enable new developments in the field of food authentication. Especially pumpkin seed oil, which is an PGI-item in DOOR and therefore a high-value food product, is prone to fraudulent intentions. Additional research in that field could provide the necessary methods and workflows. However, with the determination of the origins of the volatile substances, also the last point of the project had successfully been accomplished.

This master thesis shows how certain expectations and suspicions can lead into a false direction and influence the behaviour of whole production areas. Even professional opinion can often not correct for these wrong assumptions or, as in this case, even emphasise these wrong believes. Some of the errors which had been made during the method development would have been avoided if more time would have been spent on thinking about the nature of the product and its origins. Vegetable oil which is produced without refinement like pumpkin seed oil which has a centuries old tradition in Styria and Europe overall, is a natural products. Many regulations help natural products like these to guarantee their high quality and to protect them from fraud. However, one should be considerate with defining what is of natural origin and what is a contaminant.

5 References

Afaf, K.-E., 2003. *Lipid Oxidation Pathways.* 1. ed. Champaign, Illinois, USA: AOCS Press.

Alencar, J. W., Alves, P. B. & Craveiro, A. A., 1983. Pyrolysis of Tropical Vegetable Oils. *Journal of Agriculture and Food Chemistry*, pp. 1268-1270.

Baltes, W. & Matissek, R., 2011. *Lebensmittelchemie.* 7. ed. Heidelberg: Springer.

Belitz, H.-P., Grosch, W. & Schieberle, P., 2008. *Lehrbuch der Lebensmittelchemie.* 6. ed. Berlin Heidelberg: Springer-Verlag.

Cao, L. et al., 2013. Simultaneous Determination of Benzene and Toluene in Pesticide Emulsifiable concentrate by Headspace GC-MS. *Journal of Analytical Methods in Chemistry*, 27 February, pp. 1-5.

Chang, C.-C. & Wan, S.-W., 1947. China's Motor Fuels from Tung Oil. *Industrial and Engineering Chemistry*, pp. 1543-1548.

Chow, C. K., 2008. *Fatty Acids in Foods and their Health Implications.* 3. ed. Boca Raton: CRC Press.

deMan, J. M., 1999. *Principles of Food Chemistry.* 3. ed. Gaithersburg, Maryland, USA: Aspen Publishers, Maryland.

EFSA Panel on Contaminants in the Food Chain (CONTAM), 2013. *Scientific Opinion on Mineral Oil Hydrocarbons in Food,* Parma, Italy: Euopean Food Safety Authority (EFSA).

Forney, F. W. & Markovetz, A. J., 1971. The biology of methyl ketones. *Journal of Lipid Research*, pp. 383-395.

Frankel, E. N., 2012. *Lipid oxidation.* 2. ed. Cambridge, UK: Woodhead Publishing Limited.

Gemeinschaft Steirisches Kürbioskernöl g.g.A., 2018. *Steirisches Kürbiskernöl g.g.A.*. [Online] Available at: www.steirisches-kuerbiskernoel.eu [Accessed 7 February 2018].

Gracka, A. et al., 2016. Flavoromics approuach in monitoring changes in volatile compounds of virgin rapeseed oil caused by seed roasting. *Journal of Chromatography A,* pp. 292-304.

Gustav Heess GmbH, 2010. *gustav heess.* [Online] Available at: http://www.gustavheess.de/index.php?option=com_content&view=article&id=58&Itemid=84&lang=de [Accessed 05 Februar 2018].

Hoell, D. et al., 2012. 2-Butanon. *Ullmann's Encyclopedia of industrial chemistry*, pp. 431-444.

Krist, S., 2013. *Lexikon der pflanzlichen Fette und Öle.* 2. ed. Vienna: Springer.

Krist, S., Buchbauer, G. & Klausberger, C., 2009. *Lexikon der pflanzlichen Fette und Öle.* s.l.:Springer-Verlag.

Kubátová, A. et al., 2011. New path in the thermal cracking of triacylglycerols (canola and soybean oil). *Fuel*, pp. 2598-2608.

Kumar, N. & Gow, J. G., 1994. Residual solvent analysis by headspace gas chromatography. *Journal of Chromatography*, pp. 235-240.

Ligor, M. & Buszewski, B., 2008. The comparison of solid phase microextraction-GC and static headspace-GC for determination of solvent residues in vegetable oils. *Journal of Separation Science*, pp. 364-371.

Matsui, T., Guth, H. & Grosch, W., 1998. A comparative study of potent odorants in peanut, hazelnut, and pumpkin seed oils on the basis of aroma extraction dilution analysis (AEDA) and gas chromatography-olfactometry of headspace samples (GCOH). *European Journal of Lipid Science and Technology*, pp. 51-56.

Michulec, M. & Wardencki, W., 2004. Determination of Solvents Residues in Vegetable Oils and Pharmaceuticals by Headspace Analysis and Capillary Gas Chromatography. *Chromatographia*, pp. 273-277.

Michulec, M. & Wardencki, W., 2005. Development of headspace solid-phase microextraction-gas chromatography method for the determination of solvent residues in edible oils and pharmaceuticals. *Journal of Chromatography A*, pp. 119-124.

Montserrat-de la Paz, S., Marín-Aguilar, F., García-Giménez, M. D. & Fernández-Arche, M. A., 2014. Hemp (Cannabis sativa L.) Seed Oil: Analytical and Phytochemical Characterization of the Unsaponifiable Fraction. *Journal of Agricultural and Food Chemistry*, pp. 1105-1110.

Moreda, W., Pérez-Camino, M. C. & Cert, A., 2001. Gas and liquid chromatography of hydrocarbons in edible vegetable oils. *Journal of Chromatography A*, pp. 159-171.

Nawar, W. W., 1969. Thermal Degradation of Lipids. A Review. *Journal of Agricultural and Food Chemistry*, pp. 18-21.

Österreichisches Bundesministerium für Gesundheit, 2012. Codexkapitel / B 30 / Speisefette, Speiseöle Streichfette und andere Fetterzeugnisse. In: *Österreichisches Lebensmittelbuch.* Wien: Österreichisches Bundesministerium für Gesundheit, pp. 4-5.

Österreichisches Normungsinstitut, 2005. *Tierische und pflanzliche Fette und Öle - Bestimmung von niedrig siedenden halogenierten Kohlenwasserstoffen in Speiseölen,* Wien: Österreichisches Normungsinstitut.

OVID 2015, 2014. *OVID, Verband der Ölsaaten-verarbeitenden Industrie in Deutschland.* [Online] Available at: http://www.ovid-verband.de/index.php?id=370

Ozel, M. Z. et al., 2014. Effect of roasting method and oil reduction on volatiles of roasted Pistacia terebinthus using direct thermal desorption-GCxGC-TOF/MS. *LWT - Food Science and Technology,* pp. 283-288.

Parker, T. D. et al., 2003. Fatty Acid Composition and Oxidative Stability of Cold-pressed Edible Seed Oils. *Food Chemistry and Toxicology*, pp. 1240-1243.

Pinnel, V. & Vandegans, J., 1996. GC-MS headspace Analysis of the Volatile Components of Soya Oil without Heating the Sample. *Journal of High Resolution Chromatography*, pp. 263-266.

Poehlmann, S. & Schieberle, P., 2013. Characterization of the Aroma Signature of Styrian Pumpkin Seed Oil (Cucurbita pepo subsp. pepo var. Styriaca) by Molecular Sensory Science. *Journal of Agricultural and Food Chemistry*, pp. 2933-2942.

Prest, H. & Peterson, D. W., 2001. *New Approaches to the Development of GC/MS Selected Ion Monitoring Acquisition and Quantitation Methods,* Palo Alto: Agilent Technologies, Inc..

Rafalowski, R., Zegarska, Z., Kuncewicz, A. & Borejszo, Z., 2008. Fatty Acid Composition, Tocopherols and b-Carotene Content in Polish Commercial Vegetable Oils. *Pakistan Journal of Nutrition*, pp. 278-282.

Roth, L. & Kormann, K., 2000. *Ölpflanzen, Pflanzenöle - Fette, Wachse, Fettsäuren, Botanik, Injahltsstoffe, Analytik.* Landsberg: ecomed verlagsgesellschaft.

Schwab, A. W. et al., 1988. Diesel Fuel from thermal Decomposition of Soybean Oil. *Journal of the American Oil Chemists' Society,* Volume 65, pp. 1781-1786.

Siegmund, B. & Murkovic, M., 2004. Changes in chemical composition of pumpkin seeds during the reasting process for production of pumpkin seed oil (Part 2: volatile compounds)^. *Food Chemistry*, pp. 367-374.

Taghvaei, M. & Jafari, S. M., 2015. Application and stability of natural antioxidants in edible oils in order to substitute synthetic additives. *Journal of Food Science and Technology*, pp. 1272-1282.

The European Commission, 1996. *Database for Origin & Registration, DOOR.* [Online] Available at:

http://ec.europa.eu/agriculture/quality/door/list.html;jsessionid=pL0hLqqLXhNmFQyFl1b24mY3t9dJQPflg3xbL2YphGT4k6zdWn34%21-370879141?&recordStart=0&filter.dossierNumber=&filter.comboName=&filterMin.milestone__mask=&filterMin.milestone=&filterMax.milestone_
[Accessed 05 May 2017].

The European Commission, 2012. *Commission Regulation (EC) No 29/2012: on marketing standards for olive oil,* Brussels: European Commission.

The European Commission, 2016. *Commission Regulation (EEC) No 2568/91: on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis,* Brussels: European Commission.

The European Commission, 2017. *Commission Recommendation (EU) 2017/84 on the monitoring of mineral oil hydrocarbons in food and in materials and articles intended to come into contact with food,* Brussels: Official Journal of the European Union.

The European Parliament and the Council of the European Union, 2009. *Directive 20097327EC of the European Parliament and of the Council on the approximation of the laws of the Member States on extraction solvents used in the production of foodstuffs and food ingredients,* Strasbourg: Official Journal of the European Union.

Timmermann, F., 1990. Tocopherole - Antioxidative Wirkung bei Fetten und Ölen. *Fat Science and Technology*, pp. 201-206.

Yu, L. L., Zhou, K. K. & Parry, J., 2004. Antioxidant properties of col-pressed black caraway, carrot, cranberry, and hemp seed oils. *Food Chemistry*, pp. 723-729.

Zeb, A. & Murkovic, M., 2010. Characterization of the effects of β-carotene on the thermal oxidation of triacylglycerols using HPLC-ESI-MS. *European Journal of Lipid Science and Technology*, pp. 1218-1228.

Zhang, W. et al., 2016. Changes in volatiles of palm kernel oil before and after kernel roasting. *LWT - Food Science and Technology,* pp. 432-441.