HEADSPACE FINGERPRINTING AS A TOOL TO COMPARE THE IMPACT OF HEAT AND HIGH PRESSURE PROCESSING ON CARROT QUALITY

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Centre for Food and Microbial Technology

Master dissertation submitted in partial fulfillment of the requirements for the Degree of Master of Food Technology
By Daniel Mwangi Njoroge

July – 2011
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I wish to thank the Almighty God who gave me the strength and patience to carry out the research work to the end, may all the glory be unto Him.

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ABSTRACT

High pressure processing (HPP) is a novel processing technique that has been claimed to result in food products of better quality than their conventionally treated counterparts, namely heat processed products. This has been concluded as a result of various comparative studies that have shown that HPP inactivates vegetative microorganisms and/or enzymes, while better retaining most of the quality attributes. However, these studies often do not have an equivalent starting point. Therefore, to have a fair comparison, it is necessary to start from an equivalent impact of heat processing and HPP, such as an equivalent microbial inactivation. To maximize the chances of identifying differences, a good strategy is to analyze the evaporative fraction of the food, since volatilizable compounds are often involved in processing-induced reactions.

This study aimed at using headspace fingerprinting as a tool for the comparison of the treatment impact of heat and HP processing on the quality of carrots and to further investigate the effect of the treatment intensity. This was achieved by analyzing the volatilizable compounds of heat and HP treated carrots, processed at three different intensity levels: no treatment, mild and severe pasteurization and sterilization. Before using the headspace analysis by GC-MS for the comparative analysis, first an optimization of the sampling method was performed. SPME sampling, performed after equilibration for 20 min and with extraction for 10 min, both at 40ºC, was found to be most appropriate. Therefore, it was selected and further used for the comparative analysis of the processed carrots. After obtaining the headspace fingerprints, impact comparisons over all intensities and per intensity level were performed. A clear separation between the untreated (for the comparison over all intensities), heat and HP treated carrots was evident for both cases. Therefore, it could be concluded that heat and HP processing have a different impact on the volatilizable carrot compounds. When considering the effect of intensity, per treatment type, carrots treated at different intensities also had a clear separation between them, both for heat and HP treated carrots. Therefore, it was concluded that the effect of treatment intensity on volatilizable compounds of carrots varies with the intensity levels. Consequently, this could result in varying quality, between heat and HP treated carrots and between those treated at different intensity levels per type of treatment, with respect to the evaporative fraction. Finally, from the multivariate comparative analysis, potential markers, which were most important for the separation of the various classes, were selected and analyzed.
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List of symbols

\( \rho \) Density \((\text{kg/m}^3)\),
\( \alpha \) Volumetric expansion coefficient \((1/\text{K})\)
\( C_p \) isobaric heat capacity \((\text{J/Kg.K})\)
\( F_0 \) sterilization value \((\text{min})\)
\( m/z \) mass-to-charge ratio
\( P \) pasteurization value \((\text{min})\)
\( \rho \) pressure \((\text{MPa})\)
\( T \) temperature \((^\circ \text{C or K})\)

List of sub-/superscripts

\( i \) initial condition
\( P \) process

List of abbreviations

CAR carboxen
CI chemical ionization
CUT come-up time
DVB divinylbenzene
EI electron ionization
EU european union
GC gas chromatograph\((y)\)
GC-MS gas chromatograph\((y)\) mass spectrometry/spectrometer
HP high pressure
HPHT high pressure high temperature
HPP high pressure processing
HT holding time
LV latent variable
<table>
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<tr>
<td>MS</td>
<td>mass spectrometry/spectrometer</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>PA</td>
<td>Polyacrylate</td>
</tr>
<tr>
<td>PATS</td>
<td>pressure-assisted thermal processing</td>
</tr>
<tr>
<td>PDMS</td>
<td>poly-dimethylsiloxane</td>
</tr>
<tr>
<td>PLS-DA</td>
<td>partial least squares-discriminant analysis</td>
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<tr>
<td>SPME</td>
<td>solid phase microextraction</td>
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<td>VIP</td>
<td>variable importance in projection</td>
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Conventional methods of food processing have been utilized for food preservation by the food industry over a long period of time. The frequently used methods include heat pasteurization and sterilization, where pasteurization involves mild heat treatments at temperatures below 100ºC for a predetermined time, while sterilization is done above 100ºC. As much as these treatments lead to the desired safety of the food, by destruction of vegetative and/or pathogenic microorganisms as well as spores for sterilization, they can also cause degradation of food quality parameters such as nutrients, texture, colour and flavours. This is due to their dependence on relatively high temperatures as a way of ensuring prolonged shelf–life and food safety. On the other hand, consumers increasingly demand convenient foods of the highest quality in terms of natural flavour and taste and which are free from additives and preservatives, microbiologically safe, having an extended shelf-life and requiring minimal preparation. This has lead to the development of a new generation of conservation processes, such as high pressure processing (HPP), which supposedly results in production of higher quality foods, since they have minor impact on the quality and fresh appearance of food products (Gould, 2001, pp. 1-17; Dede et al., 2007; Rastogi et al., 2007; Wilson et al., 2008). The change in quality of the food can be attributed to the impact of the processing method. Therefore, to better understand the impact of heat and HP processing on food quality, comparative studies are necessary. However, for a fair comparison an equivalent starting point, such as equivalent microbial inactivation, is important. This work involved the comparison of the impact of heat and HP processing on the evaporative fraction of carrots, starting from an equivalent microbial impact, and further investigating the effect of intensity.

HPP involves the use of high pressures in the range of 400 - 800 MPa to inactivate microorganisms and enzymes, hence making the food safe (during refrigerated storage for low-acid products) and extending its shelf-life. In addition, the high pressures can be combined with temperature, such as in pressure assisted thermal sterilization (PATS), to result in shelf-stable products. But, unlike the conventional processing methods, HPP is claimed to have limited effects on flavours and nutrients, and hence to better maintain the quality of fresh foods. The technique of HPP is already successfully applied in Japan, the United States and Europe for pasteurization of various food products. However, HP sterilization is still in a research phase.
and hence no HP sterilized products are yet on the market (San martin et al., 2002; Rastogi et al., 2007 and Norton and Sun, 2008). The general aspects of HHP are further discussed in chapter 1.

A comparison between the impact of heat and HPP on carrot quality can be done in many ways. However, since volatilizable compounds are often involved in processing-induced reactions, the highest chance of finding differences was expected in the evaporative fraction of the food. Therefore, to maximize the chances of identifying differences, this study focuses on the use of headspace-GC/MS analysis as a fingerprinting tool to compare heat and HP processed carrots. Further information on headspace analysis and modes of sampling is described in chapter 2.

In the experimental set-up, carrots were chosen as a relevant model food product. Moreover, since they are solid and have a high pH, it is also very useful to investigate the effect of different treatment intensities. Before the comparative analysis could be performed, a selection and optimization of the method of analysis was needed. For sampling, both the technique of static headspace and SPME were investigated. The choice depended on the total peak areas and the number of peaks. The procedures and the results of this part are described in detail in chapter 4.

In chapter 5, the optimized method of analysis was used for the impact comparison of heat processing and HPP on the quality of carrot pieces. The carrot pieces were treated with heat and HP at various intensities (mild and severe pasteurization and sterilization) that would result in equivalent microbial inactivation, per intensity level. Afterwards, headspace sampling was done and the resulting fingerprints were compared using multivariate data analysis. From these comparisons, potential markers were selected that best described the differences between classes.

Finally, the inferences made from this study and the necessary future work are summarized in chapter 6 as a general conclusion.
Part 1: Literature review
1 HIGH PRESSURE PROCESSING

1.1 Introduction

High pressure processing (HPP), also referred to as high-hydrostatic pressure processing or ultra high pressure processing in literature, is an industrially tested technology that offers a more natural, environmentally friendly alternative for pasteurization or shelf-life extension of a wide range of food products, both liquid and high moisture content solid foods, by use of pressure. Although lethal to vegetative microorganisms, pressure treatment does not break covalent bonds and has a minimal effect on food chemistry. High pressure can disrupt large molecules such as proteins, lipids and cell membranes and leave small molecules such as vitamins and flavour components less affected (Linton and Patterson, 2000; Balasubramaniam et al., 2008; Barbosa-Canovas and Juliano, 2008). The idea of using pressure as a process variable is not new, since first attempts date back to the late 1800s, when Hite (1899) observed that the shelf-life of milk and other food products could be increased after pressurization. However, it is only in the last two decades that the food industry has begun using pressure for food preservation. The use of high pressure for food processing is finding increased applications within the food industry, since sensory and nutritional attributes of the processed product remain less affected, thus yielding products with better quality than those processed by traditional methods (San Martin et al., 2002; Balasubramaniam et al., 2008).

1.2 High pressure principles

The governing principles of HPP are the isostatic principle, Le chatelier’s principle and the principle of microscopic ordering.

1.2.1 Isostatic principle

According to the isostatic principle, pressure is instantaneously and uniformly transmitted throughout the sample, whether the sample is in direct contact with the pressure medium or hermetically sealed in a flexible package (Figure 1.1). Therefore, the process time for HPP is independent of sample size and shape. The compressed product is not damaged at macroscopic level by pressure, if it contains sufficient moisture and not too much air. In that case, it returns to its original shape after pressure release, as long as pressure is applied...
uniformly in all directions. However, if the food product has a porous structure that contains a lot of air, it can collapse under pressure due to differences between the compressibility of air and water (Rastogi et al., 2007; Norton and Sun, 2008; Balasubramaniam et al., 2008; Yordanov and Angelova, 2010).

Figure 1.1 The principle of isostatic processing (Olsson, 1995).

1.2.2 Le chatelier’s principle

Le chatelier’s principle governs the food chemistry and microbiology. It states that when a system at equilibrium is disturbed, the system responds in a way that tends to minimize the disturbance (Pauling, 1964). Therefore, any phenomenon, such as a chemical reaction, a change in molecular configuration or a phase transition, which is accompanied by a decrease in volume, is enhanced or stimulated by pressure. It also enhances the stabilization of proteins due to bond formation and breakage of ions, leading to volume reduction of water due to electrostriction (Norton and Sun, 2008; Balasubramaniam et al., 2008). The application of pressures higher than 400 MPa in a biological system can cause reversible and irreversible cleavage of intermolecular and intramolecular bonds, including hydrogen bonds and hydrophobic interactions. In this way, structural changes in membranes as well as the inactivation of enzymes involved in vital biochemical reactions are the key targets of microbial kill by high pressure. The primary structure of low molecular weight molecules, such as vitamins, peptides, lipids, and saccharides, is rarely affected by high pressure, because of the very low compressibility of covalent bonds at pressures below 2000 MPa. Certain macromolecules, such as starch, can change their native structure during HPP, in a manner analogous to heat treatments (Heinz and Buckow, 2010).
1.2.3 Microscopic ordering principle

The principle of microscopic ordering states that, at constant temperature, an increase in pressure increases the degree of ordering of molecules of a given substance. Therefore, pressure and temperature can exert antagonistic forces on molecular structure and chemical reactions (Balny and Masson, 1993). In this case, pressure can stabilize a molecule at high temperature, while at low temperature denaturation under influence of pressure can occur.

1.3 High pressure equipment

A high pressure system consists of a high pressure vessel and its closure(s); devices for holding closure(s) in place while the vessel is under pressure; high-pressure intensifier pumps; a system for controlling and monitoring pressure (and temperature) and a product handling system for transferring the product to and from the pressure vessel. Normally, perforated baskets are used to insert and remove pre-packaged food products from the pressure vessels. The baskets may be transported by conveyor belts (horizontal loading) or by cranes (top loading). Pressure-transmitting fluids are used in the vessel to transmit pressure uniformly and instantaneously to the product samples. Most widely used fluids are water, glycol solutions, silicone oil, sodium benzoate solutions, ethanol solutions, castor oils and inert gases (Rasanayagam et al., 2003; Balasubramaniam et al., 2008; Yaldagar et al., 2008).

The generation of pressure inside the vessel may be achieved either by direct compression or by indirect compression or heating of the pressure medium. Direct compression involves the reduction of the treatment chamber by the action of a hydraulic pressure applied over a piston (Figure 1.2). This method allows very fast compression, but the limitations of the high-pressure dynamic seal between the piston and the vessel’s internal surface restrict the use of this method to small diameter laboratory or pilot plant systems.
CHAPTER 1: HIGH PRESSURE PROCESSING

Figure 1.2 Generation of high pressure by direct compression of the pressure-transmitting medium (Mertens, 1995).

Indirect compression involves the use of an intensifier or high pressure pump to pump the pressurizing medium directly into the vessel to reach a given pressure (Figure 1.3).

Heating of the pressure medium method utilizes the expansion of the pressure fluid with increasing temperature to generate high pressure. Therefore, it is used when high pressure is applied in combination with high temperature and requires very accurate temperature control within the entire internal volume of the pressure vessel (Mertens, 1995; San martin et al., 2002; Yordanov and Angelova, 2010). It must be remarked that the last method is not often used and cannot lead to the high pressure levels usually applied for preservation purposes.

Figure 1.3 Generation of high pressure by indirect compression of the pressure-transmitting medium (Mertens, 1995).
Food can be high-pressure processed in two fundamentally different ways: in-container as a batch process (HPP is executed after filling and sealing of the food into its final or intermediate package) or in bulk as a semi-continuous process, followed by aseptic or ultraclean filling and sealing (Mertens, 1995). A typical batch HP process (Figure 1.4) uses food products packaged in a high-barrier, flexible pouch or a plastic container. The packages are loaded into the high-pressure chamber. The vessel is sealed, filled with pressure-transmitting fluid, and pressurized using a high-pressure pump, which injects additional quantities of fluid.

After holding for the desired time at a target pressure, the vessel is decompressed by releasing the pressure-transmitting fluid (Farkas and Hoover, 2000). About 5-6 cycles/h are possible, allowing time for compression, holding, decompression, loading and unloading. After pressure treatment the processed product is removed from the vessel and stored and distributed in a conventional manner (Balasubramaniam et al., 2008).

Figure 1.4 Diagram of operation of a HPP batch unit (NC Hyperbaric, 2010).

Although HPP is usually a batch process, a semi-continuous line for liquid foods may be built by assembling three or more pressure vessels in series. The vessels should be equipped with a free floating piston that allows each vessel to be divided into two chambers. One chamber is used for the liquid food while the other one is used for the pressure-transmitting liquid. The three vessels are operated in such a way that, one is loading, one is compressing and one is discharging at any point in time, hence creating a semi-continuous system capable of delivering a continuous product output (Farkas and Hoover, 2000; San martin et al., 2002; Balasubramaniam et al., 2008).
1.4 Pressure-temperature effects

1.4.1 Compression heating

The work of compression during HPP causes the food and/or pressure-transmitting medium to undergo a specific reversible temperature increase due to adiabatic heating. The temperature rise is usually not the same for all food products, since it depends on food composition as well as processing temperature and pressure and the rate of pressurization (Table 1.1). However, the maximum product temperature at process pressure is independent of the compression rate as long as the heat transfer to the surroundings is negligible. Generally, compression/decompression temperature and pressure curves are nearly linear and hence the compression rate can be assumed constant for a given interval. Once the target temperature is fixed, a constant compression rate should provide a constant compression heating (Barbosa-Canovas and Juliano, 2008). However, this depends on how the volumetric expansion coefficient \( \alpha_p \) (1/K), the density \( \rho \) (kg/m\(^3\)), and the isobaric heat capacity \( C_p \) (J/Kg.K) of both the food and liquid will change during the pressurization time, as shown by the following compression heating equation:

\[
\frac{dT}{dp} = \frac{\alpha T}{\rho C_p}
\]

with \( T \) the temperature (K) of the food or compression fluid and \( p \) the pressure (Pa) (Matser et al., 2004).

Table 1.1 Temperature changes of selected substances due to compression heating (De Heij et al., 2003).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Initial temperature (°C)</th>
<th>Temperature change(°C/100 MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>20</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>4.4</td>
</tr>
<tr>
<td>Steel</td>
<td>20</td>
<td>~0</td>
</tr>
<tr>
<td>Chicken</td>
<td>20</td>
<td>2.9</td>
</tr>
<tr>
<td>Cheese (Gouda type)</td>
<td>20</td>
<td>3.4</td>
</tr>
<tr>
<td>Milk fat</td>
<td>20</td>
<td>8.5</td>
</tr>
</tbody>
</table>
CHAPTER 1: HIGH PRESSURE PROCESSING

The pressure increase during the come up time ($t_1$-$t_2$) from an initial pressure $P_1$ to $P_2$ increases the temperature from $T_1$ to $T_2$ (**Figure 1.5**), as a result of physical compression of the food product. During the pressure holding time ($t_2$-$t_3$) the temperature of the product decreases from $T_2$ to $T_3$ due to heat loss through the pressure vessel. Upon decompression ($P_3$-$P_4$), the product will go back to its initial temperature in a perfectly insulated (adiabatic) system. However, in practice the temperature returns to a temperature ($T_4$) slightly lower than the initial temperature ($T_1$), due to the heat losses during the compression (elevated temperature) phase. The rapid heating and cooling resulting from HPP offers a unique way to increase the temperature of the product only during the treatment and to cool it rapidly thereafter (*cfr. 1.5*) (Rastogi *et al.*, 2007; Balasubramaniam *et al.*, 2008; Yordanov and Angelova, 2010).

**Figure 1.5** Pressure-temperature evolution during high pressure processing (Balasubramaniam *et al.*, 2008).

1.4.2 Temperature distribution in the vessel

In practice, the pressurization is not adiabatic since there is exchange of heat between the food, pressure-transmitting medium and the wall of the pressure vessel. Therefore, the temperature is not uniformly distributed inside the pressure vessel as well as in the product (**Figure 1.6**). On the other hand, while the temperature of the product may rise significantly, the metal vessel that surrounds the product is not subjected to significant compression heating, due to its low compressibility factor compared to the one of the product. Therefore, a
product near the steel wall of the HP vessel generally reaches a lower temperature than the ones at the centre of the vessel. This may compromise the effectiveness of the process at the sides, due to subsequent cooling near the walls. However, different technical solutions are usually implemented to improve the temperature uniformity during processing (De Heij et al., 2003; Norton and Sun, 2008).

Figure 1.6 Temperature distribution in a HPP chamber (Norton and Sun, 2008).

1.5 High pressure pasteurization versus sterilization

Use of HP to process food effects a decrease in number of vegetative microorganisms, hence resulting in pasteurized food products. At ambient temperatures, application of pressures in the range of 400 to 600 MPa inactivates vegetative micro-organisms and reduces the activity of enzymes, resulting in a pasteurized product. This product usually has a limited shelf-life, hence, needs to be stored under conditions that minimizes the growth of microorganisms and spore germination, such as refrigeration. However, pasteurized high-acid foods (pH<4.6) do not support spore germination, hence are shelf-stable for a limited period of time. The use of pressure and mild process temperatures (below 45°C) allows pasteurization of the food with minimal effects on taste, texture, appearance, or nutritional value. HP inactivation of vegetative microorganisms is caused by membrane damage, protein denaturation and decrease of intracellular pH, suggesting that pressure results in deactivation of membrane-bound
enzymes associated with an efflux of protons (Cheftel, 1995; Smelt, 1998; Balasubramaniam et al., 2008).

Low-acid foods to be stored at ambient temperature for a longer shelf-life require sterilization in order to inactivate the bacterial endospores to achieve a commercially sterile product. HP technology, as commercially defined today, is unable to produce low-acid shelf stable products, due to its inability to inactivate spores by pressure alone. Bacterial spores have shown to be very pressure resistant, capable to survive pressures up to 1200 MPa. Two mechanisms have shown to result in high-pressure inactivation of bacterial spores. At lower pressures and temperatures, pressure induces spores to germinate, and the germinated spores are subsequently inactivated by the pressure treatment. At higher temperatures, a direct spore-inactivation mechanism that bypasses germination is more likely (San Martin et al., 2002 and De Heij et al., 2003). Sterilization of low-acid foods relies on a combination of HP and other forms of relatively mild treatments. HPP can be used for sterilization of food products if applied at elevated temperatures and using the temperature increase due to compression. This is also referred to as high pressure high temperature (HPHT) processing, or pressure-assisted thermal processing (PATS). It involves the use of moderate initial chamber temperatures between 60 and 90°C in which, through internal compression heating at pressures of 600 MPa or greater, the in-process temperature can reach 90 to 130°C in only a few seconds. The temperature rise during the sterilization process and temperature difference between various positions in vessels plays an important role and requires accurate monitoring. By choosing the appropriate process conditions, it is possible to completely inactivate both vegetative cells and microbial spores resulting in food products that are shelf-stable (Matser et al., 2004; Barbosa-Canovas and Juliano, 2008).

The effect of HPHT processing is often characterized as being synergistic, not significantly different or protective compared to heat inactivation. However, the effect is strain and product dependent, and since synergy is not consistently observed in all strains and food products during inactivation of C. botulinum, high pressure sterilization should assume for the present a complete lack of synergy (Bull et al., 2009). Therefore, any HPHT process for low acid shelf-stable foods must be at least thermally equivalent to a process value ($F_o$) of 2.8 min, in line with good manufacturing practices.
The advantage of HPHT processing over conventional sterilization is realized in the shorter processing time due to the instantaneous volumetric heating and cooling, lower maximum temperatures and a more uniform radial temperature distribution. The time-temperature integration of the HPHT treatment is much smaller than that of the conventional sterilization (Figure 1.7). Because of the shorter processing time, the product is probably subjected to less damage and, consequently, a food product with better retention of colour, flavour, texture and nutrients can be obtained (Matser et al., 2004; Barbosa-Canovas and Juliano, 2008; De Roeck et al., 2010).

![Figure 1.7 Typical product temperature profiles in a retort and HPHT process. Processing steps during pressurization (Barbosa-Canovas and Juliano, 2008).](image)

1.6 Current status of high pressure processing

1.6.1 Commercial applications

The technique of HPP is currently successfully used in Japan, the United States and Europe for pasteurization of food products. The industrial application of HPP has been on an increasing trend for the last decade, as is evidenced by increased HPP equipment installations (Figure 1.8).
Currently, several HP pasteurized products are commercially available on the market. They include a wide range of food products such as fruits and vegetables, meat products, seafood, juices and beverages and dairy products, with meat and vegetable products being the most popular applications (Figure 1.9).

To date, HPP has only been used to inactivate vegetative microorganisms, but the use of high pressure in combination with heat (HPHT) to eliminate spores under conditions more favourable to quality than retorting is a topic of current research. Therefore, low acid shelf-stable products such as soups are not commercially available yet because of the limitations in killing spores with HPP (cfr. 1.5) (San martin et al., 2002; Rastogi et al., 2007; Balasubramaniam et al., 2008).

Apart from pasteurization, HPP is also applied in seafood for mollusks shucking and crustacean meat extraction. Application of pressures ranging from 200-350 MPa denatures the proteins that are responsible for holding the shells together in mollusks, while the meat of crustaceans contract and detaches from the shell. Therefore, the yield increases and the production costs decreases as the meat is easily extracted (NC Hyperbaric, 2010).
1.6.2 Novel food legislation

According to the European legislation, foods produced by HPP are considered novel foods since they fulfill two conditions. First, their history of human consumption has been negligible before 1997, since they have not been used for human consumption to a significant degree within the EU (European Union) community. Secondly, a new manufacturing process has produced them, which can give rise to significant changes in the composition or structure of the foods or food ingredients, which affect their nutritional value, metabolism or level of undesirable substances (Norton and Sun, 2008). Therefore, they fall under the “Novel Food Legislation”, Regulation (EC) 258/97 of 27 January 1997. This legislation concerns the placing on the EU market of novel foods and novel food ingredients that have not been on the EU market before its entry into force. There are several categories of novel foods that fall under this legislation in article 1(2). However, HPP products fall in the category of ‘foods and food ingredients to which has been applied a production process not currently used, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances’ (European Union, 2011). In article (3) the legislation requires that the novel foods to be placed on the EU market ‘must not present a danger for the consumers; mislead the consumer; differ from the foods or food ingredients which they are intended to replace to
such an extent that their normal consumption would be nutritionally disadvantageous to the consumer’ (European Union, 1997).

In order for a novel food to be introduced into the market, it has to be authorized through a community procedure. However, since 2001, the novel food regulation includes a simplified procedure for marketing certain types of novel foods or novel food ingredients in the EU, if they are considered substantially equivalent to an existing food or food ingredient that is already marketed within the EU. Substantial equivalence is considered as a reasonable tool to assess the nutritional composition and safety of a novel food in relation to the nutritional composition and safety of its traditional counterpart. It must be proven through scientific assessment and the characteristics verified must not be different or pose no danger for public health in comparison with conventional foods (Schauzu, 2000; Heinz and Buckow, 2010).

1.7 Conclusion

HPP can be a good alternative for heat processing since it can provide a better balance between safety and quality. Today, various studies compare the impact of HPP and heat processing on quality aspects, but often single response studies are used, such as vitamin C concentration, colour and texture degradation, enzyme activity etc. (López-Malo et al., 1998; Krebbers et al., 2002; Patras et al., 2009). In addition, the studies do not have an equivalent starting point, like equivalent microbial inactivation, and hence usually result in an unfair comparison. Therefore, there is a need for comparison of HPP products with conventionally processed products, starting from an equivalent principle, such as, safety by equivalent microbial inactivation. This would ensure that the comparison is fair and the conclusions based on the study are not biased.
2 HEADSPACE ANALYSIS

2.1 Introduction

Headspace analysis is the analysis of the components present in the gas phase in the vial above a sample. It is used for the analysis of volatile and semi-volatile organics in solid, liquid and gas samples. It is most suited for the analysis of the very light volatiles in samples that can be efficiently partitioned into the headspace gas volume from the liquid or solid matrix sample. Higher boiling compounds are not detectable with this technique due to their low partition in the gas headspace volume. Complex sample matrices, which would otherwise require sample extraction or preparation, or be difficult to analyse directly, are ideal candidates for headspace analysis since they can be placed directly in a vial with little or no preparation. The headspace can be investigated by various methods. However, gas chromatography (GC) is particularly well suited for such measurements, since it is ideal for gas (vapour) analysis. A sample of the gas in the headspace is injected into a GC system for separation of all of the volatile components (Kolb and Ettre, 1997, pp. 1-5).

The gas phase is commonly referred to as the headspace and lies above the condensed sample phase, while the sample phase contains the compound(s) of interest. The sample phase is usually in the form of a liquid or a solid in combination with a dilution solvent or a matrix modifier. The sample is usually introduced into a vial and the vial is tightly sealed. If the system contains volatile analytes that are soluble in the matrix, they will distribute among the two phases according to a thermodynamically controlled equilibrium, as shown by the arrows in Figure 2.1. Several methods can be used to take a sample from the headspace and introduce it into the GC, such as taking a direct gaseous sample (static headspace analysis) or trapping the volatile material on a solid phase microextraction (SPME) fiber (Smith, 2003; Kolb and Ettre, 1997, pp. 1-5).

![Volume of the gas phase.](https://example.com/figure2.png)

**Figure 2.1** A headspace vial containing a sample (Kolb and Ettre, 1997, p. 14).
2.2 Gas Chromatography-Mass Spectrometry (GC-MS) principles

Gas chromatography mass spectrometry (GC-MS) is an important tool for the identification and quantitation of volatile and semivolatile organic compounds in complex mixtures (Figure 2.2). As such, it is very useful for the determination of molecular weights and (sometimes) the elemental compositions of unknown organic compounds in complex mixtures. With the GC component chemical mixtures can be separated while with the MS part the components can be identified at a molecular level. Volatile compounds are an important part of the food, since they contribute to the aroma and flavour of the final food product. In addition, process-induced reactions also affect the volatiles, leading to increases or decreases and production of new volatiles. To achieve an identification, three distinct steps are involved; that is injection, separation and detection. The sample volatiles are heated in the injection port and are carried through a column, coated with fluid or solid support (stationary phase), with an inert gas (mobile phase), where they are separated. Carrier gases commonly used include nitrogen, helium, argon and carbon dioxide. As the separated substances emerge from the column opening, they flow into the mass spectrometer, at which the compounds are detected (Hites, 1997; McNair and Miller, 1998, pp. 153-164).

![Scheme of a typical capillary gas chromatograph-mass spectrometer](image)

**Figure 2.2** Scheme of a typical capillary gas chromatograph-mass spectrometer (Masucci and Caldwell, 2004).
2.2.1 Injection

The sample is usually injected through a septum into the injector port above the column. The temperature of the injector port is usually higher than the temperature of the analyte with the highest boiling point to avoid condensation. The amount of sample injected into the GC depends on the type of column. Packed columns can take a relatively higher amount of sample (tenths of a microliter up to 20 microliters) as compared to capillary columns ($10^{-3}$ µL). Injection can either be done in split or splitless mode. In split mode, some of the sample is carried away by the carrier gas and goes to the waste (Figure 2.3), while in splitless mode all the sample enters the column. Split mode ensures that there is no overloading of the column and due to the high flow rate of the carrier gas, there is rapid injection and hence the initial bandwidth of the column is narrower. Consequently, for analyses that require rapid injection and separation it is preferred. Splitless mode may result in broadening of peaks and overload of the column, since 95% of the sample enters the column. However, also other techniques can be used to solve these problems, such as control of flow rate, thermal and solvent effects (Snow, 2004; McNair and Miller, 1998, pp. 86-99).

![Figure 2.3 Cross section of a typical split injector (McNair and Miller 1998, p. 98).](image)

2.2.2 Separation

The separation of the analytes occurs within the chromatographic column. Columns can be of two types; either packed columns or capillary columns. Packed columns are usually filled...
with inert solid support material, coated with a liquid stationary phase. Separation depends on
the interaction intensity of the analyte components carried by the gas (mobile phase) with the
column matrix (stationary phase). Particles with high affinity take longer time to elude from
the column, while those with low affinity take shorter time. The elution profile of the analyte
also correlates with the boiling point of the substances, where the substances with low boiling
points elude fast, while those with high boiling points take a longer time, hence the need for
control of the column temperature. The carrier gas may have a significant effect on the
separation process. Adsorbents with high specific surface areas adsorb the carrier gas, thus
decreasing the number of active sites (adsorption centers) available to the components of
interest. (Barry, 2004; Grob, 2004; Kaiser and Dybowski, 2004).

2.2.3 Detection

Detection of the sample occurs in the mass spectrometer (MS) (Figure 2.4). First, ions are
formed by electron ionization (EI) or chemical ionization (CI). In EI, analyte gaseous
molecules leaving the GC and entering the MS are bombarded by a strong beam of electrons,
therefore ionizing and fragmenting them. In CI, a reagent gas is ionized first, followed by a
transfer of charge to the sample molecule by a chemical process. A reagent gas, such as
methane, is introduced into the ionization chamber, where it is ionized first, instead of the
sample molecule. The cation formed undergoes further reaction to form secondary ions,
which serves as a reagent that gently ionizes the sample molecule by proton donation. It
usually results in less fragmentation and simpler mass spectra (Colon and Baird, 2004;
McNair and Miller, 1998, pp. 156-159).

The charged particles (molecular ions and fragment ions) formed are required for the mass
analysis. Manipulation of the charged particles by the ion analyzer (quadrupole or magnetic
sector) accelerates them through the mass spectrometer, depending on the mass to charge
(m/z) ratio, while the uncharged particles are pumped away. In the quadrupole mass analyzer,
different radiofrequencies and direct current potentials are applied to the rods, enabling ions
of specific m/z ratio to have a stable trajectory and pass through to the detector. Since the EI
produces particles with single charge, the path followed by the ions depends on the mass. The
voltage on the ion analyzer is not fixed, therefore, different masses can find a successful path
to follow to the detector. Once the ion strikes the detector an electronic signal is produced.
Through timing mechanisms that integrate the signals produced with the voltage, the instrument reports the m/z ratio that strikes the detector. The mass analyzer sorts the ions according to m/z and the detector records the abundance of each m/z (Colon and Baird, 2004; McNair and Miller, 1998, pp. 156-159).

![General scheme of a mass spectrometer](image)

**Figure 2.4** General scheme of a mass spectrometer (McNair and Miller, 1998, p. 154).

### 2.3 Types of headspace sampling

#### 2.3.1 Static headspace

Static headspace analysis consists of three steps (**Figure 2.5**). First, the sample, a liquid or a solid, is placed in a vessel, having a gas volume above it, and next, the vessel, usually a vial, is closed. The vial is thermostated at a constant temperature until equilibrium is reached between the two phases. Secondly, an aliquot of the vial’s gas phase is extracted, either manually or by use of an autosampler, both with a gas-tight syringe. Finally, it is introduced into the injector, where the carrier gas stream carries it into the column, where it is analyzed. Although static headspace analysis is the simplest method of analyzing the headspace, it lacks sensitivity and allows only the quantification of the most abundant compounds, due to the lack of a concentrating effect (Pawliszyn, 1995; Kolb and Ettre, 1997).
Figure 2.5 Steps in static headspace injection. (Labhut, 2011).

2.3.1.1 Optimization of static headspace sampling conditions

Equilibration time and temperature
Equilibration is important to have a representative sample of the food. The time and temperature combination required to achieve equilibration depends on diffusion of the volatile sample components from the sample matrix to the headspace and vice versa. To establish it, several vials are prepared with the same sample and thermostated at different time and temperature combinations, and finally analyzed under identical conditions. The total peak areas and/or the peak numbers are plotted against the thermostating time. The time and temperature required for the equilibration, is the time and temperature combination at which a constant peak area is obtained. However, care should be taken to avoid prolonged thermostatting at high temperatures, since some components may be sensitive to prolonged heating (Kolb and Ettre, 1997, pp. 118-123).

Salting
Addition of a salting-out agent, such as sodium chloride, to the sample matrix reduces the solubility of polar compounds by lowering the water activity, hence lowering the partition coefficient. Therefore, there is an increase in concentration of analyte in the headspace. Consequently, the headspace sensitivity increases. However, the effect depends on the particular analyte and salt concentration in the sample. To be effective, high salt
concentrations are needed (Kolb and Ettre, 1997 pp. 30-33; Yang and Peppard, 1999; Wardencki *et al.*, 2004).

### 2.3.2 Solid phase microextraction

Solid phase microextraction (SPME), followed by GC-MS, is a modern, rapid, solvent-free sample preparation technique, commonly used in trace analysis, and an easy-to-optimize fractionation technique for characterization of volatiles. It integrates sampling, extraction, concentration, and sample introduction to GC. It offers the benefits of short sample preparation times; small sample volumes; analyte concentration from liquid, gaseous and solid samples and easily automated to allow the high-throughput analysis (Soria *et al.*, 2008; Risticevic *et al.*, 2009).

#### 2.3.2.1 SPME apparatus

The SPME fiber device consists of a fiber holder and a fiber assembly with a built-in fiber inside the needle, which looks like a modified syringe. Two types of fiber assemblies exist on the market: one for use in a manual holder (*Figure 2.6*) and the other for use in a holder for an autosampler. The major difference between the two is the presence of a spring in the manual holder that keeps the fiber retracted while not in use, which is not necessary in the automated version. The fiber holder consists of a plunger, a stainless-steel barrel and an adjustable depth gauge with needle, and is designed to be used with reusable and replaceable fiber assemblies. The chemically inert fused-silica optical fiber is very stable, even at high temperatures. It is externally coated with a thin film polymeric [e.g. Carbowax, divinylbenzene (DVB), polydimethylsiloxane (PDMS), Polyacrylate (PA)] stationary phase or a mixture of polymers blended with a porous carbon-based solid material (e.g. PDMS–Carboxen, DVB-Carboxen-PDMS). The film concentrates the organic analytes on its surface during absorption or adsorption from the sample matrix. The fiber can either be used manually or by an autosampler to deliver the sample from the vial to the GC (Wercinski and Pawliszyn, 1999; Shirey, 1999; Mills and Walker, 2000; Kataoka *et al.*, 2000).
2.3.2.2 SPME sampling procedure

The SPME technique involves exposure of a small amount of extracting phase dispersed on a solid support to the sample matrix. Two basic steps are involved; (i) the analyte is partitioned between the extraction phase and the sample matrix and (ii) concentrated extracts are desorbed into an analytical instrument. The sample is put in a vial and thermostated to achieve equilibrium and then the fiber is exposed to the sample for extraction, either by direct dipping the SPME fiber into the sample solution (direct extraction) or from the headspace (HS-SPME) (Figure 2.7). The latter method prolongs the life of the fiber used, because it is not in direct contact with the sample matrix. After adsorption, the fiber is introduced into the hot GC injector for thermal desorption of the analyte. Desorption should be carried out within the shortest time possible and hence the temperature of the injector should be higher than the boiling point of the compound with the highest boiling point. As the temperature increases, the coating/gas coefficient decreases and the fiber coating ability to retain analytes quickly diminishes, hence releasing them (Wercinski and Pawliszyn, 1999; Mills and Walker, 2000; Wardencki et al., 2004; Risticevic et al., 2009).
2.3.2.3 Optimization of SPME conditions

Selection of fiber type

SPME has been used routinely in combination with GC-MS and successfully applied to a wide variety of compounds, especially for the extraction of volatile and semi-volatile organic compounds from complex sample matrices. Two types of fibers can be distinguished based on their method of analyte extraction. First, fibers coated with liquid coating substrate such as polydimethylsiloxane (PDMS) that extract analyte by partitioning it in the liquid phase. Second, fibers with a porous substrate such as carbowax or divinylbenzene (DVB) that adsorbs the analyte by physically trapping or reacting with them through the hydrogen bonding or van der waals interactions. Selectivity of the SPME fractionation towards different analytes can be controlled by selecting the most appropriate fiber coating. The sensitivity can be improved by increasing the coating/water distribution constant of the analyte by changing to a fiber coating that is more selective for the target analytes (polar fibers are used for polar analytes and non-polar types for non-polar analytes). Since the majority of flavour components are apolar in nature, commercially available SPME fibers (Table 2.1), which are generally apolar or slightly polar can be used for extracting them from the food matrices. Thickness of the film is also important. Increasing the fiber coating thickness increases the
mass of analyte absorbed at equilibrium, since a larger phase volume can retain more analytes. However, it increases the equilibration time, as opposed to a thinner fiber. Naturally, the thinnest fiber should be used to obtain the sensitivity desired within the minimum extraction time (Wercinski and Pawliszyn, 1999; Shirey, 1999; Mills and Walker, 2000; Vas and Vekey, 2004; Wardencki et al., 2004; Risticevic et al., 2009).

Table 2.1 Examples of standard fibers available on the market (Mills and Walker, 2000).

<table>
<thead>
<tr>
<th>Fiber Coating</th>
<th>Film thickness (µm)</th>
<th>Polarity</th>
<th>Coating stability</th>
<th>Maximum temperature (°C)</th>
<th>Analytical application</th>
<th>Recommended use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polydimethylsiloxane (PDMS)</td>
<td>100</td>
<td>Non-polar</td>
<td>Non-bonded</td>
<td>280</td>
<td>GC/HPLC</td>
<td>Volatiles</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Non-polar</td>
<td>Non-bonded</td>
<td>280</td>
<td>GC/HPLC</td>
<td>Non-polar semivolatile analytes</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Non-polar</td>
<td>bonded</td>
<td>340</td>
<td>GC/HPLC</td>
<td>Mid-to non-polar semivolatile analytes</td>
</tr>
<tr>
<td>PDMS-Divinylbenzene (DVB)</td>
<td>65</td>
<td>Bi-polar</td>
<td>Cross-linked</td>
<td>270</td>
<td>GC</td>
<td>Polar volatiles</td>
</tr>
<tr>
<td>(StableFlex fiber)</td>
<td>60</td>
<td>Bi-polar</td>
<td>Cross-linked</td>
<td>270</td>
<td>HPLC</td>
<td>General purpose</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>Bi-polar</td>
<td>Cross-linked</td>
<td>270</td>
<td>GC</td>
<td>Polar volatiles</td>
</tr>
<tr>
<td>Polyacrylate (PA)</td>
<td>85</td>
<td>polar</td>
<td>Cross-linked</td>
<td>320</td>
<td>GC/HPLC</td>
<td>Polar semivolatile analytes (phenols)</td>
</tr>
<tr>
<td>Carboxen-PDMS (StableFlex fiber)</td>
<td>75</td>
<td>Bi-polar</td>
<td>Cross-linked</td>
<td>320</td>
<td>GC</td>
<td>Gas and volatiles</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>Bi-polar</td>
<td>Cross-linked</td>
<td>320</td>
<td>GC</td>
<td>Gas and volatiles</td>
</tr>
<tr>
<td>Carbowax/DVB (StableFlex fiber)</td>
<td>65</td>
<td>polar</td>
<td>Cross-linked</td>
<td>265</td>
<td>GC</td>
<td>Polar analytes (alcohols)</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>polar</td>
<td>Cross-linked</td>
<td>265</td>
<td>GC</td>
<td>Polar analytes (alcohols)</td>
</tr>
<tr>
<td>Carbowax/templated resin (TPR)</td>
<td>50</td>
<td>polar</td>
<td>Cross-linked</td>
<td>240</td>
<td>HPLC</td>
<td>Surfactants</td>
</tr>
<tr>
<td>DVB-PDMS-Carboxen</td>
<td>50/30</td>
<td>Bi-polar</td>
<td>Cross-linked</td>
<td>270</td>
<td>GC</td>
<td>Odours and flavours</td>
</tr>
</tbody>
</table>

Equilibrium time and temperature

Just as for static headspace sampling, it is important to establish an equilibrium phase between the sample and its headspace before introducing the SPME fiber, in order to have a representative sample of the food. The necessary steps to determine time and temperature required to achieve this equilibrium are the same as for the static headspace sampling (cfr. 2.3.1.1).
Extraction time and temperature

The extraction time is directly proportional to the amount of analyte adsorbed to the SPME fiber until equilibrium between the headspace and fiber coating is reached. However, for many volatile and semivolatile compounds this equilibrium is usually not achieved in a reasonable time, but, if the extraction time is precisely controlled, good precision can still be achieved, even without attaining equilibrium. The distribution constant of a substance between the fiber and sample also depends on temperature. Optimizing the sample temperature changes the distribution constant, hence increasing the sensitivity of higher boiling components, while decreasing the sensitivity for the lower boiling components. The higher the distribution constant of a compound, the higher the affinity of that compound for the SPME fiber coating. However, excessive increase of temperature may cause premature desorption of the more volatile analytes since the distribution constant decreases at higher temperature (Wercinski and Pawliszyn, 1999; Penton, 1999; Wardencki et al., 2004).

Salting

Just as for static headspace sampling, the addition of salts can improve the release of volatile and semivolatile compounds into the headspace, and, consequently, more analytes can be extracted by the fiber (cfr. 2.3.1.1).

2.4 Volatilizable carrot compounds and the effect of processing

2.4.1 Volatilizable compounds in carrots

Volatilizable components have been reported to be among the most influential parameters on consumer acceptance of carrots (Rosenfield et al., 2002). Varming et al. (2004) indicated that sensory profiling and consumer likings are well correlated in various carrot varieties. The eating quality of carrots can be measured directly by chemical, mechanical or optical measurements. The sensory quality of carrots is dependent on the amount of volatilizable compounds and non-volatilizable bitter taste compounds and sugars. The intensity of the aroma is variety-specific with the orange carrots having a higher intensity than the yellow varieties (Kreutzmann et al., 2008.)

Volatilizable carrot compounds include terpenoids, phenylpropanoids and fatty acid derivatives. Terpenoids are divided into monoterpenes (including α-pinene, sabinene, β-
myrcene, limonene, γ-terpinene, ρ-cymene, and terpinolene), sesquiterpenes (including β-caryophyllene copaene, cuparene, β-bisabolene, α-humulene, and (E)- and (Z)-γ-bisabolene) and irregular monoterpenes such as 6-methyl-5 hepeten-2-one and β-ionone, which are most likely degradation products of carotenoids (Table 2.2). Mono- and sesquiterpenes are the two most abundant classes accounting for approximately 99% of the total volatile mass and have been described to contribute significantly to carrot aroma. Phenylpropanoids include eugenol methyl ether, eugenol, elemicin, and myristicin, while fatty acid derivatives include aldehydes, such as octanal (Alasalvar et al., 1999; Kjeldsen et al., 2001; Kjeldsen et al., 2003).

**Table 2.2** Main volatilizable raw carrot compounds and their odour description (Alasalvar et al., 1999; Kjeldsen et al., 2003).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Oduor description</th>
<th>Compound</th>
<th>Oduor description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-pinene</td>
<td>Carrot top</td>
<td>2,5 Dimethyl styrene</td>
<td>-</td>
</tr>
<tr>
<td>Camphene</td>
<td>-</td>
<td>Undecane</td>
<td>-</td>
</tr>
<tr>
<td>Sabinene</td>
<td>Carrot-like, fresh green</td>
<td>Camphor</td>
<td>-</td>
</tr>
<tr>
<td>β-pinene</td>
<td>Fresh green</td>
<td>Bornyl acetate</td>
<td>Green</td>
</tr>
<tr>
<td>Myrcene</td>
<td>Green</td>
<td>β-caryophyllene</td>
<td>Terpene-like, spicy, woody</td>
</tr>
<tr>
<td>α-phellandrene</td>
<td>Herbaceous, green, carrot top</td>
<td>Trans-α-Bergamotene</td>
<td>-</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>Citrus</td>
<td>α-Humulene</td>
<td>Woody</td>
</tr>
<tr>
<td>β-cymene</td>
<td>Carrot top</td>
<td>Cis-β-Farnesene</td>
<td>Floral</td>
</tr>
<tr>
<td>Limonene</td>
<td>Citrus, fruity</td>
<td>Valencene</td>
<td>-</td>
</tr>
<tr>
<td>Cis-Ocimene</td>
<td>-</td>
<td>β-Bisabolene</td>
<td>Sweet</td>
</tr>
<tr>
<td>Trans-Ocimene</td>
<td>-</td>
<td>(E)-γ-Bisabolene</td>
<td>Soapy, spicy</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>-</td>
<td>(Z)-γ-Bisabolene</td>
<td>Fatty, woody</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>Sweet, fruity, citrus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 2.4.2 Effect of processing on volatilizable carrot compounds

Volatile carrot compounds are affected by various processing methods. As a result of heat treatment they either decrease or increase due to process-induced reactions. Trejo Araya et al. (2009) reported that all monoterpenes were still present after a heat treatment of 5 min at 90°C (sous-vide) and after 20 min at 100°C (boiling). On the other hand, according to Shamaila et al. (1996) most carrot volatiles, in particular terpenoids (sabinene, β-pinene, β-myrcene, limonene, trans-caryophyllene, α-humulene, β-bisabolene and α-farnesene), decreased by at least 50% within 60 sec of blanching, while the cooked aroma increased. In
addition, Alasalvar et al. (1999) found that cooking for 10, 20 and 30 min resulted in 88.6, 93.0 and 95.5% loss in total volatiles, respectively. Severe heat treatment, such as canning of carrots, can result in transformation of compounds, such as styrene, to form ethanethiol, dimethyl sulfide and dimethyl substituted styrene compounds, which give rise to the typical canned carrot flavour. Heatherbell et al. (1971) reported that canning resulted in a loss of approximately 50% of “higher boiling” compounds. However, it produced an increase in “lower boiling” compounds, particularly methanol, which increased from 0.05 to 60 ppm.

The effect of HPP on carrot volatiles was only reported once: according to Trejo Araya et al. (2009), all monoterpenes were still present after treating carrot pieces at 600 MPa for 2 min, meaning that the intrinsic carrot compounds were not lost and in some cases were even increased in processed carrots relative to raw samples. Some additional studies on the impact of HPP on volatiles of other vegetables exist: in HP-treated onions (300 MPa/25°C/30 min) and tomato juice (500, 700 or 900 MPa/room temperature/3, 6 or 9 min) there was an increase in hexanal concentration. This was probably due to HP-induced oxidation of free fatty acids, such as linoleic and linolenic acids. In garlic treated at 200, 400 and 600 MPa for 20 min at room temperature, the flavour intensity of odor-active compounds decreased with increasing pressure (Oey et al., 2008; Ma et al., in press).

2.5 Conclusion

GC-MS is used in many applications to study the composition of a certain fraction of foods. However, it is only rarely used to study the impact on processing-induced changes or to compare the impact of heat processing and HPP on foods. For many processing-induced reactions volatilizable compounds are involved. Therefore, the highest chance to find differences is in the evaporative fraction of a food product, hence the use of headspace-GC/MS analysis to compare the impact of HPP and conventional heat processing can be very useful.
Part 2: Experimental part
3 OBJECTIVE

HPP can be a good alternative for heat processing since it can provide a better balance between safety and quality. However, there is a need to perform a fair comparison between HP-treated food products and their heat treated counterparts, in order to investigate the impact of this processing on the quality of the food product. This is only possible by starting from an equivalent point, such as equivalent microbial inactivation. Since volatilizable compounds are often involved in processing-induced reactions, the highest chance of finding differences is in the evaporative fraction of the food. Therefore, headspace-GC/MS analysis can be a very useful tool to compare the impact of HPP and heat processing on food quality.

The objective of this study was to use headspace-GC/MS analysis to make a fingerprint of the evaporative fraction of a HP treated food product and to compare this with the one of the conventionally treated counterpart.

The experimental set-up was as depicted in Figure 3.1. First, carrot pieces were selected as a relevant model food product. Since they are a low-acid solid food product, the effect of different treatment intensities could also be investigated. To have a fair comparison, the starting point was to select HP and heat processing conditions with equivalent microbial inactivation. This was done for three intensity levels: mild pasteurization, severe pasteurization and sterilization. After processing, the carrot pieces were analyzed by headspace-GC/MS, in order to make a fingerprint of the evaporative fraction. In a last step, the resulting fingerprints of the untreated, HP processed and heat processed carrots were compared, using multivariate statistical data analysis, to look for specific differences.

Before these carrot pieces could be analyzed, first the method for headspace analysis had to be optimized. For sampling, both the technique of static headspace and headspace-SPME were investigated. Chapter 4 describes this optimization and provides a conclusion on the best method for the comparative analysis of the carrot pieces. In chapter 5, this method was used for the impact comparison of HPP and heat processing on the quality of carrot pieces, as described in Figure 3.1.
This comparison can be an important first step in evaluating the substantial equivalence of HP and heat treated foods, and thus providing important information for legislators and the food industry, within the framework of the novel food legislation (*cfr.* 1.6.2).

![Diagram](image_url)

**Figure 3.1** General scheme of the experimental set-up.
4 METHOD OPTIMIZATION FOR HEADSPACE ANALYSIS OF CARROTS

4.1 Introduction

The objective of this chapter was to select and optimize experimental conditions, such as sample equilibration time and temperature, for the sampling method to be used during the comparative analyses of volatilizable compounds of carrots in chapter 5. First, preliminary tests (investigating the effect of salt addition, testing the sample stability in the cooling tray of the autosampler and reproducibility tests) were performed and then, two types of headspace sampling were investigated, namely static headspace and SPME. In both cases, sample equilibration time and temperature were optimized and identical GC-MS conditions were used for analysis, apart from the mode of injection (splitless for static headspace and split for SPME). For SPME sampling, also the extraction time had to be optimized. Finally, the most appropriate sampling method for the comparative analysis of volatilizable compounds of carrots was selected.

4.2 Materials and methods

4.2.1 Sample preparation

Untreated (raw) carrots (Daucus carota cv. Nerac) from a local vegetable distributor were cut in homogeneous pieces (cfr. Chapter 5) and commercially sterilized carrots (Printana, Colruyt Group, Halle, Belgium) from a local shop were separated from the brine, after which they were both frozen in liquid nitrogen and stored at -40°C until analysis. The untreated carrots were used for preliminary tests, while the commercially sterilized carrots were used for the optimization of the sampling methods. For headspace analysis, a portion of frozen carrot pieces was cooled in liquid nitrogen and mixed for 20 s at 7500 rpm (Grindomix GM 200, Retsch, Haan Germany), resulting in a fine powder. 2.5 ± 0.1 grams of the carrot powder was immediately put into a 10 ml glass vial (screw neck, amber, VWR International, Dublin, Ireland) and 2.5 ml of saturated sodium chloride solution at 25°C was added. The vial was tightly closed and the sample mixed thoroughly and then transferred to the GC-MS cooling tray (10°C) for sampling.
4.2.2 Headspace sampling

4.2.2.1 Static headspace

Static headspace sampling was used to perform preliminary tests. They included investigating the effect of salt addition, testing the stability of the sample in the cooling tray of the autosampler and reproducibility tests. For these tests, untreated carrot powder samples, prepared as described in section 4.2.1, were used. Samples were transferred from the cooling tray to an equilibration oven, by an autosampler. In the oven, samples were subjected to an equilibration temperature of 40°C for 30 min. After equilibration of the sample, the headspace was sampled using a gas-tight syringe, and 2 ml was injected in splitless mode into the GC injection port, set at 230°C. All samples were analyzed using identical GC-MS conditions.

To optimize the static headspace sampling method, commercially sterilized carrot samples, prepared as described in section 4.2.1, were used. In the equilibration oven, samples were subjected to varying equilibration temperatures (30, 40 and 50°C) and times (10, 20, 30 and 45 min) in order to determine the best equilibration conditions. Further sampling and injection were done as described above. All analyses were replicated six times.

4.2.2.2 SPME

In a first step, five SPME fibers of diverse polarity and extraction mechanisms were tested under identical experimental conditions, in order to select the best SPME fiber for the analyses. Polydimethylsiloxane (PDMS) (100 µm, low polarity, partition), Carboxen™/polydimethylsiloxane (CAR/PDMS) (85 µm, medium polarity, adsorption/partition), polydimethylsiloxane/divinlybenzene (PDMS/DVB) (65 µm, bi-polar, adsorption/partition), polyacrylate (PA) (85 µm, high polarity, partition) and DVB/CAR/PDMS (50/30 µm, bi-polar, adsorption/partition) SPME fibers were tested. All fibers were purchased from Supelco (Bellefonte, PA, USA). Before analysis they were conditioned at the manufacturer’s recommended conditioning temperature and time. For fiber selection, equilibrium between the samples and their headspace was done at the optimum conditions selected for static headspace sampling, namely 30 min at 40°C and extraction was performed by inserting the SPME fiber in the vial for 30 min.
In a second step, equilibration and extraction times were optimized for the selected fiber. First, extraction times of 10, 20, 30 and 45 min were tested, after which the equilibration time was reassessed for 10, 20, 30 and 45 min. Both steps were performed at 40°C.

The volatiles were desorbed from the fiber for 2 min in the GC injection port, set at 230°C, after which the fiber was regenerated in a regeneration oven for 5 min at the conditioning temperature. All analyses were replicated six times.

### 4.2.3 GC-MS analysis

GC-MS analyses were performed on a gas chromatograph (6890N GC system, Agilent technologies, Diegem, Belgium), coupled to a mass selective detector (5973N inert MS, Agilent Technologies, Diegem, Belgium) and equipped with an autosampler (CombiPAL, Agilent Technologies, Diegem, Belgium) ([Figure 4.1](#figure4_1)).

![Figure 4.1 Gas chromatograph (6890N GC system, Agilent technologies, Diegem, Belgium), coupled to a mass selective detector (5973N inert MS, Agilent Technologies, Diegem, Belgium) and equipped with an autosampler (CombiPAL, Agilent Technologies, Diegem, Belgium).](#figure4_1)

Injection of the samples was performed in split mode with a split ratio of 1/5 for SPME analyses, and in splitless mode for the static headspace with an injection volume of 2 ml. The
injection port temperature was kept at 230°C. Chromatographic separation was carried out on a 30 m long, 0.25 mm internal diameter and 0.25 μm film thickness, non polar, capillary column (HP-5MS, Agilent Technologies, Diegem, Belgium) having (5%-phenyl)-methylpolysiloxane as a stationary phase and -60 to 325/350°C temperature limits. The column was conditioned before analysis according to the manufacturer’s recommended temperature and time. Helium with a flow rate of 1.5 ml/min was used as a carrier gas. The oven temperature was programmed from an initial temperature of 40°C (2 min) to 160°C at 4°C/min, then increased to 300°C at 20°C/min and kept constant at 300°C for 2 min before cooling back to 40°C. Mass spectra were recorded in electronic ionization (EI) mode at 200 eV, with a scanning range of 35 to 400 m/z. The interface and source temperature were 260 and 230°C, respectively.

4.2.4 Data analysis

The chromatograms and mass spectra were evaluated using the MSD ChemStation software (Version E.02.01. 1177, 2010, Agilent Technologies, Diegem, Belgium). The sum of the areas of all the peaks in the chromatogram and the number of peaks were used for the data analysis. Further analyses, such as t-tests were performed to determine significant differences at a significance level of 95% using MS Excel. The error bars on the figures represent the standard error of the means.

4.3 Results and discussion

4.3.1 Static headspace sampling

4.3.1.1 Preliminary tests

Effect of salts

Addition of salt to the sample reduces the solubility of polar compounds, hence, they are more easily released from the food matrix to the headspace (cfr. 2.3.1.1). This increases the chances of extracting the volatilizable components by concentrating them in the headspace, hence more compounds and larger amounts can be sampled. More and larger peaks in the chromatograms of different samples provide larger chances to identify differences among them. Four samples from different mixes were prepared as described in section 4.2.1, 2.5 ml of saturated sodium chloride at 25°C was added to each sample and placed in the cooling tray.
of the autosampler after thorough mixing. An equal number of control samples were prepared without sodium chloride.

Addition of saturated sodium chloride to the sample enhanced the peak size, as shown in Figure 4.2. This was due to the salting-out effect of the sodium chloride. It enabled more volatilizable compounds to be sampled, as indicated by the enhanced peaks. Therefore, saturated sodium chloride was added to all samples used in further analyses.

![Figure 4.2](image)

**Figure 4.2** GC-MS total ion chromatograms of volatilizable carrot compounds from untreated samples with (----) and without (—) saturated sodium chloride solution.

**Sample stability**

Samples to be analyzed are required to be stable within the sampling period in order to acquire results that are fairly comparable. Therefore, there was a need to establish how long the mixed carrot powder was stable in the cooling tray of the autosampler. Different samples were placed in the cooling tray and sequentially analyzed over a period of more than 48 h. After analysis, samples were found to be stable for at least 48 h.

It was also tested whether the mixed carrot powder could be stored in the freezer at -40°C. A freshly prepared untreated carrot powder was analyzed, while a portion of it was stored at -40°C and tested after 3 days. Three samples were taken from the freshly prepared powder and three from the stored powder. The freshly prepared carrot powder had a higher average total peak area than the stored carrot powder, as shown in (Figure 4.3). This could be due to loss of volatilizable compounds during storage. Therefore, to minimize the loss, a fresh mix was always prepared for all subsequent analyses.
Reproducibility

The credibility of the experimental results depends on the ability of reproducing the results every time the experiment is performed with similar conditions. The reproducibility of results indicates whether the method applied for the analysis was reliable or not. Three mixes were prepared from the commercially sterilized carrots and from each mix three samples were tested, using similar conditions (cfr. 4.2.2.1). The chromatograms from the analyses showed that there was a larger variation between samples of different mixes (mix 1 sample a, mix 2 sample a, and mix 3 sample a) than between samples of the same mix (mix 1 sample a, mix 1 sample b and mix 1 sample c) (Figure 4.4). This indicated that the variation in the carrot samples themselves was larger than the variation in the method of analysis. Therefore, to have a representative sampling for comparative analyses, different mixes were prepared, instead of preparing one mix from which several samples are made.

Figure 4.3 Average total peak area of volatilizable compounds for freshly prepared carrot powder (■) and carrot powder stored at -40°C (■).
In a second experiment, the reproducibility of the analysis of untreated carrots was compared with the one of sterilized carrots. Ten mixes of carrot powder, both for untreated and commercially sterilized carrots were prepared and from each mix, one sample was analyzed. The results showed that the sterilized carrots had a standard error of 4.98%, while the untreated carrots had a standard error of 19.36%. For this, two possible explanations can be proposed. First, in the sterilized carrots enzymes were inactivated, which could have an influence on the volatilizable compounds composition. Second, the commercially sterilized carrots were small baby carrots, almost equal in size, while the untreated carrots were large.
carrots, of different sizes, which were divided in pieces, coming from different parts of the carrots. Consequently, sampling a number of these pieces resulted in more variation between samples compared to the baby carrots. Therefore, the commercially sterilized carrots were selected for optimization of the sampling method.

4.3.1.2 Equilibration temperature and time

Equilibration of the sample and the headspace is important for achieving a representative sample during sampling of the headspace. It is majorly influenced by the temperature and time, among other factors, and hence an optimum equilibration temperature and time combination is necessary. Equilibration temperatures of 30, 40 and 50°C, for 10, 20, 30 and 45 min were tested. Six mixes were prepared for testing a specific equilibration temperature and time combination. The differences in the total peak area and individual peaks were used to find the optimum temperature and time combination. Initially, the amount of volatilizable compounds increased as temperature and time increased, as expected. However, it reached a plateau after a certain equilibration time for all temperatures as shown in Figure 4.5. Although a higher total area was reached at 50°C, a temperature of 40°C was selected as the best equilibration temperature. At 50°C, extra peaks occurred in the chromatogram. Because of the possibility that these peaks are due to components formed during reactions induced by the relatively high equilibration temperature, and because 50°C is very close to the mild pasteurization temperature of 70°C that was used for processing in chapter 5, a prudent selection of 40°C was made. Thirty minutes was selected as equilibration time, because after that time, no significant increase in total area occurred anymore.
CHAPTER 4: METHOD OPTIMIZATION FOR HEADSPACE ANALYSIS OF CARROTS

4.3.2 SPME sampling

4.3.2.1 Fiber selection

The PDMS, PA, PDMS/DVB, CAR/PDMS and DVB/CAR/PDMS fibers were tested in order to select the best fiber for volatilizable carrot compounds extraction. At first, equilibration of the commercially sterilized sample and headspace was done at the optimum conditions found for static headspace sampling, for 30 min at 40°C. Secondly, the fiber was inserted into the headspace for 30 min for extraction of the volatiles. The fibers showed varying extraction yields for the total carrot volatiles, with the PA fiber having the lowest extraction yield, while the CAR/PDMS fiber had the highest (Figure 4.6). There was no significant difference between the total peak area of the CAR/PDMS fiber and that of the DVB/CAR/PDMS fiber; however, the DVB/CAR/PDMS fiber had a significantly higher number of peaks. Since the objective of this study was to find whether there are differences in impact of heat and HP processing on quality, and because with a higher number of peaks, there is a higher chance of identifying differences among the carrot samples tested, the DVB/CAR/PDMS fiber was selected as the best fiber for extraction of volatilizable carrot compounds.

Figure 4.5 Average total peak area of volatilizable compounds from commercially sterilized carrots sampled with static headspace and analyzed at 30 (■), 40 (■) and 50°C (■) for 10, 20, 30 and 45 min.
CHAPTER 4: METHOD OPTIMIZATION FOR HEADSPACE ANALYSIS OF CARROTS

4.3.2.2 Extraction time and temperature

The amount of analytes extracted by the SPME fiber depends on the equilibration status of the three phases involved, the sample, headspace and the fiber coating. This equilibrium is influenced by the temperature and time combination used. Therefore, the extraction time at a particular temperature allows the SPME fiber coating to reach equilibrium with the other two phases, if it’s adequate. However, precision can also be achieved in the analyses without attaining equilibrium, if the extraction time is precisely controlled, which is possible by use of an autosampler. At first, different extraction times of 10, 20, 30 and 45 min at 40°C were tested. From this experiment, 30 min could be selected as the optimal extraction time.

Figure 4.6 Total peak areas (top) and total number of peaks (bottom) of volatilizable carrot compounds, extracted by various SPME fibers, after 30 min equilibration time and 30 min extraction time at 40°C.
However, at this time an overload of the fiber and column occurred. Because of this, a relatively fast degradation of the fiber was found and hence the fiber could not last long enough for analyses of all the samples. In addition, if all adsorption sites are occupied, detecting differences in concentrations of compounds, present in a relatively large amount in the headspace, is not possible. On the other hand, overload of the column could result in broadened and asymmetrical peaks. As noted earlier, precision could still be attained by precisely controlling the extraction time used, even without the three phases reaching equilibrium. Therefore, a shorter extraction time of 10 min was selected, which provided a solution for the above mentioned issues.

4.3.2.3 Confirmation of headspace equilibration time

In the first step, where different SPME fibers had been tested with 30 min extraction time, the equilibration time had been set at 30 minutes, as was derived from the static headspace analysis. After having selected the appropriate SPME fiber (DVB/CAR/PDMS) and set the appropriate extraction time (10 min) at 40°C, the equilibration time was reconfirmed. For this, 10, 20, 30, and 45 min equilibration time were tested.

The total peak area and number of peaks of the volatilizable carrot compounds extracted after 10 min equilibration time was significantly lower than for 20 min. However, there was no significant difference between 20, 30 and 45 min (Figure 4.7). Therefore, 20 min was selected as the appropriate equilibration time for the volatilizable carrot compounds extraction, with the DVB/CAR/PDMS fiber and 10 min extraction time.

![Figure 4.7](image)

Figure 4.7 Total peak areas (left) and total number of peaks (right) of volatilizable carrot compounds, extracted by DVB/CAR/PDMS fiber at 10, 20, 30 and 45 min equilibration time, after 10 min extraction time at 40°C.
4.3.3 Conclusion

The samples could be left in the cooling tray of the autosampler for at least 48 h. Sodium chloride was added to the samples to enhance the release of components from the food matrix to the headspace. This could allow volatile components with small peaks that would otherwise fall below the detection threshold, to be extracted in larger quantities. To minimize losses of volatilizable compounds, a fresh mix was to be prepared for each analysis. The commercially sterilized carrots were best for optimization of the sampling method, since they had a better reproducibility than the untreated carrots.

For static headspace sampling, the temperature and time combination of 40°C for 30 min was selected as the best equilibration condition for the sample.

For SPME sampling, the DVB/CAR/PDMS fiber was selected as the best fiber for the comparative analysis of volatilizable carrot compounds in chapter 5. Twenty minutes was selected as the best equilibration time at 40°C. Ten minutes was selected as the best extraction time, because longer extraction times resulted in overload and fast degradation of the fiber.

In order to choose between the static headspace and SPME method of sampling, their respective chromatograms obtained from the results of the analyses, performed at the selected optimum conditions were compared (Figure 4.8). Static headspace sampling was performed after equilibration of the sample for 30 min at 40°C, while SPME sampling was done after equilibration for 20 min at 40°C and with an extraction time of 10 min at 40°C. Sample injection was done in splitless mode for static headspace, while it was done in split mode (ratio 1/5) for SPME, all other GC-MS conditions were identical. Since the mode of injection was not the same for both methods, in theory the two methods cannot be fairly compared. However, the total peak area as well as the number of peaks were far much greater and more for SPME sampling than for static headspace (Figure 4.9). Even if both sampling methods were performed with the same injection mode, SPME would still result in much more and larger peaks compared to static headspace. Since a higher number of peaks increases the chances of identifying differences between carrot samples subjected to different processing intensities, which was the objective of this study, it was concluded that the SPME method was the most appropriate sampling method for this study.
CHAPTER 4: METHOD OPTIMIZATION FOR HEADSPACE ANALYSIS OF CARROTS

**Figure 4.8** GC-MS total ion chromatograms of volatilizable compounds from commercially sterilized carrots sampled by static headspace after equilibration of the sample for 30 min at 40°C (top) and SPME after equilibration of the sample for 20 min at 40°C and an extraction time of 10 min (bottom).

**Figure 4.9** Total peak areas (left) and number of peaks (right) of volatilizable compounds from commercially sterilized carrots sampled with static headspace (■) after equilibration of the sample for 30 min at 40°C and sampled with SPME (■) after equilibration of the sample for 20 min at 40°C and 10 min extraction time.
5 IMPACT COMPARISON OF HEAT AND HIGH PRESSURE PROCESSING ON VOLATILIZABLE CARROT COMPOUNDS

5.1 Introduction

As described in the literature review, HP processing can result in a better quality retention of food as compared to heat treatment. However, a fair comparison of these two processing techniques can only be made by starting from an equivalent point, such as equivalent microbial inactivation. Because volatilizable compounds are very often involved in processing-induced reactions, the highest chance to find differences in impact of the processing methods is in the evaporative fraction of a food product. This chapter entails the use of the SPME sampling method selected and optimized in chapter 4, coupled with GC-MS, for the analyses of volatilizable carrot compounds. Fingerprints of the volatilizable compounds were used to compare the impact of heat and HP processing on the quality of carrots. Three categories of carrot samples were analyzed: untreated, heat and HP processed. Carrots were treated using three processing intensity levels: mild pasteurization, severe pasteurization and sterilization. The processing conditions were chosen to achieve equivalent microbial inactivation for heat and HP treatment within the same intensity level.

5.2 Materials and methods

5.2.1 Sample preparation

A single batch of fresh carrots (*Daucus carota cv. Nerac*) was purchased from a local vegetable distributor and stored at 4°C. On a day prior to treatment, carrots were cut into small pieces as homogeneous as possible, packed in plastic bags (15/75 oriented polyamide/polypropylene, inner dimensions: 8 x 28 cm for mild pasteurization and 5.5 x 28 cm for severe pasteurization and sterilization) for HP treatment or glass jars (99 mm height and 80 mm diameter) for heat treatment, and stored again at 4°C. At the start of a treatment day, a brine of deionised water was added to the packed carrots. All treatments were done within 10 days, in which heat and HP treatments of the same intensity were performed on the same day. A portion of the carrot pieces was frozen in liquid nitrogen and stored at -40°C as untreated (raw) sample.
5.2.2 Sample processing

5.2.2.1 Selection of processing conditions

A selection of heat and HP processing conditions was made to result in equivalent microbial safety, for three different intensity levels: mild pasteurization, severe pasteurization and sterilization. In Table 5.1, an overview of the selected conditions is given with the corresponding intended reduction in target pathogen, per intensity level. Each treatment was repeated six times.

Table 5.1 Heat and HP processing conditions per intensity level with P and F process values, CUT the come-up time of the retort, HT the holding time used, $T_p$ the process temperature of the retort and $T_i$ the initial temperature in the HP vessel.

<table>
<thead>
<tr>
<th>Intensity level</th>
<th>Processing conditions Heat</th>
<th>Processing conditions HP</th>
<th>Reduction target pathogen</th>
<th>Reference HP conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild pasteurization</td>
<td>$P_{90}^{10} = 2$ min CUT = 8.5 min HT = 7.5 min $T_p = 70^\circ C$</td>
<td>600 MPa $T_i = 10^\circ C$ HT = 10 min</td>
<td>6-log <em>Listeria monocytogenes</em></td>
<td>(Dogan and Erkmen, 2004)</td>
</tr>
<tr>
<td>Severe pasteurization</td>
<td>$P_{90}^{10} = 10$ min CUT = 8.5 min HT = 7.5 min $T_p = 90^\circ C$</td>
<td>700 MPa, $T_i = 38^\circ C$ HT = 5 min</td>
<td>6-log spores non-proteolytic <em>Clostridium botulinum</em> type E</td>
<td>(Wilson et al., 2008)</td>
</tr>
<tr>
<td>Sterilization</td>
<td>$F_0 = 5$ min CUT = 8.5 min HT = 23 min $T_p = 117^\circ C$</td>
<td>700 MPa $T_i = 90^\circ C$ HT = 3 min</td>
<td>12-log spores proteolytic <em>C. botulinum</em> type A</td>
<td>(Bull et al., 2009)</td>
</tr>
</tbody>
</table>

5.2.2.2 Heat treatment

Heat treatment was carried out at the Laboratory of Food Technology, Katholieke Universiteit Leuven, in a water-cascading retort (Barriquand Steriflow retort, France). The treatment conditions were as indicated in Table 5.1. During the heating and holding phase, the external heat exchanger was supplied with steam, while cold water was used for the cooling phase. The product temperature was recorded in the coldest zone in two different jars, using thermocouples (Type T) connected to a thermocouple box (TR9216, Ellab, Hilleroed, Denmark) and a CMC-92 data acquisition system (Ellab, Hilleroed, Denmark).

5.2.2.3 High pressure treatment

Mild HP pasteurization was performed in the industrial-scale equipment (Wave 6000/55, NC Hyperbaric, Spain) of Fruity Line, The Netherlands, while severe HP pasteurization and
sterilization were performed in the pilot-scale equipment (Resato, The Netherlands) of Wageningen UR Food & Biobased Research, The Netherlands. Severe pasteurization and sterilization were preceded by preheating of the carrot pieces for 10 min at 38°C and 10 min at 90°C respectively. The treatment conditions used were as indicated Table 5.1. The packed carrots were transported to and from The Netherlands with a cooling truck.

5.2.3 Post-processing sample handling

After treatment, the packed carrots were stored overnight at 4°C. The following day, the carrot pieces were separated from the brine, frozen in liquid nitrogen and stored at -40°C.

5.2.4 Headspace analysis

The headspace analysis was carried out using the SPME sampling method as selected in chapter 4. The DVB/CAR/PDMS fiber was used at 20 min equilibration time and 10 min extraction time, both at 40°C. GC-MS conditions used are described in section 4.2.3.

5.2.5 Data analysis

The GC-MS total ion chromatograms were evaluated and integrated using the MSD ChemStation software (Version E.02.01.1177, 2010, Agilent Technologies, Diegem, Belgium). The resulting peak areas were used for multivariate data analysis. This consisted of partial least squares discriminant analysis (PLS-DA), in which the different peaks were considered as X variables and the different treatment conditions as categorical variables or Y variables. The data were mean-centered and the variables were weighed by their standard deviation to give them equal variance. For model selection, the lowest number of latent variables, resulting in a separation of the classes, was used. Variable importance in projection (VIP) scores were calculated and plotted as a tool to rank the different evaporative components in order of importance for class separation. Multivariate data analysis was carried out in Solo (Version 6.2, 2011, Eigenvector Research, Wenatchee, WA, USA).

To select possible markers for grouping, the ten components with the highest VIP scores, for each class, were plotted individually and tested for significant differences ($p < 0.05$) between the different treatment conditions. For this, Tukey multiple comparison tests were performed in SigmaPlot (Version 11.0, 2008, Systat Software Inc, San Jose, CA, USA). Compounds for which significant differences were found, were identified by comparison of their mass
spectrum with the NIST08 mass spectral database (National Institute of Standards and Technology, Gaithersburg, MD, USA). A threshold match of 700 (on a scale of 0 to 1000) was employed and further visual inspection of the spectral matching was conducted for acceptance.

### 5.3 Results and discussion

Given the objective of this study, which was to compare the impact of heat and HP processing on the evaporative fraction of carrot pieces, and to investigate the effect of treatment intensity, this section details the outcome of their respective comparative data analyses. As described in section 5.2, the carrot pieces were subjected to heat and HP treatments at three intensity levels: mild pasteurization, severe pasteurization and sterilization. The processing conditions for each intensity level were selected such that they would result in equivalent microbial inactivation for heat and HP treatment. This ensured that the results could be fairly compared. Headspace analysis was carried out and the resulting data were used for the comparative analyses at three levels, as shown in **Figure 5.1**.

At first, the comparison of treatment impact was done in two phases, as described in section 5.3.1. The first one consisted of the impact comparison of the three classes of untreated, heat treated and HP treated carrots over all intensities (□) (cfr. 5.3.1.1) and the second one of the comparison of the treatment impact per intensity level (↔) (cfr. 5.3.1.2). Secondly, the comparison of effect of intensity per type of treatment (↓) was done as described in section 5.3.2, first for the heat treatments (cfr. 5.3.2.1) and second for the HP treatments (cfr. 5.3.2.2).

From GC-MS, chromatograms of volatilizable carrot compounds, such as in **Figure 5.2**, were obtained. In total, 169 peaks were detected, which is an indication that the carrot variety used is a rich source of evaporative compounds.
Figure 5.1 General scheme of comparative analyses performed in this chapter: comparison of treatment impact over all intensity levels (□) and per intensity level (→) and the effect of intensity per treatment (↓).

Figure 5.2 GC-MS total ion chromatogram of volatilizable compounds from untreated carrots.

5.3.1 Comparison of treatment impact

5.3.1.1 Comparison of treatment impact over all intensity levels

In a first step, a comparison was made between the untreated, all heat treated and all HP treated carrots, not taking into account the different intensity levels in treatment. Figure 5.3 represents the biplot of the first two latent variables based on the PLS-DA analysis of the fingerprints of volatilizable carrot compounds from untreated, heat treated and HP treated
carrots. These two latent variables explained together 26.04% of the variance in the X variables (volatilizable compounds) and 73.92% of the variance in the Y variables (treatments). A separation between the heat treated and HP treated carrots can be seen along the first latent variable, while, a separation between the treated and untreated carrots can be seen along the second latent variable. Because a separation between the heat and HP processed carrots was possible with the PLS-DA analysis, differences in the volatilizable compounds must be present after treatment. Therefore, it can be concluded that heat and HP treatments have a varying impact on the evaporative fraction of the carrots. To identify which variables were responsible for this separation, VIP scores were calculated. These indicate the importance of a certain variable in discriminating one class from the other(s). A VIP plot is an interesting tool, in which a ranked list is created of the variables with the highest discriminative power. Figure 5.4 displays the first twenty variables with the highest VIP scores, for each class.

Figure 5.3 Biplot based on the PLS-DA analysis of the headspace fingerprints of untreated (♦), heat treated (□) and HP treated (☆) carrots. The circles represent the different peaks of the headspace fingerprints.
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Figure 5.4 VIP plots based on the PLS-DA analysis of the headspace fingerprints of untreated (A), heat treated (B) and HP treated (C) carrots, indicating the twenty most important peaks for separation.

5.3.1.2 Comparison of treatment impact per intensity level

In a second step, the comparison of treatment (heat and HP) impact was done at the three intensity levels separately i.e. mild and severe pasteurization, and sterilization.

Mild pasteurization

At first, a comparison between mild heat and mild HP pasteurization impact on volatilizable carrot compounds was done. Figure 5.5 represents the biplot of the first two latent variables based on the PLS-DA analysis of the fingerprints of the volatilizable compounds from mild heat pasteurized and mild HP pasteurized carrots. The first latent variable (LV1) already explained 31.19% of the variance in the X variables and 88.82% of the variance in the Y
variables, which was adequate for the clear separation of the two treatments. This clear separation indicates that the impact of mild heat pasteurization on volatilizable carrot compounds is different from the impact of mild HP pasteurization. Figure 5.6 shows the VIP plot of the twenty most important peaks for the separation.

![Biplot based on the PLS-DA analysis of the headspace fingerprints of mild heat pasteurized (Δ) and mild HP pasteurized (★) carrots. The circles represent the different peaks of the headspace fingerprints.](image1)

**Figure 5.5** Biplot based on the PLS-DA analysis of the headspace fingerprints of mild heat pasteurized (Δ) and mild HP pasteurized (★) carrots. The circles represent the different peaks of the headspace fingerprints.

![VIP plot, based on the PLS-DA analysis of the headspace fingerprints of mild heat and mild HP pasteurized carrots, indicating the twenty most important peaks for separation.](image2)

**Figure 5.6** VIP plot, based on the PLS-DA analysis of the headspace fingerprints of mild heat and mild HP pasteurized carrots, indicating the twenty most important peaks for separation.

**Severe pasteurization**

Secondly, a comparison between severe heat and severe HP pasteurization impact on volatilizable compounds of carrots was done. In the biplot in **Figure 5.7**, the first latent
variable explained 23.07% of the variance in the X variables and 93.35% of the variance in Y variables. The variance in Y variables explained by the first latent variable was high enough to enable a clear separation of the two classes. Therefore, it can be concluded that severe heat pasteurization has a different impact on the volatilizable compounds of carrots than HP pasteurization. The twenty most important peaks for the separation of severe heat pasteurized and severe HP pasteurized carrots are shown in the VIP plot in Figure 5.8.

**Figure 5.7** Biplot based on the PLS-DA analysis of the headspace fingerprints of severe heat pasteurized (▲) and severe HP pasteurized (x) carrots. The circles represent the different peaks of the headspace fingerprints.

**Figure 5.8** VIP plot, based on the PLS-DA analysis of the headspace fingerprints of severe heat and severe HP pasteurized carrots, indicating the twenty most important peaks for separation.
**Sterilization**

Thirdly, the impact of heat and HP sterilization on volatilizable compounds of carrots was compared. In Figure 5.9, a biplot of the first two latent variables is shown; the first latent variable already explained 42.34% of the variance in X variables and 95.24% of the variance in Y variables. One latent variable was again sufficient in providing a clear separation between the classes. This separation indicates that the impact of heat sterilization on the volatilizable compounds of carrots is different from the impact of HP sterilization. In Figure 5.10 the VIP plot, indicating the twenty most important peaks for the separation of the heat and HP sterilized carrots, is shown.

**Figure 5.9** Biplot based on the PLS-DA analysis of the headspace fingerprints of heat sterilized (□) and HP sterilized (+) carrots. The circles represent the different peaks of the headspace fingerprints.

**Figure 5.10** VIP plot based on the PLS-DA analysis of the headspace fingerprints of heat and HP sterilized carrots, indicating the twenty most important peaks for separation.
5.3.1.3 Selection of potential markers

The first ten most important peaks for separation of the different classes from the VIP plots were analyzed, in order to find interesting compounds that could be selected as potential markers. Peaks that were too small for identification or which did not have a clear trend within the treatments were left out of consideration.

Potential markers for untreated carrots, in comparison to treated carrots

Volatile carrot compounds that were considered interesting because they were significantly different for the class of untreated carrots, compared to treated carrots, were selected as potential markers. Figure 5.11 indicates the peak areas of peaks 112 and 145, which were identified as 2-nonenal and geranylacetone respectively. 2-Nonenal was significantly higher in untreated carrots than in treated carrots, possibly indicating a breakdown during processing. In the heat sterilized carrots, however, 2-nonenal appeared again in higher concentrations, compared to the other treated carrots. Geranylacetone on the other hand, was significantly lower in untreated carrots as compared to the severe pasteurized and sterilized carrots, probably indicating a formation. However, there was no significant difference between the untreated and mild treated carrots for geranylacetone. Because of these significant differences, both 2-nonenal and geranylacetone can be selected as potential markers for untreated carrots.

![Figure 5.11](image-url)  
**Figure 5.11** Peak areas of potential markers for untreated carrots (untreated (●), heat (■) and HP (■) treated carrots). Areas with the same letter are not significantly different from each other.

Potential markers to distinguish heat and HP treated carrots

Compounds that had significantly different peak areas for heat and HP treated carrots can be selected as potential markers to distinguish between heat processing and HPP. At first,
potential markers which were present in higher concentrations after heat treatment, compared to HP treatment, were selected. These are shown in Figure 5.12 and include dimethylsulphide (P11), heptanal (P54), 2,6,6-trimethyl-cyclohexanone (P82), α-cyclocitral (P103) and β-homocyclocitral (P125). There were significant differences in the peak areas of all these compounds between heat and HP treated carrots at various treatment intensities. The peak areas of dimethylsulphide, heptanal, and α-cyclocitral were significantly higher for severe heat pasteurized and heat sterilized carrots than for the corresponding HP treated carrots.

Figure 5.12 Peak areas for volatilizable compounds of untreated (■), heat (■) and HP (■) treated carrots, higher for heat treated carrots. Areas with the same letter are not significantly different from each other.
addition, for heptanal, differences between the two types of treatment increased as the intensity increased. For 2,6,6-trimethyl-cyclohexanone, however, the difference between heat and HP treated carrots was only significant for severe pasteurized carrots. At the pasteurization levels, β-homocyclocitral was only present in the heat treated carrots. At the level of sterilization however, β-homocyclocitral could also be found in the HP treated carrots. Nevertheless, its presence in HP sterilized carrots could possibly be attributed to the heat treatment that is accompanied by HP sterilization, i.e. the heat pretreatment of the carrots at 90°C and the temperature increase due to compression heating. Because the peak areas of these volatilizable carrot compounds (dimethylsulphide, heptanal, 2,6,6-trimethyl-cyclohexanone, α-cyclocitral and β-homocyclocitral) were always higher in the heat treated carrots, with significant differences for at least one intensity level, they could serve as potential markers to discriminate heat treated carrots from HP treated carrots (at certain intensity levels).

Secondly, potential markers which were present in higher concentrations in HP treated carrots were selected. Figure 5.13 shows compounds that always had a higher peak area in HP treated carrots, namely ethanol (P7), 2,4-dimethylheptane (P41), 2,4-dimethyl-1-heptene (P44) and 4-methyloctane (49). The peak area of ethanol was significantly higher in severe HP pasteurized and HP sterilized carrots than in the heat treated counterparts, hence can be selected as a potential marker for higher intensity levels. The latter three, were of special interest because they only occurred in HP treated carrots. This would mean that these compounds could be excellent markers for HPP. However, in literature (Suman, 2001) it was found that these compounds are probably coming from the packaging material used for HP treatment, which contained polypropylene. Therefore, further analysis is necessary to confirm their source. Nevertheless, it was verified whether the separations described in the previous paragraphs were still as successful when these three variables are excluded. Figure 5.14 shows a biplot of the first two latent variables based on the PLS-DA analysis of the fingerprints of the evaporative fraction from untreated, heat treated and HP treated carrots, over all intensity levels, after exclusion of peaks 41, 44 and 49. The two latent variables explained together 25.13% of the variance in the X variables and 72.63% of the variance in the Y variables, which was close to the 26.04% and 73.92% respectively, for the comparative analysis with all peaks included (cfr. 5.3.1.1). The biplot showed that there was still a clear
separation between the volatilizable carrot compounds of the different treatments, even when peaks 41, 44, and 49 were left out. The same conclusion can be made for the comparative analyses per intensity level. Table 5.2 indicates the amounts of variance explained by the first latent variable, based on the PLS-DA analysis after exclusion of peaks 41, 44 and 49. These were not much lower than for the analyses with all peaks included (cfr. 5.3.1.2) and the different treatments within the same intensity were still well separated. Because of their likely source, it was decided to also exclude peaks 41, 44 and 49 in the successive comparative analyses of the effect of intensity per type of treatment (cfr. 5.3.2).

![Graphs showing peak areas for volatilizable compounds of untreated, heat, and HP treated carrots, with areas marked with the same letter indicating no significant difference.](Figure 5.13 Peak areas for volatilizable compounds of untreated (■), heat (■) and HP (■) treated carrots, higher for HP treated carrots. Areas with the same letter are not significantly different from each other.)
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**Figure 5.14** Biplot based on the PLS-DA analysis of the headspace fingerprints of untreated (♦), heat treated (□) and HP treated (★) carrots, without peaks 41, 44 and 49. The circles represent the different peaks of the headspace fingerprints.

**Table 5.2** Amount of variance explained by the first latent variable based on PLS-DA analysis of the fingerprints of untreated, heat treated and HP treated carrots, with exclusion of peaks 41, 42 and 49.

<table>
<thead>
<tr>
<th>Intensity</th>
<th>% X explained</th>
<th>% Y explained</th>
<th>Groups separated?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild pasteurization</td>
<td>30.17</td>
<td>87.43</td>
<td>Yes</td>
</tr>
<tr>
<td>Severe pasteurization</td>
<td>21.97</td>
<td>92.12</td>
<td>Yes</td>
</tr>
<tr>
<td>Sterilization</td>
<td>41.47</td>
<td>95.00</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**5.3.2 Effect of intensity per type of treatment**

The second part in the objective of this study was to investigate what the effect is of treatment intensity on the evaporative fraction of carrots. This was done for heat treatments and HP treatments. For both, 4 levels were studied: no treatment, mild pasteurization, severe pasteurization and sterilization.
5.3.2.1 Effect of intensity for heat treatments

Figure 5.15 represents the biplot of the first two latent variables based on the PLS-DA analysis of fingerprints of the evaporative fraction of untreated, mild heat pasteurized, severe heat pasteurized and heat sterilized carrots. The two latent variables explained together 39.25% of the variance in the X variables and 62.73% of the variance in the Y variables. The first latent variable separated sterilized carrots from the pasteurized and the untreated carrots, while the second latent variable made a separation between the untreated carrots, the pasteurized carrots and the sterilized carrots. The separation was clear between all intensities, however, it was only minimal between mild and severe pasteurization. In other words, the discrimination power was largest for the untreated and sterilized carrots. From the biplot it is clear that the various intensities of heat treatment had different impact on the volatilizable compounds of carrots. The VIP plots in Figure 5.16 indicate the twenty peaks that were most important for the separation of the four classes.

**Figure 5.15** Biplot based on the PLS-DA analysis of the headspace fingerprints of untreated (♦), mild heat pasteurized (△), severe heat pasteurized (▲) and heat sterilized (□) carrots. The circles represent the different peaks of the headspace fingerprints.
Figure 5.16 VIP plots based on the PLS-DA analysis of the headspace fingerprints of untreated (A), mild heat pasteurized (B), severe heat pasteurized (C) and heat sterilized (D) carrots, indicating the twenty most important peaks for separation.
5.3.2.2 Effect of intensity for HP treatments

The four classes of intensities analyzed for HP treated carrots (untreated, mild and severe pasteurized and sterilized carrots) are shown in Figure 5.17, representing the biplot of the first two latent variables based on the PLS-DA analysis of the fingerprints of their volatilizable carrot compounds. 46.34% of the variance in the X variables and 61.76% of the variance in the Y variables was explained by the two latent variables together. The sterilized carrots were separated from the pasteurized and the untreated carrots by the first latent variable, while a separation between the untreated and treated carrots was realized by the second latent variable. Between all four intensity levels there was a clear separation. Just as for the heat treatment, the separation was most pronounced for the untreated and sterilized carrots, while the difference between mild and severe pasteurized carrots was rather minimal. The twenty peaks that were most important for the separation are shown in the VIP plots in Figure 5.18. From the separation, we can conclude that, the various intensity levels within HP treatments had different impact on the volatilizable compounds of the carrots.

Figure 5.17 Biplot based on the PLS-DA analysis of the headspace fingerprints of untreated (♦), mild HP pasteurized (♦), severe HP pasteurized (x) and HP sterilized (+) carrots. The circles represent the different peaks of the headspace fingerprints.
Figure 5.18 VIP plots based on the PLS-DA analysis of the headspace fingerprints of untreated (A), mild HP pasteurized (B), severe HP pasteurized (C) and HP sterilized (D) carrots, indicating the twenty most important peaks for separation.
5.3.2.3 Selection of potential markers

Just as for the comparison of treatment impact, the first ten most important peaks from the VIP plots (cfr. Figure 5.18) were analyzed for selection of potential markers for the effect of treatment intensity. The compounds that were of interest are those of which the peak area either decreased or increased with increasing intensity. However, within the first ten peaks from the VIP plots, no compounds were found with decreasing peak areas. On the other hand, eight compounds were found to have a clear trend of increasing peak area. Figure 5.19 indicates the plots of four of these components, namely 5,5-dimethylcyclopenta-1,3-diene (P45), octanal (P74) and two components which were both identified as theaspirane (P128 and P130). The latter two are perhaps isomers and further research is necessary for decisive identification. The plots for the remaining four; heptanal, 2,6,6-trimethyl-cyclohexanone, α-cyclocitral and β-homocyclocitral, were already shown in the section of potential markers for distinction between heat and HP treatment (cfr. 5.3.1.3). These eight compounds were significantly increasing with the increase in intensity level, both for heat treated and HP treated carrots. Therefore, because of this trend, they can serve as potential markers for the effect of intensity.

**Figure 5.19** Peak areas for volatilizable compounds of untreated (●), heat (◼) and HP (■) treated carrots, increasing with increasing treatment intensity. Areas with the same letter are not significantly different from each other.
5.3.3 Conclusion

From the analyses for comparison of treatment impact over all intensity levels, it can be concluded that heat processing and HPP have a different impact on the evaporative fraction of carrots. When considering intensity levels that would result in the equivalent microbial inactivation, also a different impact on this fraction was found for heat and HPP. Consequently, it can be concluded that this would probably result in carrots with different quality, in terms of volatilizable compounds.

2-nonenal and geranylacetone were selected as potential markers for untreated carrots, compared to treated carrots. For heat treated carrots, dimethylsulphide, heptanal, 2,6,6-trimethyl-cyclohexanone, α-cyclocitral and β-homocyclocitral were present in high concentrations, while, ethanol, 2,4-dimethylheptane, 2,4-dimethyl-1-heptene and 4-methyloctane were more found in HP treated carrots. Consequently, these compounds could be suggested as a potential markers to distinguish between heat processed and HPP carrots. However, to conclusively decide on the latter three compounds as potential markers, further analysis are needed to confirm whether they are products of migration from the packaging material used for HP treatment.

For comparison of the effect of intensity per type of treatment, it can be concluded that the evaporative fraction of the carrots treated by the four different intensity levels (untreated, mild and severe pasteurization and sterilization) was different for the four classes, both for heat treated carrots and HP treated carrots. This probably results in a different quality of the carrots, with respect to the volatilizable compounds.

For potential markers for the effect of intensity, 5,5-dimethylcyclopenta-1,3-diene, heptanal, octanal, 2,6,6-trimethyl-cyclohexanone, α-cyclocitral, β-homocyclocitral and two components identified as theaspirane were selected. All of them increased in concentration with increasing treatment intensity.
6 GENERAL CONCLUSION

The objective of this study was to compare treatment impact of heat and HP processing on the evaporative fraction of carrot pieces and further to investigate the effect of treatment intensity. This was achieved by utilization of headspace fingerprinting as a tool for the comparison.

The first step was to determine the most appropriate sampling method. Based on the total peak area and number of peaks, it was concluded that SPME sampling was the best method, since it provided a higher number of and larger peaks, compared to static headspace sampling. The SPME sampling conditions were: equilibration for 20 min at 40°C, extraction for 10 min at 40°C and split mode (ratio 1/5) injection. This method was further used for sampling of the volatilizable carrot compounds for the comparative analysis of carrots processed by heat and HP.

In the second step, the fingerprints, obtained from GC-MS analysis of carrots subjected to different treatments, were compared and analyzed for specific differences. Both a comparison of treatment impact and an investigation of the effect of intensity per type of treatment were performed. From these comparative analyses it was found that the treatment impact of heat processing on the volatilizable carrot compounds was different from that of HPP. Furthermore, it was found that the effect of treatment intensity on the volatilizable compounds of carrots varies with the intensity levels.

Finally, potential markers were selected for distinguishing untreated from treated carrots, between heat and HP treated carrots and for the effect of intensity. The peak areas for 2-nonenal and geranylacetone in untreated carrots were significantly different from those in the treated carrots. 2-nonenal was significantly more present in untreated carrots, while geranylacetone less. Therefore, they were selected as potential markers for untreated carrots. For heat treated carrots, dimethylsulphide, heptanal, 2,6,6-trimethyl-cyclohexanone, α-cyclocitral and β-homocyclocitral occurred in higher amounts, while the peak areas of ethanol, 2,4-dimethylheptane, 2,4-dimethyl-1-heptene and 4-methylloctane were higher in HP treated carrots. Due to their significantly higher peak areas in the heat or HP processed carrots, it was concluded that they can serve as potential markers for distinguishing between the two processes. In addition, 2,4-dimethylheptane, 2,4-dimethyl-1-heptene and
4-methyloctane occurred exclusively in HP treated carrots and could serve as specific markers for HPP. However, according to literature records their source could probably be the packaging material (containing polypropylene) used for the HP treatments, rather than the carrots themselves. Therefore, further investigation is necessary to confirm their source.

For the effect of intensity, compounds whose peak areas had a clear trend (increasing or decreasing) with increasing treatment intensity, were selected. However, within the first ten compounds of the VIP plots, none were found with a decreasing trend. The ones with an increasing trend included 5,5-dimethylcyclopenta-1,3-diene, heptanal, octanal, 2,6,6-trimethyl-cyclohexanone, α-cyclocitral, β-homocyclocitral and two components which were both identified as theaspirane. It was concluded that these eight compounds could serve as the potential markers for the effect of intensity.

In this work, potential markers for differences in treatment impact of heat and HPP on the evaporative fraction of carrots have been selected. Future work will consist of a further investigation of these markers. At first, confirmation of the identity and quantification of the selected compounds by use of standard solutions of the selected markers must be done. Secondly, the nature and origin of the compounds must be unraveled. Families of compounds that are related to each other and reactions involved should become known. This information should be interpreted in the context of substantial equivalence and the novel food legislation (cfr. 1.6.2), which means investigating the possible involvement of risk components, the metabolism after consumption and nutritional aspects. In this respect, a shelf life study of the processed carrots would also be of great value, since reactions that are influenced by processing can also manifest themselves during storage. Last, kinetic studies can be conducted for a further characterization of the selected compounds. This would reveal more of the behavior of the compound under different treatment conditions.

Furthermore, in this study, HPP carrots were compared with conventionally processed carrots by investigating their evaporative fractions, since this fraction was expected to provide the highest chance of finding differences in impact. In a next phase, other fractions should be investigated as well, e.g. by Liquid Chromatography-Mass Spectrometry. This could reveal other potential markers, for which the same procedure as described above can be followed.
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