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Development of an analytical method for the screening of meaty flavors in commercial foodstuff by SPME-GC/O-MS

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I Abstract

The aim of the project was to develop a methodology for the chromatographic analysis able to identify the key odorants in meaty food products. The approach was based on the analysis of an already in market product and the theoretical background of the meaty flavors sensorially. Firstly, a custom-made flavor was created for the experiments covering as much as possible a broad area of compounds precipitated as meaty odors. Secondly, the extraction method was optimized to obtain a rich in compounds headspace. The extraction method used was the solid phase micro-extraction (SPME). Finally, the SPME-GC/O-MS method was holistically optimized using the previously identified odorants as target compounds.

The tested parameters were eight. Specifically, four different fibers and three different values for sample quantity, solute quantity, solute's salt concentration, incubation temperature, incubation time, exposure time, desorption temperature. The procedure consisted of the preparation of the samples within their respective quantities of solute and sample. Also, the gas chromatography methods were changed, in order to examine the different parameters.

The results of GC-olfactometry revealed the presence of eighteen odors. More than half of the odorants were successfully identified from all three identification parameters, exactly ten odorants. Among them 1-Octen-3-one (mushroom-like), 2-Methyl-3-furanthiol (meat-like) 2-Furfurylthiol (coffee), 2-Methoxyphenol (smoky), *(E)*-2-nonenal (fatty, greeny), dimethyltrisulfide (cabbage-like), Furaneol (caramel), 2-Ethyl-3,6-dimethylpyrazine (roasty) and 2-Ethyl-3,5-dimethylpyrazine (roasty). The majority of odorants are either Maillard reaction products or lipid degradation products.

Combination of SPME with GC/O-MS allowed to identify 10 out of 18 detected odorants. These odorants together were used as marker compounds for the optimization of the SPME method. The optimization was done in two steps: First the sample preparation, incubation time, incubation temperature, fiber type, exposure time and desorption temperature were selected. In order to optimize incubation time (10,15,20 min), incubation temperature temperature (40,60,80°C), exposure time (10,20,30min), desorption temperature (100,160,220°C), a fractional factorial design was used.

Analyzing the results, is revealed that both the number of detected compounds as well as their peak area are mainly affected by fiber type, sample preparation, incubation time and temperature. The best results were generally achieved with PDMS/CAR/DVB 2cm fiber after hydration of the sample with water (20% of sodium chloride). The peak areas significantly

increased with incubation time and temperature. The majority of compounds showed maximum peak areas after incubation for 10 min at 80°C (5 out of 10) or 15 min at 80°C (3 out of 10).

II Acknowledgements

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III Abbreviations

AEDA aroma extract dilution analysis

DVB/CAR/PDMS divinylbenzen/carboxen/polydimethylsiloxane

FD-factor flavor dilution factor

GC gas chromatography

GC-O gas chromatography-olfactometry

HPLC high pressure liquid chromatography

LC liquid chromatography

LOX lipoxygenase

MS mass spectrometry

RI retention index

SAFE solvent assisted flavor evaporation

SDE simultaneous distillation extraction

SPE solid phase extraction

SPME solid phase micro extraction

IMP Inosine Monophosphate

GMP Guanosine Monophosphate

T1R1 One Transmit Antenna, One Receive Antenna

T1R3 One Transmit Antenna, Three Receive Antenna

OAV Odor Activity Values

GHARM Combined Hedonic Aroma Response Measurement

FD Flavor Dilution

ppt Parts per trillion

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1 Introduction

Throughout the years flavors haves been tried to be explored in detail. Specifically, for the meaty flavors. Analytical chemistry is applied in sciences (biology, chemistry, geology) but in industry (chemical engineering) and medicine (clinical chemistry) as well. Specifically, it is used to quantify or identify or separate and any possible combination of these methods (Skoog, 2014). Firstly, with the separation method the analyte is separated from the sample. Secondly, to identify an analyte qualitative analysis is used. Thirdly, in order to determine the quantity or concentration. Analytical methods are separated to classic (wet chemical methods) and the modern (instrumental methods) (Skoog, Holler and Crouch, 2007). Chromatography is one of the instrumental methods and it is separated further to liquid and gas chromatography (Poole, 2000). The principal idea behind these techniques is the same, to separate a compound from a mixture. The type will be used depends on the compound should be separated from its mixture.

In general, the method of identification of odor active compounds in food products is strongly dependent on the development of research techniques such as isolation, detection and identification of them in volatile mixtures.

The gas chromatography- olfactometry (GC/O) is the most commonly used method (Poole, 2000) for odor active compounds. It combines the sensitivity of the human nose with the compounds' separation of GC, which provides an idea of the volatiles and their contribution in the food product aroma-taste profile. Also, using the mass spectroscopy (MS) can quantify these volatiles and possibly find their thresholds (Tao et al., 2014).

Aromas and flavors are a field with high importance. They could be used in many different fields. For instance, cosmetics and food industry. Specially in food industry the last years, the need for natural elements in food products is high importance. The reason could be current recipes' renovation, launch of new products etc.

The first aim of the study was to gain a deeper insight into the composition of aroma active volatiles creating the meaty flavor, determined mainly by roast and meaty notes. Following, aim of this project is to develop an analytical method for screening the meaty flavors of commercial food products. There are several techniques for isolating compounds in foods. For instance, liquid-liquid extraction (LLE), solid-liquid extraction (SLE), solvent assisted flavor evaporation (SAFE), solid phase extraction (SPE), solid phase micro extraction (SPME). Among these techniques SAFE and SPME are especially interesting as high throughput and

volatile compounds isolating techniques. In contrast with SAFE, SPME is quite environmentally friendly as no solvents used. The isolation of volatiles using SPME needs extensive method development as the efficiency of the extraction depends on many parameters including polarity and volatility of analyzed compounds, sample preparation (dry vs. wet, pH) (Ma et al., 2013), type of fiber coating (Lecanu et al., 2002), extraction conditions (incubation time, extraction temperature/time, agitation) (Ma et al., 2013), and desorption conditions (time, temperature) (Ma et al., 2013). Finally, several trials were performed in order to understand the impact of different SMPE parameters' combinations are possible and as the different parameters may interact several trials were performed in order to understand the impact, they have on the aroma active volatiles generated during cooking.

2 Theoretical Background

2.1 Flavor and Aroma

2.1.1 Flavor

Flavor is the characteristic sensory sensation of aroma and taste of a substance (drink or food) making it recognizable (Flavor | meaning in the Cambridge English Dictionary, 2020). This combined sensation occurs due to the chemical irritation in the mouth and nose. Specifically, taste is the sense perceived in the taste receptors of the tongue (Taste bud | anatomy, 2020). As for the aroma, it is the sense the smell perceives from the olfactory receptors in nose (Olfactory receptor | anatomy, 2020).

2.1.2 Meat Flavor

Meat flavor is thermally produced, since uncooked meat has only a blood-like taste and more less no aroma. Only after cooking and a series of thermal reactions does meat become flavorsome, which occur between different non-volatile compounds of the lean and fatty tissues (Calkins and Hodgen, 2007; Mottram, 1998). Hundreds of odiferous volatiles in combination with the basic flavors including “Umami” are forming what is known as “meaty” flavors. Nowadays, there has been discovered more than 1000 flavor volatiles, which are closely connected with the meat (Melton, 1999). Although, only a few have the “meaty” aroma the impact of sulfur containing compounds in meat aroma was of main importance, but the contribution of other aroma compounds should not be underestimated due to possible synergisms among aroma components (O’Sullivan and Kerry, 2012). The majority of, the precursors include cysteine reacting with reducing sugars during Maillard reaction and products from thiamin thermal degradation (Tai, Yang and Ho, 1999). The broad array of flavor compounds found in meat includes hydrocarbons, aldehydes, ketones, alcohols, furans, thriphenes, pyrrols, pyridines, pyrazines, oxazoles, thiazoles, sulphurous compounds and many others (MacLeod, 1994). Sulphurous compounds exist in low concentrations, but their very low odor thresholds make them potent aroma compounds and high contribution to cooked meat aromas. (Mottram, 1998). Sulphurous, onion-like and, sometimes, meaty aromas derive from the Many of these sulphur compounds contribution in aroma profile (Fors, 1983). The presence of heterocyclic compounds such as thiazoles, pyrazines and oxazoles associates roast flavors in foods. (Mottram, 1998).

2.1.3 Meaty taste

Taste is one of the five human sense and being critical for the food quality perception (Briand and Salles, 2016). As meaty taste is described as the savory taste called Umami. Basic tastes are 5 and Umami is one of them. Generally, defined as the taste of the monosodium glutamate (MSG) and 5'-ribonucleotides. For instance, sodium salts of 5'adenosine monophosphate (AMP), 5'-inosine monophosphate (IMP) and 5'guanonsine monophosphate (GMP) (Fuke and Shimizu, 1993). Specifically, MSG has been extensively used in the food supply as a sodium salt or exemplified to other glutamate-rich ingredients such as hydrolyzed yeast extract in order to enhance flavor of savory products (soups, meats, cheese, etc.). In theory, the taste would be able to indicate sources of amino acids, vital for human growth and development. However, the main reason behind the predominantly stimulation of the taste from only one amino acid the glutamate, is unclear. Moreover, even if this taste is characterized as "pleasant" it is also inconsistent and possibly depends on the medium (food) was added. Although, the taste itself maybe not be as pleasant is believed. (Weiland, Ellgring, & Macht, 2010). The umami receptor is a heterodimer. The dimer consists of T1R1/T1R3 subunits, so the T1R3 subunit is expressed in both the sweet and umami receptors (Running and Hayes, 2016). T1R1 and T1R3 are different types of taste receptors (Crowe et al., 2019). Sensitivity to umami taste could be subjective, because of the large differences have been found such as some individuals may even be unable to taste umami sensations (Lugaz, Pillias, & Faurion, 2002).

2.1.4 Chicken aroma

Poultry meat is probably the most consumed meat all around the globe. Through the years a lot of reviews on chicken flavor have been published (Wilson and Katz, 1972; Steverink, 1981; Ramaswamy and Richards, 1982). According to Mottram (1991), more than 450 components have been identified in cooked poultry meat. The formation of volatile compounds corelated with the chicken aroma is happening due to chemical reactions. Chicken meat flavor is derived from thermal reactions as the Maillard reaction, thermal degradation of lipids, and interaction between the previously mentioned reactions are mainly responsible for the flavor and aroma compounds generation (Jayasena, Ahn, Nam and Jo, 2013). The reaction of cysteine and sugar will cause characteristic meat flavor specially for chicken and pork. Also, volatile compounds as well as 2-methyl-3-furanthiol, 2-furfurylthiol, methionol, 2,4,5-trimethyl-thiazole, nonanol, 2-trans-nonenal, and other compounds are known as necessary for the flavor of chicken (Jayasena, Ahn, Nam and Jo, 2013). Specifically, Gasser and Grosch (1990) identified 2-

methyl-3-furanthiol as the flavor compound with most importance on the meaty perception of chicken broth. Which has been recognized as a characteristic compound in the aroma of cooked beef (Gasser and Grosch, 1988) and canned tuna fish (Withycombe and Mussinan, 1988). In line with Evers et al. (1976) the 2-methyl-3-furanthiol and its oxidative dimer, bis-(2-methyl-3-furyl) disulphide, have characteristic meat flavor notes, among the volatile products from heating thiamine hydrochloride with cysteine hydrochloride and hydrolysed vegetable protein. These two compounds were also found in heated yeast extract (Ames and MacLeod, 1985) and in volatile products of thiamine degradation (van der Linde et al., 1979; Hartman et al., 1984; Reineccius and Liardon, 1985). For that reason, thiamine has been recognized as the precursor for forming the meaty aroma compounds, 2-methyl-3-furanthiol and bis-(2-methyl-3-furyl) disulphide. Although, thiamine is not the only source of 2-methyl-3-furanthiol. It was found that a significant quantity of 2-methyl-3-furanthiol is formed when ribose or IMP react with glutathione or cysteine (Farmer and Mottram, 1990; Grosch et al., 1990; Zhang and Ho, 1991). Using ribose or IMP to form of 2-methyl-3-furanthiol requires interaction with one of the sulphur-containing amino acids, cysteine, peptide, glutathione. The 2-methyl-3-(methylthio) furan and 2-methyl-3-(ethylthio) furan were identified as the volatile compounds of chicken (Werkhoff et al., 1993). Werkoff et al. in 1993 thought that the 2-Methyl-3-(methylthio) furan is one of the meaty flavor contributors because of its low threshold value of 5 ppt. Because Due to the of structure of similarities to 2-methyl-3-furanthiol, Gasser and Grosch in 1990, identified 2-furfurylthiol as a primary odorant in chicken broth. This compound has roasted and sulphury aroma qualities, which have been recognized as the most important components of roasted coffee (Tressl, 1989) and possesses a threshold of 5 ppt. Compounds such as this are thermally generated from the reaction of furfural and cysteine (Tressl and Silwar, 1981) Several heterocyclic compounds are formed when low moisture and high temperatures conditions are used during certain chicken cooking methods. For example, grilling, frying, roasting or pressure cooking compared to boiled poultry meat. The most important volatile compounds corelated with fried chicken are 3,5-dimethyl-1,2,4-trithiolanes, 2,4,6-trimethylperhydro-1,3,5-dithiazines, 3,5-diisobutyl-1,2,4-trithiolane, 3-methyl-5-butyl-1,2,4-trithiolane, 3-methyl-5-pentyl-1,2,4-trithiolane, 2,4-decadienal and trans-4,5-epoxy-trans-2-decenal (Jayasena, Ahn, Nam and Jo, 2013). In addition, the thermal degradation of cysteine and cystine in aqueous solution has been studied by Shu etal. (1985 a,b).

2.1.5 Beef aroma

Beef flavor has been investigated more than any other meat flavor. Probably, because of beef's huge consuming popularity and hence its commercial significance in meat flavorings creation. Literature reports from the last 30 years show that the beef flavor is quite complex. In its simplest form, beef flavor consists of taste-active compounds, aroma components and flavor enhancers.

Aroma components are generated in beef from nonvolatile precursors during cooking. The reactions occurring are first lipid oxidation/degradation, second thermal degradation and inter-reactions of proteins, peptides, amino acids, sugars and ribonucleotides and third thermal degradation of vitamin B1. However, reaction products become reactants, and therefore the outcome may be an advanced and intertwining network of reactions. With such many potential contributors to the sensory perception of cooked beef aroma, the critical question is, 'What is the relative sensory significance of these volatiles?' The solution is not all clear, but three conclusions can be drawn. First, many are relatively unimportant. Second, meatiness term can be sensorially into about ten different odor qualities (Gait and MacLeod, 1983), in which case, many of the identified volatiles are acting as 'aroma modifiers' contributing buttery, caramel, roast, burnt, sulphurous, green, nutty, fragrant and fatty perceptions. Structure-activity correlations, or at least associations, do exist in the literature for many of these odor qualities. Third, some aroma components do contribute to a truly specific 'meaty' odor and are therefore character impact compounds. Many potent, key and trace meaty compounds are present in natural cooked beef aromas and lots of them remain unidentified.

The beef flavor profile relies on meaty/beefy compounds and the reactions which generate them. Strecker degradations and Maillard reactions are the main contributors, both chemically and sensorially. Furthermore, secondary reactions can occur involving the products of the above reactions (e.g. H_2S, NH_3, thiols and simple carbonyl compounds), thereby increasing quite considerably the variety of compounds which may be formed. Several Strecker degradations aldehydes are famous cooked beef aroma components, e.g. acetaldehyde (from alanine), methylpropanal (from valine), 2-methylbutanal (from isoleucine), 3-methybutanal (from leucine), phenylacetaldehyde (from phenylalanine) and methional, which readily decomposes into methanethiol, dimethyl sulphide, dimethyl disulphide and propenal (from methionine). The Maillard reaction between compounds containing a free radical (e.g. amino acids, amines, peptides, proteins, ammonia) and carbonyl compounds (e.g. aldehydes, reducing

sugars) contains a series of inter-reactions so is an advanced reaction, decomposition and leads to several volatile products. Firstly, an Amadori or Heyns compound is formed, respectively for the reaction of an a-amino acid with an aldose or ketose. Both are non-volatile but they are heat labile and they decompose under high temperatures involving rearrangement and subsequent decomposition of these compounds (MacLeod and Ames, 1988). Thus, 2-furaldehyde forms from pentoses, and 5-hydroxymethyl-2- furaldehyde from hexoses. Additionally, several dicarbonyl and hydroxycarbonyl fragmentation products can be formed. For instance, glyoxal, glycolaldehyde, glyceraldehyde, pyruvaldehyde, hydroxyacetone, dihydroxyacetone, diacetyl, acetoin and hydroxydiacetyl.

Yayalan had proposed that, since the currently accepted mechanisms of 1,2- and 2,3-enolizations of the open chain form of the Amadori compounds, and their subsequent dehydration (as just described), do not adequately account for many products observed in model systems and in foods, alternative mechanisms should be considered, e.g. direct dehydrations from cyclic forms of Amadori compounds (Yayalan, 1990). By whichever mechanism, the final stage reactions are extremely large in number and varied in nature and cannot be generalized. Examples are as follows. Furanoids often arise, via the 1,2-enolization pathway, 2,3-enolization route. An exception is 2-acetylfuran which is derived from a 1-deoxyhexosone intermediate (Tressl et aL, 1979). The 2- Furaldehyde is an important precursor of other furanoids, other heterocyclic compounds such as thiophenes and pyrroles. In the presence of H_2S or NH_3, the oxygen of the furan ring might be substituted by sulphur or nitrogen, forming the thiophenes or pyrrole derivatives. The most likely pathway for the formation of alkyl-pyrazines is by self-condensation of the a,p-aminoketones formed during Strecker degradation. While alkylpyrazines are frequently occurring volatiles in many heated foods, the bicyclic pyrazines, namely the alkyl-(5//)6,7-dihydrocyclopenta[6]pyrazines and the pyrrolo[l,2-0] pyrazines, are unique to grilled and roasted beef aromas (Maarse and Visscher, 1989).

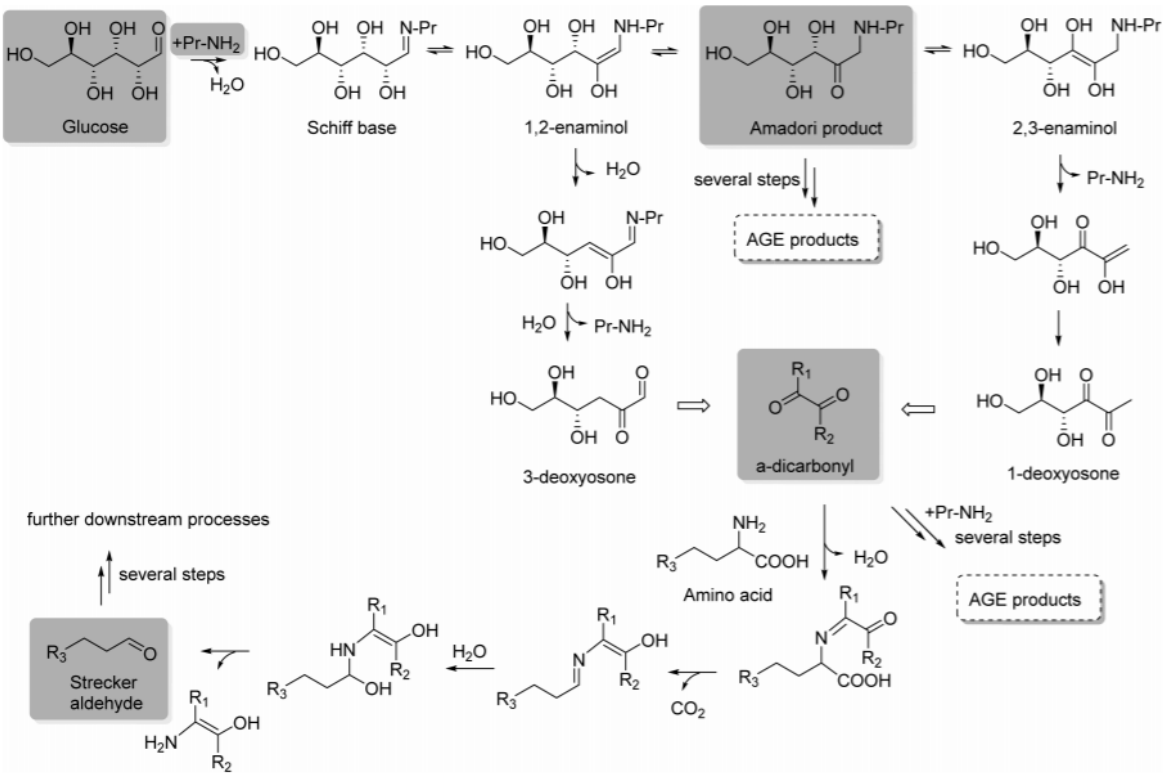


Figure 1: Amandori rearrangement and Strecker degradation. (Figure was adapted from Lund and Ray, 2017)

2.2 Extraction Methods

2.2.1 Solid Phase Micro-extraction

The solid phase micro-extraction (SPME) technique is relatively new and developed to sample volatiles and semi-volatiles organic compounds. SMPE is a simple and cost-effective technique (Wilson, 2000) (Marsili, 1997, p 82). It is based on the principle of using a stationary phase coated onto silica fibers that traps the volatiles in contact with the surface taking advantage of the partitioning effect between the vapor phase and the aqueous phase (Marsili, 1997, p 82). Volatiles and semi-volatiles firstly are released from their matrices and then absorbed or adsorbed, depending on the polymer, on the fiber (Ecoursesonline.iasri.res.in, 2020). Specific time is predetermined for the silica fiber for removal and placement directly into the GC and LC for analysis. Phases used commonly are polydimethylsiloxane (PDMS) and polyacrylate (PA) (Wilson, 2000). Figure 2 (a) illustrates SPME and its capability to adsorb of volatiles on the exposed fiber. Parameters like temperature and time during the process play important role.

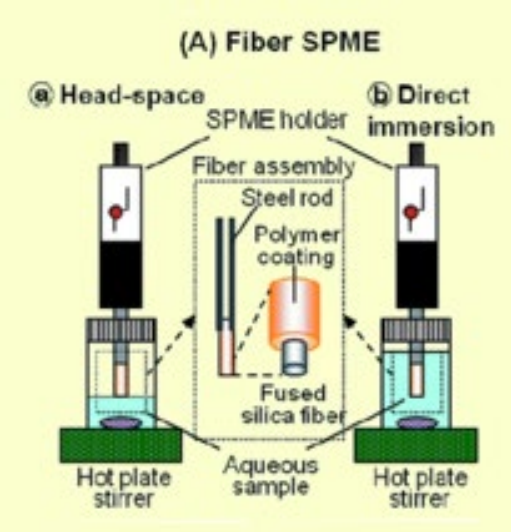


Figure 2:Representation of SPME method using (a) the headspace or (b) direct immersion for the fiber exposure (adapted from Kataoka, Ishizaki and Saito, 2016)

2.2.2 Solvent extraction

Solvent extraction is a selective separation procedure for isolating and concentrating a valuable material from aqueous solution with the aid of organic acids. The extraction process depends on the solubility of the flavoring compounds in the solvent. A compound can be separated from impurities in solution.

2.2.3 Liquid-Liquid Extraction

Liquid-liquid extraction is a separation process a solute transfer from one solvent to another. The two solvents being pure or partially unmixed with each other. Usually, one of the solvents is water or a water-based mixture and the other is a nonpolar organic liquid. As in all extraction processes, LLE includes the first step of mixing, followed by the phase separation step. It is necessary to consider both steps for selecting solvents and modes of operation. Otherwise, while strong mixing will transfer the extract from one solvent to another but might affect phase separation by forming emulsions.

Equilibrium is reached when the chemical potential of the extractable solute is the same in the two phases (Berk, 2018). In LLE the most common solvents used today are diethyl ether, diethyl ether – peptane mixtures, hydrocarbons, freons, methylene chloride with the last two having the capability of not being flammable. Solvent selection is a really important factor for the extraction outcome (Schnaak, 1986). Each type of solvent is used for different purposes. For instance, freons and hydrocarbons are nonpolar solvents and used for samples containing

alcohol. Also, for general purposes the solvents are commonly used are diethyl, ether, and methylene chloride (Marsili, 1997, p 6).

2.2.4 Solid-Liquid Extraction

Another separation process is Solid-Liquid extraction and is based on the dissolution of one or more components of a solid in liquid solvent consisting mixture (Berk, 2009). SLE is used to extract the most possible solute quantity with the least solvent quantity and collect a concentrated extract (Berk, 2009). These types of techniques rely on mechanical or temperature enhancement such as dilution and 'Soxhlet', respectively, which are expensive as they require high volumes of solvents and long processing time (Wang and Weller, 2006). The extraction method in the industry involves the combination of high temperature and chemical solvent. Although, even if the techniques are efficient during the transfer, thermo-labile compounds may be damaged (Barba et al., 2016). For example, conventional extraction techniques have been shown to damage heat-sensitive aromas (Galanakis, 2012) (McDonnell and Tiwari, 2017)

2.2.5 Solvent Assisted Flavor Evaporation

Solvent-assisted flavor evaporation (SAFE) is considered as the best method to produce a "clean" aroma extract avoiding the loss of labile aroma compounds or during GC analysis the formation of thermally created artifacts. However, SAFE is both labor intensive and time consuming. With SAFE, odor-active components are distilled under high vacuum, thus, the extraction bias during SAFE is based on volatility rather than polarity, such as in the case of solvent extraction.

2.3 Analysis Measurements Methods

2.3.1 Gas Chromatography

In 1951 Anthony Trafford James and Archer John Porter Martin, at the National Institute for Medical Research in London, UK, introduced gas chromatography (James and Martin,1941) (James and Martin,1942) , which was based on work done by Martin and Richard Laurence Millington Synge in 1941 (Martin and Synge,1941). In 1952 they were awarded the Nobel Prize in chemistry for inventing the partition chromatography.

In GC, procedure starts with the sample vaporization. Then the vapor is carried by the mobile gas phase through the column. Pure helium is usually used for a basic GC system, also hydrogen could be used, but due to its explosive nature it is avoided. Helium is used due to its

inertness, non-reactive nature in most analyses, samples partition (equilibrate) into and out of the stationary liquid phase, based on their solubilities in the stationary phase at the given temperature (McNair, Miller and Snow, 2019, p1-14). Sample's analytes separate from one another based on affinities for the stationary phase and their relative vapor pressures. Subclassification within GC is made according to the state of the stationary phase. If the stationary phase is a solid, the technique is called gas–solid chromatography (GSC); and if it is a liquid, the technique is called gas–liquid chromatography (GLC). Note that the names used to describe open tubular (OT or capillary) GC columns and LC columns include more detail than the simple guidelines just presented (McNair, Miller and Snow, 2019, p1-14).

2.3.2 Gas Chromatography/ Mass spectroscopy

GC is the most used analytical technique for separating volatile compounds. Its advantages are speed analysis, ease to operate, quantitative results etc. makes it important technique. Although, it cannot identify the structure and so the identity of compounds (McNair, Miller and Snow, 2019). The use of GC alone may lead to questionable results because of ambiguities in relying only on a retention index for identification of various sample components. Moreover, using only MS for an impure sample analyzing, superimposed mass spectra of two or more compounds are obtained; the results can be confusing (Watson and Sparkman, 2007: 573). On the other hand, if an impure sample analyzed by GC/MS the impurities will be separated from the analyte and obtain the mass spectrum of each of the components individually (Watson and Sparkman, 2007: 573).

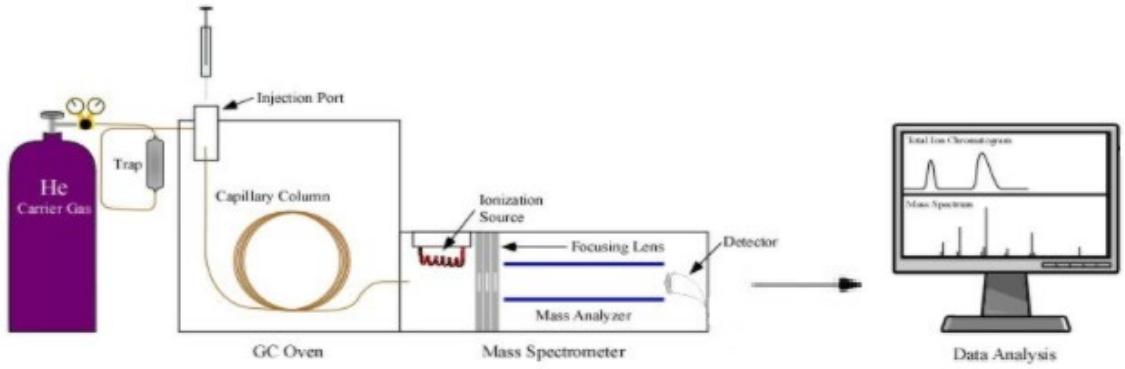


Figure 3:GC/MS equipment (GC/MS, 2020)

2.3.3 Liquid Chromatography

Liquid chromatography (LC) separates compounds in a liquid mixture on a chromatography column based on compounds' polarity of interest in the sample and the interaction they have with the column. LC introduced at the beginning of 1900s. Initially, a glass cylinder was packed with layers of fine powder such as chalk and then the sample is applied to the top of the column. Afterwards, a solvent is poured onto the column and due to gravity, the solvent flows down to the column. The components of the sample because of different size move through the column at different speeds and finally separate. In its initial form, samples were colored in order to the results being visual observed on the column. Afterwards, the solvent leaving the column were collected, then evaporated, and finally the separated compounds were recovered for quantitative analysis or other use.

2.3.4 High Performance Liquid Chromatography

High performance liquid chromatography is a form of column chromatography used to separate compounds with depending on their polarity. The early 1960s a general purpose HPLC was developed by Horvath in US and Huber in Europe. In the late 1960s, equipment for the commercial use of HPLC was introduced.

Firstly, the pressure was delivered by a large syringe. Although, this approach limited the solvent volume that would pass through a column and the analysis time as well. Because of these limitations the following years syringes were later replaced by a single reciprocating pump. However, problems were detected in delivery systems due to flow surges because of the movement of pistons interfering with stable detector baselines. The placement of two reciprocating pump, operating opposite to each other with respect to flow, lead to lower flow fluctuations which were completely removed the next few years by adding a pulse damper.

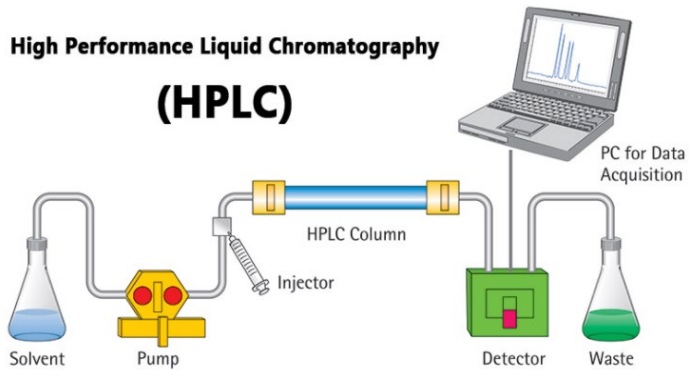


Figure 4:HPLC equipment (Toppr. 2020)

2.3.5 Thin Layer Chromatography

Thin layer chromatography (TLC) is a form of chromatography used to sperate compounds with varied polarity, based on a stationery and mobile phases on silica plate. Nikolai Izmailov and Maria Schreiber originally developed the TLC in 1938 for pharmaceutical preparations. At the beginning, TLC was conducted with calcium, magnesium, and aluminum oxides coated microscope slides. TLC advance throughout the following years was slow. In 1956 Egon Stahl made a major improvement when he attempted to standardize the preparation of the sorbents used to make the plates. For today's use, a small spot of solution is placed on one end of the slide, the slide is dipped into a solvent, and then the analytes migrate at different rates through the coatings where they can now be detected by a UV lamp or chemical stain.

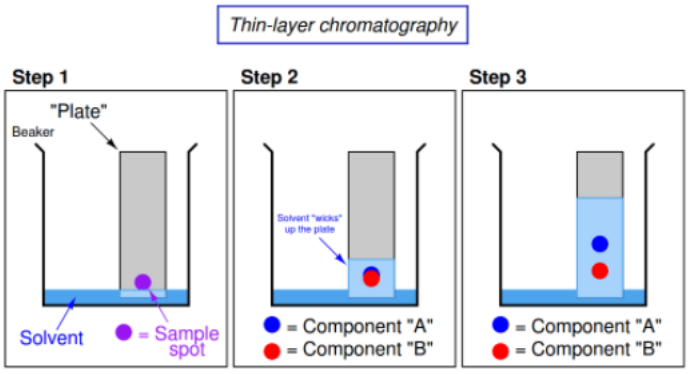


Figure 5:Separation of compounds using TLC (Thin-Layer Chromatography : Manual Method - Instrumentation Tools, 2020)

2.3.6 Aroma Extract Dilution Analysis

Aroma Extract Dilution Analysis is a quantitative procedure of gas chromatography olfactometry (GC-O), where the food odorants' activity is determined (Grosch, 1993). Specifically, in AEDA the previously distillated odorants concentrate is separated on a capillary column by gas chromatography. A single GC-O run cannot be sensory assessed, because it meets quantity limitations (Belitz, Grosch and Schieberle, 2009, p 351). The original extract is diluted in different steps (Feng et al., 2015). So, stepwise dilution gives the opportunity to determine different aroma substances appeared on gas chromatogram and flavor

dilution (FD) factors be provided (Belitz, Grosch and Schieberle, 2009, p 350) (Feng et al., 2015).

2.3.7 Flavor Dilution

Flavor dilution (FD) factor is defined as the number of parts of solvent is needed to dilute the aroma extract until its value becomes equal to one. FD factor is represented by 2^n, where n is the number of 1 + number of dilutions until GC-O cannot detect any aroma substance (Belitz, Grosch and Schieberle, 2009, p 350).

2.3.8 Odor Activity Values

In 1957 Patton and Josephson introduced the odor activity value. OAV is the ratio of a single compound's concentration to the odor threshold of that compound. Depending on the compounds' OAV value, it is determined their contribution in the odor profile of complex odor mixture (Song and Liu, 2018). OAV has been used in the past to determine important aroma-active compounds in foods e.g. fruit (Du, Plotto, Baldwin & Rouseff, 2011), wine (Escudero et al., 2004). cheese (Qian & Reineccius, 2003). AEDA use both parameters FD factor and OAV to identify the significance of the role of every compound in the overall food aroma. Even the OAV is used to determine the key odorants in varied foods, it faces some limitations. The main limitation is the odor thresholds between the dominant compounds in air and in medium are not correlated (Marsili, 1997, p 285).

2.3.9 Combined Hedonic Aroma Measurement

Combined Hedonic Aroma Response Measurement analysis is a both quantitative and qualitative method for gas chromatography analysis. The CHARM term is used to define the peaks on a chromatogram. As the AEDA analysis, extract's stepwise dilution is followed until GC-O is unable to detect any odor (Marsili, 1997, p 276). Even the main procedure of AEDA and CHARM is similar, there is a significant difference. In contrary with AEDA's maximum dilution value detection, CHARM analysis takes under consideration the duration of detection time of the odor by the gas chromatography. Also, in AEDA analysis its FD factor has the same significance as the peak in the CHARM analysis. Although the final results taken from both methods are completely different (Marsili, 1997, p 278-279).

3 Material and Methods

3.1 Material

3.1.1 Materials

The raw materials used in the preparation of the custom meaty flavor were obtained from the warehouse at the Nestle Product Technology Center Singen. Water for analysis was purified by means of a PURELAB Option-S 7/15 system (ELGA, Vivendi Water Systems Ltd, Marlow, United Kingdom).

Figure 6: PURELAB Option-S 7/15 water purification system (Elga Purelab. 2020)

Samples of an already in market product were obtained from Nestle's factory in Guatemala as the reference. The samples were stored in their original packaging at room temperature prior to opening. Once opened, the samples were closed in a small plastic container with an intermediate plastic lid and stored in a refrigerator (5°C) for further use.

3.1.2 Meaty flavor preparation

For the purpose of this study various meaty flavor recipes comprising amino acids, water, fat, salt was created. The exact composition and material names are not given due to confidentiality reasons.

The ingredients were weighed into Duran flasks and partially homogenized shaking the bottle before boiling. Final homogenization occurred during the boiling step.

The meaty flavors were boiled in an autoclave for 1h at 120 °C. The flavors were removed and allowed to cool to room temperature. Afterwards, stored in a freezer (-18 °C) until further use.

3.2 Methods

3.2.1 Impact of sodium chloride addition on extraction of volatiles from meaty flavors

Preliminary trials performed with a custom-made meaty flavor showed that the yield of e.g. Trimethylpyrazine and 2-Ethyl-3,6-dimethylpyrazine is increased significantly by using a 20 % NaCl solution instead of water as illustrated by their MS peak areas. Particularly regarding the detection of odorants of low volatility which usually are hardly represented in the headspace, the salting out effect is useful as illustrated by the dramatically increased peak areas.

3.2.2 Isolation of volatiles from the meaty flavor by solid phase micro-extraction (SPME)

A specific quantity of the sample was mixed in a 20 mL vial with screw cap with predetermined mL of a solution of sodium chloride in water (20%). The vial was tightly sealed, the sample vigorously shaken to obtain a homogenous suspension. All the quantities used according to tables 2,3 and 4. Also, different parameters affect the extraction step. Solid phase micro-extraction (SPME) was performed automatically using a MPS2XL autosampler (Gerstel, Mühlheim an der Ruhr, Germany) with incubation time and temperatures as shown tables 2,3 and 4 at 250 rpm.

Then, one of the fibers in table 1 being preconditioned at 250°C for 5 min, was exposed to the headspace. Sample extraction was performed according to tables 2,3 and 4 at 250 rpm

Determinations of the odor active regions were performed by two trained sniffers in duplicate repetition. The detection of an odor active region was denoted as positive if the aroma was detected in at least 3 of 4 runs of the reference sample.

Table 1: Fibers' specification table

Coating Material		CAR/PDMS carboxen/ polydimethylsiloxane	PDMS/DVB divinylbenzen/ polydimethylsiloxane	PDMS/CAR/DVB divinylbenzen/ carboxen/polydimethylsiloxane
Fibers' properties	Hub-color	Light Blue[2]	Pink[2]	Grey[2]
	Way of extraction	Adsorbent[2]	Adsorbent[2]	Adsorbent[2]
	Length in cm	1[2]	1[2]	1&2[2]
	Coating in um	85[2]	65[2]	50/30[2]
Compounds' properties information	Polarity	Bipolar[2]	Bipolar[2]	Bipolar[2]
	Polarity	Polar[2]	Polar[1,3]	-
	Volatility	Volatiles[3]	Low volatility[1]	Volatiles and semi-volatiles C3-C20[4]
	Molecular weight	Mw 30-225[4]	Mw 50-300[4]	Mw 40-275[4]
	Concentration range	Low ppt to high ppb[2]	High ppt to low ppm[2]	Low ppt to high ppb[2]
	Specific Affinities	Low molecular compounds[4], volatile free acids, polar pyrazines and furfural[1]	Volatile amines, nitroaromatic compounds[4]	Trace compounds (in the 2 cm fiber)[2]

1(Roberts et al. 2000),2Information of Supelco (Bellefonte, PA, USA), 3 (Paschke and Popp 2003), 4Information of Sigma Aldrich, 5 (Setkova et al. 2007b)

3.2.3 Identification of odor active compounds and Aroma Extract Dilution Analysis

Odor active compounds were identified based on odor qualities, retention indices on a DB-WAX column and mass spectral data. The determination of retention indices was achieved using a series of n-alkanes from C7 to C30. Retention indices were compared with those published in literature.

3.2.4 Gas chromatography-olfactometry/mass spectrometry (GC-O/MS)

An Agilent 7890 A (Waldbronn, Germany) gas chromatograph coupled to a mass spectrometer and a sniffing port was used. Volatiles were separated on a DB-WAX column (60 m length, 0.250 mm i.D., 0.25 µm film thickness, Agilent, UK). At the end of the column the effluent

was split 1:1 (by volume) into the mass spectrometer operating in electron ionization mode (EI) at 70 eV in mass range 30-230 amu (5975 C inert XL MSD, Agilent, Waldbronn, Germany) and the Olfactory Detection Port (ODP 3, Gerstel, Muelheim an der Ruhr, Germany). An additional flow of humidified air was added to the GC effluent prior detection at the outlet of the ODP3.

The samples were injected in either split less an inlet temperature of 230 °C and an oven temperature of 40 °C. After 3 min, the temperature of the oven was raised at 6°C/min to 180 °C, and finally at a rate of 10 °C/min up to 240 °C, where the temperature was kept constant for 15 min. Helium was used as a carrier gas with a constant flow of 2 mL/min. The effluent was split 1:1 into the sniffing port and the mass spectrometer. The temperature of the transfer line to the sniffing port was kept constant at 240 °C.

Retention indices were pre-determined from the lab staff. They injected manually 0.2 μL of a commercially available series of n-alkanes in hexane (C7 to C30). Liquid injection and split mode with a ratio of 1:10 was applied while using the same conditions mentioned above. The retention indices were calculated by application of the formula below.

$RI(X) = 100\left[\frac{Log\ t(x) - Log\ t(u)}{Log\ t(u+z) - Log\ t(u)}\right] * z * u$, Kovac retention index

RI(X): Retention index of compound X

t (x): Retention time of compound X in minutes

t (u): Retention time of the n-alkane eluting before the peak of interest

t (u +z): Retention time of the n-alkane eluting after the peak of interest

u Number of the C-atoms of the n-alkane eluting before the compound of interest

z Difference in the numbers of the C-atoms of n-alkanes used for calculation

3.2.5 Samples

As mentioned above the already in market reference flavor recipe used for the odor active compounds identification in meaty flavors. Initially, they were created 14 different custom flavors. Studying the effect of 12 different parameters on one flavor, in total there are 3.584. In order to minimize the number of tested samples only one meaty flavor used, the which translates into 256 experiments. However, after internal discussion assumptions took place. Theoretically, there is no interaction effects and for that reason a fractional factorial design was followed with 16 runs per fiber and one center point to check for linearities.

So, the flavor had the most natural and complete flavor profile was chosen. As tables 2,3 and 4 show varying quantities of the custom-made flavor (50, 125, 200 µL) were mixed in a 20 mL headspace vial with screw cap with (1, 3, 5) mL of either purified water or a solution of sodium chloride in water (10%, 20%). The vials were tightly sealed, then the sample vigorously shaken to obtain a homogenous suspension. The flavor was baked for an hour in the autoclave at 120 °C. The different suspensions were analyzed by means of SPME-GC-O/MS. As the performance of SPME fibers in the extraction of flavor compounds is enhanced by salting out effects (e.g. demonstrated by *Pinho et al.*) the meaty flavor samples were either mixed with ultrapure water or with a sodium chloride solution prior analysis as mentioned previously.
The experiments were conducted as is stated in tables 2,3 and 4. The liberated volatiles were isolated by means of SPME, using different types of coated fiber. According to *Majcher et al*. the highest extraction efficiency occurs when using DVB/CAR/PDMS. Furthermore, the different fibers can cover a broad range of different odorants. Analysis was performed by means of GC-O by two trained assessors. Altogether six sniffing were performed on the GC using the DB-WAX column.

3.2.6 Fiber selection

The fiber selection was performed by testing the efficiency of the four commercially available fibers obtained from Supelco (Bellefonte, PA, USA) in table one and mentioned below:

- Carboxen/ polydimethylsiloxane (CAR/PDMS, 85µm, fused silica/ SS)
- Polydimethylsiloxane/ divinylbenzene (PDMS/DVB, 65 µm, fused silica/ SS)
- Divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm, stable flex/ SS, 2 cm)
- Divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm, stable flex/ SS, 1 cm)

All fibers were conditioned according to manufacturer's recommendation prior to first use.

3.2.7 SPME parameters selection

Optimization of other SPME parameters by multivariate experimental design. It was assumed that the parameters being important for this study would be the mentioned below. Firstly, the different values of pH, will show how the volatility could be affected. Secondly, the quantities of sample and the solvent will determine the amount of volatiles. Also, the rest of the parameters will help to the determination of the volatiles' amount. In general the objective

behind the different parameters' values is to understand how these affect the reactions needed for the volatiles' production.

Multivariate design was used to optimize eight SPME parameters in order to maximize the peak areas of the marker compounds:

- pH (4, 6, 8)
- Sample quantity mL (50, 125, 200)
- Solvent quantity mL (1, 3, 5)
- Salt concentration (0%, 10%, 20%)
- Incubation temperature (40°C, 60°C, 80°C)
- Incubation time (10 min, 15min, 20min)
- Exposure time (10min, 20min, 30min)
- Desorption temperature (100 °C, 160 °C, 220 °C)

The peak areas are assumed to be the result of a function of these eight factors. The reduced fractional factorial design required the performance of 52 experiments. The original idea was to test different values for the different parameters. However, the experiments were too many so the need for less, led to choose three values per parameter with center point only one experiment without replicates. The values were selected according to previous Nestle studies and in literature. The outline of the design was built under statistical software

3.2.8 Experimental design

The optimum goal of experiment's design was to estimate the direct effect of each factor on response and certainly each fiber's type effect.

Table 2: 85 µm CAR/PDMS experimental design

85 µm CAR/PDMS, Lt.Blue/Plain								
Sample	pH	Sample_mL	Solvent_ml	Salt_conc	Temp_incub	Time_incub	Time_expos	Temp_desorb
A	8	200	1	20	40	20	10	100
J	8	50	1	0	80	20	30	100
K	8	50	1	20	80	10	10	220
B	8	50	5	20	40	10	30	100
L	4	200	1	0	80	20	10	220
M	8	200	5	0	80	10	10	100
C	4	200	5	20	40	10	10	220
D	4	50	1	20	40	20	30	220
E	8	50	5	0	40	20	10	220
F	8	200	1	0	40	10	30	220
N	8	50	5	20	80	20	10	100
O	8	200	5	20	80	20	30	220
P	4	200	1	20	80	10	30	100
Q	4	50	5	0	80	10	30	220
G	4	50	1	0	40	10	10	100
H	4	200	5	0	40	20	30	100
I	6	125	3	10	60	15	20	160

Table 3: 65 µm CAR/PDMS experimental design

65 µm PDMS/DVB, Pink/Plain								
Sample	pH	Sample_mL	Solvent_ml	Salt_conc	Temp_incub	Time_incub	Time_expos	Temp_desorb
A	8	200	1	20	40	20	10	100
B	8	50	1	20	80	10	10	220
C	8	50	1	0	80	20	30	100
D	4	200	5	0	40	20	30	100
E	4	50	1	20	40	20	30	220
F	4	200	1	20	80	10	30	100
G	8	50	5	0	40	20	10	220
H	8	200	5	20	80	20	30	220
I	4	50	5	20	80	20	10	100
J	8	200	5	0	80	10	10	100
K	4	50	5	0	80	10	30	220
L	4	200	1	0	80	20	10	220
M	4	50	1	0	40	10	10	100
N	8	200	1	0	40	10	30	220
O	4	200	5	20	40	10	10	220
P	8	50	5	20	40	10	30	100
Q	6	125	3	10	60	15	20	160

Table 4: 50/30 µm PDMS/CAR/DVB, Gray/Plain 1 & 2 cm experimental design

50/30 µm PDMS/CAR/DVB, Gray/Plain 1 & 2 cm									
Sample	Fibr size	pH	Sample_mL	Salt_ml	Salt_conc	Temp_incub	Time_incub	Time_expos	Temp_desorb
E	1	6	125	3	10	60	15	20	160
A	1	4	200	5	20	40	10	10	220
A2	2	4	50	1	0	40	10	10	100
F	1	8	50	1	0	80	20	30	100
B2	2	8	50	5	20	40	20	10	100
C2	2	4	50	5	0	40	20	30	220
B	1	8	200	1	0	40	20	10	220
C	1	4	200	1	20	40	20	30	100
D	1	8	200	5	0	40	10	30	100
G	1	4	50	1	20	80	20	10	220
D2	2	8	200	1	20	80	10	10	100
H	1	4	50	5	20	80	10	30	100
E2	2	4	200	1	0	80	10	30	220
I	1	8	50	5	0	80	10	10	220
F2	2	8	200	5	20	80	20	30	220
G2	2	4	200	5	0	80	20	10	100
H2	2	8	50	1	20	40	10	30	220
I2	2	6	125	3	10	60	15	20	160

4 Results and discussion

4.1 Results Identification of potent odorants in meaty flavors

4.1.1 Identification of potent

In order to identify odor active compounds in meaty flavors, the custom meaty flavor mentioned above was used. The recipe comprised water, oil, amino acids, salt and yeast. Firstly, the flavor was heated in the autoclave for one hour at 120 °C. Following this, the flavor was suspended in different aqueous solutions, so the odorants created from the process above would be released in vial's headspace. The odorants were analyzed by means of SPME-GC-O/MS. As the performance of the SPME fibers in the extraction of flavor precursors showed that the yield of e.g. Trimethylpyrazine and 2-Ethyl-3,6-dimethylpyrazine increased significantly. As illustrated in figure 7, odors that are usually difficult to detect, with salting out effect they release to the headspace.

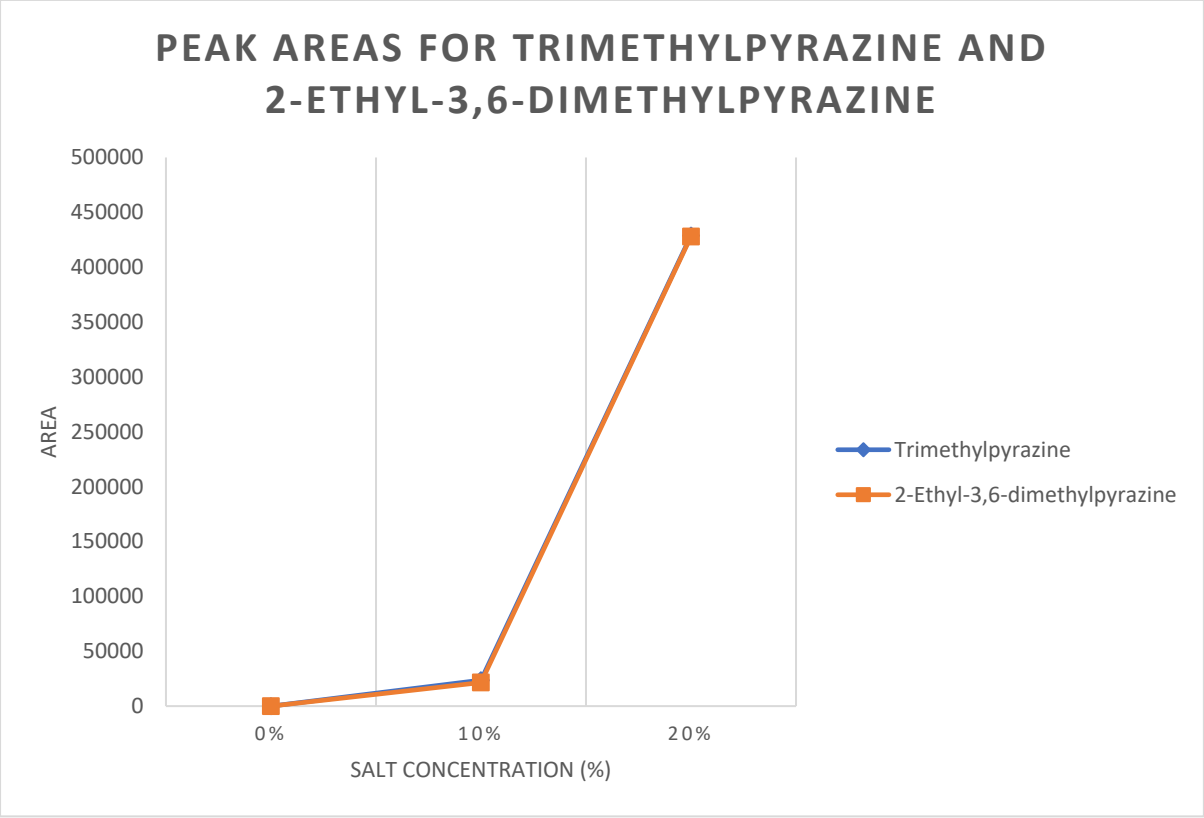


Figure 7:Peak areas for Trimethylpyrazine and 2-Ethyl-3,6-dimethylpyrazine

In order to extract sufficient quantities performing the experiments by GC-O/MS, maximum of 200 µL of the sample was suspended in 5 mL of salt solution (20%). The volatiles were

trapped on a DVB/CAR/PDMS 2 cm fiber. Analysis was performed by two trained assessors. In total four sniffing test were run, performed by means of GC-O/MS and a DB-WAX column.

Among 18 detected odor active regions, 10 were successfully identified using RI, odor perception and mass spectra. The rest 8 odors were not identified from all three parameters. Further down, table 5 illustrates the identified odors used in the tables below.

Table 5:Identified aroma active compounds in custom flavor with their corresponding odor qualities, retention indices (RI) on DB-WAX, mass spectroscopy being the criteria used for identification.

No	Compound	RT	RI experimental	RI literature	Odor experimental	Odor literature	Identification Odor	Identification RI	Identification MS
1	1-Octen-3-one	12.36	1292	1298	mushroom	mushroom	+	+	+
2	2-Methyl-3-furanthiol	12.55	1300	1305	meaty	meat-like, sulphurous	+	+	+
3	1-Hexanol	13.78	1360	1359	greeny,fatty	grass, marzipan-like	-	+	-
4	Dimethyl trisulfide	14.02	1371	1368	cabbage	cabbage-like	+	+	+
5	Nonanal	14.29	1383	1389	soapy	citrus-like, soapy	+	+	+
6	Trimethylpyrazine	14.73	1404	1405	roasty	roasty,popcorn-like	+	+	+
7	2-Propionyl-1-pyrroline	14.88	1411	1434	burned, roasty	roasty,popcorn-like	+	-	+
8	2-Furfurylthiol	15.32	1433	1430	roasty coffee	coffee	+	+	+
9	2-Ethyl-3,6-dimethylpyrazine	15.85	1459	1462	roasty coffee	roasty,burned	+	+	+
10	2-Ethyl-3,5-dimethylpyrazine	16.01	1467	1462	meaty, roasty (chicken)	roasty,burned	+	+	+
11	E-2-Nonenal	17.52	1543	1542	greeny,fatty	greeny,fatty	+	+	+
12	Phenylacetylaldehyde	18.78	1605	1644	flower	honey,bee wax	+	-	+
13	2-Phenylethylacetate	22.35	1814	1820	sweet	honey-like,flowery	-	+	-
14	2-Methoxyphenol	23.34	1865	1862	smoky	smoky	+	+	+
15	Furaneol	26.08	2028	2033	caramel	caramel	+	+	+
16	Cinnamaldehyde	26.26	2040	2025	sweaty	cinnamon-like	-	-	-
17	5-Vinyl-2-methoxyphenol	28.97	2220	2223	marzipan	smokey,clove-like	-	+	-
18	Myristicin	29.58	2262	2257	celery,carrot	carrot,nutmeg	+	+	-

1(Pozo-Bayon M.A., Ruiz-Rodriguez A., et al., 2007), 2(Dreher, Rouseff, et al., 2003), 3(Tatsuka, Suekane, et al., 1990), 4(Kim, 2001), 5(Tatsuka, Suekane, et al., 1990), 6(Kim, 2001), 7(Mahajan, Goddik, et al., 2004), 8(Ruther and Baltes, 1994), 9(Shimoda, Shiratsuchi, et al., 1996), 10(Umano and Shibamoto, 1987), 11(Choi, Kim. M.-S.L., et al., 2002), 12(Choi, Kim. M.-S.L., et al., 2002), 13(Carunchia Whetstine, Croissant, et al., 2005), 14(Botelho, Caldeira, et al., 2007), 15(Schwambach and Peterson, 2006), 16(Möllenbeck, König, et al., 1997), 17(internal Nestle database reference),18(Lee, Umano, et al., 2005)

4.1.2 Maillard reaction

Five odorants, namely 2-propiony1-pyrroline, 2-methyl-3-furanthiol, 2-Furfurythiol, 2-ethyl-3,6-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine were identified exhibiting roasty and meaty odor notes. As mentioned earlier, (in section 2.1.2) these type chemical compounds are contributing to the roasty and meaty aroma.

Specially, the meaty aroma deriving from 2-methyl-3-furanthiol is essential for cooked meat (Cerny and Davidek, 2003, Grosch et al., 1990, Hofmann and Schieberle, 1995, Mottram and Nobrega, 2002), despite its small mass fraction as a Maillard reaction volatile (Cerny and Guntz-Dubini, 2013). It must be mentioned its really small odor threshold in water 0,007 μg/Kg (Hoffman, 1995)

Furthermore, the 2-ethyl-3,6-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine have slightly higher odor threshold in water 0.4 (Grosch, 2001) and 0.16 (Guadagni et al. 1972) μg/Kg, respectively. Also, these compounds are known as for their sensory essential contribution to bread (Rychlik and Grosh,1996), French fries (Wagner and Grosch.1997), cocoa (Schnerman and Schieberle, 1997), chocolate (Schnerman and Schieberle, 1997), popcorn (Schieberle and Grosch,1994). Additionaly, the 2-ethyl-3,5-dimethylpyrazine is detected in beef (Kerler and grosch,1996; Cerny and Grosch, 1993) and chicken products (Kerler,1996).

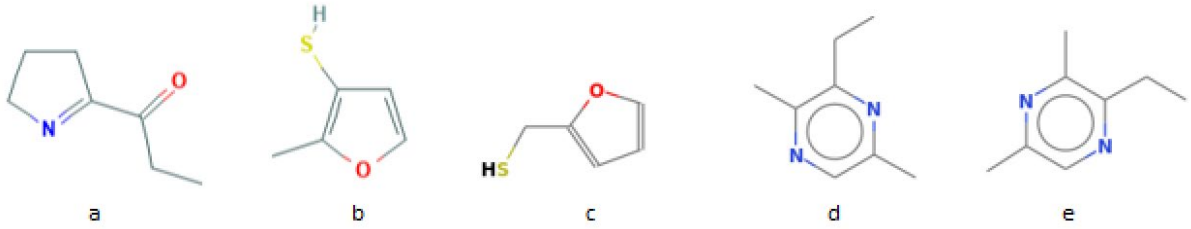


Figure 8: Chemical structure of a) Propionyl-1-pyrooline, b) 2-Methyl-3-furanthiol, c) 2-Furfurylthiol, d) 2-ethyl-3,5-dimethylpyrazine, e)2-ethyl-3,6-dimethylpyrazine (Figures were adapopted from "National Institute of Standarts and Technology, U.S. Department of Commerce" and from "PubChem,National Library of Medicine, U.S. Department of Health and Human Services")

4.1.3 Strecker aldehydes and degradation products of methional

During the olfactometric analysis Strecker aldehydes were detected. The compounds found are phenylacetylaldehyde and cinnamaldehyde. Strecker's reaction mechanism is when amino acids are degraded by α-dicarbonyls, via decarboxylation and deamination. While amino acids are intrinsically present in numerous foods, the dicarbonyls are formed by degradation of reducing sugars in the course of the Maillard reaction.

For instance, according to Guadagni et al., 1972 methional is described as cooked potato-like odor at a very low odor threshold in water 0.2 µg/kg. It is reported to be one of the most potent odorants in cereal products and is detected in reacted flavors containing methionine, ribose (Meyner and Mottram,1995) or thiamine, methionine (Güntert et al. 1996).

In figure 9 the aldehyde (I) is an aroma compound itself, but also a potent aroma precursor. Upon heating putrid smelling methanethiol (II) is released by β-elimination from methional. Methanethiol further oxidizes easily to form dimethyldisulfide (II) which is disproportionate to odorous dimethylsulfide (IV) and dimethyltrisulfide (V). Related to the lower odor threshold of dimethyltrisulfide it is more sensory relevant (Belitz, Grosch and Schieberle, 2009). Dimethyltrisulfide was detected by GC-olfactomery.

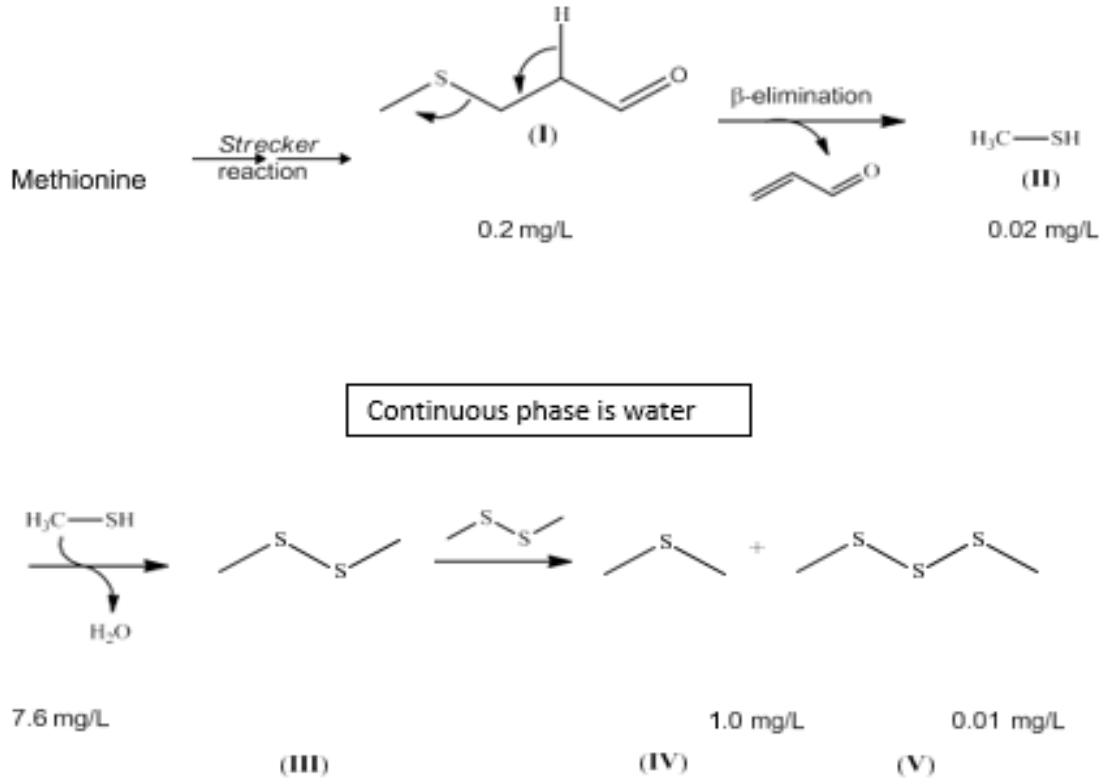


Figure 9: Degradation of methional leading to methanethiol (II), dimethylsulfide (IV) and dimethyltrisulfide (V); odour thresholds in water (Belitz, Grosch and Schieberle, 2009)

4.1.4 Lipid degradation products

Some lipid derived products were identified within the odor active volatiles of meaty flavors. Most of them belong to the chemical group of aldehydes. Nonanal which provides a green and soapy note and hexanal exhibiting typical green odor that resembles the smell of freshly cut grass are saturated aldehydes. The latter serves as an off-flavor indicator compound in re-

heated meat after storage (Belitz, Grosch and Schieberle, 2009).

Lipid derived odorant was identified within the chemical group of ketones. The 1-octen-3-one was the most powerful exhibiting mushroom-like odor. It is characterized by a significantly low threshold of 0.05 μg/L water (van Gemert, 1999). Linoleic acid is the precursor for 1-octen-3-ol (Belitz, Grosch and Schieberle, 2009), which is oxidized further its ketone derivative. Considering the use of the sunflower used for the creation of the custom flavor the linoleic acid in the oil can vary from 9-70 %, it affects significantly the final concentration of the 1-octen-3-one in the final product.

Furthermore, as for the E-2-nonenal and its cis isomer Z-2-nonenal studies has shown that they derive from the autoxidation of palmitoleic acid (Swift, 1999). Palmitoleic acid has been produced firstly, by the desaturation of the palmitic acid (Parveez et al., 2012) which can be naturally located in sunflower oil. Its quantity is quit solid varying from 4-6 % of oil's total mass.

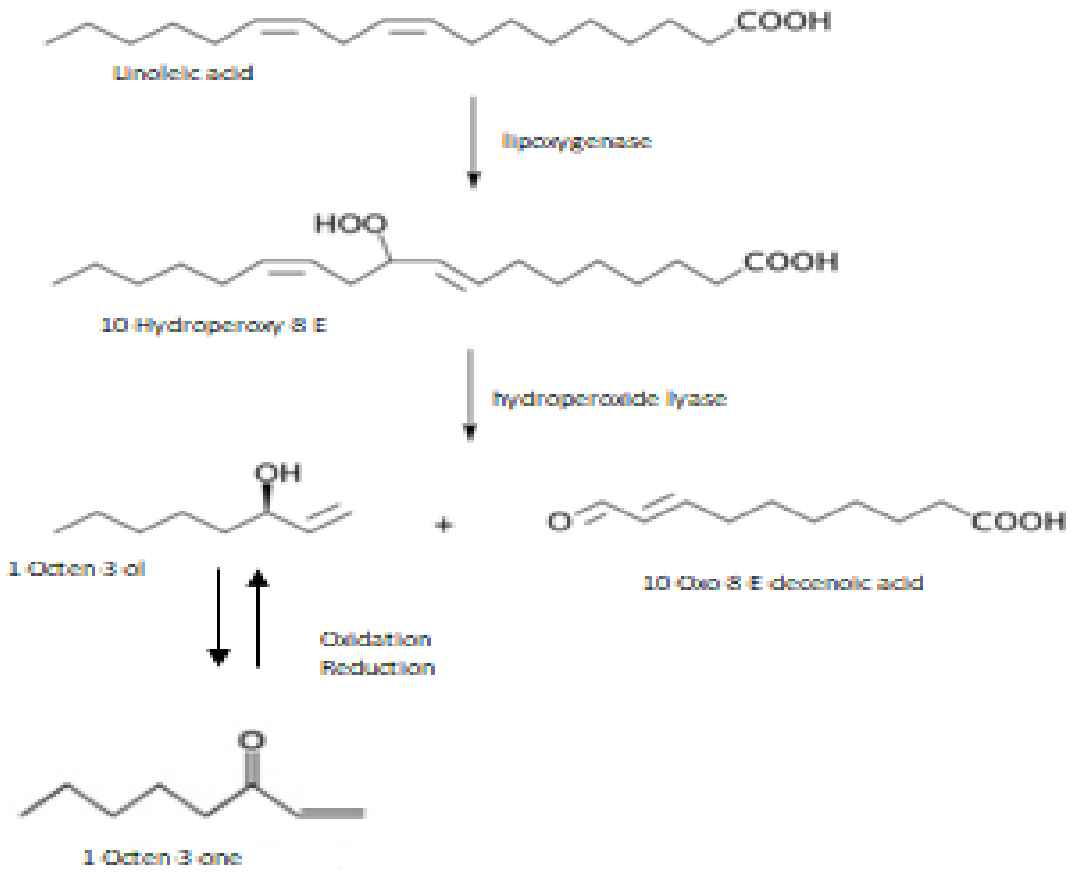


Figure 10: Transition from Linoleic acid to 1-Octen-3-one

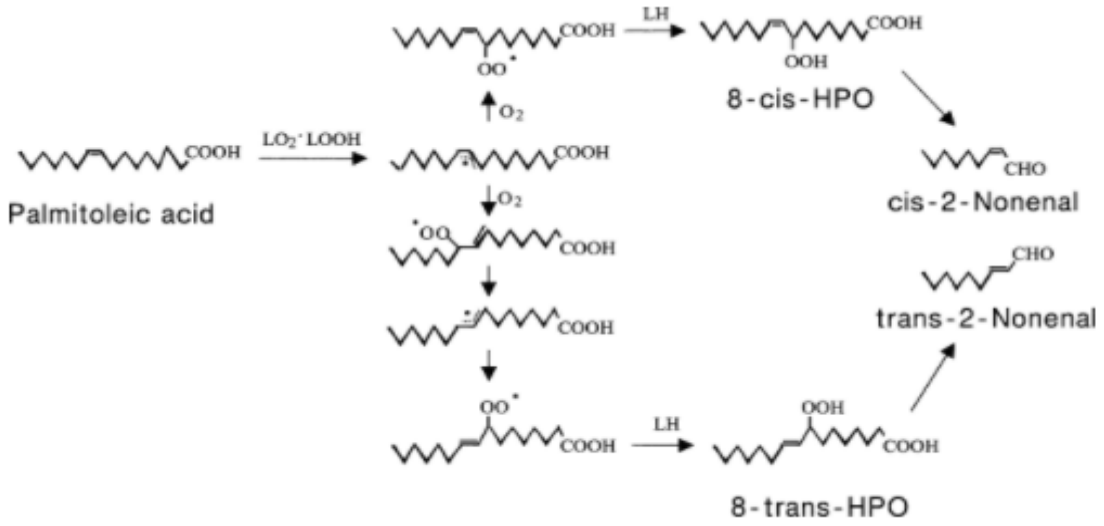


Figure 11: Oxidative degradation of the palmitoleic acid to lipid peroxides and specifically to Z-2-nonenal and E-2-nonenal (adapted from Haze et al., 2001)

4.1.5 Phenolic compounds

Apparently, the detection of phenolic compounds in the custom meaty flavor was limited. The compound 2-methoxyphenol was detected during the GC/O-MS. It contributes to the custom-made flavor with its characteristic smoky aroma. Other compounds such as vanillin that were expected to contribute, could not be perceived by sniffing the SPME extract.

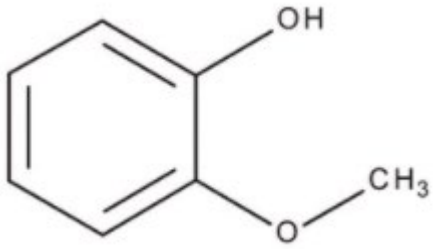


Figure 12: Chemical structure 2-methoxyphenol, (Sigmaaldrich. 2020)

4.2 Optimization of SPME preparation parameters

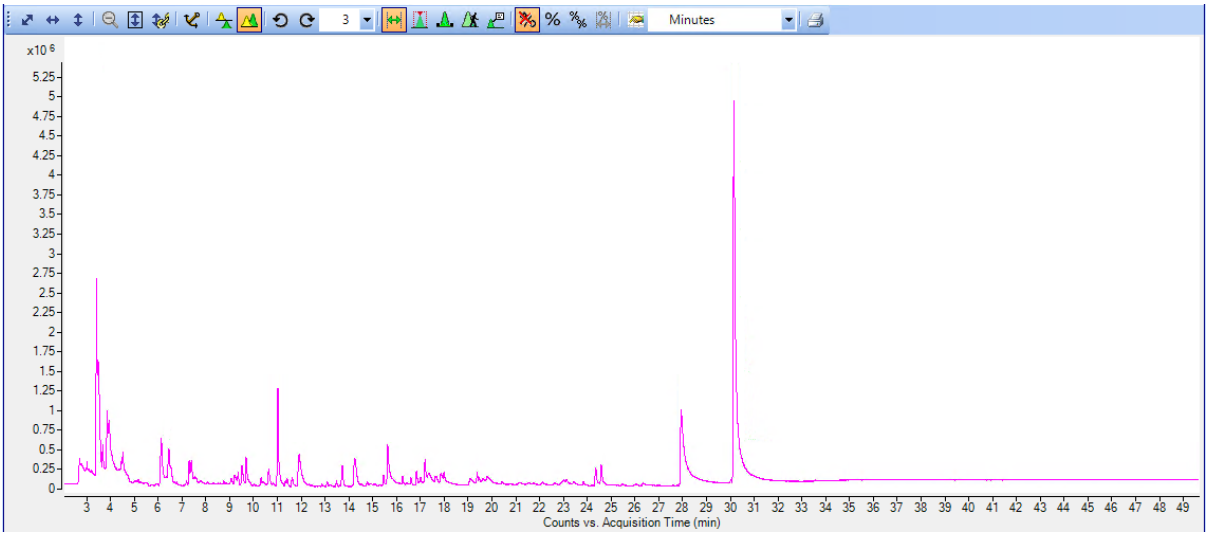
4.2.1 Fiber coating

The choice of the SPME fiber coating is known to affect the amounts and the type of the extracted compounds (Roberts *et al.* 2000; Paschke and Popp 2003). Therefore, four commercially available fiber coatings were compared for their ability to extract volatiles. As expected, the fiber, extraction and data processing method for all fibers, CAR-PDMS-DVB fiber can extract the highest number of compounds followed by PDMS-DVB and CAR-PDMS. The triple coated CAR-PDMS-DVB fiber showed the best overall results. According the studies of Ho *et al.* 2006, Setkova *et al.* 2007a and Risticevic *et al.* 2008a, it is confirmed that this type of fiber can detect the most compounds.

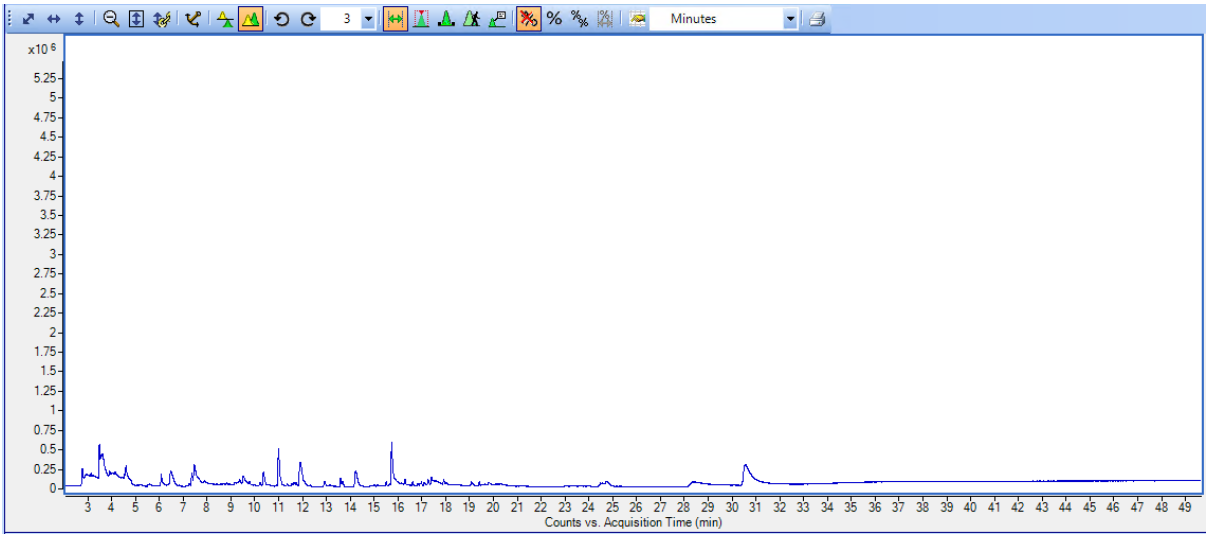
The CAR/PDMS fiber might be considered for the extraction of acids and compounds like dimethyl-trisulfide, which provides higher yield for these compounds compared to CAR-PDMS-DVB fiber.

The PDMS/DVB fiber according to Setkova *et al.* 2007a; Roberts *et al.* 2000 has higher affinity for middle to low volatiles, such as 2-methoxy-phenol. Although, this type of fiber showed a broad absorption range (Ho *et al.* 2006; Cramer *et al.* 2005) the fiber didn't have the ability of PDMS/CAR/DVB fiber for absorbing a wide range of volatiles.

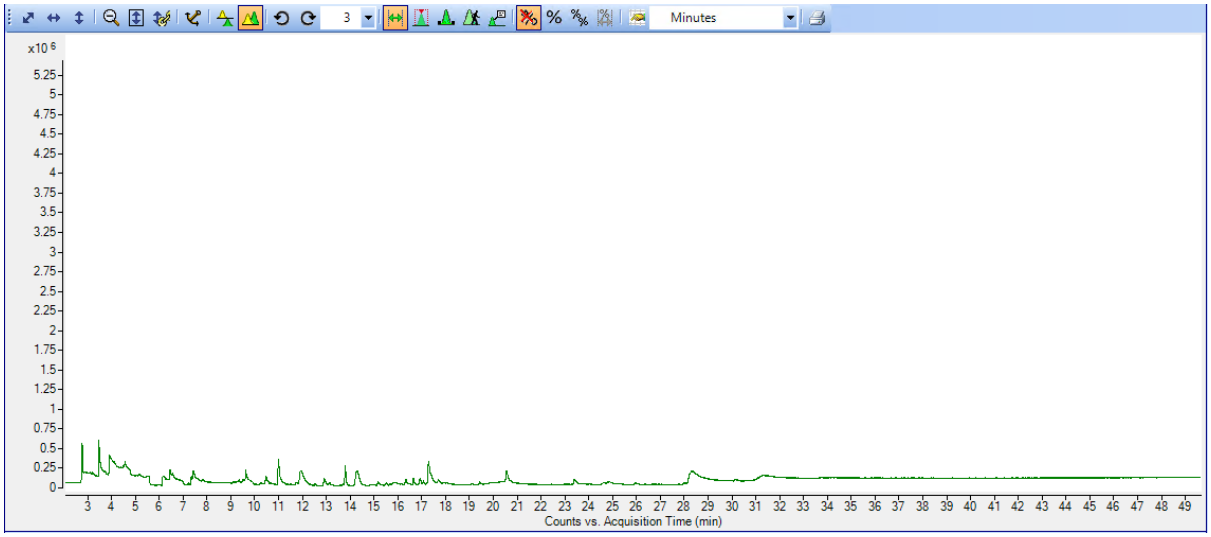
Figure 16 illustrates the chromatograms of the four different fibers under the same parameters. As it was expected the fiber PDMS/CAR/DVB with 2 cm length is the most capable fiber for the compounds' adsorption. The compounds' peak areas are higher comparing with those from the rest of the fibers.



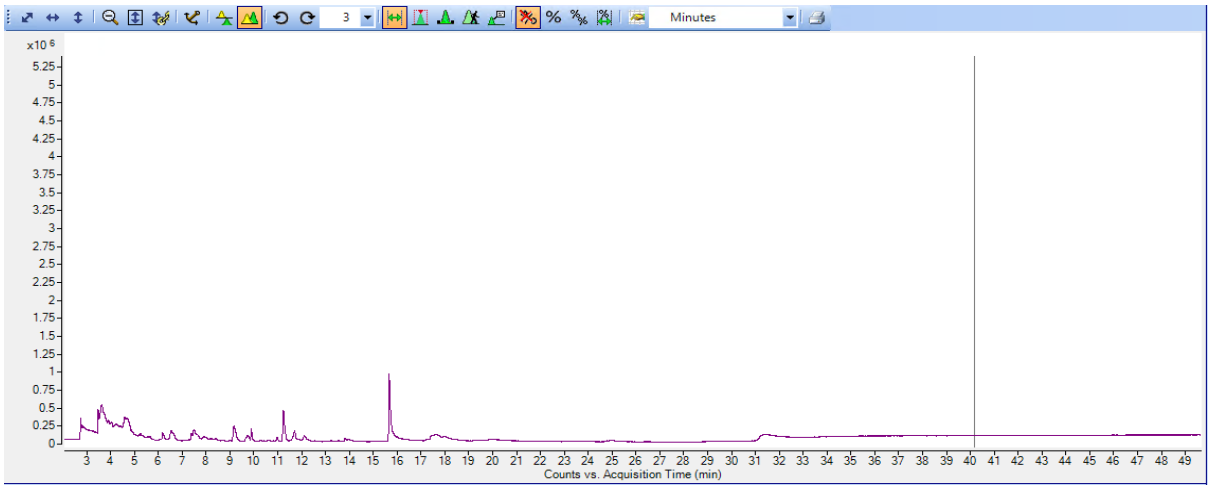
PDMS/CAR/DVB 2cm



PDMS/CAR/DVB 1cm



PDMS/DVB 1cm



CAR/PDMS 1cm

Figure 13: Comparison of chromatograms obtained with different SPME fiber-coatings.

4.2.2 Effect of incubation time

According to the literature, the incubation time has a minor effect on the detection of the compounds (Risticevic *et al.* 2008a; Setkova *et al.* 2007a). Nevertheless, a too short or too long time gives a different distribution of the compounds and leads to undesirable effects. On one hand, if the incubation time is too short, the low volatiles such as Furaneol cannot be detected as much as high volatiles. On the other hand, too high incubation time, especially at higher temperatures may lead to the degradation of some compounds in the sample.

As shown in Figure 14 the incubation time impact is significant for the majority of the compounds. However, if it is considered that small incubation time would lead to samples not being equilibrated, the incubation time of 20 minutes seems the best analysis option.

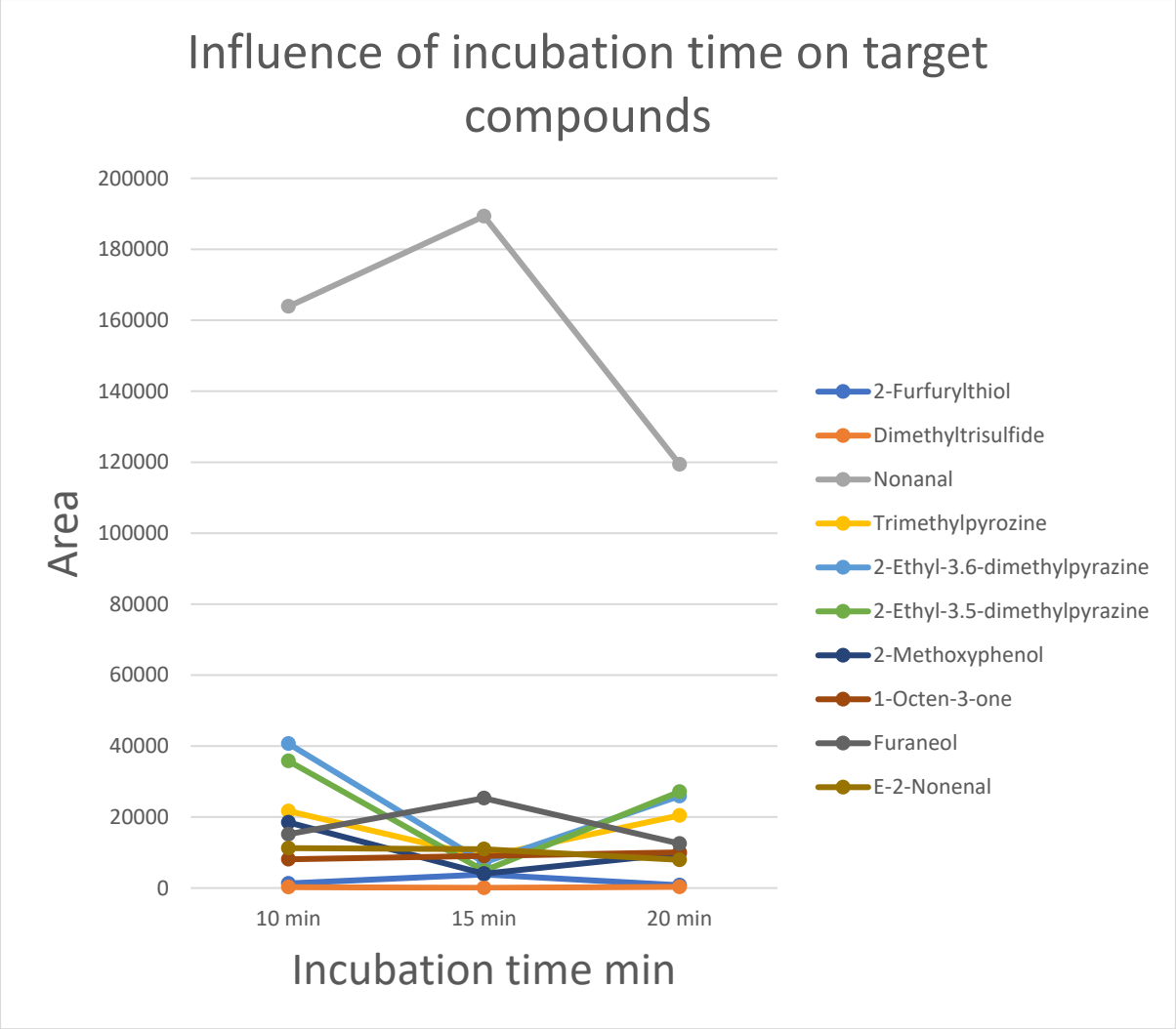


Figure 14: Evaluation of the influence of incubation time on target compounds

4.2.3 Effect of incubation temperature

The average peak areas obtained for different incubation temperatures are illustrated in figure 15. The graph in figure 15 shows how the peak areas change depending on the incubation temperature. Firstly, Nonanal the compound with the largest peak area multiplies its area as the temperature increases 20 degrees from 40°C to 60°C. Secondly, the next compound's peak area went up is Furaneol. A slight increase it is observed to the rest of the compound except the Dimethyltrisulfide. As the incubation temperature raises another 20 degrees from 60°C to 80°C, all the compounds' average peaks exceed even more. This leads to a safe conclusion that the incubation temperature plays a significant role of volatiles' release in vial's headspace. So, the higher the temperature is, more volatiles will be in the headspace. However, it is worth mentioning that the incubation temperature should have an upper limit, because after 80°C boiling starts to occur. Also, degradation of specific compounds can occur if the temperature exceeds that limit, giving unpleasant results.

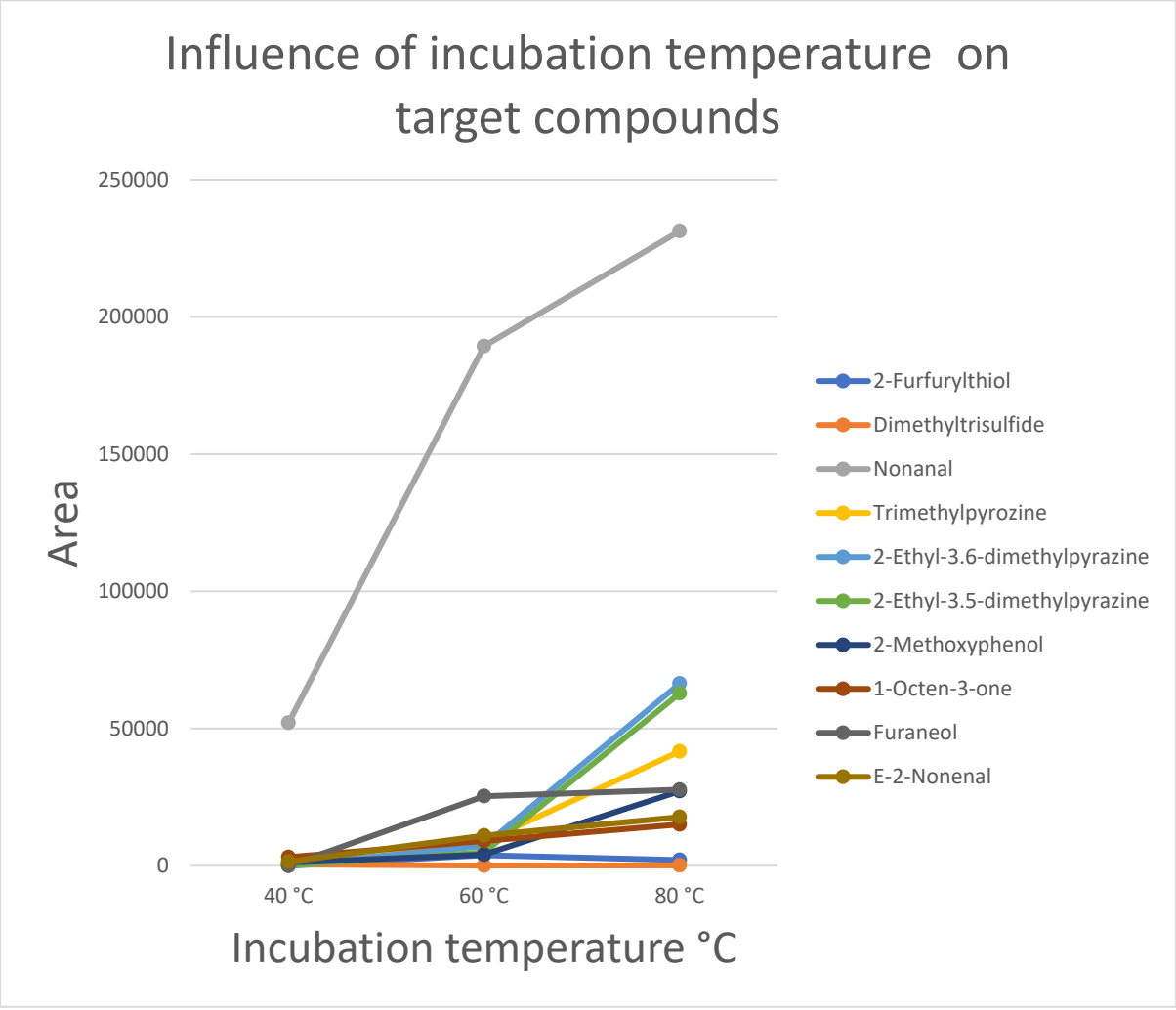


Figure 15: Evaluation of the influence of incubation temperature on target compounds

4.2.4 Effect of pH

The prisonization plays a role on compound's release into headspace. So, the pH of the samples was investigated to understand how affects the headspace. The pH parameter was checked in 3 different values. Specifically, pH=4 used for the acidic pH, pH=8 used for the alkaline pH and pH=6 as the average value of these two.

As illustrated in Figure 16 Nonanal, Furaneol and 2-Furfurythiol are favored from the pH increase, in comparison with the rest of the compounds' peak areas staying stable or decreasing like 2-Ethyl-3,6-dimethylpyrazine and 2-Ethyl-3,5-dimethylpyrazine. Further change of pH had only a small impact. Acidification namely to pH 4, had a slight positive impact on the peak areas of (*E*)-2-nonenal and 1-octen-3- one.

On the other hand, decrease of pH had negative impact on key compounds including 2-Ethyl-3,5-dimethylpyrazine. So, it is possible the optimum pH be neither of the three values used. The pH 7 seems to be the most appropriate pH according to the graph in figure 19, as the peaks of the compounds haven't completely reached their maximum or minimum area.

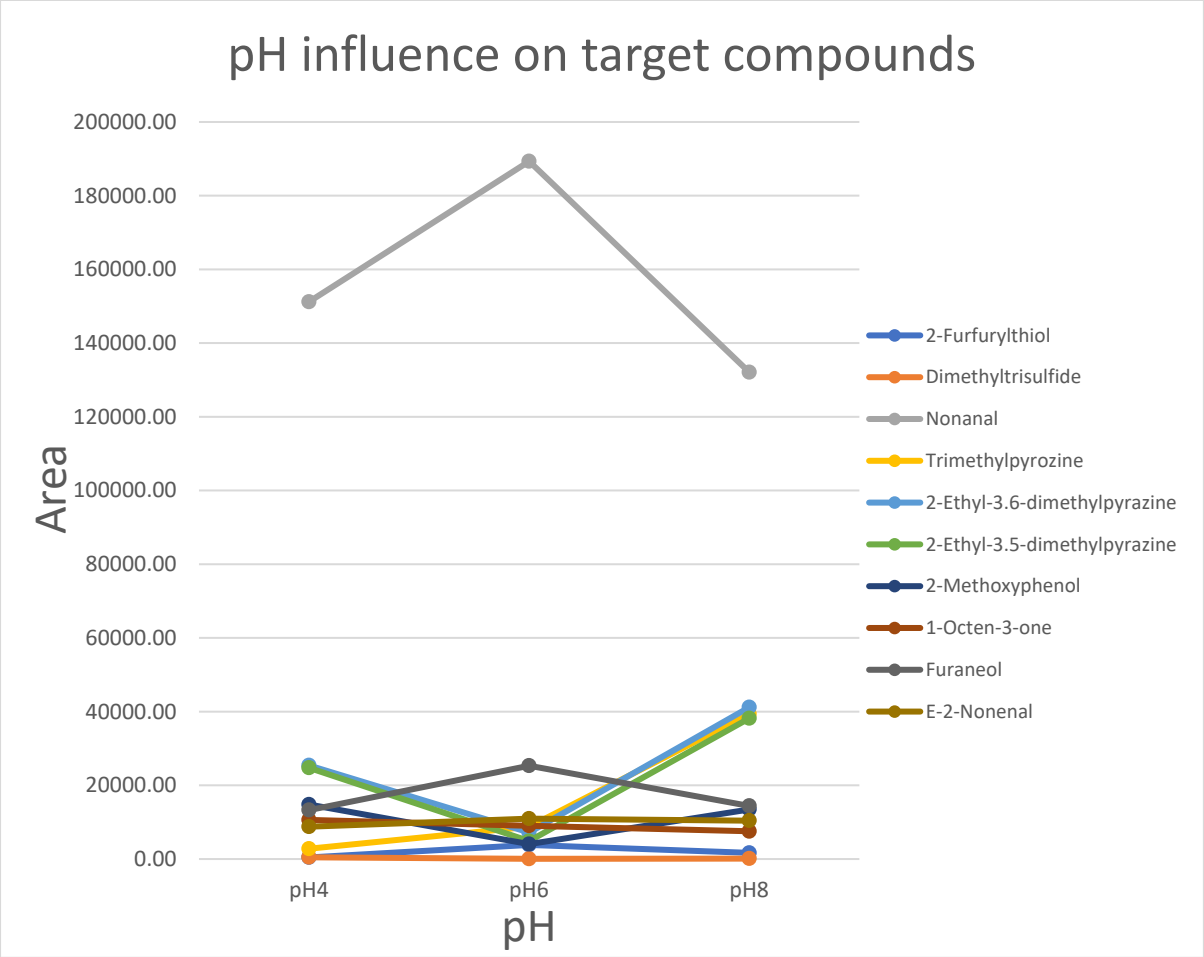


Figure 16: Evaluation of the influence of pH on target compounds

4.2.5 Effect of salt addition

The addition of sodium chloride may lead to improved release of volatiles due to the named salting out effect.

During this effect two different trends were observed. Initially, the addition of salt to increase its concentration to 10% led four compounds' peak areas to increase respectively. These compounds are Nonanal, 2-Furfurythiol, 2-Furfurythiol and Furaneol. In contrast, the peak areas of the rest compounds remained stable or reduced such as 2-Ethyl-3,6-dimethylpyrazine and 2-Ethyl-3,5-dimethylpyrazine.

Continuing, the increase of salt concentration to 20% leads the 2-Ethyl-3,6-dimethylpyrazine, 2-Ethyl-3,5-dimethylpyrazine and 2-Furfurythiol peak areas rapidly increase. On the contrary, Nonanal and Furaneol peak areas decrease significantly. Also, it is observed a slight decrease of E-2-Nonenal and 1-Octen-3-one. It is possible the salting out effect doesn't affect the same way all the compounds.

Nonanal is the compound with the biggest peak area in the figure 17, even the downfall from the 10% salt concentration sample. So, the best salt concentration is the 20% as most of the rest compounds' peak areas increase and the compounds can easier release to headspace and adsorb on the fiber.

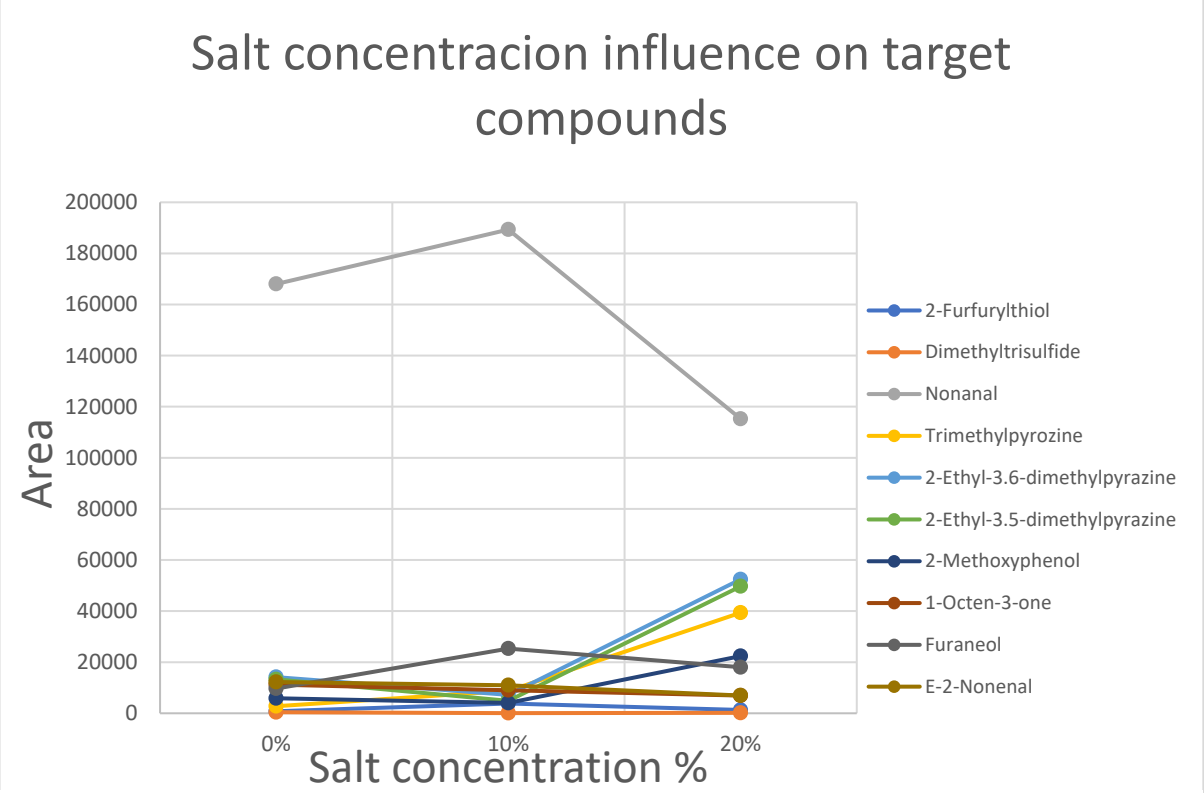


Figure 17: Evaluation of the influence of salt concentration on target compounds

4.2.6 Effect of flavor quantity

Different quantities could give different compound composition in the headspace. The purpose of what figure 18 illustrates, is to identify if the additional quantity of the samples helps the identification of the different compounds. Theoretically, more sample quantity could give potentially more volatiles into the vial's headspace. Looking carefully figure 18, becomes clear that the most compounds' peak areas increase. Furthermore, the peak areas are increasing even more while the quantity changes from 125µL to 200µL.

Consequently, the best flavor quantity added in the sample is the 200µL, because the higher quantity permits the release of higher volatile compound quantity.

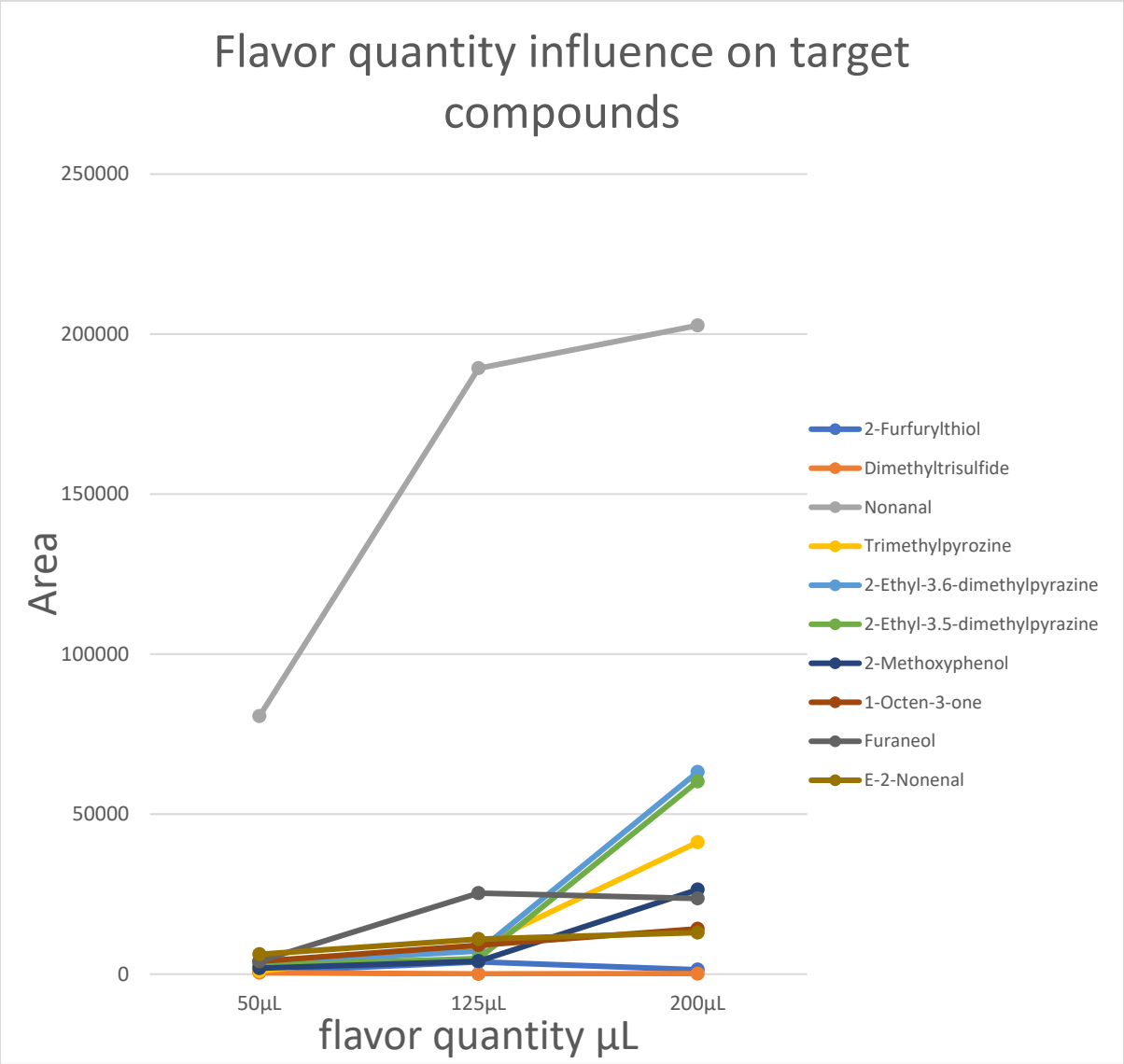


Figure 18: Evaluation of the influence of flavor quantity on target compounds

4.2.7 Effect of desorption temperature

The desorption temperature one of the SPME parameters is illustrated in figure 19. The higher desorption temperature finally led to an increase of average peak area comparing with the initial one. These results are valid for all the compounds except the dimethyl-trisulfide, which doesn't seem to be affected of any of the different parameters. However, three of the compounds showed slightly better results at lower desorption temperature (160°C). Specifically, those compounds are Nonanal, Furaneol and 2- furanmethanethiol. The impact of desorption temperature (230-270°C) has been studied in 2007 by Setkova et al., showing similarities with the temperature increase. The compounds' peak areas in 220°C are similar to 100°C. So, following the example in pH parameter evaluation, maybe an intermediate temperature of 200°C is the right temperature for desorption.

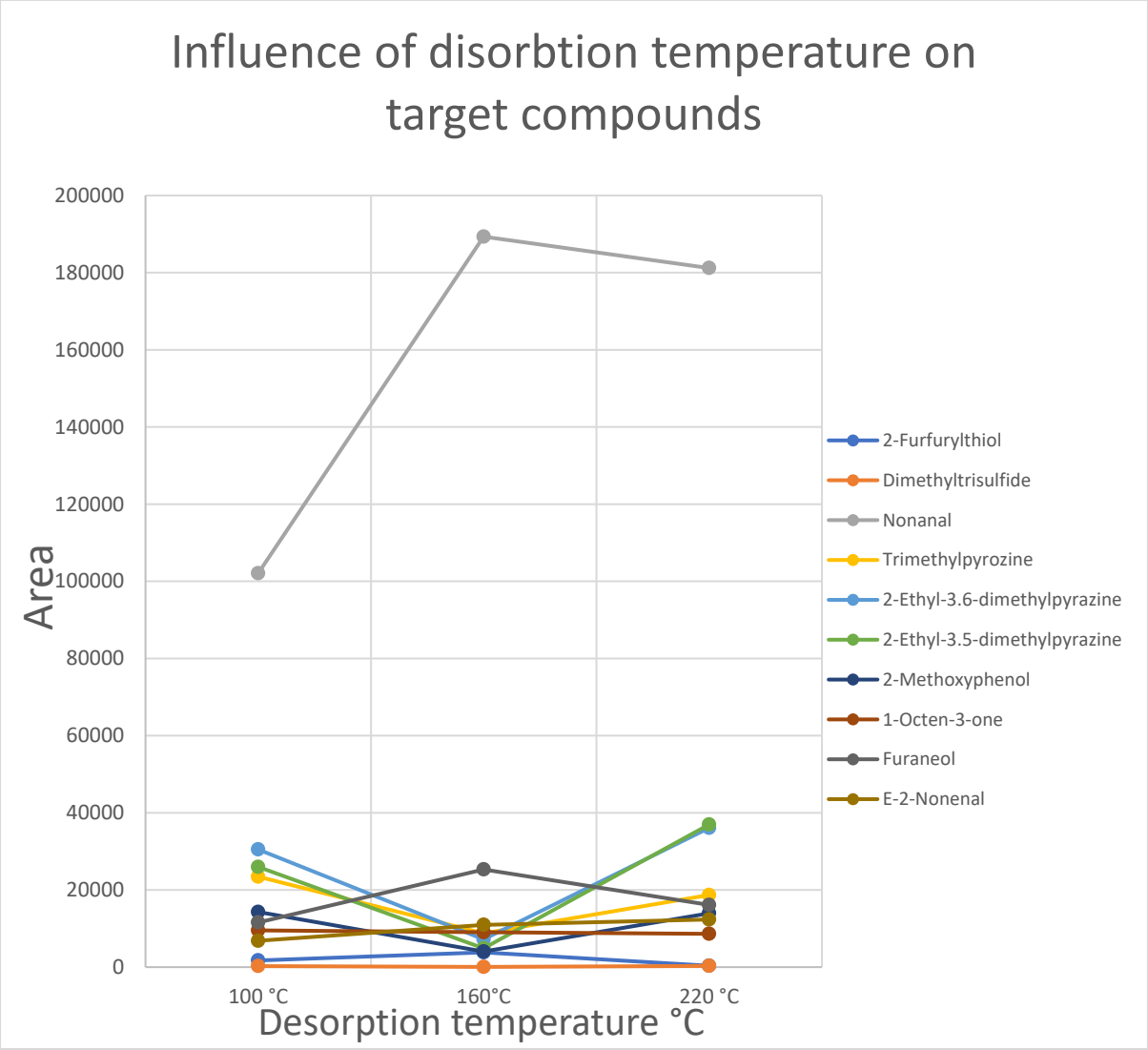


Figure 19: Evaluation of the influence of desorption temperature on target compounds

4.2.8 Effect of solute quantity

The figure 20 display the effect of the solute quantity on the selected compounds. The used quantities of 1,3 and 5 mL being implemented for these measurements. Instantly, it becomes clear that as long as the quantity increases the peak areas decrease for most of the compounds. The compounds can be separated into two different categories. Those that decrease in the intermediate solute quantity and those that increase such as Nonanal and Furaneol. As mentioned above the final peak areas are significantly less than the initials, which could be due to higher dilution of the flavor sample and making it more difficult for the compounds to transfer to headspace. Then, the quantity of 1 mL of solute provides condenser samples and is easier for the compounds transfer to the headspace.

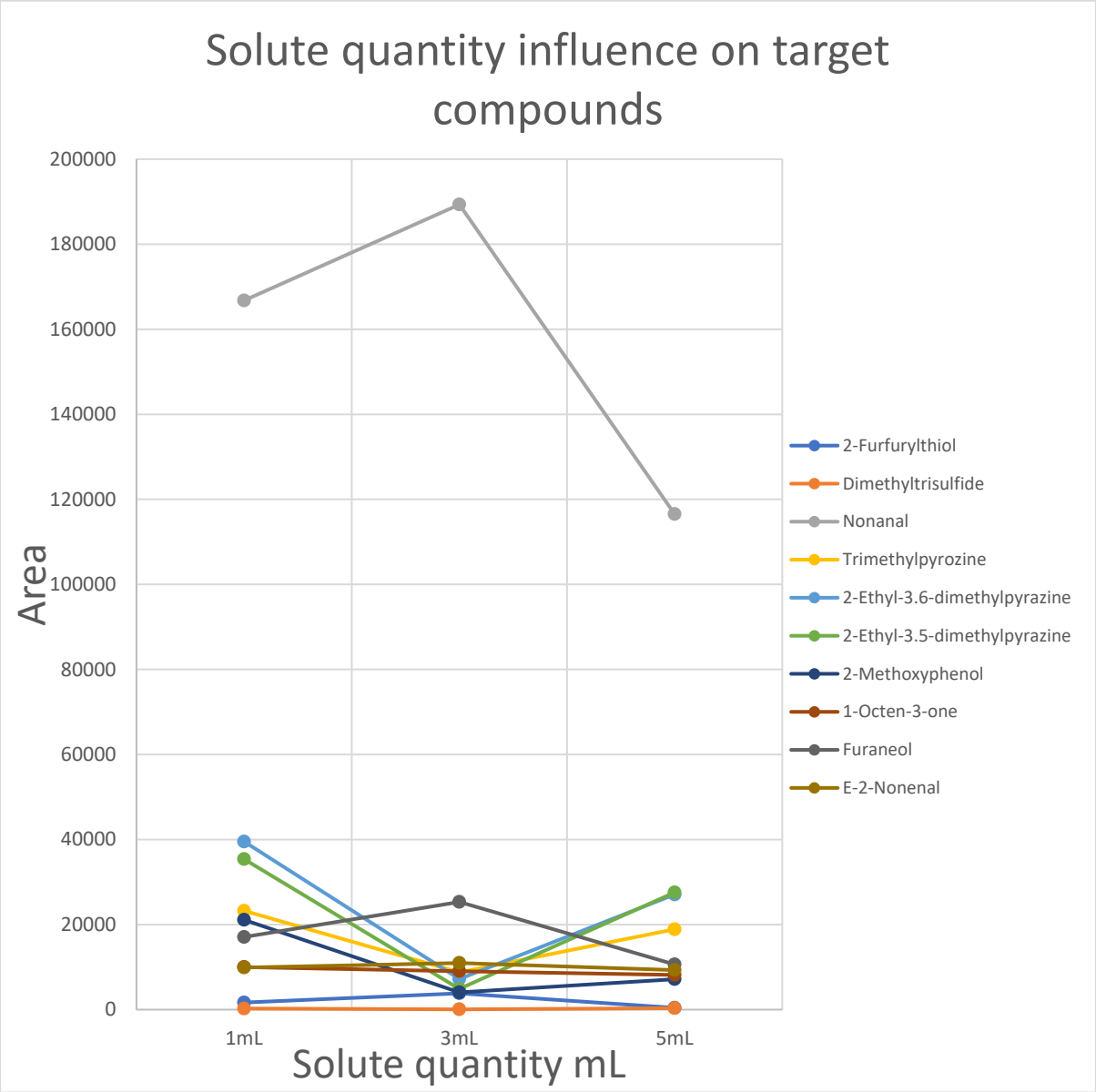


Figure 20: Evaluation of the influence of solute quantity on target compounds

4.2.9 Effect of exposure time

The exposure time represents the time that fiber is exposed in vial's headspace. In figure 21 it is illustrated its effect on the selected compounds. Fiber's exposure time is highly importance parameter for SMPE. Specifically, the exposure time determines fiber's saturation.

Nonanal increases rapidly, almost doubles its peak area as the time doubles from ten to twenty minutes. Also, Furaneol's, E-2-Nonenal's and 2-Furfurythiol's peak areas slightly increase. As for the rest of the compounds they slightly decrease. In comparison, when the exposure time increases another ten minutes; the same compounds behave differently as their peak areas rise faster. On the other hand, Nonanal's, 2-Furfurythiol's and furaneol's peak areas seem to have a small fall. Hence, the 30 min of exposure time is the time needed to redeem as much as possible of the compounds where previously adsorbed on the fiber.

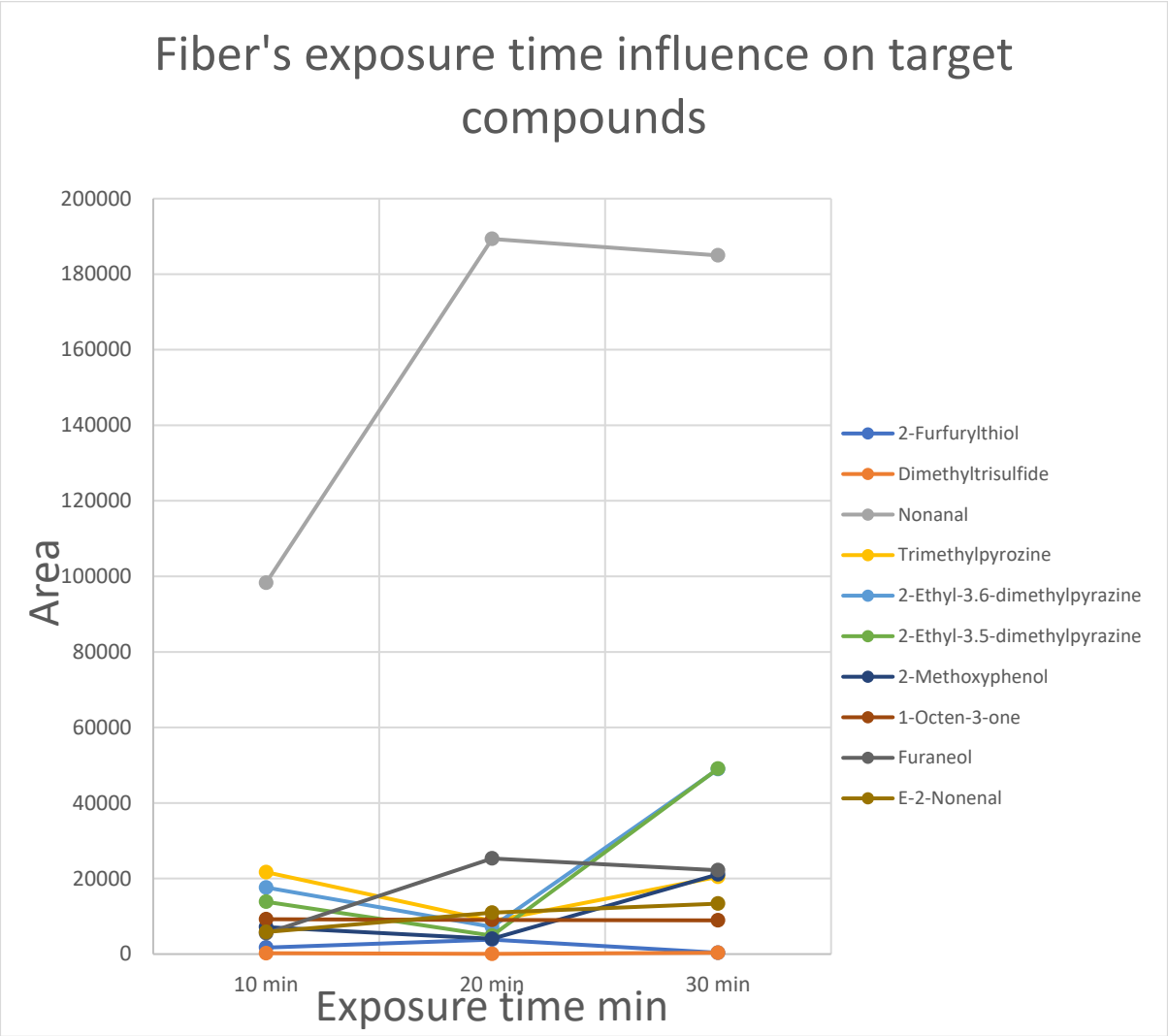


Figure 21: Evaluation of the influence of fiber's exposure time on target compounds

4.2.10 Individual parameters' graphs

Generally, the above graphs were expected to be either exponential or linear. However, because of the poor results in the center points of the experiments, there was always a minima. Thus, the graphs decline in the middle and raise again in the highest values of the parameters.

4.2.11 Experimental design

An experimental design was used to optimize all the different parameters: type of fiber, pH, sample quantity, solvent quantity, salt concentration, incubation temperature, incubation time, desorption temperature and exposure time. The bar chart in figure 22 shows that the factors with the positive effect in the experiment, are the type of the fiber, sample quantity and incubation temperature. In contrast, the solvent quantity and the incubation time has almost no to negative effect on the samples. The rest of the parameters (pH, salt concentration, desorption temperature and exposure time) have minor effect. All the parameters were assessed at three levels (values).

Further down in the figure, are presented only the results obtained for average compound (i.e. results based on average peak area). However, these results have a good reflection of the effect of parameters on most individual compounds. It is important to mention that the error bars are possibly big because of the presence of Nonanal and it's big numbers as in the poor results retrieved in the center points of the parameters.

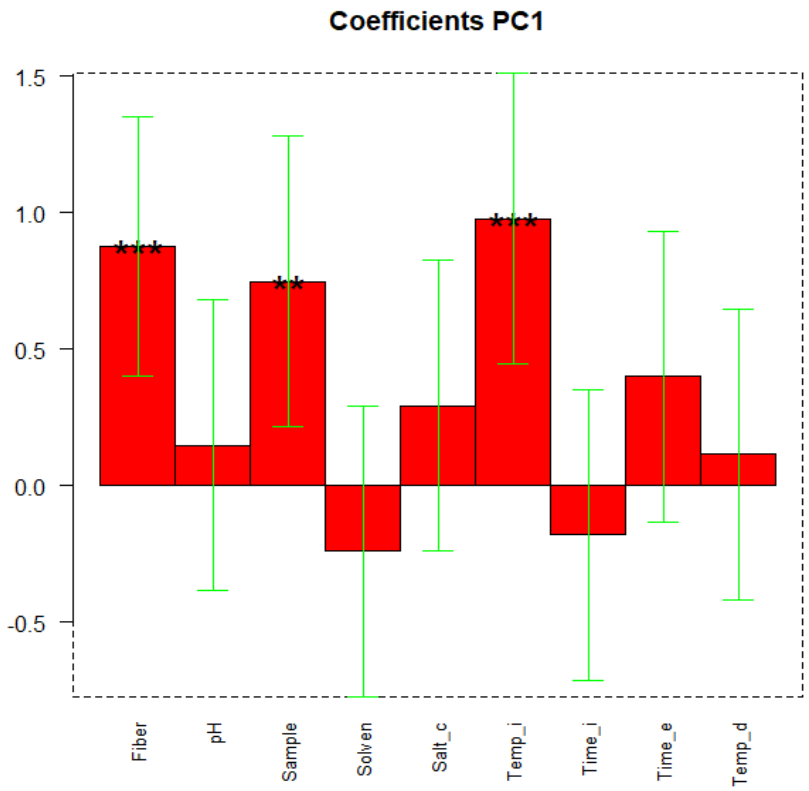


Figure 22 Variability of all the coefficients

5 Conclusion

Thesis conduction permitted its original aim to develop and optimize extraction procedures necessary for the aroma characterization of meaty flavors in commercial food products.

The final results indicate the most characteristic aroma being extracted for characterization of key odorants by GC-olfactometry is obtained when 200µL product is dissolved in 1mL 20% salt concentration water, the obtained suspension is separated from non-volatile compounds by solid phase microextraction (SPME) and finally the aroma compounds are isolated with the use of PDMS/CAR/DVB 2cm

The thesis successfully demonstrated that SPME can be very useful tool to examine the trace odorants. However, the method's parameters optimization plays a crucial role and SPME should be coupled to a gas chromatograph equipped with mass detector (SPME-GC/O-MS). Mass spectrometer should be used as a complementary way of verifying the compounds where previously identified with olfactometry and RI.

The optimization of SPME-GC/O-MS method showed that both the number of detected compounds as well as their peak area are mainly affected by fiber type, sample preparation, incubation time and incubation temperature. The best results were generally achieved with PDMS/CAR/DVB 2cm fiber after dilution of the custom-flavor sample with 1mL 20% salt concentration water (the salt is sodium chloride). The peak areas were significantly increased with incubation time and temperature. The majority of the compounds showed maximum peak areas after incubation time of 10 min at 80°C (5 out of 18). Specifically, two compounds Nonanal and Furaneol showed different behavior as the maximum peak area was obtained at the incubation time of 15 min and peak areas decreased with increasing incubation time.

All the successful parameters:

- Flavor quantity 200μL
- Solvent 1mL
- Salt concentration 20%
- pH 8
- PDMS/CAR/DVB 2cm fiber
- Incubation time of 10 min
- Incubation temperature at 80°C
- Exposure time 30 min
- Desorbtion temperature 220°C

The developed methods are suitable for qualitative but also quantitative assessment of key odorants in meaty flavors in commercial food products that are eaten after reconstitution. Small adaptation may be necessary for other type of meaty flavored products, e.g. ready to eat products.

These methods will allow further studies on the impact of a recipe structure and/or the process parameters on key odorants in meaty flavored commercial products. The knowledge extracted from this thesis-project, will become the basis for meaty flavored foodstuff development from the company. Consumers' preferences are priority for every company. In order to follow the rapid changing food markets, the flavor profiles changing according to these preferences will now become faster, efficient and reliable.

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