STABILITY AND RELEASE OF MODEL AROMA COMPOUNDS

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Abstract

Overall, this thesis has two parts: The first evaluated the use of complex coacervation for aroma encapsulation to control aroma release. The second part focused on improving the storage stability of encapsulated aroma compounds by different techniques.

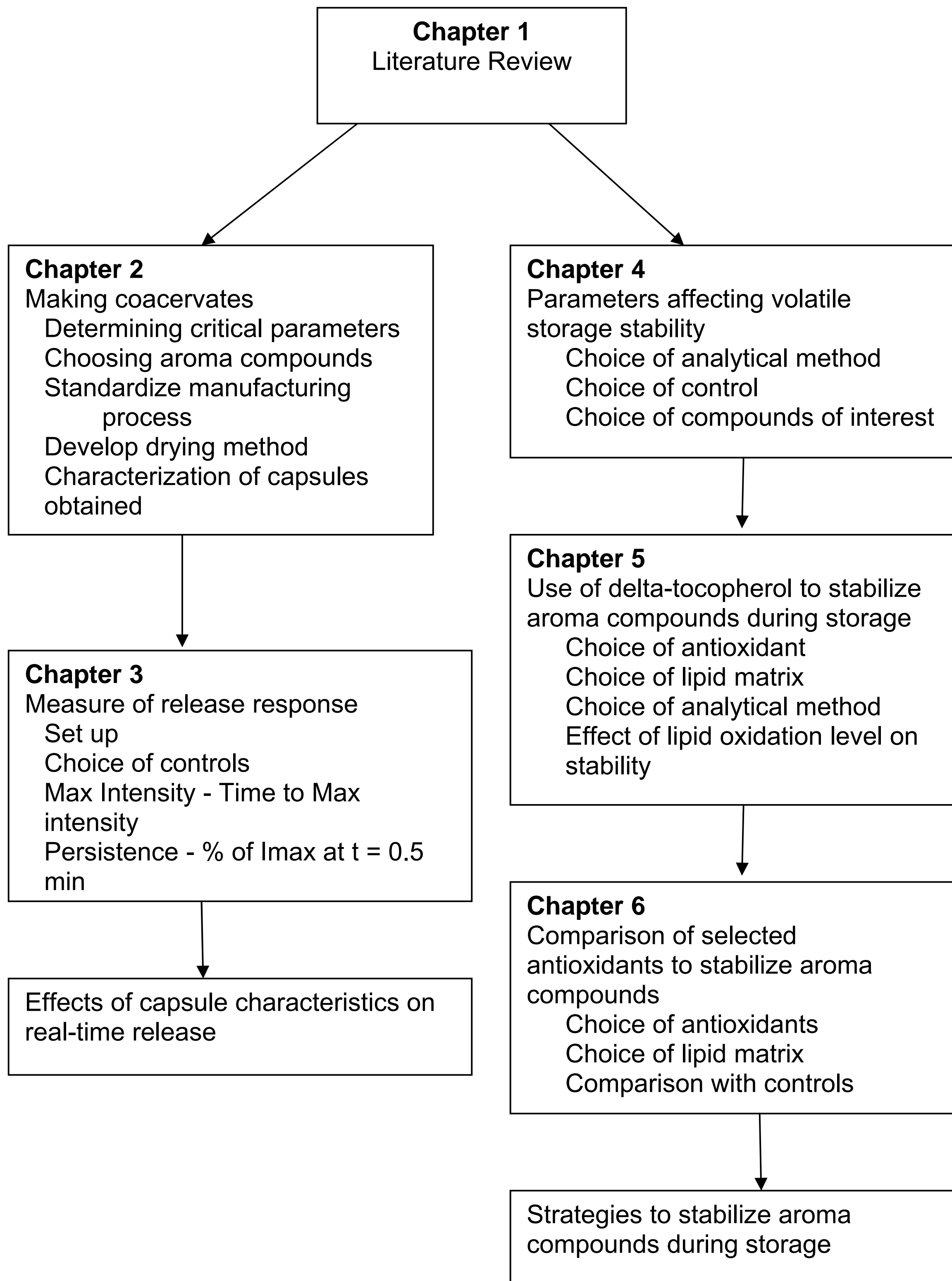
In part 1 coacervate capsules were formed using gelatin and gum acacia as wall materials and either a liquid or solid core (aroma compounds). A brief storage study compared the oxidation barrier properties of coacervate capsules and spray dried powder. No significant oxidation of limonene was detected in coacervate capsules after 25 days, whereas significant oxidation was found in spray dried powder.

Then the effect of coacervate capsule properties on volatile release (measured using proton transfer reaction MS) was studied. No significant effect of glutaraldehyde cross-linking or wall:core ratio on aroma release was found. Comparing aroma release data from coacervates to their release from a spray dried material, no significant difference in release pattern could be found. However, testing temperature, aroma volatility and hydrophobicity were found to be significant but not predictive factors.

In the second part of this thesis, the storage stability (12 weeks at 30 ˚C) of volatiles was found to be significantly decreased by the presence of oxygen and the type of matrix in which compounds are diluted (water or various edible oils). Medium chain triglycerides, sunflower oil and soy bean oil were evaluated as the edible oils. Water was found to have a highly significant detrimental effect on volatile stability compared to the oils. The type of oil matrix was also found to significantly affect storage stability. No correlation could be detected between the oxidation level of the oil and the oxidation pattern of the volatiles.

The stabilization of volatiles during storage was attempted by physically removing oxygen from the storage environment (< 0.5 %) and by adding selected antioxidants (rosemary extract, vitamin E, vitamin C, BHA and BHT). The addition of antioxidants in oil matrices showed compound specific action and a significant detrimental effect in some cases. Overall, no system would offer optimal protection for all aroma compounds studied simultaneously.

Thesis Flow Chart



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Chapter 1: Literature Review

1. Encapsulation

Encapsulation generally refers to techniques by which a sensitive material is coated or entrapped within another material which forms a protective shell or wall (1, 2). Many terms are commonly employed to describe the coated material, such as core, active material, internal phase, or filling. The shell is referred to as the wall, carrier, encapsulant or coating material (3). Encapsulation technologies are commonly used in industries such as food, pharmaceutical, cosmetics, chemical or printing, each being controlled in terms of materials by various regulatory agencies (2). In addition, each industry has its preferred techniques of encapsulation.

The purposes of encapsulating flavors are principally to protect them from oxidative environment, evaporation and convert a liquid flavoring into a free-flowing, and easy to handle powder after drying (1-4).

The major encapsulation techniques used in the flavor and drug industries can be summarized as follow.

1.1. Plating

One of the oldest methods, it consists in simply mixing the sensitive material with a dry carrier matrix, such as carbohydrates, whey proteins, salts or silica. The porosity of the dry matrix retains the sensitive material mostly by absorption.

This process has been typically used in confectionary products, using sugar as coating material, or more recently, coupled with a fluidized bed to coat tablets, chewing gums or nuts using waxes, oils or starches as wall material (5). Relatively inexpensive, this technique offers however limited protection to aroma compounds (3).

1.2. Spray chilling and spray cooling

These techniques rely on the rapid cooling of melted matrix, usually lipids, containing the core material upon atomization in a cooled chamber (-50˚C or 0˚C for spray chilling or cooling respectively) (1, 4). The main disadvantage of these techniques is the poor flavor retention. Hydrophilic compounds do not mix well with the matrix and are therefore more likely to be lost during the atomization. Lipophilic compounds on the other hand diffuse into the lipids and modify the final crystalline structure, increasing its fragility and reducing the barrier to losses (4, 6).

1.3. Liposome

The principle of liposome is to form a double layer of amphiphilic material to produce a "pocket" of hydrophilic material within the micelle, using emulsion technologies. The encapsulated material can be dispersed either in aqueous or fatty phase (3). The main disadvantages of this method are that the inclusions can be relatively fragile to rupture depending on their size and that there are limited barrier properties against losses of small molecules. This technique is mostly used in the cosmetic industry, but there are some limited applications for flavor encapsulation (2, 4). There is very limited data available on the storage stability properties or the release properties of this encapsulation method for volatile molecules.

1.4. Molecular inclusion, also called inclusion complexation

This technique can be described as the inclusion of a molecule of the sensitive material into a cyclic protective material by chemical interactions. The most common inclusion of flavor chemical is in cyclodextrins (3, 6, 7) . This encapsulation is truly a one to one encapsulation (a molecule of cyclodextrin for a molecule of flavor compound) and is therefore used mostly for highly unstable and expensive chemicals. Numerous studies have investigated the

characteristics of these inclusions and the protection they confer, as summarized in the review by Goubet *et al.* (7).

1.5. Spray drying

While the process of spray drying was initially invented for non encapsulation purposes, it became rapidly a preferred method of flavor encapsulation due to high retention of sensitive material, ease of manufacturing, low costs and the production of small particles (6). Spray drying consists in drying an emulsion of the sensitive material and a carbohydrate-based matrix, using equipment similar to that used for drying milk ingredients (2). The most common matrix types used in spray drying of volatiles are hydrolyzed starches, modified starches and gum acacia, due to their good emulsifying properties (8, 9).

Extensive research has been conducted on parameters optimization, storage stability and release of aromas in powders obtained by spray drying, as summarized in the reviews by Gibbs (3), Thies (2), Gouin *et al.* (10) and Madene *et al.* (1).

1.6. Coacervation

This technique consists in a true encapsulation of a core droplet by entangled colloid material, forming a shell. The present research will use complex coacervation and will therefore be detailed below. For ease of writing, the term coacervation mentioned from now on will refer to complex coacervation.

2. Coacervation

2.1. Principle

Coacervation describes the general phenomenon of phase separation of biopolymers in water systems (11). The coacervation can be “simple” when one polymer separates from the water phase due to the addition of salt. This can be

reversible. The coacervation is “complex” when it is based on electrostatic interactions between one or more water-immiscible oppositely charged polymers around an emulsified phase (2). It is a non- reversible separation of two distinct phases, namely a dense phase (coacervate) and a dilute phase (equilibrium phase) (12). This is generally done using proteins and polysaccharides, and has been extensively studied in particularly with gelatin and gum acacia.

2.2. Materials used

The materials typically used in the manufacturing of coacervate microcapsules and therefore in our investigations are gelatin and gum acacia, because of their specific characteristics.

2.2.1. Gelatin

Gelatin refers to the product of irreversible hydrolysis of collagen, a macroprotein from animal skin and bones. There are two main type of processing of collagen into gelatin, called type “A” and type “B”. Type A typically signifies an acid treatment of pigskin, and type B an alkaline treatment of cattle bones (13). A new technology using enzymatic treatment also exists (14) but is not commercially available for food applications and therefore has not been studied for its properties compared to the two other types (15).

Gelatin is a heterogeneous mixture of peptide chains of 300 to 400 amino acids each. Overall, gelatin contains high levels of glycine and proline (16, 17). A typical sequence has been published elsewhere (17). The composition of gelatin will depend on the type of process and the animal species, i.e pigskin or cattle bone. It has been shown for example that asparagine and glutamine are transformed into their acid residues during the alkaline treatment, giving the final product a high viscosity (18). In addition, different origins of the collagen imply different amino acid compositions, and therefore different reactions to treatment (18). The properties and characteristics of gelatin are consequently dependent on the type of gelatin considered.

Gelatin has been used in the pharmaceutical, cosmetic and food industries for over 150 years. Its characteristics and properties have been widely studied and described in the literature. A brief overview of these characteristics is presented here, focusing on those having a significant impact on the manufacturing of coacervate microcapsules.

The first notable characteristic of gelatin is its distinctive solubility in water, with different properties depending on the concentration of gelatin in solution. We will focus here only on dilute solutions as are used in manufacturing coacervates. Gelatin dissolves readily in water at temperatures slightly above room temperature, or around 35 ˚C (19). Below this temperature, gelatin forms a gel, except in dilute solutions, in which it aggregates in a very stable manner. This property is used during the manufacturing of coacervate capsules.

A second key property is the effect of pH on gelatin structure. Due to amino acid residues, gelatin is affected by pH. The isoelectric pH of gelatin is dependent on the type of processing: type A gelatin typically has an isoelectric point between 6-8, whereas type B's is around 4- 5 (18). In addition, the charges carried by the macromolecule will affect the type of bonds and interactions possible. This property is particularly used to expose certain residues to form intermolecular bonds, such as mentioned above, in the cross-linking process of coacervates, binding lysine residues to glutaraldehyde (20).

2.2.2. Gum acacia (gum Arabic)

Gum acacia, also known as gum Arabic is the natural product harvested from acacia trees of the species *Acacia senegal* and *Acacia seyal*. The trees are native to the sub-Saharan regions and most of the production comes from Sudan, Chad, Nigeria, Mali and Senegal (21). The harvest consists of either collecting naturally exudated sap from the trees, as for "gum Acacia *seyal*" or in collecting exudates from man-made wounds in the tree for "gum Acacia *senegal*". In either case, the location, climate, season of harvest and mode of production will affect the quantities collected and the composition of the gum (22).

The gross composition of gum acacia (*A. senegal)* has been established (22). Overall, gum acacia is a hetero polymer of polysaccharides containing galactose, arabinose, rhamnose, and glucuronic acid and glycoproteins. The ratios and types of polysaccharides vary depending on the location, climate and age of the tree. Still, the sequence and full chemical structure of the polymer is still under investigation, as extraction techniques modify slightly the intermolecular bonds and therefore give biased data. Examples of commonly accepted sequences have been reported by Defaye *et al.* (23) and Qi *et al.* (24). The main characteristic of gum acacia of importance for coacervation is the presence of polysaccharide residues and glycoprotein, which confer negative charges to the polymer in solution.

Gum acacia is widely used in the food industry as a texturing agent, a thickener, and an emulsifier, such as in the beverage industry. The manufacturing of coacervate microcapsules also takes advantage of the unique properties of this gum.

Gum acacia disperses in water, in a wide range of temperature and forms a low viscosity solution when present at low concentration (25). The typical pH of a gum solution is around 4- 5, where it carries a net negative charge. The excellent emulsifying functionality comes from the mixture of the hydrophilic polysaccharides and the hydrophobic glycoproteins (25). The polypeptide fraction interacts with the oil-water interface of the emulsion, while the polysaccharide portion stabilizes the emulsion by its steric presence.

During the coacervation process, the emulsion is stabilized by the presence of gum Arabic, which in turn will interact with the gelatin and form the complex wall-core structure.

2.3. Formation of microcapsules by complex coacervation: state of the literature

The formation of microcapsules by coacervation has been extensively studied. There is however a lot of controversy and disagreement among the manufacturing recommendations in the literature as well as a lack of practical

details. For this reason, the production of microcapsules by coacervation has been called “a mix of science and art” (2, 6).

As mentioned above, the fundamental principle of formation of capsules by coacervation relies on electrostatic interactions between oppositely charged colloids. These interactions will therefore depend on the charges themselves, i.e. the net charge carried by both colloids. This, in turn, will be modified depending on the pH, on the type and amount of colloid, ratio of the two colloids (positive charges vs. negative charges), and accessibility of the charges for interaction. Numerous works have investigated several main parameters, such as the influence of pH, the choice of material, stirring conditions and particle sizes, but few papers investigate the influences of each parameter in the process as a whole.

In addition, the majority of published literature on encapsulation by complex coacervation focuses on encapsulation of large, non – volatile molecules such as proteins, genes and drugs (12, 26-33). However, flavor compounds are typically small molecules, very volatile and can be hydrophilic. These very characteristics make encapsulation of flavor compounds different from encapsulation of drugs and proteins. The manufacturing processes for these two types of sensitive material are therefore likely to be different and make comparisons between them difficult.

2.4. Applications and uses of coacervated microcapsules

The very first historical use of coacervated capsules was in carbonless copy paper, where fine droplets containing the ink coated the back of the carbon paper.

More recently, applications using coacervated microcapsules have been quite diverse. As mentioned above, microcapsules are typically found in pharmaceutical applications, encapsulating drugs, proteins or even genes. Their major advantage here is their storage stabilizing property and the facilitated absorption in the gastro-intestinal track (34, 35). Complex coacervation is also

used as an encapsulation technique for fertilizer and insecticides (36), allowing a slow release depending on rainfall and exposure time (37, 38). Encapsulation by complex coacervation is used in the food industry as well, to encapsulate vitamins and antioxidants (2). However, limited published literature is available in this field, providing limited details.

3. Aroma release

3.1. Importance of aroma release

It is generally accepted that aroma is a key factor for food acceptance. Many methods exist to analyze flavor components in a given food system, such as trapping, extraction and chromatographic methods. These methods provide information on the presence of aroma compounds in the air above the whole food (headspace) or the overall aroma composition within the food (39). However, the key to aroma perception is not so much the presence of the volatiles but their adequate release rate and intensity (40, 41). Indeed, as soon as the food is eaten, dramatic changes occur, such as hydration by contact with saliva, destruction of the texture by mastication, swallowing of small pieces, etc. (42). All these affect the aroma profile in the mouth and nasal cavity, affecting the overall perception (40). For this reason, the analysis of aroma perception needs to be done in a dynamic manner. Similarly, to fully describe a flavor encapsulation system it is desirable to monitor the release of the compounds from a given application in addition to more traditional measurements of composition, protection or stability.

3.2. Release from encapsulated material

There are two main mechanisms for aroma delivery from microcapsules. First, the release occurs after a mechanical destruction of the capsule wall leading to leaks of the encapsulated material into the surrounding system. A second

possible mechanism is by diffusion of the active component from the core through the wall. Both approaches are used in pharmaceutical products.

For food applications, if capsules are above a certain size, they can be chewed and therefore degraded, liberating the encapsulated material in the mouth. For all other configurations, the release principally occurs by diffusion from a solubilization of the wall material or increase in porosity (4, 43). This solubilization occurs in the presence of water. The diffusion mechanisms of water into the dried polymer systems have been described previously, and been shown to follow several possible diffusion models such as the Fickian- diffusion model (i.e. polymer is in glassy state, “simple” water diffusion), Case-II transport model (relaxation of the polymer at transition between glassy and rubbery state), or “anomalous”, when the phenomenon is in-between the two other situations (44).

Diffusion is affected mostly by the shape and speed of the water front entering the outer shell and the structure of the polymer, i.e. glass transition, strength and cohesiveness of the network. For example, for a strongly cross-linked polymer, the relaxation and water diffusion will occur to a small extent, and slowly compared to the non cross-linked polymer (26). Release from such cross-linked particles will then be more long-term. As a result, the dynamic swelling or rate of water (solvent) uptake of dried particles is a critical parameter for release. The size of the particles and thickness of the encapsulating material will also affect the particle swelling rate.

Diffusional release depends also on the ease and rate at which the encapsulated material can migrate through the porous wall material. For this reason, the works published in the pharmaceutical area do not necessarily apply in flavor applications due to reduced molecular size and chemical properties (e.g. water solubility and volatility) of aroma molecules. Release patterns and properties of aroma compounds from coacervate capsules are therefore expected to be different from published data on drug molecules.

In most applications and more particularly in food applications, the powder containing the encapsulated material is hydrated before any release would occur.

The aroma needs then to diffuse from the encapsulating matrix into first its surrounding environment (solid matrix in solid food or water in beverages), and second from that environment into the air or headspace. Only once in the headspace can the aroma be perceived. This “diffusion” or equilibrium between the various phases can be described by partition coefficients.

There are two partition coefficients of interest: between octanol and water (mimicking an oil- water interface) and between water and air (headspace) (45, 46). Combining the two coefficients describes with greater precision phenomenon occurring in complex food systems, as well as the overall phenomenon occurring in release from hydrated powders.

3.3. Methods for measuring release

In the past, attempts to measure and predict aroma release have been conducted using different analytical techniques. However, traditional analytical methods (e.g. direct measures of headspace, using adsorption techniques, prediction using partition coefficients…) do not provide information on the dynamic aspect of release, since measures are done at equilibrium.

The use of trapping devices such as Tenax Traps can provide a good understanding of the dynamic aspects of release when used consecutively in determined overlapping sampling periods (47). The main advantage is their ease of usage, and systematic analysis that can be performed using GC/MS for identification and quantification. However, the aroma profile obtained will be dependent on the amount and type of trapping material used, which provides a biased representation of the overall profile. In the last decade, technological developments in analytical instruments allow on-line, real-time measurements via direct mass spectrometry (MS) (48, 49).

Recent technologies using direct mass spectrometry permit the measurement of real-time release of volatile compounds. There is no chromatographic separation involved with compounds separated for quantification solely by their mass. The two most common technologies are called “Atmospheric Pressure Chemical Ionization – Mass Spectrometry” (APCI- MS) (50) and “Proton Transfer Reaction-

Mass Spectrometry" (PTR-MS) (49, 51). These techniques are based on the ionization of volatile compounds (aroma compounds) by transfer of a proton from H_3O^+ ions and detection of these ions by MS. A scheme showing the principle of a PTR-MS instrument is detailed in **Figure 1-1.** The air stream used to purge the sample enters the instrument at the beginning of the drift tube, where the ionization of any volatiles released from the sample takes place. The ionized molecules are then brought to the detection system. The amount of reagent ions is controlled by the ionization source, and is critical for detection sensitivity (49). One disadvantage of such instrumentation is that the ions formed can be of same nominal mass for different volatiles. For this reason, unique ions need to be determined for each compound individually and in mixture so as to record appropriately the signal without overlap.

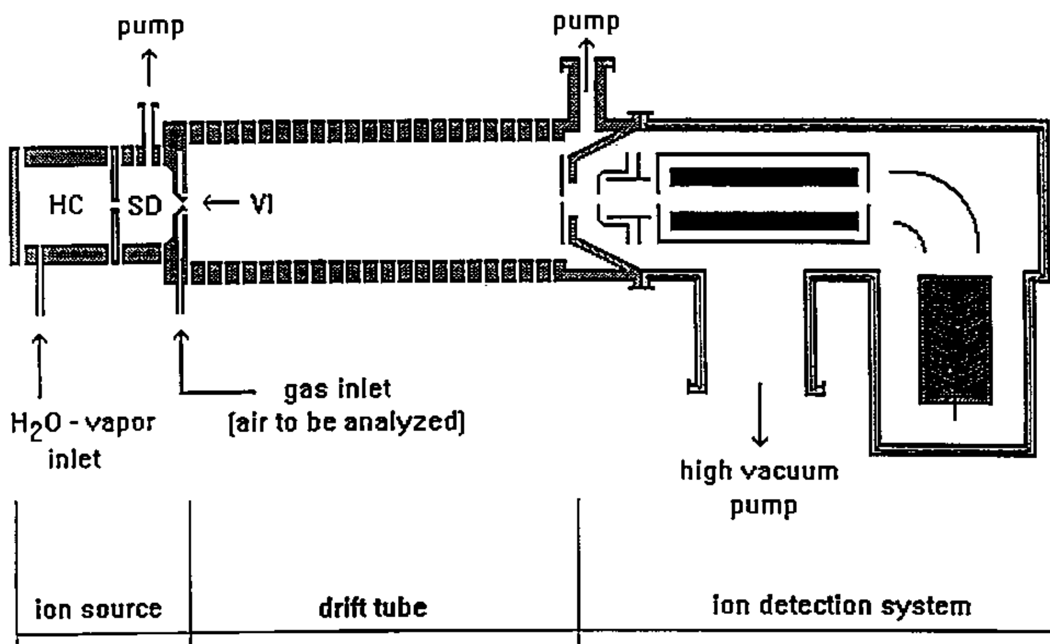

Figure 1-1: Schematic representation of the PTR–MS apparatus. HC, hollowcathode; SD, source drift region; VI, Venturi-type inlet. Copyright © Ionicon Analytik GmbH. Reproduced with permission.

Since the reagent ions (H_3O^+ ions) are produced from water, water can be used as a solvent in samples analyzed and is constantly monitored (ion 37). This particularity has been extensively used for medical breath analysis (49) studies of volatiles in mouth (40), in nose and sensory perceptions (52-55). The principles, advantages and limitations of such an instrument have been discussed in depth in the available literature, including instrument optimization, sensitivity optimization, data transformation and calculations (49, 51, 56-58).

4. Storage Stability of Aroma Compounds

4.1. Storage Stability of Foods

A major issue in the food industry is the storage stability of the different constituents and in particular stability against oxidation during storage. Numerous studies have investigated the oxidation mechanisms of various food components such as lipids, vitamins, polyphenols, and color compounds, leading to quality loss.

Oxidation mechanisms of food constituents can be divided into two broad categories: enzymatic and non-enzymatic oxidation (59-61). The occurrence of each type depends greatly on the food product (e.g. natural vs. processed) and the food component considered.

Enzymatic oxidation has been extensively studied in fruits and fruit juices, in particular the action of polyphenol oxidase on fruit and fruit juice quality (including wine) (61-64). This type of oxidation can be in great part controlled by processing conditions, for example by adjusting the pH, temperature or physically removing the enzymes by filtration.

Non-enzymatic oxidation, however, depends only on the presence of oxygen and subsequent reactions. Non-enzymatic oxidation has mostly been studied in lipids, which induces important quality deterioration in foods.

4.2. Lipid Oxidation: a model for organic oxidation

Non-enzymatic oxidation has been extensively studied in lipids, because of its common occurrence in food systems and because of the possible modeling from lipids to other similar food components.

4.2.1. Mechanism

Lipid autooxidation mechanisms have been studied in great detail, from reaction mechanisms, significant factors and kinetics (65-67). Only a broad overview will be given here. While the information is based on lipid oxidation, the mechanisms have been studied in other organic compounds and are very similar.

Autooxidation is a free-radical reaction, which can be described in 3 steps. The first step is the initiation period, where free radicals are formed by reaction with reactive species (e.g. hydroperoxides). The second step is the propagation stage, where the free radical is transformed into an alkyl radical, by abstraction of an hydrogen in the presence of oxygen. The last phase is the termination step, where radicals react to form new, non-radical compounds, called “oxidation products”, such as aldehydes, ketones and alcohols in the case of lipid oxidation, leading to sensory deterioration of the food product (67-69).

4.2.2. Factors influencing oxidative deterioration

The occurrence of oxidative reactions depends on several factors. The external factors are, for example, the amount of oxygen in the environment, the presence of catalysts (e.g. minerals) and temperature. The intrinsic factors affecting autooxidation consist mainly on the types of molecules present and the state of the system, i.e. monolayer or emulsion state, which increases the surface area in contact with the oxidative agents (70).

As described earlier, the reaction propagates by forming highly reactive intermediaries, such as free radicals. It has been shown that the structure of the

fatty acids affects the propagation rate, as reviewed by Porter *et al.* (71). The presence, number and location of double bonds in the fatty acid chain have been shown to increase oxidation rate due to possible resonance (60, 72). The presence of double bonds and resonance systems not only occur in lipids but in many volatile compounds, such as terpenes. Oxidation of such compounds has been studied showing the importance of resonance, for example in the oxidation of d-limonene into carvone (69). The non-enzymatic oxidation kinetics of encapsulated orange oil have been described as being a function of the initial composition (monounsaturated and sesquiterpene hydrocarbons balance), the porosity of the matrix material surrounding the oil, its water activity, the availability of oxygen in the environment, the presence of sensitizers such as trace mineral compounds, and the presence of potential antioxidants such as vitamin C or flavonoids (8, 73, 74). While extensive research has been conducted on orange oil and its degradation products, little is published on the oxidation of other aroma compounds. Some recent data has focused on degradation under extreme oxidative environment, known as Fenton- reaction conditions (75), but little is available on the storage stability of complex aroma systems. Still, it is reasonable to hypothesize that some aroma compounds might undergo oxidation reactions similar to these described in the literature for lipids. Very little is published to this date, besides for terpenes.

In addition to the very structure of the compounds involved, it has been shown that the oxidative reactions could propagate from fatty acids to other chemical components present, such as proteins. Mottram and Edwards showed the effect of lipid oxidation on the deterioration of proteins and amino acids present in cooked meat (76). In addition, some studies showed the propagation of lipid oxidation inducing the degradation of lipoproteins in vivo (77, 78). Limited work has been published on the impact of lipid oxidation on the oxidation of other oxidation sensitive food constituents.

4.3. Food Antioxidants

Oxidation can be retarded or modulated in three ways. It can be delayed by limiting the influence of physical factors, and preventing reactions from starting: storing foods in the dark at low temperature will limit the photooxidation kinetics; physically removing oxidative enzymes (blanching fruits and vegetables) and transition metal ions (distillation of water) will prevent their action; physically removing ambient oxygen, reactant (e.g. in modified atmosphere packages) will retard the reactions. However, in autooxidation, the only action possible is to retard the propagation stage and therefore limit the undesirable by-products. This is typically done in the industry by adding antioxidant molecules (67, 68).

Antioxidants are defined as "*substances capable of delaying, retarding or preventing the development in food of rancidity or other flavour deterioration due to oxidation*" (67). Antioxidants operate by either breaking the propagation stage (chain – breaking action), or by competing as reactant, i.e. by reduction reactions or hydrogen donator. Extensive literature and excellent reviews are available on antioxidant reaction mechanisms (66, 67, 79-85). The principles will not be detailed here.

Antioxidants are classified in numerous ways, either by the action mechanisms, or by their origin, that is if they are synthetic or natural antioxidants.

4.3.1. Synthetic antioxidants

a) Structure

Several synthetic antioxidants are allowed for use in the food industry. The most common are butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), esters of gallic acids (propyl-, octyl- and dodecyl- gallate, PG) and tert-butylhydroquinone (TBHQ). (**Figures 1-2**) Due to their differences in chemical structure, these antioxidants will have different properties. For instance, BHA and BHT are steam distillable as opposed to TBHQ and PG. Therefore BHA and BHT

are poor choices for foods undergoing heat processing such as spray drying, drum drying, or vacuum drying (82).

b) Toxicology

The use of antioxidants in the food industry is strictly controlled. Furthermore, they are also strictly regulated by World Health Organization: the current authorized levels are 0.01 % - 0.02 % of total fat or oil content (WHO, 2001). Still, consumers have expressed concern regarding their safety, and some studies have suggested a long term carcinogenic potential (86, 87).

c) Antioxidant action

The efficacy of these antioxidants has been thoroughly tested and reported in the literature (82, 88, 89). A great number of lipid types and emulsions have been tested: lard, butter, chicken fat, fish oil, mashed potato, sunflower oil, vegetable oil, soybean oil, etc. The efficacy of each of the synthetic antioxidants varies from model to model. However, there is an agreement in the literature to suggest a synergetic effect between BHA and BHT, and BHA and PG (84, 90).

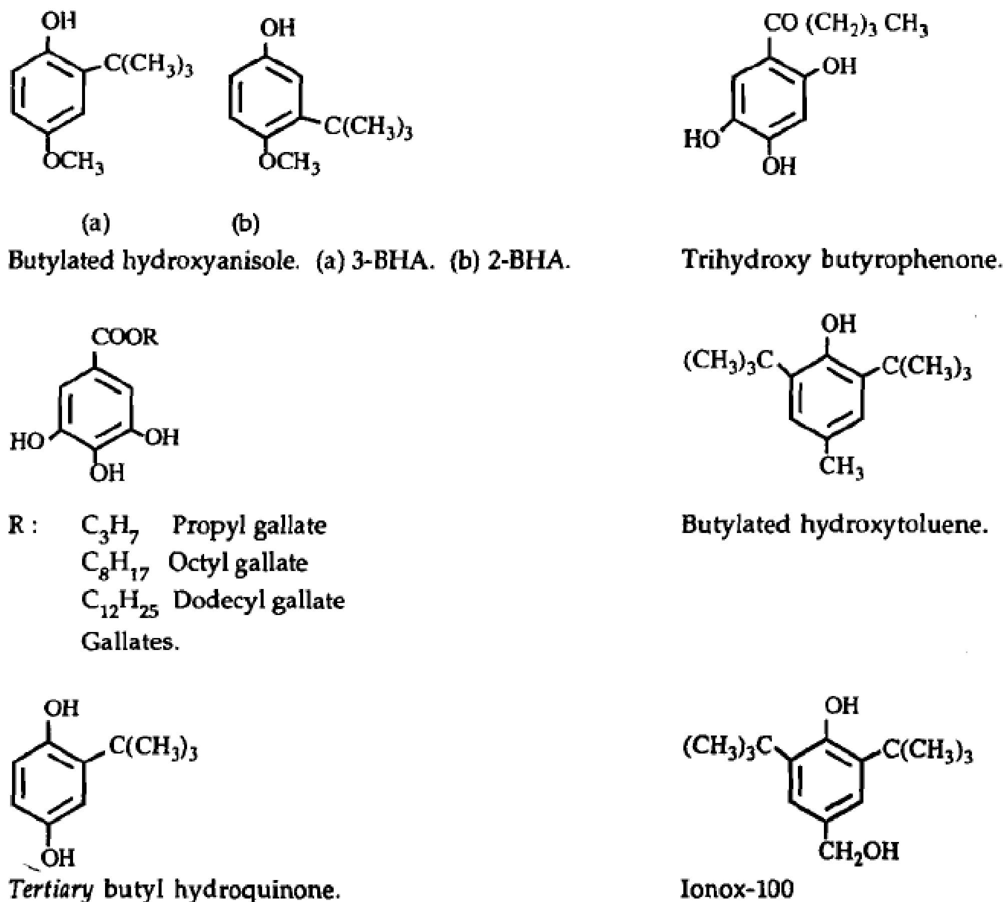

Figure 1-2: Chemical structure of most commonly used synthetic food antioxidants.

4.3.2. Natural

Natural antioxidants are either biologically active molecules, such as vitamins, carotene, flavones or short chain organic acids, or extracted compounds from plant sources, such as spices, herbs, grains, tea or vegetables (91). Although synthetic antioxidants have been proven efficient, there is an important consumer demand for natural-based additives (92). Intensive research is occurring to identify new natural sources of antioxidants, and a list of them would therefore be

outdated as soon as published. The following review will focus principally on selected, commonly used natural antioxidants.

a) Tocopherols

Tocopherols are a group of monophenolic compounds found abundantly in nuts, seeds, grains, leafy vegetable and fruits. There exist 4 structures of tocopherols, referred to as α-, β-, γ- and δ- tocopherol, depending on the position and number of methyl groups on the ring (C1, C2 and C3, **Figure 1-3**).

The antioxidant effect of the various homologs has been intensively studied in lipid systems and emulsion systems (93-97). Tocopherols act as either free radical formation inhibitor (i.e. “preventive antioxidant”), or a chain propagation breaker (scavenging effect) (92). Overall, α-tocopherol is considered to have the highest antioxidant activity at lower temperature (20 ˚C) and the δ form at higher temperature (> 50 ˚C). The antioxidant property extend of each homolog was found to be not only temperature dependent but also matrix dependent (bulk oil vs. emulsion, oil type) (98). Therefore stabilizing properties reported for example in sunflower oil are not directly comparable to these reported in beef muscle.

The homologs have been described as “weak” compared to synthetic antioxidants such as BHA or TBHQ, which lead to studies on mixtures (82). Some synergetic effects have been reported using a racemic mixture of tocopherols (94, 96, 99), in conjunction with synthetic antioxidants (BHA, sodium phosphates) (100, 101) or complemented with ascorbyl palmitate (lipophilic substitute of vitamin C) (102).

Very limited literature is available on the antioxidant properties of tocopherols on aroma compounds oxidation. A patent by the Wrigley Company (103) mentions high protection of mint oil by a mixture of tocopherols, without providing sufficient data for a critical review. Other authors mention an increase in stability of orange oil in the presence of tocopherol mixtures (85) but failed to provide enough detail for complete review.

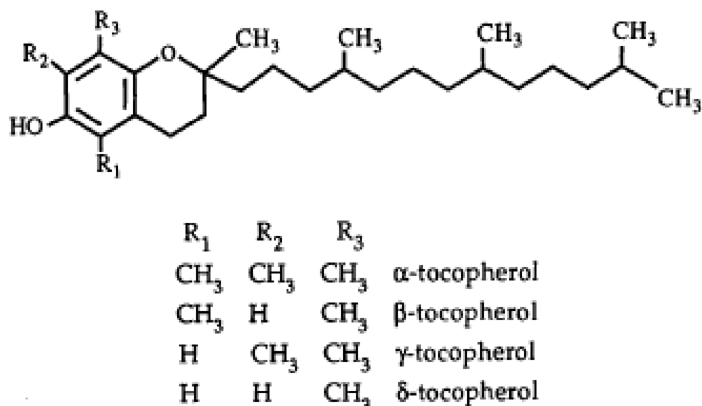

Figure 1-3: Chemical structure of tocopherols

b) Vitamin C

Another commonly used natural antioxidant is L- ascorbic acid (aka Vitamin C), or its fat-soluble homolog, ascorbyl palmitate (**Figure 1-4**). Although ascorbyl palmitate is not found directly in nature, it is still regarded as "natural" (reaction of ascorbic acid and palmitic acid, at room temperature and in the presence of excess sulfuric acid).

The dominant mechanism of this antioxidant is of an oxygen scavenger (104) . The main role is to shift the system redox potential to reduction and regenerate primary antioxidants. Some studies have shown a higher antioxidant power of Vitamin C in vegetable oils than BHA, BHT or PG (105). They are either used alone, requiring lower levels than the synthetic equivalent (0.01% vs. 0.02% to obtain similar antioxidant property) (82) or in conjunction with other antioxidants, as mentioned previously with tocopherols.

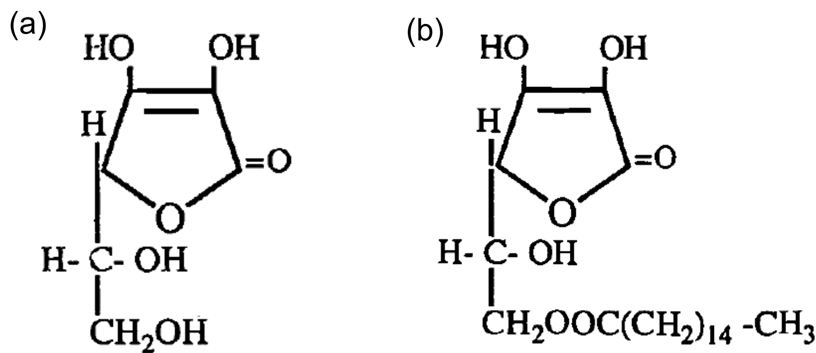

Figure 1-4: Chemical structure of ascorbic acid (a) and ascorbyl palmitate (b).

Ascorbyl palmitate is mostly used to stabilize fried products, while ascorbic acid is commonly used in canned foods, frozen fruits, fruit beverages and soft drinks.

c) Plant extracts

As interest in synthetic antioxidants is decreasing, attention and research on natural compounds exhibiting antioxidant properties has significantly increased. For example, to date, 15 out of the 20 most cited articles from *J. Ag. Food Chem.* since 1997 report findings on plant-based antioxidants.

Screening for natural antioxidants takes place in a variety of sources beyond the traditional spices (cumin, pepper, coriander…), herbs (marjoram, rosemary, sage, clove…) and fruits (blueberries, cranberries, citrus fruits…), to also include grains (barley, wheat, oats…), leafy vegetables (mustards, celery…), nuts (almond, hazelnut, pecan…), tea, coffee and chocolate extracts (93, 106-119). Recent technological advancements in isolation, identification and activity measurements have allowed finding specific molecules with high antioxidant properties (80, 110). For instance, not only could rosemary be detected as one of the most effective antioxidants sources, but the very specific fractions of rosemary extracts responsible for the antioxidant properties could be identified, evaluated and compared with other products (109). An overall commonality between all the antioxidant components identified is that they are phenolic

compounds with hydroxyl groups located in either the C3 or C5 position. Flavonoids have been recognized as higher antioxidants due to the increase number of hydroxyl groups and double bonds in their structure, allowing scavenging activity, hydrogen donating and metal chelating (79).

Some comparisons between the measured antioxidant activities are reported in the literature, as for example in **Figure 1-5**. Yanishlieva and Heimonen (120) concluded that natural antioxidants could cover a wide range of antioxidant activities, alone or synergistically, add limited sensory impact when diluted at low levels in food products and would satisfy consumer's nature-based ingredients expectations.

Source	**Substrate tested**	**Relative Activity**
Marjoram, nutmeg, white pepper, rosemary, sage, coriander, black pepper	Lard	Rosemary> sage> nutmeg> white pepper> marjoram
Herbs (> 30 material)	Lard	Rosemary> sage> oregano> thyme
Herbs and Spices	Oil in Water emulsion	Clove> cinnamon> sage> oregano
Spices	Oil in Water emulsion	Clove>turmeric> allspice> cinnamon> ginger
Allspice, paprika,marjoram, black pepper, white pepper, coriander	Sausage, water	Allspice> red paprika> marjoram> black pepper
Herbs	Ground chicken	Marjoram> caraway> peppermint> clove

Figure 1-5: Relative antioxidative effectiveness of various plant extracts in selected matrices, adapted from Yanislieva and Heinonen (120).

Numerous components have been investigated to limit oxidation in lipid matrices and *in vivo* systems, each providing specificity (activity, origin, sensory impact). However the use of such antioxidants to limit oxidation of other organic materials, such as aroma compounds has been only rarely reported in the literature.

5. Research hypotheses

The literature review led us to formulate the following research hypotheses and objectives. They constitute the overall framework for the research presented in the thesis.

Chapter 2 and 3 will explore the following research hypotheses:

- The physical and chemical characteristics of microcapsules influence the release of aroma compounds encapsulated therein.
- Chemical properties of aroma compounds affect their release properties from capsules.

Chapter 4, 5 and 6 will relate specifically to the following general hypothese:

- The environment conditions influence the storage stability of aroma compounds.
- Chemical properties of the storage matrix affect the volatiles storage stability.

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Chapter 2: Formation and Characterization of Microcapsules by Complex Coacervation with Liquid or Solid Aroma Cores

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1. Abstract

The process parameters typically reported in the literature for the encapsulation of aroma compounds via coacervation are reviewed and their effects on capsule formation discussed. We then report on our approach to produce coacervates (liquid [limonene or medium chain triglycerides] or solid core [menthol]) using gum-acacia/ gelatin as wall materials. Manufacturing parameters were optimized to allow the production of consistent batches of coacervate microcapsules. Capsules were cross-linked with glutaraldehyde and freeze dried. Coacervates were characterized for their structure and shape, size distribution, flavor load and water uptake rate. In addition, a brief storage study compared the ability of coacervate capsules and spray dried capsules (using modified starch as carrier material) to protect limonene from oxidation. No detectable increase in limonene oxide could be detected in capsules made by coacervation over 25 days in storage at 45 ˚C whereas a significant increase in limonene oxide was detected in spray dried powder over the same period. Encapsulation by coacervation appears to be an effective technique to encapsulate aroma compounds and provides a good barrier against oxidation of sensitive material.

2. Introduction

Complex coacervation was first described by Bungenberg de Jong (1) as a spontaneous phenomenon occurring between two oppositely charged polymers in aqueous solution. The neutralization of these carried charges induces a phase separation (polymer rich phase vs. aqueous phase) (2,3). Encapsulation by complex coacervation also involves a reduction in surface tension in the emulsion system, which leads to the coating of the core material by the entangled neutralized polymer phase (forming the wall) (4). Although spontaneous, this phenomenon only occurs under very specific conditions. In particular, it will depend principally on the charges of the polymers, their charge density, the surface tension in the system, the temperature at which the system is maintained and the dynamics of the system (stirring, cooling). Each of these parameters has been widely studied (5-11). However, they generally have been studied independently rather than part of a whole process. A few studies have shown interactions between parameters, but none have covered the entire manufacturing procedure. In addition, some steps in the manufacturing process are poorly detailed or no justification is provided for the given recommendations.

The majority of the literature available on the encapsulation of sensitive materials by complex coacervation refers to pharmaceutical applications. However, encapsulation of aroma volatiles differs significantly from the encapsulation of drugs, peptides or genes because of the different intrinsic physical properties of aroma compounds, including small molecular weight, high volatility, sensitivity to oxidation and degradation. Limited literature is available on aroma encapsulation by complex coacervation.

The objectives of the present paper are to: 1) review the critical factors affecting the coacervation process in its entirety; 2) propose a standardized method to produce a high yield of complex coacervate capsules with good volatile retention and spherical shape after drying; and 3) characterize the physical properties and storage stability of the capsules produced by the proposed standardized method.

3. Materials and Methods

3.1. Preparation of microcapsules

3.1.1. Materials

Gelatin 250 Bloom strength, 20 Mesh, type A provided by PB–Leiner (Davenport, IA, USA) and gum acacia (*Acacia seyal,* FT powder, TIC Gums, Belcamp, MD, USA) were used as wall material in the formation of microcapsules.

The core materials used in this study included limonene and menthol powder. Limonene was obtained from Aldrich Chemicals (St Louis, MO, USA) at the highest purity available. Pure synthetic menthol pellets (L-menthol, Takasago, Rockleigh, NJ, USA) were ground and sieved to obtain a 200 - 350 µm particle size. Vegetable oil (Medium chain triglyceride oil, Lumulse CC-33K, Lambent Technologies, Gurnee, IL) was also used as core material to compare to limonene.

3.1.2. Encapsulation process

The manufacturing parameters suggested below were determined by experimentation. Intermediate non-ideal conditions are not reported.

Eight g of gum acacia and 12 g of gelatin were dispersed in 450 mL DI water (45 ˚C) in a stainless steel beaker, with an overhead stirrer (RW20 digital, IKA works, Wilmington, NC, USA) – 350 rpm.

- pH was adjusted to 4.5 with hydrochloric acid (10% aqueous solution) and solution temperature was reduced to 42 ˚C to lessen losses by volatilization.
- Eighty g of liquid core material were emulsified into the hydrocolloid “solution” by stirring at 600 rpm for 25 min, maintaining the temperature at 42 ˚C. For menthol (solid core ground to the desired particle core size), no emulsion was needed. Thus, parameters were slightly adjusted: this step

was carried on for 15 minutes at 37 ˚C, to avoid melting the menthol crystals.

- After emulsification, 400 mL of dilution DI water at 35 ˚C were added, the stirring reduced to 300 rpm and the system cooled gradually to 13 ˚C: first to room temperature (about 25 ˚C, in about 2 hrs), and secondly using a water bath filled with ice water (cooling from 25 ˚C to 13 ˚C in about 1.5 hr).
- Maintaining the system at about 13 ˚C and stirred at 300 rpm, pH was adjusted to 9 with sodium hydroxide (5% aqueous solution) and 2 g of cross-linking agent were added (glutaraldehyde, 50% solution in water, Aldrich Chemicals). The cross-linking stage was continued for about 2 hrs at 13 ˚C and then the system was allowed to reach room temperature for the next 12 hrs.
- Capsules were collected (rose to the top of the system) and rinsed with DI water.
- The wet slurry was then freeze dried as detailed below.

A summary of the steps is presented in **Figure 2-1.**

Material		Conditions		Stage
8 g gum acacia 12 g gelatin 450 mL DI water		350 rpm 45 ˚C		***Dispersed System***
		↓		
hydrochloric acid (10%)		pH = 4.5 30 min		
		↓		
80 g core material		600 rpm 25 min		***Emulsification***
		↓		
400 mL DI water heating source removed		300 rpm		
		↓		
		T = 25 ˚C		***Coacervation***
ice water bath		↓		
		T = 13 ˚C		
		↓		
sodium hydroxide (5%) 2g cross-linking agent		pH = 9.0 T = 13 ˚C - 2 hours T = 25 ˚C - 12 hours		***Cross- Linking***
		↓		
Collect Capsules				

Figure 2-1: Scheme of process steps to form microcapsules by complex coacervation, with liquid or solid core

3.1.3. Drying

Preliminary studies were conducted to determine the optimum drying method of the wet coacervate slurry. These methods included simple filtering to recover the coacervates and then dehydrating this coacervate-rich phase in alcohol (anhydrous methanol, golden grade, 99% purity, Sigma-Aldrich), by adding silica (Syloid 244, Grace Davison, Columbia, MD), by spray drying (Niro Atomizer BRAND AND LOCATION, two fluid nozzle) and freeze drying (FTS Systems, Stone Ridge, NY, USA). While spray drying yielded an acceptable dry coacervate, freeze drying gave a higher quality (more intact capsules) product and thus, was chosen for this study. Product quality was evaluated in terms of capsule shape, volatile loss (after extraction) and final yield.

For freeze drying, the capsule-rich coacervate slurry was deposited on stainless steel trays and frozen at -30 ˚C in a built-in blast freezer of the University of Minnesota Food Science department Pilot Plant. After 24 hrs, frozen capsules were transferred to a freeze drier (FTS Systems, Stone Ridge, NY, USA) for 48 hrs, and using the following dryer parameters: condenser temperature -30 ˚C and drying chamber vacuum (100 mTorr).

3.2. Evaluation of microcapsules

3.2.1. Efficiency of process

a) Capsule Yield

The overall yield of the process was defined as the ratio of mass of final product obtained to initial mass of wall and core materials used in formulation. The mass used in calculation is an adjusted mass, i.e. $total\ mass - water\ content$. Moisture content was determined using the Karl Fisher procedure, as detailed below. Yield reported is the average of triplicate processes for each type of capsule formed (solid or liquid core).

b) Encapsulation efficiency

A second measure of process efficiency is the amount of aroma material recovered in the dry coacervates. Therefore, the total aroma load of the capsules was determined after drying. The aroma compounds were extracted from the coacervates as follows: 0.3 g of dry capsules were placed in a 20 mL headspace vial with 7 mL of DI water. Protease (0.025 g, Validase BNP L, Valley Research, South Bend, IN, USA) was then added. The closed vials were heated to 65 ˚C and stirred at 100 rpm for 18 hrs. Three ml of dichloromethane (GC grade, Aldrich Chemicals) containing 1000 ppm of internal standard (2-octanone, Aldrich Chemicals) were added directly to the vial and mixed well. One µL of the solvent was then injected in a gas chromatograph (GC, model 5890, Hewlett Packard, Wilmington, DE, USA). The GC was equipped with a 30m x 0.25 mm x 0.25 um DB-5 column (J&W Scientific, Folsom, CA, USA). The GC operating parameters were: injection port 225 ˚C, detector 250 ˚C, 12 PSI column head pressure, inlet split ratio 1: 50, oven temperature program 90 ˚C / 10 ˚C.min^{-1} / 140 ˚C / 20 ˚C.min^{-1} / 200 ˚C / 3 min hold. Quantification was done by dividing the peak area of the aroma compound by that of the internal standard, and comparing to a pre-established calibration curve created under the same analytical conditions. Data reported are the average of triplicate extractions (one injection per solvent extraction).

3.2.2. Microscopy

The structure, shape and formation of microcapsules were determined by mounting the capsules on a microscope slide and observed using a bright field microscope (Carl Zeiss Inc., Thornwood, NY), 10x lense mounted with a digital camera (Olympus Evolt E330, Japan). Images were analyzed with ImageJ software (National Institute of Health, USA). The images obtained were used to determine the structure of capsules (mononuclear vs. aggregates or polynuclear), the shape of the capsule, and estimate the wall thickness. Images presented in **Figures 2-2 a and b** are representative of the respective sample, in spite of the presence of a limited number of capsules in each frame.

3.2.3. Size Distribution

Particle size distribution of capsules was determined by light-scattering using a Malvern Series 2600 Particle Size Analyzer (Malvern Instruments Inc., Malvern, Worcestershire, UK): methanol was used as solvent (spectrophotometric grade, 99% purity, Sigma- Aldrich). The size distribution was characterized by its mean diameter, standard deviation (reflecting the particle size distribution around the mean, not the variation in means across replicates) and type of distribution (i.e. unimodal or bimodal). Data gathered are the De Broucker means, measured by the laser scattering instrument. Results reported are the average of triplicate samples.

3.2.4. Moisture content

Moisture content of capsules after drying was determined by Karl Fisher using an Aquatest CMA titration unit (Seradyn, Japan). Capsules (0.3 g, dry) were placed in 20 ml headspace vials and 11 g of anhydrous methanol (highest purity grade, Sigma- Aldrich) was added. Vials were sealed with Teflon septa and shaken for 18 hrs on a shaking table at 150 rpm and then allowed to “rest” 1 hr before analysis. Results are reported as % moisture (total mass) and represent the average of triplicate samples.

3.2.5. Wettability

Wettability of dry capsules is defined as the capacity to swell in the presence of solvent or water. Dry particles were fixed onto double faced tape on a microscope slide. A drop of deionized water (room temperature) was added to the slide, and a slip cover was added, moving the water onto the capsules. To determine the time to complete hydration of the dry particles, the microscope was mounted with a digital camera in “video” mode. Time to complete hydration is defined as the time difference between the addition of the water to the slide and the moment the capsules ceased swelling, judged visually.

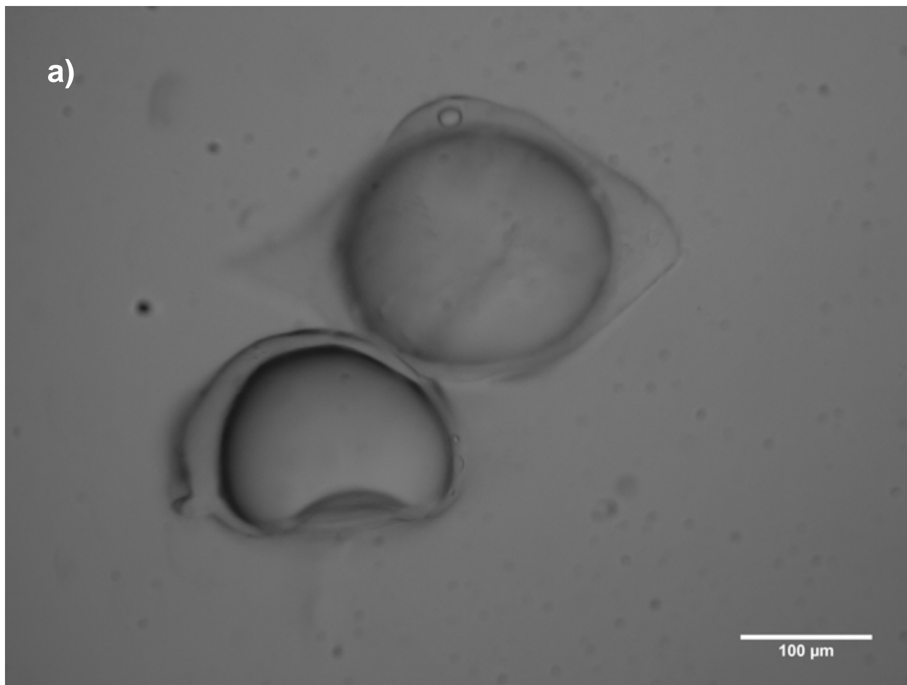

Figure 2-2: Microcapsules formed by complex coacervation with liquid core (a) or solid core (b) by light microscopy.

3.3. Storage Study

A short storage study was conducted to evaluate the ability of complex coacervates to prevent limonene oxidation. A comparison was conducted against spray dried limonene powder. The spray dried powder was manufactured using Capsul ®, a chemically modified starch, as carrier and 25 % (w/w of solid content) limonene loading. Spray drying was done using a Niro Atomizer (Utility model, Copenhagen, Denmark), maintaining an inlet temperature of ca. 175 ˚C and an outlet temperature of ca. 85 ˚C (by adjusting the infeed flow rate to ca. 300 ml/min).

Even though the two powders do not have the same initial flavor load, they both represent typical loads obtained in the industry for each type of encapsulation method. The two powders were equilibrated for 48 hrs in chambers above saturated salt solution to standardize their water activity (a_w= 0.53) prior to placing the samples in the storage study.

Two g of each equilibrated powder was placed into 10 ml glass headspace vials, caped and sealed with Teflon septa. Samples were placed in a 45 ˚C incubator. Sampling times were 0, 2, 4, 6, 8, 12, 16, 20, 25 and 30 days in storage. At each sampling time, samples were taken and placed into a -20 ˚C freezer until analysis. Limonene and limonene oxide in coacervates were extracted and quantified by GC as detailed above.

Aroma load in the spray dried powder was determined as follows: 0.7 g of spray dried capsules were placed in a headspace vial, and 7 ml of DI water was added. The closed vial was heated to 80 ˚C for 1 min with shaking to dissolve the starch matrix. The sample was then allowed to equilibrate for 1 hr at ambient temperature. Three ml of solvent with internal standard was added followed by the procedure detailed above for coacervate load determination (adding enzyme). Results are reported as the ratio of the amount of limonene oxide/ total amount of aroma volatiles, as determined by a calibration curve.

4. Results and Discussion

4.1. Encapsulation process

The fundamental principle of capsule formation by coacervation relies on electrostatic interactions between oppositely charged hydrocolloids. These interactions will therefore, depend on the charges themselves, i.e. the net charge carried by both colloids. This, in turn, will be modified depending on the pH, the type and amount of colloid, the ratio of the two colloids (positive charges vs. negative charges), and accessibility of the charges for interaction. Numerous studies have investigated several of these parameters, such as the influence of pH, the choice of wall material, stirring conditions and particle size, but most often single out one or two of these key parameters, without providing a discussion of the overall process. In addition, very few papers mention details about methods of drying capsules and retention of the flavoring.

4.1.1. pH

During the emulsion and coacervation phases, obtaining the proper pH is probably the most critical parameter for the formation of coacervate microcapsules. Numerous studies have investigated the pH at which the process should be carried out, when using a protein-polysaccharide system. A study reported by Burgess (7) noted the best coacervate yield at a pH below the gelatin isoelectric point (3.8 or 3.5 for type A gelatin and type B, respectively), using a gelatin/ gum acacia system. A similar finding was reported for a whey protein/ gum acacia system by Weinbreck *et al.* (18). The fact that the optimum pH is below the isoelectric point has been explained by the hydrocolloids carrying multiple ionizable functional side groups. Therefore the isoelectric point of the protein alone is a poor indicator of the charge density of the polymer. (6, 9) In

addition, pHs below the isoelectric point maximize the surface tension of gelatin, which is critical during the coacervation stage (19). Our trials with various pH levels (3.8 to 4.9) did not suggest significant yield reduction (wet basis), unless the pH was very low (< 3.8, data not shown). In the process presented herein, the pH was maintained around 4.5 (i.e. significantly lower than the isoelectric point of gelatin) during the emulsion and coacervation phases and good yields were obtained.

4.1.2. Colloid amount and ratio

There is little agreement in the literature regarding the ratio of the two polymers to be used. Some publications suggested the ratio should be calculated depending on the charge the polymers carry, while others have shown that the ratio had little influence on capsule formation. Our trials covered ratios of gelatin to gum acacia ranging from 1:1 to 1:3. These trials suggest that the ratios of the individual polymers influenced the ease of emulsion formation, and therefore the final size distribution of the formed capsules. However, the final yield (total core encapsulated and total yield on wet basis) produced appears to be influenced more by the pH for each ratio than the ratio itself.

The available literature is very vague on the optimal amount of oil phase or the core:wall ratio. Recently, Dong *et al.* (20) indicated a significant size distribution difference in the multinuclear capsules formed depending on the core:wall ratio used. They also suggested a positive relationship between the ratio and the final loading, but they only determined this loading theoretically and not experimentally. In addition, the limited literature is unclear whether all of the oil was indeed encapsulated and/or if the entire amount of polymer present was included in the final particle walls or alternatively, if much was lost with the dilution water. Based on initial trials (core:wall ratios varying between 1:2 and 1:6) and given the amount and ratio of polymer used (20 g), we determined that 80 g of total core material (or a core: wall ratio of 4:1, representing 80% of core) resulted in an optimal amount of, and adequately shaped capsules. However,

this ratio is clearly dependent on the emulsion parameters, which affect the droplet size (core size) and the formation of aggregates during the coacervation stage.

In addition, the physical state and properties of the core have not been discussed in detail in the published literature. Arneodo *et al.* (4) and Guzey *et al.* (21) described surface tension as a critical factor affecting the formation of the colloid wall around an emulsified droplet core. Although the surface tension was not measured in the present study, it would be expected that differences in types of oil (limonene and medium chain triglycerides) and the difference between liquid and solid state (menthol) would affect the formation of coacervate capsules, by modifying the surface tension of the system. To the authors' knowledge, no data on the influence of the core's physical state has been reported.

4.1.3. Size and stirring rate during formation of the emulsion

Capsules formed by coacervation have been reported to range in size, from 10 µm to 1000 µm (22-24). The final size of the capsules depends on the core size and the wall thickness. The particle core size can be changed when using a liquid core by modifying the shear applied in emulsification. The literature indicates incredibly variable emulsification parameters, reported either as stirring time, stirring rate and time, or shear rate, which makes comparisons very difficult (9). No clear consensus exists in the literature regarding a relationship between emulsion formation parameters and final coacervate size. In the present study, stirring rates during the emulsification stage were varied between 300 rpm and 800 rpm, lasting between 15 and 30 min. Shear rates were not measured. The effects of stirring rate and time were found to be interrelated with the core:wall ratio and the cooling rate during the coacervation stage. The parameters presented represent the optimal conditions found to obtain the highest yields and best individualized capsule shapes.

Size distributions of the coacervates are presented as the mean volume average, as given by the instrument software. Results are summarized in **Table 2-1**. Final

capsule size distributions were consistent with the emulsion droplet size (liquid core). As discussed earlier, when the emulsion was too fine (<100 μm), aggregates as opposed to individual particles were formed. Producing capsules between 250 – 350 μm allowed the manufacture of consistent batches of capsules. No significant differences could be determined between the average particle size before (data not shown) and after drying, which suggests that wall shrinkage during drying is negligible compared to the core size.

Table 2-1: Size distribution obtained for capsules after drying formed with each type of core material and estimate of wall thickness on moist capsules.

	Type of Core		
	Liquid	**Solid**	
		Top Layer	*Bottom Layer*
Size Distribution	325 μm (± 112 μm)^{a}	273 μm (± 136 μm)^{b}	219 μm (± 132 μm) ^{c}
Wall thickness (moist capsules)	21 μm (± 7 μm)	20 μm (± 6 μm)	21 μm (± 3 μm)

Values in the table are the average of measures made on triplicate batches, made with each core material (mean (standard deviation)). Subscripts represent statistical differences after Fischer's LSD ($P < 0.05$)

Capsules formed with a solid core (menthol) separated into two subgroups, as mentioned earlier. Each subgroup (precipitate or supernatant) also had consistent size distributions across batches. However, the three capsule types (different core materials) had significantly different size distributions. The differences are likely due to the means of making the core particles (forming an emulsion vs. grinding and sieving).

The second factor affecting the final particle size is the thickness of the capsule shell. As mentioned earlier, this thickness is theoretically variable, depending on the concentration of colloid, the ratio of colloid compared to core material, the

stirring rate during the coacervation stage and the quality of interactions between the colloids. Unfortunately, this aspect is rarely discussed in the current literature. Jégat and Taverdet (22) investigated the relationship between the stirring rate during the formation of drug-loaded coacervates and drug release. Their study showed that the particle size was inversely proportional to the stirring rate. Particle size, however, was not found to be as important as wall thickness and capsule structure (i.e. polynuclear or mononuclear) in influencing the rate of drug release. Since wall thickness, particle size and capsule structure are influenced by the stirring rate, it is difficult to determine the influence of either parameter from this work.

4.1.4. Temperature

Few papers mention the temperature at which the coacervation process was carried out. When mentioned, there is a great discrepancy in what is thought to be optimal, i.e. optimal temperatures between 5 ˚C and 50 ˚C are reported (18, 25). Temperature might not be as critical for drug encapsulation as it is for flavor encapsulation because of losses due to volatilization, which might explain this wide variation.

Trials were conducted to determine optimal temperature profile, starting at 45 ˚C (necessary to maintain gelatin in solution). We determined that maintaining ca. 45 ˚C at the beginning of emulsification and then gradually lowering the temperature to 42 ˚C in the last 10 min seemed optimal in it limited volatilization of our volatiles and still allowed adequate formation of microcapsules.

Coacervation occurs when the entangled wall materials aggregate around the oil droplets. For this to happen, the system needs to cool, while stirring from 45˚C to about 25˚C, the temperature at which the colloid material is gelled. There is limited guidance in the published literature on how fast this cooling should be, or the importance of the stirring rate while cooling. Thimma and Tammishetti (26) mentioned that a slow cooling rate is critical and suggested a cooling rate of about 1 ˚C. min^{-1} without providing further justification. The following combinations were tested. Fast cooling (1.5 ˚C. min^{-1}) coupled with rapid stirring

(350 rpm) led to few, elongated capsules with thin walls and little residual material in solution. A slower cooling (0.2 ˚C. min^{-1}) coupled with a slow stirring (100 rpm) led to aggregates instead of individual capsules. We determined that the cooling rate and stirring rate after emulsification are interrelated. The best compromise was found to be a slow cooling (0.2 ˚C) and rapid stirring (about 300 rpm). To the authors' knowledge, studies on this process parameter are lacking in the available literature, even though it is a key factor in producing consistent, adequately formed and shaped coacervate capsules.

All microcapsules formed in the present study portrayed similar particle shapes. As seen in **Figures 2-2a and 2-2b**, the microcapsules obtained are mononuclear, with a thin layer of wall material around the droplet or crystal core. A similar structure was obtained regardless of using a solid or liquid core, as well as independently of the type of oil (limonene vs. vegetable oil, with different surface tension, data not shown). Surface tension was described by Arneodo *et al.* (4) as a critical factor affecting the formation of the colloid wall around the emulsified droplet core. Therefore we expected that the core material would affect the formation of the capsules and the process parameters would have to be adjusted. However, only the parameters used in the coacervation stage (cooling) were changed when the type of core was varied (process was observed by light microscopy during all phases).

As is evident from figures, the wall is not uniformly distributed around the core but assumes a slightly "rugby ball" shape. This elongated shape results from fast stirring during the coacervation phase, inducing an alignment of the capsules with the water flow. Attempts to reduce the stirring rate did not permit forming individual microcapsules but aggregates and were, therefore, not used.

Many papers have reported data on polynuclear coacervate microcapsules. We expect that their properties would be quite different from mononuclear capsules, particularly in terms of core release and stability and thus, have avoided this structure. A few recent studies have shown that core release of material was

dependent on the aroma droplet size, such as the work presented by Jégat and Taverdet (10) and de Roos (32).

4.1.5. Cross-linking

Cross-linking consists of the formation of non-soluble networks via the reaction of aldehyde residues from the cross-linking agent and amino groups from the colloid (protein). Literature suggests the use of a cross-linking agent to strengthen the capsule wall, improve the ease of drying, and increase storage stability. The most commonly used cross-linking agents are glutaraldehyde, formaldehyde (1% of colloid material w/w) or poly-amines, and anecdotally some natural compounds are being used, such as fruit extracts as reported in the recent literature (14, 27, 28). However, contradictory details are given on the optimal conditions for the cross-linking reaction to happen. Recently, Dong *et al.* (20) reported that the reaction was optimized at elevated pH (pH = 9) and intermediate temperature (T = 15 ˚C) when conducted for 6 hrs, while others suggested room temperature and did not mention pH (12,13,29). The reaction mechanism (reaction between an aldehyde group and amino group) would suggest that the reaction is optimized at extreme pH. Our preliminary cross-linking experiments were conducted with glutaraldehyde at pH 9.

Three experiments were done to determine the best temperature profile for cross-linking: 1) the system was held at refrigeration temperature during the whole time (8 ˚C), 2) maintained at 15 ˚C for the first few hrs and then returned to room temperature, or 3) held constant at room temperature (25 ˚C). The robustness of the capsules cross-linked using each temperature profile was qualitatively assessed by placing the capsules between a slide and cover glass and observing the time until they ruptured after applying pressure on the cover.

No difference in capsule sturdiness was observed when cross-linked at 8 ˚C and 15 ˚C (data not shown). However, capsules were more durable then when cross-linked at 25 ˚C upon application of pressure on the glass cover. For ease of experimentation, all subsequent cross-linking was done using a cool water bath

for about 2 hrs after addition of glutaraldehyde, and brought back to room temperature after that. The effectiveness of cross-linking on storage stability or its effect on release properties has not been evaluated.

4.1.6. Drying

While most of the literature on microcapsules formed by complex coacervation has generally focused on capsule formation, there is a lack of detail on capsule drying. While drying at elevated temperature might not be critical for drug encapsulation, significant losses of volatiles would occur and high temperatures are therefore not recommended for aroma encapsulation. Our trials included several methods of drying the wet slurry collected, such as simple filtering, dehydrating in alcohol, adding silica, spray drying and freeze drying. Using the aforementioned methods, clumps (aggregates) of capsules were formed (filtering, and adding silica) or capsules were disrupted (spray drying, and sieving after adding anhydrous alcohol). Volatile retention was measured only when particle structure was considered acceptable (intact, non-clumped capsules).

A study by Palmieri *et al.* (30) compared the efficiency of three methods to dry capsules containing either a solid drug core or an oily core (containing the dissolved drug). They concluded that spray drying was optimum overall for capsule shape and load retention. However, a lack of detail in their process parameters did not allow us to reproduce their conditions. As noted, we did not find spray drying to be satisfactory for our purposes because of excessive losses of flavoring and substantial capsule breakage. Of the methods tried, the only satisfactory process yielding free-flowing, individual, low moisture (ca. 3%) particles was freeze drying.

4.2. Encapsulation efficiency and aroma load

Jiang *et al.* (31) reported an efficiency of slightly above 90% for complex coacervates formed using gelatin and various polyanions, encapsulating protein

cores. Madene *et al.* (23) reported loads varying between 60 and 90% without specifying whether these loads are of volatiles or large molecules such as drugs, and before or after drying. To the authors' knowledge, limited information is provided regarding the efficiency of complex coacervation as an aroma encapsulation technique.

The capsules we have made when using a liquid core contained approximately 80% core material and 20% wall material. Since oil is less dense than water, the formed capsules rose to the water surface when stirring is stopped. This supernatant was considered for all of the following measurements.

The capsules containing a solid core behaved differently. Depending on the core size (and ultimately the entire capsule size), the capsules rose to the surface or precipitated. The two "types" of capsules (found on the bottom and top) were pooled under the overall **Table 2-1** heading "solid core". The yield was determined by measuring the masses of water, capsules and material not encapsulated (mostly colloid material). Data presented are largely an estimate of non-encapsulated material remaining in the bottom phase. Yields we observed using optimal conditions are summarized in **Table 2-2.** No substantial difference could be noted between the yields obtained for the different core materials. The process steps allow for forming consistent microcapsules, regardless of the type of core material encapsulated.

The flavor load was determined in the capsules formed with liquid and solid cores after drying. Results are presented in **Table 2-3**. The flavor loads of each type of microcapsule were significantly different from one another, varying between 70 and 85% (masss/mass). Given the core to wall material ratio used in formulation, the theoretical load should have been about 80% core. The two aroma compounds used in these examples are very hydrophobic, and therefore, losses due to partitioning into the aqueous phase were very limited. One could argue that some losses might occur due to volatilization during the process which was carried out at 45 ˚C. These losses were not determined analytically. In addition, it is expected that some losses would occur during the drying stage. Overall, the

load of aroma in the final microcapsules formed by coacervation is high compared to other encapsulating methods such as spray drying (20%) or plating (2-7%) (34, 35).

Table 2-2: Yield obtained for each type of core material (mass dry capsules / mass of material added).

	Type of core	
	Liquid	**Solid**
Material Encapsulated	85.3 ± 6.9 %	>90%
Material Non- Encapsulated	13.8 ± 2.9 %	<10%

4.3. Water uptake dynamics

Water uptake is a key factor determining the release kinetics of core material. Robert *et al.* (12) detailed the effect of water front dynamics on the transport mechanism of the core material through the encapsulant wall (i.e. Fickian mechanism or Case- II transport). Results presented here are time (in sec) until no visible change in shape is visible for at least 5 capsules per trial (**Table 2-3**).

The initial moisture content of capsules (dry) is also reported in **Table 2-3** to give readers an idea of the amount of water absorbed by the capsules. Moisture contents were consistent between surface capsules (liquid and “top layer, solid”) but were significantly different from the bottom layer capsules. This can be explained by the difference in particle size and wall to core proportion (see **Figures 2-2 a and b**). Based on microscopy, no significant difference in wall thickness could be determined across capsules (measured on 61 capsules in 10 different images; average thickness: 21.7 µm). However, since the bottom layer capsule cores were significantly smaller, these particles had a higher ratio of core: wall compared to the liquid core and top layer capsules. If the results were

reported as moisture content per g of wall material only, there would be no significant difference between the samples.

No difference could be detected in the time to complete swelling, with an average of 4 to 5 sec across all particles. It is possible that there was a difference in swelling time but the difference was too small to be found using the methodology chosen. Furthermore, since moisture uptake was very rapid (< 10 sec), dynamic of water uptake cannot be considered a limiting factor for core release in a water environment. A recent study (33) indicates similar findings (rapid swelling) for freeze dried microcapsules formed by complex coacervation when in the presence of aqueous solutions containing surfactants.

Table 2-3: Moisture content of capsules after freeze drying, time to complete rehydration (complete water uptake, water at room temperature) and flavor load.

	Type of Core		
	Liquid	**Solid**	
		Top Layer	*Bottom Layer*
Moisture content (% per weight)	0.63% (±0.04)^{a}	0.68% (±0.03)^{a}	1.39% (±0.14) ^{b}
Time to complete rehydration (in seconds, after addition of water)	5.3 (±1.2)	3.5 (±0.7)	5.3 (±1.3)
Flavor load (%, m/m)	70% (±2.2)^{c}	85.7% (±5.0)^{a}	74.6% (±3.8)^{b}

Values are the average of measures made on triplicate batches, made with each core material (mean (standard deviation)). Subscripts on a same row represent statistical differences after Fischer's LSD (P < 0.05).

4.4. Storage stability

A brief storage study was conducted between coacervation and spray drying using limonene (liquid) as the core material. Limonene was chosen since its

oxidation products are easy to identify and quantify by analytical methods. The objective of this short study was to evaluate the oxidation protection provided by the two types of encapsulant matrices, but not to determine oxidation kinetics. As mentioned earlier, the two powders did not contain the same initial load of limonene (70% and 15% for coacervates and spray dried powder, respectively). For this reason results are reported in terms of limonene oxide compared to the total aroma load (limonene content + limonene oxide content).

Results are presented in **Figure 2-3**. It is noteworthy that the limonene oxide level was high initially suggesting the oil was not as fresh as desired. While this would tend to reduce the induction period, it would not affect the outcome of the study: greater protection against oxidation by a wall material would still result in slower rates of oxidation.

No significant increase in limonene oxide level was detected in the coacervated samples during storage. However, a statistically significant increase in limonene oxide, 5.7 mg/g oil to 10.2 mg/g oil, was observed in the spray dried powder over the study period. The differences between the two oxidation rates have been explained by the structural differences between the two types of encapsulation techniques (36). Spray dried powder is more porous and can have pores through which oxygen can easily move and reach the oil. In addition, the oil is not uniformly distributed in the powder but might be on the surface or close to pores. Conversely, in coacervate capsules, the oil core is entirely surrounded by the wall, thus limiting oxygen passage ways and porosity.

Risch *et al.* (37) reported significant increases in oxidation products of orange oil when encapsulated in gum Arabic and modified starch when stored under similar conditions (30 days at 45 ˚C) as used in our study. The increase we observed is substantially lower than that reported by Risch. However, they also showed that processing parameters such as emulsion size and type of material had a significant influence on product shelf life. Since our spray drying parameters and matrix are different from theirs, these differences might explain the observed data.

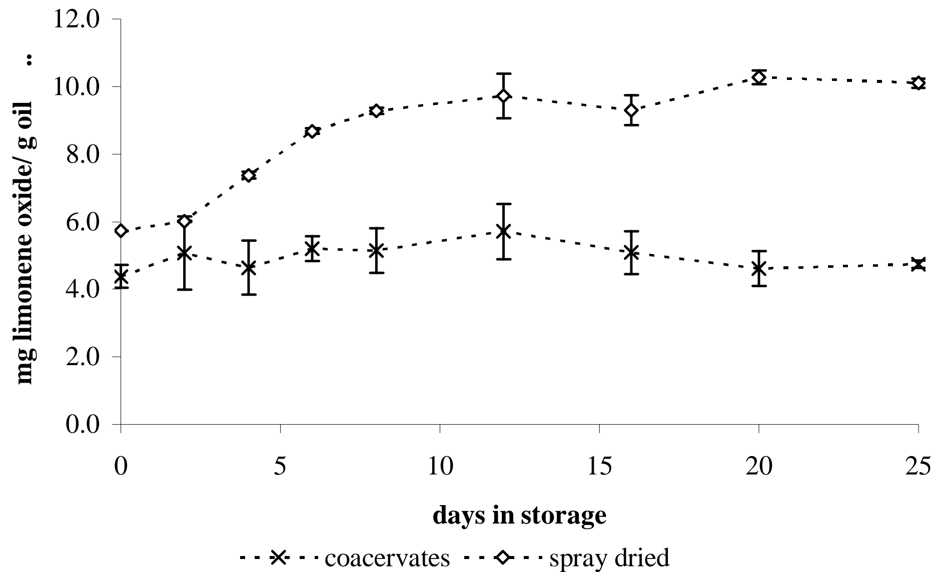

Figure 2-3: Influence of encapsulation method on storage stability of limonene

5. Conclusions

This study has reviewed and discussed the influences of processing parameters for volatile encapsulation by complex coacervation. The proposed protocol allowed the formation of consistent batches of microcapsules containing gum acacia / gelatin as wall material and either liquid or solid aroma cores. The performance of the coacervation process was evaluated based on the core load obtained, achieving the desired particle structure (not aggregated) and the protection provided against oxidation.

Coacervation provided good results in each of these performance criteria. We should note that our study did not include any water soluble actives which would be problematic to incorporate in this process due to the large volumes of water used in manufacturing. Despite this limitation, coacervation offers a unique method for the encapsulation of flavors that will find use in the industry. Additional work needs to be conducted in order

to gain knowledge on release mechanisms and factors affecting release from microcapsules made by complex coacervation.

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Chapter 3: Effects of Cross-Linking, Capsule Wall Thickness and Compound Hydrophobicity on Aroma Release From Complex Coacervate Microcapsules

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1. Abstract

Microcapsules were produced by complex coacervation with a gelatin- gum acacia wall and medium-chain-triglyceride core. Dry capsules were partially rehydrated and then loaded with model aroma compounds covering a range of volatility, hydrophobicity and molecular structure. An experimental design was prepared to evaluate the effects of cross-linking, wall: core ratio, and volatile load level on aroma release from capsules in a hot, aqueous environment. The real-time release on rehydration was measured by monitoring the headspace of a vessel containing the capsules to a PTR-MS. Data collected showed no effects of cross-linking or wall:core ratio on volatile release in hot water for any of the volatiles studied. When comparing real time release of the prepared coacervates to a spray dried equivalent, there was no difference in release from hot water but release was slower when coacervates were added to ambient temperature water. We found volatile release to be primarily determined by compound partition coefficients (oil to water and water to air) and temperature.

2. Introduction

Encapsulation refers to techniques by which a material is coated or entrapped within another material forming a protective shell or wall (1, 2). The main purposes of producing dry flavorings are to convert liquid compounds into a powder easy to handle, and to provide a protection against oxidation and evaporation (3, 4). The materials composing the wall or coating vary from technique to technique as well as with the ultimate application. The most common wall materials are carbohydrates (e.g. maltodextrins, modified starches, and gum acacia), proteins (e.g. gelatin, or whey protein), cellulose, or combinations of these materials. The flavoring material can either be entrapped as such or diluted in a matrix such as oil (3). Several literature reviews detail the various encapsulation methods along with their strengths and weaknesses (3-5).

Several techniques exist to manufacture dry flavorings through a variety of processes each providing unique characteristics. This study focuses on encapsulation via complex coacervation. Complex coacervation is a “true” encapsulation (shell- single core structure) of oil droplets into a colloidal material in solution. Coacervation is based on electrostatic interactions between one or more polymers formed around an emulsified phase. Complex coacervate formulations and process parameters have been extensively studied. Schmitt et al. (6) and Burgess (5) have provided in depth reviews regarding the optimization of several manufacturing parameters in forming complex protein-polysaccharide coacervates.

Cross-linking in capsule formation is an optional process which can modify the structure and properties of the coacervate microcapsules. The role of cross-linking is described as to harden the wall material after formation of the capsules (7). The goal of hardening the capsules is to make them more stable during drying and also to confer some unique properties to the wall material, such as modifying the physical state (change of glass transition temperature). The chemical cross-linking agents used link hydroxyl residues on polysaccharides and/or amine residues on the protein polymer. Typically, formaldehyde and

glutaraldehyde have been used as cross-linking agent in the fertilizer and pharmaceutical industries (8, 9). There are some toxicology issues on using formaldehyde and glutaraldehyde in food applications. No published data could be found on the effects of cross-linking on the release of encapsulated material, in particular, for encapsulated volatiles.

Complex coacervation has been investigated intensively for pharmaceutical applications and as drug carriers for targeted delivery (10). For these applications, capsule formation parameters have been optimized to obtain a desired drug release profile. The parameters focused on include particle size, water transport dynamics (8), wall composition and ratio (11), effect of degree of cross-linking (7) and drug solubility (12). While there is extensive literature available on the release of drugs, genes or proteins from complex coacervates, limited published data are available to date on the release of volatiles (i.e. aroma compounds) from such microcapsules.

It is generally accepted that aroma is a key factor determining food acceptance. However, it is well recognized that it is not the absolute presence of volatiles in a food that determines their perception but their release (rate and quantity) (13, 14). For this reason, to fully characterize a flavor encapsulation system it is desirable to characterize the release of aroma compounds from it in a given application. In the last decade, technological developments in analytical instruments allow on-line, real-time measurements via mass spectrometry (MS) (15, 16) such as with Proton Transfer Reaction Mass Spectrometry (PTR-MS). The principles behind this method have been described in detail elsewhere (16-18). PTR-MS has been extensively used in such applications including breath analysis (medical applications) (16), aroma release during eating (13), and in nose and sensory perceptions (19-21).

The two main mechanisms for aroma release from coacervate microcapsules are the mechanical destruction of the capsule wall leading to rapid leakage of the encapsulated material into the surrounding system, and by the slower diffusion of the active component from the core through the intact wall. Both approaches are

used in pharmaceutical applications. For food applications, if capsules are above a certain size, they maybe degraded by chewing, liberating the encapsulated material in the mouth. For all other situations, release principally occurs via diffusion, i.e. hydration of the wall such that it becomes permeable to the core material (4). Diffusion rate will be affected primarily by the shape and speed of the water front entering the particle shell and the characteristics of the shell polymer, i.e. glass transition, strength and cohesiveness of the network. As a result, the dynamic swelling or rate of water (solvent) uptake of dried particles is a critical parameter for release. One would also expect particle size and thickness of the capsule wall to affect the particle swelling rate.

Diffusional release rate also depends on the ease and rate at which the encapsulated material can migrate through the porous wall material. For this reason, works published in the pharmaceutical area do not necessarily apply in flavor applications since aroma compounds are relatively small molecules and differ greatly in chemical properties (e.g. water solubility and volatility) vs. typical pharmaceuticals. The release profiles of aroma compounds from coacervate capsules are, therefore, expected to be very different from published data on drug molecules.

In the work presented herein, we report on the dynamic release of aroma compounds from capsules produced by complex coacervation. This study focused on determining the influence of capsule manufacturing parameters, such as wall thickness and cross-linking of wall materials on the release profiles of various aroma compounds differing in chemical properties.

3. Material and Methods

3.1. Materials and chemicals

Gelatin 250 Bloom strength, 20 Mesh, type A provided by PB – Leiner (Davenport, IA, USA) and gum Arabic (Acacia seyal, FT powder, TIC Gums, Belcamp, MD, USA) were used as wall materials in the formation of microcapsules. Medium chain triglyceride oil (MCT, Lumulse CC-33K, Lambent Technologies, Gurnee, IL) was used as core material.

Capsul ®, a octanyl-succinate- anhydrous substituted starch (National Starch Corp, Bridgewater, NJ) was used as encapsulation matrix in spray drying.

The aroma compounds used were purchased from Aldrich Chemicals (St Louis, MO) at the highest purity available except for β-damascenone which was provided by Robertet Flavors, Inc. (Piscataway, NJ). 2-butanone, β-damascenone and methyl-pyrrole were used at 100 ppm (i.e. each 12.5 % of the pure compounds mixture) and methyl-propanal at 500 ppm (w/w of oil present, 62.5% of the pure compounds mixture).

3.2. Preparation of microcapsules

Microcapsules were prepared by complex coacervation using the following process. 8 g of gum acacia and 12 g of gelatin were dispersed in 450 mL DI water heated at 45 ˚C in a stainless steel beaker using an overhead stirrer (RW20 digital, IKA works, Wilmington, NC, USA) rotating at 350 rpm. pH was adjusted to 4.5 with hydrochloric acid (10% aqueous solution). The unflavored liquid core material (MCT, 40, 80 or 120 g for the 3 wall-thickness levels) was emulsified into the hydrocolloid dispersion (600 rpm on stirrer) for 25 min, maintaining the temperature at 45 ˚C. After emulsification, 400 mL of DI water (35 ˚C) were added, the stirring rate was reduced to 300 rpm and the system was slowly cooled to 13 ˚C: first to room temperature (about 25 ˚C, in about 2 hrs), and secondly using a water bath filled with ice water (cooling from 25 ˚C to 13 ˚C in about 1.5 hr).

In the case of cross-linked capsules, the following step was added: while maintaining the system at about 13 ˚C and stirring at 300 rpm, the pH was adjusted to 9 with sodium hydroxide (5 % aqueous solution) and 2 g of cross-linking agent were added (glutaraldehyde, 50 % solution in water, Aldrich Chemicals). The cross-linking stage was continued for about 2 hrs at 13 ˚C and then allowed to reach room temperature for the next 12 hrs.

Capsules were collected: scooped from the surface of the vessel and rinsed with DI water. Collected capsules were deposited on stainless steel trays and cooled to -30 ˚C in a blast freezer. After 24 hrs, the frozen capsule slurry was freeze dried (FTS Systems, Stone Ridge, NY, USA) for 48 hrs, under the following parameters: chamber temperature -30 ˚C and vacuum of 100 mTorr.

3.3. Flavor loading method

Based on preliminary experiments, loading aroma compounds into the microcapsules during their formation led to significant losses. This was due to volatilization (the process is carried out at 45 ˚C for at least 30 min) and partitioning into the water phase (which is discarded). An alternative loading method based on the procedure detailed in patent # US 6,106,875(22) was used. The procedure consisted of spreading 10 g of freeze dried capsules on a sieve (#140, mesh 106 µm) over a steam flow (2 m.s-1) until all capsules were moist. Capsules were then transferred into a 50 ml glass jar with a Teflon lid. Twenty µl of a mixture of pure compounds (listed above and in the proportion desired) were added to the jar which was then closed, shaken vigorously for 5 min and allowed to equilibrate for 24 hrs. One g of finely ground silica (Syloid 244, Grace Davison, Columbia, MD) was then added to the jar and mixed well to absorb moisture from the capsule walls thereby sealing them from volatile loss. Capsules remained in the closed jar until analysis.

3.4. Capsule characterization

3.4.1. Microscopy

The structure, shape and formation of microcapsules during manufacture were observed by microscopy using a bright field microscope (Carl Zeiss Inc., Thornwood, NY), mounted with a digital camera (Olympus Evolt E330, Japan). Images were analyzed with ImageJ software (National Institute of Health, USA). The images obtained were used to determine the structure of capsules (mononuclear vs. aggregates or polynuclear), wall shape and estimate the wall thickness.

3.4.2. Wettability

Wettability of dry capsules is being defined as the capacity to swell in the presence of a solvent or water. Dry particles were fixed onto double faced tape on a microscopy slide. A drop of DI water (room temperature) was added to the slide, and a slip cover was added, moving the water onto the capsules. To determine the time to complete hydration of the dry particles, the microscope was mounted with a digital camera in "video" mode. Time to complete hydration is reported as the time difference between the addition of the water to the slide and the moment the capsules cease swelling, judge visually.

3.4.3. Size Distribution

Particle size distribution of capsules was determined using light - scattering (Malvern Series 2600 Particle Size Analyzer, Malvern Instruments Inc., Malvern, Worcestershire, UK) using methanol as solvent (spectrophotometric grade, 99% purity, Sigma- Aldrich). The size distribution was characterized by its mean diameter, standard deviation and type of distribution (i.e. unimodal or bimodal). Data gathered are the De Broucker means, measured by the laser scattering

instrument. Results reported are the average of triplicate samples. Particle size measurements were confirmed by data collected by microscopy.

3.4.4. Flavor load

Surface "oil" of capsules was determined by first weighing 0.5 g of capsules into a 20 ml headspace vial. Five ml of dodecane (Aldrich Chemicals) containing 1000 ppm of internal standard (heptane, Aldrich Chemicals) was added to the vial, which was then capped with a Teflon septa and shaken at 2000 rpm for 2 min (Table Shaker Lab Line Orbit No 3590, Lab-Line Instruments Inc, Melrose, IL). Three ml of solvent was removed with a 3-ml glass syringe mounted with a syringe filter (0.45 µm pores, nylon, Fisher Scientific, Pittsburgh, PE) to remove any floating capsules. One µL of the solvent was then injected into gas chromatograph (GC, 5890, Hewlett Packard, Wilmington, DE, USA). The GC-FID was equipped with a DB-5 column (J&W Scientific, Folsom, CA, USA) - 30 m x 0.25 mm x 0.25 µm. The GC operating parameters were: injection port 225 ˚C, detector 250 ˚C, 12 PSI column head pressure, split ratio 1: 50, oven temperature program 43 ˚C / 6 min /15 ˚C.min-1 / 110 ˚C / 20 ˚C.min-1 / 200 ˚C / 2 min. Quantification was done by dividing the peak area of the aroma compound by that of the internal standard, and comparing this ratio to a pre-established calibration curve created under the same analytical conditions. Data reported represent the average of triplicate extractions (one injection per solvent extraction).

Total flavor load of the capsules was determined by first weighing 2 g of capsules into a 20 ml headspace vial, and then adding 7 ml of DI water containing 0.025g of protease (Validase BNP L, Valley Research, South Bend, IN, USA). The vial was sealed with a Teflon septum, heated at 60 ˚C for 5 min, and then placed on the shaker table (1500 rpm) at room temperature for 18 hrs. The sample was allowed to rest for 1 hr after shaking and then 3 ml was transferred into a new 20 ml headspace vial. Three ml of propylene glycol (Aldrich Chemicals) containing 1000 ppm of internal standard (heptane, Aldrich Chemicals) was added to the

vial, which was then sealed and vortexed for 1 min. One µl of this extract was then injected into the GC-FID set up as detailed above. Quantification was done by dividing the peak area of the aroma compound by that of the internal standard, and comparing this ratio to a pre-established calibration curve created under the same analytical conditions. Data reported represent the average of triplicate extractions (one injection per solvent extraction).

3.4. Dynamic Release: PTR – MS set up

The objective of this part of the study was to evaluate the release of encapsulated volatiles from the prepared capsules in the presence of water. For this determination, the following headspace purging system was utilized.

Fifty mg of capsules were weighed into a water jacketed glass cell (total volume 250 ml), thermostated at 70 ˚C. The glass cell was closed at the top by a stainless steel lid which also supported a heated, double-jacketed burette (100 ml total volume) set up to empty its contents into the sample cell. The burette – cell system was placed in an oven (85 ˚C) to maintain temperatures while manipulating the samples and avoiding any condensation of released volatiles. Sample purge gas (150 sccm) entered the burette and then flowed through the sample vessel (when opened to allow water to enter the sample cell). The purge gas coming from the cell (loaded with any volatiles released from the sample) was diluted by air (2000 sccm) to avoid overloading of the PTR-MS. This diluted sample effluent was directly sampled by the PTR-MS (Ionicon Analityk, Innsbruck, Austria). Only about 20 sccm of the diluted sample gas (2150 sccm) was introduced into the PTR-MS.

The PTR-MS parameters were set as follows: drift tube voltage: 600V; drift tube temperature: 60 ˚C; drift tube pressure 2.1 mbar; quadrupole (SEM) voltage: 2800V; quadrupole pressure: 3.5 x 10 -5 mbar.

The best ion (based on abundance and uniqueness) for each volatile compound monitored was selected in preliminary experiments. The instrument was set-up using Multiple - Ion- Detection, using 0.1 s dwell time on each mass. The

following m/z were monitored in the study: 21, 37 (water cluster), 73 (methyl-propanal), 82 (N-methyl pyrrole), 87 (diacetyl) and 191 (beta-damascenone).

3.5. Data Analysis (from PTR-MS)

The PTR-MS instrument software provides data in counts – per- second for each mass recorded. The counts are then transformed into concentration as given by the following equation (18, 23). The equation takes into account the operating parameters of the reaction chamber.

$$(RH^+)_{ppb} = \frac{(RH^+)_{counts} \cdot 10^9 \cdot U_{drift} \cdot 2.8 \cdot 22400 \cdot P_{atm}^2 \cdot T_{drift}^2}{k \cdot 9.2^2 \cdot (H_3O^+)_{corr.counts} \cdot P_{drift}^2 \cdot N \cdot 273.15^2 \cdot transm._{(RH^+)}}$$

Where:

(RH+) ppb : concentration of the compound in the gas phase

(RH+) counts : counts- per-second of the ion representing the compound

(H3O+) corr. Counts: counts-per-second of ion 21 corrected with m/z 21 transmission factor and multiplied with the isotopic factor (500)

Udrift: voltage in the drift tube (V)

Patm: 1013 mbar

Tdrift: temperature in the drift tube (333.15 ˚K)

k: reaction rate constant (≈ 2 x 10-9 cm3.s-1)

Pdrift: pressure in the drift tube (2.1 mbar)

N: Avogadro number (6.022 x 1023 mol -1)

Transm (RH+): transmission factor in quadrupole of m/z value of RH+

The transformed data were then plotted in terms of ppb vs. time.

3.6. Statistical analysis

Analysis of variance (ANOVA) was conducted on the time to maximum intensity and relative intensity at 0.5 min to determine the effects of capsule cross-linking,

wall thickness and aroma properties. Analyses were performed with the R package software (R-2.7.1, http://www.r-project.org/). Modeling of decay curves was done using the R package software as well.

4. Results and Discussion

The four compounds studied in this study were chosen because of their differences in physical and chemical properties, namely molecular structure and size, volatility and hydrophobicity. A summary of these properties is presented in **Table 3-1**.

The release of the noted volatiles from different encapsulation systems is being presented. Release is considered only in the context of a hot beverage application thus, time zero on all figures is when hot water (75 ˚C) was added to hydrate the microcapsules. Release was monitored for a total of 5 min after water addition. Release is typically characterized by the maximum intensity, time to maximum intensity, and persistence (or burst). We hypothesized that the difference in chemical and physical properties of the volatile compounds would affect their release.

Table 3-1: Aroma compounds used in this study and their physical-chemical properties.

Compound (abbr.)	Molecular Mass (ion used in PTR-MS measurement)	Vapor Pressure (mm Hg at 75 ˚C)	Log P value
Diacetyl	86 (87)	23	-1.34
Methyl Propanal (m.prop)	72 (73)	33	0.74
N-Methyl Pyrrole (m.pyrr)	81 (82)	1.1	1.43
β-damascenone (damas.)	191 (191)	<1	4.21

4.1. Effect of aroma compound properties on release

A typical release profile from cross-linked coacervated capsules is presented in **Figure 3-1a)**. First of all, one should note that the maximum intensity (Imax) is reached almost instantaneously for the four compounds after addition of hot water. Looking more closely, there are differences in Imax between the four compounds, even though methyl pyrrole (m.pyrr), diacetyl and β-damascenone (damas.) were present in similar initial concentrations (surface and total load). However, the time to Imax is not significantly different for any of the compounds, even if slightly longer in the case of damas. (3-4 s vs. 6-8 s). The difference in volatility and hydrophobicity of the compounds does not affect the initial release profile.

The differences in amounts of the compounds released makes comparisons of release persistence difficult. For this reason, data have been converted to an I/Imax format and then plotted against time (**Figure 3-1b**) and following). This presentation format allows us to determine more confidently that the release occurs as a burst for the four compounds. Most of the release occurs within 0.5 min of addition of hot water. There is a substantial difference between the compounds regarding the length of the burst. At 0.5 min the relative intensity of diacetyl and m.pyrr is about 10% of Imax, whereas it is about 15% and 20% for m.prop and damas., respectively.

However, the difference between the two groups cannot be explained by either the difference in volatility (m.prop and damas. are the most and least volatile compounds, respectively), molecular mass or structure, and only partially by their hydrophobicity (damas. has highest hydrophobicity and highest persistence at 0.5 min). It is important to note as well that the complete purging of the headspace volume occurs within 1 min of water addition (150 ml of headspace purged at 150 sccm). This indicates that the release is mostly immediate.

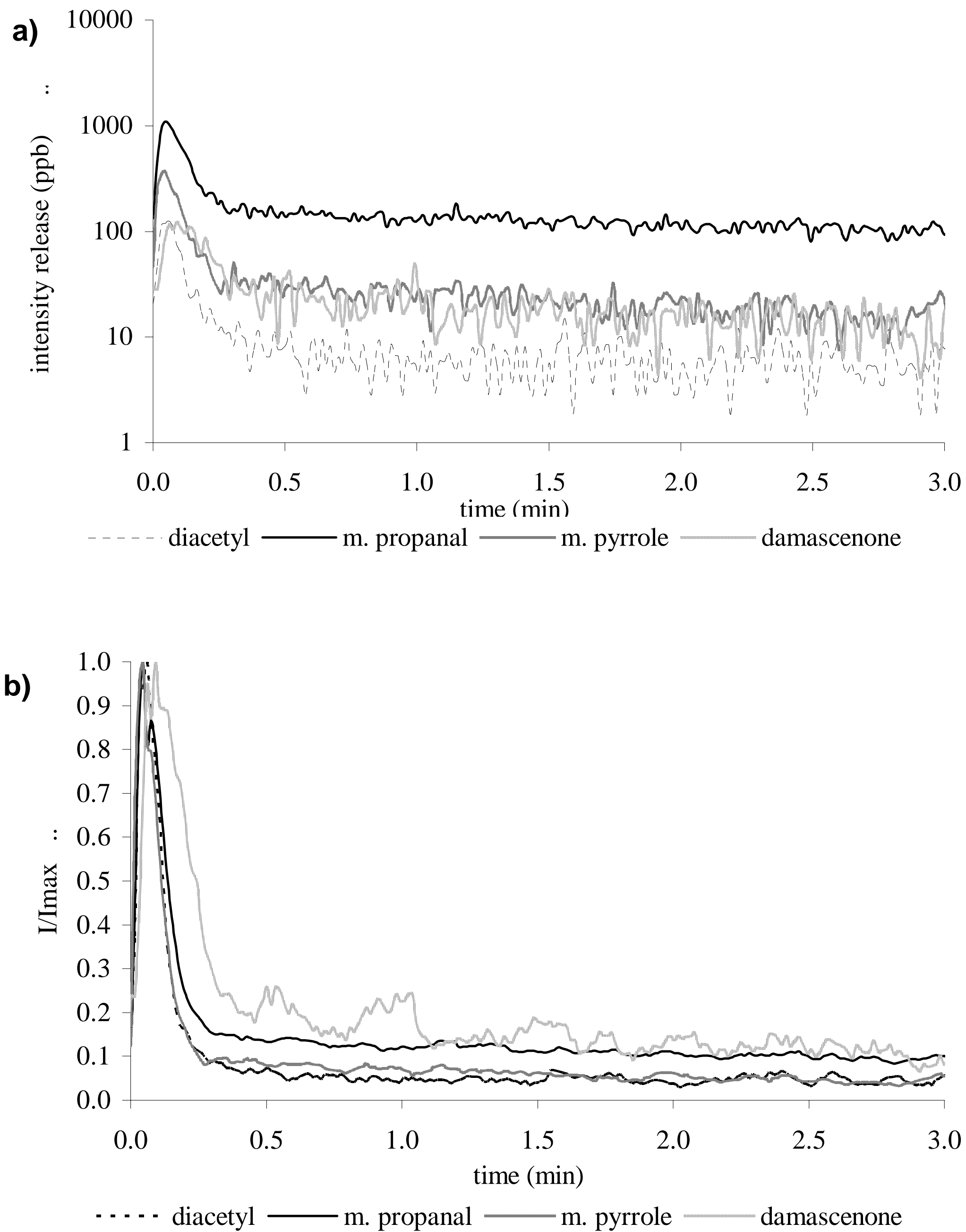


Figure 3-1 : a) Absolute release, and b) relative release (I/Imax) of volatiles from coacervate microcapsules, cross-linked and intermediate wall thickness (made with 80g of oil). Time 0 is moment when hot water was added.

In summary, volatile release from cross-linked, intermediate shell thickness coacervated capsules is similar for the 4 compounds studied regarding the Imax, time to Imax and burst pattern. There are some differences in length of burst but they are small and thus, of questionable significant in influencing flavor perception. It appears that differences in volatile release that one may expect across compounds due to their differing chemical and physical properties are minimized by the use of very hot water.

4.2. Effect of cross-linking

Extensive literature is available on the effects of various cross-linking agents on coacervate capsule structure and water holding capacity (6, 8, 9, 24-27). For our study, non cross-linked (clk) capsules were prepared following the same manufacturing steps as the cross-linked capsules but the cross-linking agent was omitted. Relative release pattern from the non clk capsules is presented in **Figure 3-2.**

One can observe that the release burst from non clk capsules is similar to that from clk capsules **(Figure 3-1b**). Time to Imax for all 4 compounds in non clk capsules does not significantly differ from the clk capsules. The absolute values for Imax were also similar in the two cases. In addition, the burst lasted about the same time (10% - 25% of Imax at 0.5 min for all 4 compounds) as for clk capsules. These observations indicate that for the two types of capsules, the release was immediate and not significantly influenced by the cross-linking of the wall polymers.

In addition, the times to maximum swelling on water addition showed no differences between clk and non clk capsules (3-5 s from addition of water until no more visible increase in size). This indicates that the addition of cross-linking agent did not affect the water uptake kinetics, and that therefore, this mechanism does not limit volatile release. This observation is in agreement with some previous findings, but in contradiction to others. For example, Nixon et al. (28)

reported no slowing of drug release between cross-linked and non-cross-linked, polynuclear microcapsules. They indicated that release could be explained by a model assuming simple diffusion through a thin membrane. Factors such as particle size and surface area in contact with the aqueous environment were key. However, Robert and Buri (7) and Kumbar et al. (29) found that the degree of cross-linking significantly slowed drug release from capsules made by simple coacervation, i.e. using only one polymer (polyacrylamide grafted chitosan). The difference in wall and capsule structure (simple coacervation vs. complex coacervation) as well as potential for cross-linking might be responsible for the differences observed regarding the effect of cross-linking.

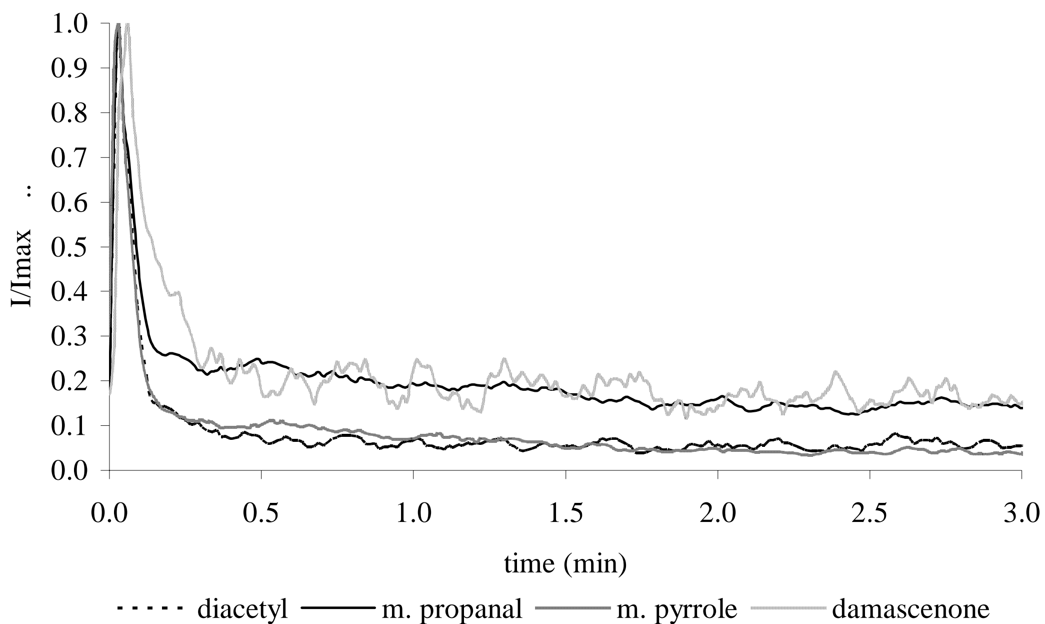


Figure 3-2: Relative release of volatiles from coacervate microcapsules, non cross-linked, and intermediate wall-thickness (made with 80g of oil). Time 0 is moment when hot water was added.

4.3. Effect of wall thickness and volatile load

In this study we assume that release from coacervate microcapsules occurred by diffusion of aroma from the core through the wall, into the aqueous environment. For this reason, we hypothesized that a thicker wall would slow overall release. Capsules with 3 wall thicknesses were produced. The wall thicknesses were 50 µm (± 12), 16 µm (± 4) and 8.5 µm (± 3) for capsules made with 40, 80 and 120 g oil, respectively. Since the particle size distributions were similar for these three capsules, only the overall wall:core ratio was varied. The relative release of aroma from the thickest wall capsules is presented in **Figure 3-3.**

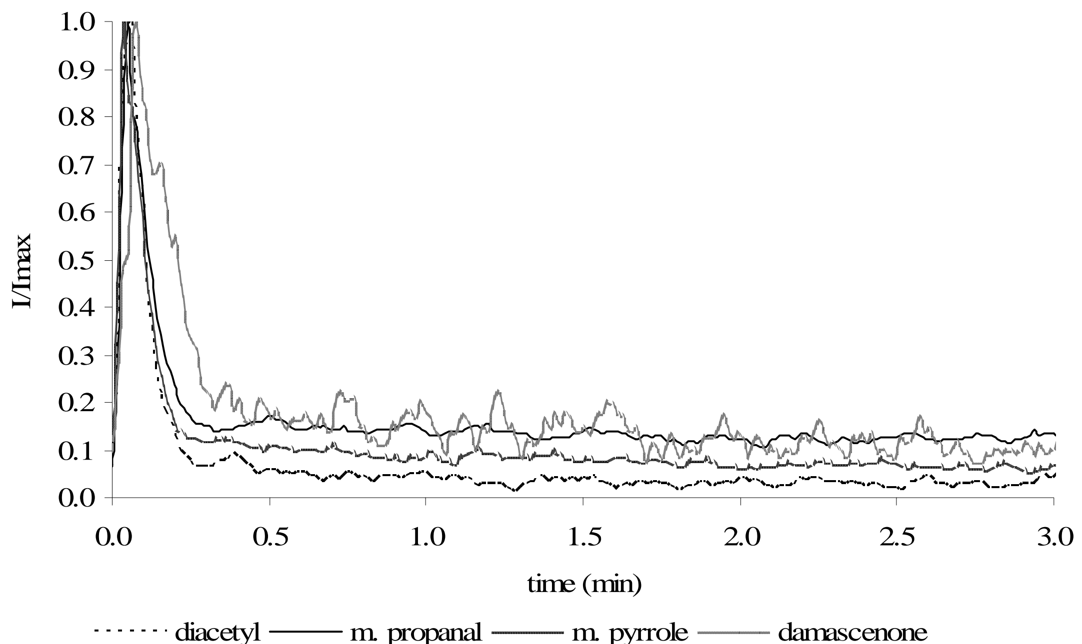


Figure 3-3: Relative release of aroma from coacervate microcapsules, cross-linked, and thickest wall (made with 40g of oil). Time 0 is moment when hot water was added.

Compared to **Figures 3-1b) and 3-2,** no significant differences can be observed for any of the 4 compounds, in terms of burst time and duration, or in terms of

persistence. Relative release from capsules with a thinner wall (made with 120g of oil for 20g of wall material, data not shown) also did not show any significant differences compared to the 2 other thicknesses. Wall thickness and degree of clk are related factors. One can argue that the greater the wall thickness, the greater the cross-linking effect would be. However, the addition of clk agent on various wall-thicknesses did not influence volatile release. In summary, neither clk nor wall thickness was found to significantly alter aroma release from coacervate microcapsules. In addition, no statistical interaction between these two factors could be detected on burst or persistence.

Wall thickness and wall:core ratio are also expected to have different influences irrespective of capsule structure (mono- or poly- nuclear). Our data are consistent with those of Nixon et al., who also found no statistical effect of wall thickness on drug release from mono-nuclear capsules.

Jégat and Taverdet (30) investigated the effect of stirring speed during manufacturing on drug release in water. Stirring speed would lead to differences in capsule structure (polynuclear vs. mononuclear). They reported that release was significantly faster from mono-nuclear compared to poly-nuclear capsules. However, their results were not reported in terms of wall:core ratio, so it is possible that the variation in wall:core ratio and variation in structure might be confounded in their conclusions. Several papers also indicate a slower, controlled release of hydrophobic drugs when encapsulated in poly-nuclear structures (31-33). The difference of release profiles might be due to the small molecular size of the aroma compounds (in our study) as opposed to large non-volatile drugs (in the literature). A recent study by Hasan et al. (34) suggested the use of multilayer emulsions to slow the release of nanoparticles from coacervated microcapsules to reduce the burst effect and obtain a controlled, persistent release. More work in this area should be conducted to evaluate this technique with volatile molecules.

We also prepared capsules with a 10-fold higher load of volatiles. Relative release from 10x load capsules was similar to its equivalent lower load, in terms

of time to Imax and relative persistence. This observation confirms that release was not influenced by volatile concentration but rather simple diffusion, and therefore, the structure of the capsules was not the limiting factor.

4.4. Comparison of release from coacervates and spray dried powder

To evaluate if coacervate capsules have any effect on aroma release in a hot, aqueous environment, volatile release from a spray dried powder (made with modified starch and pure compounds) was also determined. Since spray dried particles are readily water soluble, one might expect a more rapid release than observed for the coacervates particles since the coacervates are not soluble.

The relative release of our model volatiles from spray dried powder is presented in **Figure 3-4.** One notes that the release occurs as a burst with this type of encapsulation as well. The times to Imax are very comparable to those obtained with coacervate capsules, except for the slower release of damas. and perhaps m.prop. A summary of times to maximum intensity is presented in **Figure 3-5**. This figure also includes data collected when only an equivalent amount of flavored oil (MCT) was added to the vessel (no encapsulation), instead of a dry powder. This figure illustrates that there is no statistical difference between the various samples (i.e. between not-clk and spray dried), but that there is a substantial difference between damas. and the other 3 compounds. This reinforces the idea that although volatility and hydrophobicity play a substantial role in the release, they cannot be used as predictors of release pattern.

The difference in release between damas. and the other compounds is also found in the persistence from spray dried powder (**Figure 3-4**): at 0.5 min, about 30% of Imax was still being released from the spray dried powder compared to 15-20% from the coacervate. The persistences for the 3 other compounds are similar across all encapsulation systems. Comparing the relative release from coacervate powder and spray dried powder, it appears that the matrix did not affect the release. In addition, neither particle size (average 350 µm particle size

for coacervates vs. 45 μm for spray dried powder) nor surface area of the capsules was found to have an effect on release.

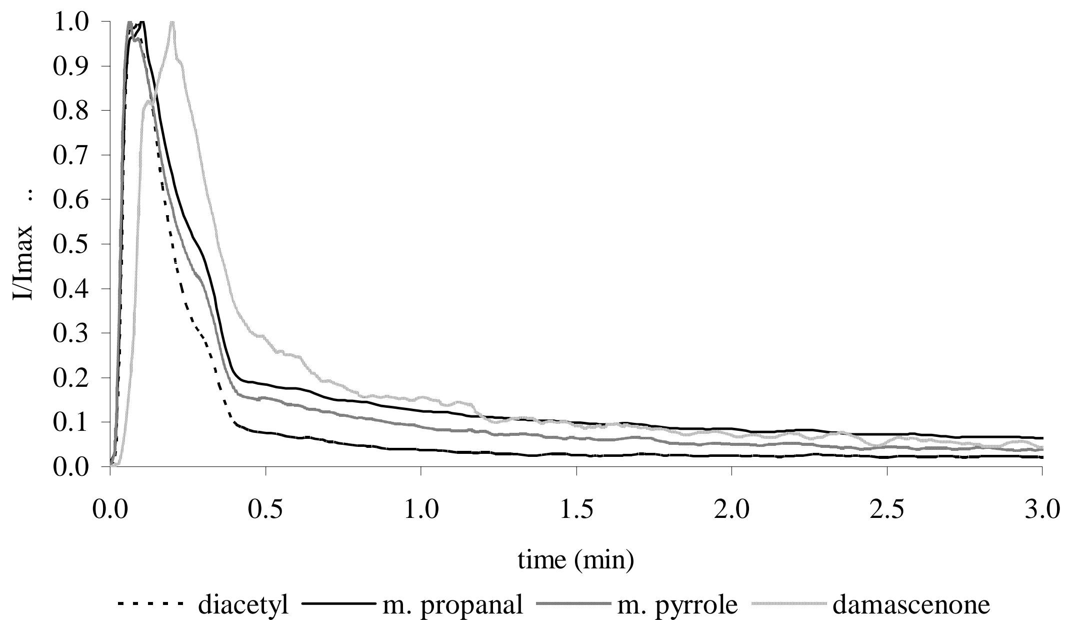


Figure 3-4: Relative release of aroma from spray dried powder. Time 0 is moment when hot water was added.

We also evaluated volatile release "without matrix", i.e. using only flavored oil (MCT) with the same 4 compounds. The release occurred as burst (data not shown), similar to the release from the various systems presented above. The same slight variability between the compounds was also noted, in terms of time to maximum intensity (**Figure 3-5**).

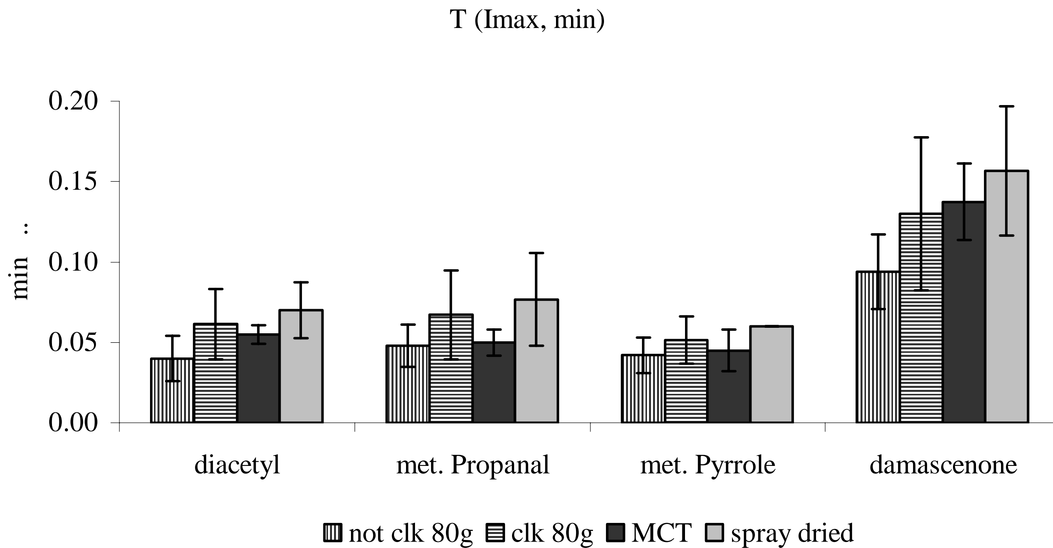


Figure 3-5: Time to Imax for various types of capsules tested: coacervates, intermediate wall thickness (80g oil) not cross-linked (not clk), and cross-linked (clk), flavored oil only (no matrix, MCT) and spray dried powder.

The decay, however, was sharper when only oil was present compared to the encapsulated products. I/Imax reached about 10% -15% at about 0.25 min for the 4 compounds, i.e. half the time compared to relative release from the encapsulated materials. This implies that some additional “reservoir” or controlling system is present when using encapsulated material.

This observation led us to model the overall release system. The amount of a given volatile in the sample headspace is a function of compound partitioning between water (continuous phase) and air, and partitioning between the capsule reservoir and water. Since the water/air partition coefficients are constants (one water/air partition coefficient for each compound at a given temperature), this suggests that the capsule reservoir/water partitioning was similar across the various encapsulants. A mathematical model using a bi-exponential function fit the observed decays very well. The model used was as follows:

$$f = K_1 \times \exp^{(-K_2 \times t)} + K_3 \times \exp^{(-K_4 \times t)}.$$

The parameters extracted (K2 and K4) from these models support the hypothesis that there was no effect of the type of encapsulating matrix on the release, but that there was a difference with the “oil only” system (which followed a single exponential decay model). This suggests that the partition coefficients played a significant role in the observed release. To confirm this hypothesis, all samples were run at ambient temperature (25 ˚C). The underlying reason is that partition coefficients are temperature dependent, and therefore varying the environment temperature should affect the release substantially more than the differences between compounds. Relative releases collected from spray dried powder and coacervates (clk, intermediate wall thickness) are presented in **Figure 6 a) and b),** respectively. In both cases, release of all compounds at ambient temperature (25 ˚C) is significantly different from the release observed at the higher temperature (75 ˚C). In addition, a significant difference exists between the two encapsulation methods, i.e. coacervates and spray dried powder. One can note that release from coacervates still occurs in some type of burst (within 1 min) for all compounds except damas. (no detectable release until after 1.5 min after addition of water). In spray dried powder, the release lasts longer (2 min up to > 3.5 min) but is different for each compound. This suggests that release from an encapsulated complex aroma would not be constant over time, and would potentially lead to sensory imbalances.

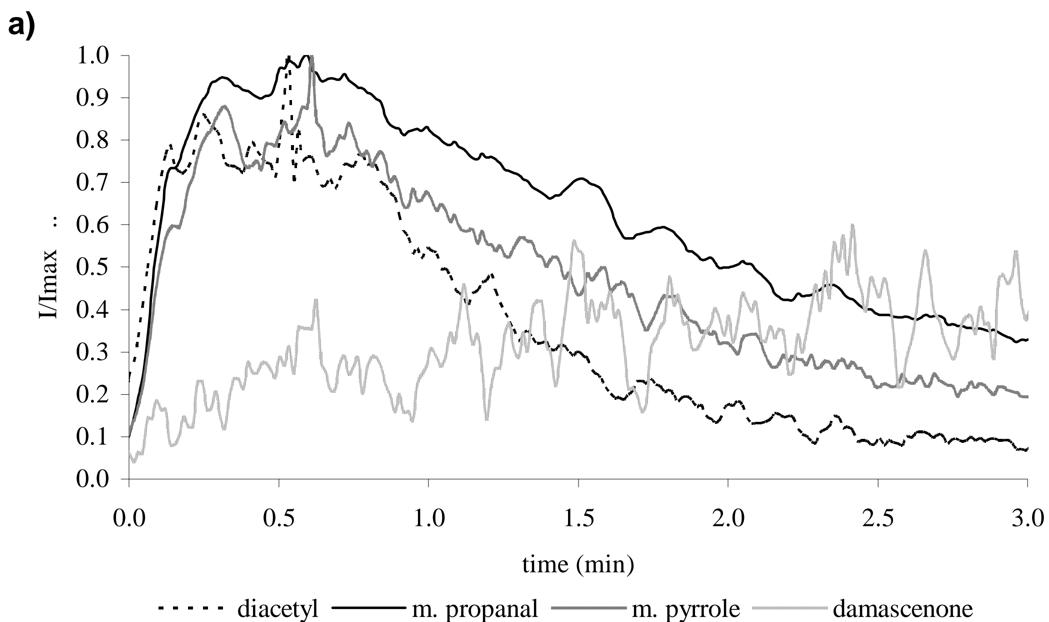
Castelli et al. (35) also found a similar effect of media temperature on drug release from coacervate capsules. However, they were studying the release in biological lipidic membranes, which could explain some of the differences observed in terms of persistence duration.

To conclude, there was no significant difference in volatile release profiles from hot aqueous systems when volatiles were prepared via coacervation vs. spray drying. However, a significant difference in release profiles occurred when release was studied at room temperature. Coacervate capsules offered a uniform

burst release for hydrophilic to slight hydrophobic compounds, whereas spray dried powder presented a long- lasting, non –burst release, but was non uniform across aroma compounds.

5. Conclusions

In summary, this study investigated volatile release from microcapsules prepared by complex coacervation and spray drying. Coacervate wall thickness and chemical cross-linking were manufacturing variables. No effect of cross-linking or wall thickness on volatile release was found. Furthermore, within the study limits, no correlation between the physical/chemical properties of the test volatile compounds and their release was observed. And finally, no significant differences in volatile release were observed between coacervates and spray dried powders under high temperature release conditions. However, testing temperature had a highly significant effect on the overall release most likely by altering the partition coefficients (oil/water and water/air).



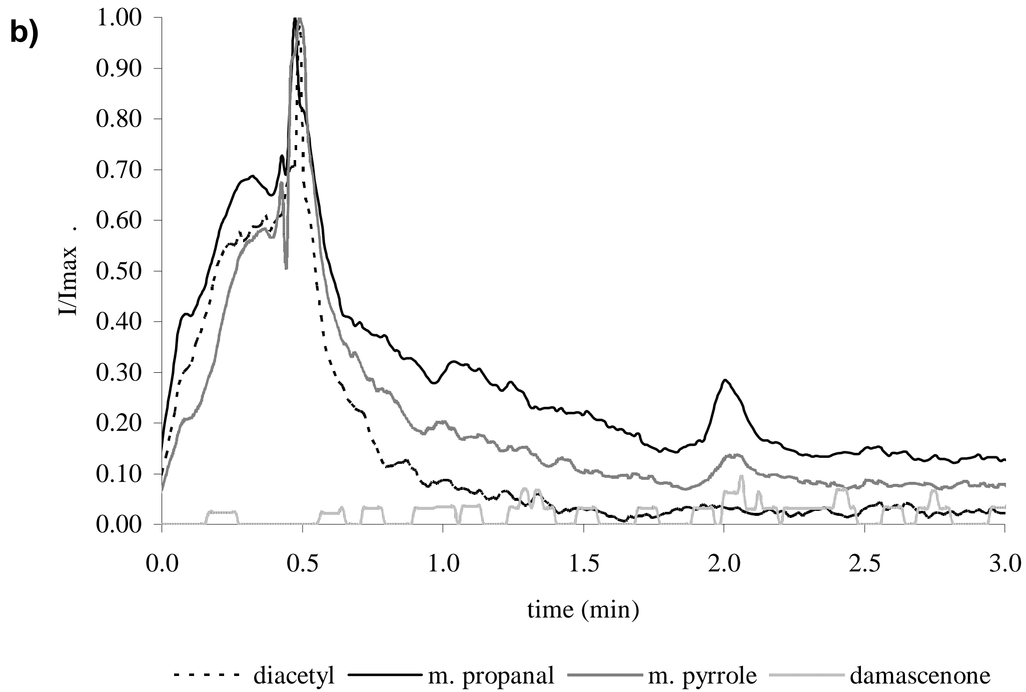


Figure 3-6: Relative release of volatiles at room temperature (air and water) from spray dried powder a) and coacervates b). Time 0 is moment when water (room temperature) was added.

Acknowledgments:

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Chapter 4: Model Studies on the Influence of Matrix Type and Storage Environment on the Stability of a Model Aroma Mixture During Storage

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1. Abstract

The objective of this study was to investigate the effect of oxygen in the storage atmosphere on the degradation of model compounds when present in water or a medium chain triglyceride (MCT) matrix. A model aroma compound mixture was prepared in oil (MCT) or water, and stored under either an ambient air or argon atmosphere containing respectively ca. 20% and <0.5% residual oxygen. Samples were analyzed by SPME - GC/MS to determine the relative stability over time of different classes of aroma compounds. The low oxygen atmosphere appeared to have a significant protective effect on sulfur compounds, aldehydes and ketones in oils but a detrimental influence on pyrroles. Data showed little influence of the atmosphere for these compounds in water. In addition, the type of matrix had a significant effect ($P < 0.05$) on the stability of aldehydic, ester and pyrrole compounds. These compounds were more stable in MCT than in water.

2. Introduction

Flavor deterioration in a food during storage has been extensively investigated in sensory studies, focusing mainly on the appearance of undesirable flavors. These off-flavors can result from various mechanisms involving: lipid or terpene oxidation (e.g. in snack foods (1) or citrus beverages (2), respectively), non-enzymatic browning (e.g. in fruit juices (3)), enzymatic reactions (e.g. bitter notes in dairy products (2, 4)) or light induced reactions (e.g. beer staling (5)). It is, however, most probable that the staling of a food is not only due to the formation of off-flavors but also to the disappearance of desirable flavor. Nevertheless, there is limited literature on the degradation of desirable aroma compounds during storage. Williams et al. (6) found significant losses of several key components of roasted peanuts over time resulting in an increase in negative sensory attributes. Their study showed that staling can be due to combined effects of losses of desirable flavors and the appearance of undesirable ones.

The characteristics of the food matrix, such as presence of proteins or quantity of lipids in specific solid matrices, have been shown to influence the stability of flavor compounds during storage (7, 8). However, there is little literature published at this time investigating the effect of storage conditions on the stability of flavorings in a liquid matrix other than the degradation of lemon flavor components in low pH beverages (3).

The primary objective of the present study was to investigate the influence of the liquid carrier (oil vs. water) on the stability of aroma compounds over time. One would anticipate that different degradation mechanisms would occur in a water solvent vs. a lipid. A secondary objective was to determine the effect of oxygen level on aroma stability. Many foods are packaged under reduced oxygen to limit oxidative reactions leading to off-flavors. However, one must also recognize that degradation reactions leading to the loss of desirable flavor components may also involve oxidative steps and be influenced by the food environment, i.e. oxygen level. This study reports on the role of oxygen level on the stability of

several classes of potentially desirable aroma compounds during storage in a water and oil medium.

3. Materials and Methods

3.1. Aroma compounds

A selection of nine aroma compounds was studied (**Table 4-1**). These compounds were selected as representing different chemical classes of compounds. The selection of aroma compounds included a thiol, an aldehyde, a diketone, a phenol, a pyrrole, an ester, and a pyrazine. Chemicals used were purchased at the highest purity from Sigma-Aldrich Chemicals. Since one objective of this work was to study the influence of matrix type on our model compounds, the compounds chosen covered a wide range of water solubility, expressed as the log of the octanol-water partition coefficient (log P). The log P values (9) for each compound are presented in **Table 4-1**. The concentrations of these compounds was chosen to be close to those found in processed food systems and are shown in **Table 4-1**. Solutions of model compounds were prepared by adding calculated volumes of each aroma compound to the desired matrix volume while being stirred. Five ml of this stock solution was then pipetted into 20ml headspace vials for storage. Four vials of each sample for each sampling period were prepared three of which were analyzed. The fourth sample was held in reserve for other analysis if needed.

3.2. Solutions of the model aroma compounds

Two solvents were studied: water (distilled water, pH ~7, abbreviated W) and medium chain triglycerides oil (MCT, Delios V from Cognis / Grünau; fully saturated, shelf-stable oil.).

Table 4-1: Model system composition (MCT or water matrix) being stored and ions used for SIM monitoring of each compound.

Aroma Compound	Abbrev. Used In Figures	Log P	Concentration (parts per million, v/v)	Ions Monitored (MS, SIM mode)	
Butanedione	DIAC	-1.34	1,538	86	43
Acetaldehyde	ACET	-0.17	3,846	44	29
2-Ethylpyrazine	ETHPYR	0.98	154	107	80
2-Methylbutanal	2-METB	1.23	769	57	41
Ethanethiol	ETSH	1.27	231	62	47
Furfuryl acetate	FURFACET	1.45	769	140	98
Dimethyldisulfide	DMDS	1.87	154	94	79
4-Vinylguaiacol	VINYLGUA	2.24	2,000	150	135
Furfuryl pyrrole	FURFPYR	2.5	385	147	81

Log P values obtained from (10).

3.3. Sample packaging for storage and analysis

Vials containing sample to be stored in ambient air were immediately closed with septa previously baked to avoid any odor contamination, and then Gerstel Autosampler caps. Vials containing sample to be stored in a low oxygen environment were taken quickly to an anaerobic glove box for gas flushing and similar closure. The glove box chamber had been flushed twice with pure Argon and a third time with a mix of Argon and Hydrogen (90 : 10, respectively). The presence of a catalyst system (alumina coated palladium chloride, Stak-Pak, Coy Laboratory Products, Grass Lake, MI) insured a low oxygen level in the chamber by reacting any residual oxygen with hydrogen to form water, which was absorbed by Drierite. The O_2 level in the glove box was monitored by gas chromatography equipped with a thermal conductivity detector (GC-TCD) during sample preparation and did not exceed 0.5 %.

Previous research has shown that while the sample vial closures (septa) are very impermeable to organic volatiles, they are quite permeable to oxygen. Therefore, a second oxygen barrier was used by packaging the sealed sample vials in metallized polyester foil pouches (3 side seal Malipak, 16.5 cm x 20.32 cm OD, Karpak, Minneapolis MN, USA). The vial loaded foil pouches were vacuum treated and then argon flushed before final sealing. The sample environment ultimately contained < 0.5 % oxygen, the remainder being small amounts of nitrogen and primarily argon.

3.4. Storage of vials

Samples in air (no pouches) and low oxygen environments (aluminum sealed pouches) were stored standing in an incubator at 30 ˚C. Reference samples to include in analysis to monitor the stability of the analytical system were frozen immediately. Sampling times were 0, 1, 2, 4, 8 and 12 weeks of storage. At sampling time, samples were transferred to a -46 ˚C freezer until analysis. Vials were carefully frozen in standing positions in order to avoid contamination of the septum with the liquid inside.

3.5. Method for gas analysis

Gas analysis of the anaerobic chamber, pouches and vials was performed using a Hewlett Packard gas chromatograph (HP- 5890) equipped with thermal conductivity detector (TCD) and an HP-Molesieve column 30 m x 0.53 mm x 50 µm (J&W Scientific, Folsom, CA, USA). The operating parameters were: injection port 150 ˚C, isothermal oven at 40 ˚C, detector 175 ˚C, column head pressure 5 psi, and column flow 5 $ml.min^{-1}$. Ten µl samples were taken with a gas tight syringe (Hamilton, Switzerland) from either pouches or vial headspace for analysis.

3.6. Analytical method for volatile analysis

3.6.1. Extraction method

Automated Solid Phase Micro Extraction (SPME) was used to isolate volatile compounds from the samples (Gerstell Combipal MPS 2). A 75 μm PDMS/CBX/DVB fiber was used (Supelco, Belefonte, PA, USA). The extraction parameters were as follows: 60 min equilibration of the sample at 55 ˚C, 10 min SPME sampling at 55 ˚C, 5 min desorption in a gas chromatograph (GC) inlet at 225 ˚C. The same procedure was used through the whole experiment.

3.6.2. Separation and identification

An Agilent gas chromatograph (HP- 6890) equipped with a 30 m x 0.25 mm x 0.5 μm DB-Wax column (J&W Scientific, Folsom, CA, USA) was used in analysis. The operating parameters were as follows: constant column flow control at 1 ml min^{-1}; helium as carrier gas, column head pressure 6.86 psi; split-less mode 5 ml min^{-1} for 3 min; oven program 42 ˚C/5 min/6 ˚C min-1 /135 ˚C/20 ˚C min^{-1}/190 ˚C/8 min. A mass spectrometer (Hewlett Packard-5972 Mass Selective Detector) was used coupled with Hewlett Packard ChemStation software. The parameters were set with 0.5 min solvent delay and 1.84 scan. sec^{-1}.

3.6.3. Quantification

Quantification of compounds during storage was done by MS in SIM mode. Two abundant but yet unique ions for each compound were chosen. Their respective peak areas at the GC elution time corresponding to that of the pure reference compound were summed. Ions monitored and used for integration are presented in **Table 4-1**. The quantitative data reported are peak areas relative to those of the corresponding compound at week 0. Samples were analyzed randomly in blocks corresponding to weeks of storage and compared to reference samples (stored frozen until analysis - week 0) analyzed within the block. Triplicate samples were analyzed.

3.7. Data analysis

Data presented represent the average peak areas obtained at each sampling period. Data are generally expressed in terms of percentage remaining based on the peak area at time 0. In a second data treatment, a regression function was calculated. In this treatment the data were linearized using logarithmic values. Results were then presented as log (% remaining) vs. time. For comparison purposes, linear regression parameters (rate of loss) are presented in bar graphs for a given matrix or a given compound.

In addition, Analyses of Variances (ANOVA) were conducted with the R.2.0.1 package on the rate of losses obtained for each system. We studied the influence of the type of matrix, atmosphere, and compound as sources of variability.

4. Results and Discussion

4.1. Efficiency of gas flushing

All pouches were tested for oxygen content and at least two vials in each pouch were also tested. If one vial had a high oxygen level (> 0.5%), a third vial was analyzed. Due to the low level of oxygen (< 0.5%) in the samples and some tailing of the argon peak, the oxygen peak could not be directly measured. The oxygen content of a sample was, therefore, calculated based on the quantity of nitrogen detected: assuming the amount of oxygen was approximately equivalent to a quarter of the amount of nitrogen. Consequently, these calculated values are maximum possible amounts since the oxygen in the anaerobic hood should have been consumed by the catalyst system and thus, not be 25% of the nitrogen level but less. A sampling of the results is shown in Table 2.

As presented in Table 2, maximum calculated oxygen levels (a quarter of the highest Nitrogen value) were consistently below 0.5% oxygen. The results were consistent between pouches and vials of a given week, and throughout storage.

4.2. Initial headspace levels of model volatiles

Since all of the loss data to be reported and discussed later have been normalized to percentage remaining over time, it is useful to present an overview of the GC profile of the model compounds at time 0. As one would expect, the peak areas varied greatly with model compound and matrix. This is partly because the model had varying amounts of individual volatiles (**Table 4-1**). However, peak areas also reflect the solubility and volatility of each compound in each matrix (water or oil), the extraction efficiency of the SPME fiber for each compound, and the competitive binding of aroma compounds for the SPME fiber (matrix effect on SPME recovery). It is well known that fibers will preferentially bind certain volatiles at the expense of others (11, 12).

The effect of the system matrix on peak area is related to the log P of each volatile. Compounds such as 4-vinylguaiacol (log P= 2.24) will show much lower peak areas in an oil vs. a water system (**Figure 4-1**). Compounds that have higher Log Ps have less solubility in water and thus, are forced into the headspace thereby giving higher headspace responses in the water matrix, and the converse in the oil system. Compounds with Log Ps close to 0 would be expected to show similar peak areas across matrices (assuming they have equal affinities for the SPME fiber and volatility) (**Figure 4-1**). Compounds with such Log Ps have approximately equal solubility in water and oil systems. However, this is not observed for acetaldehyde (log P = -0.17). This reflects that, as noted earlier, the peak area response is not influence solely by the solubility of each compound but also influenced by the SPME fiber affinity for each volatile as well as the total volatile load and composition on the fiber, as documented by Nongonierma et al. (10) and Roberts et al. (11). The use of a single chemical

property cannot predict the initial peak area values obtained by static headspace SPME extraction.

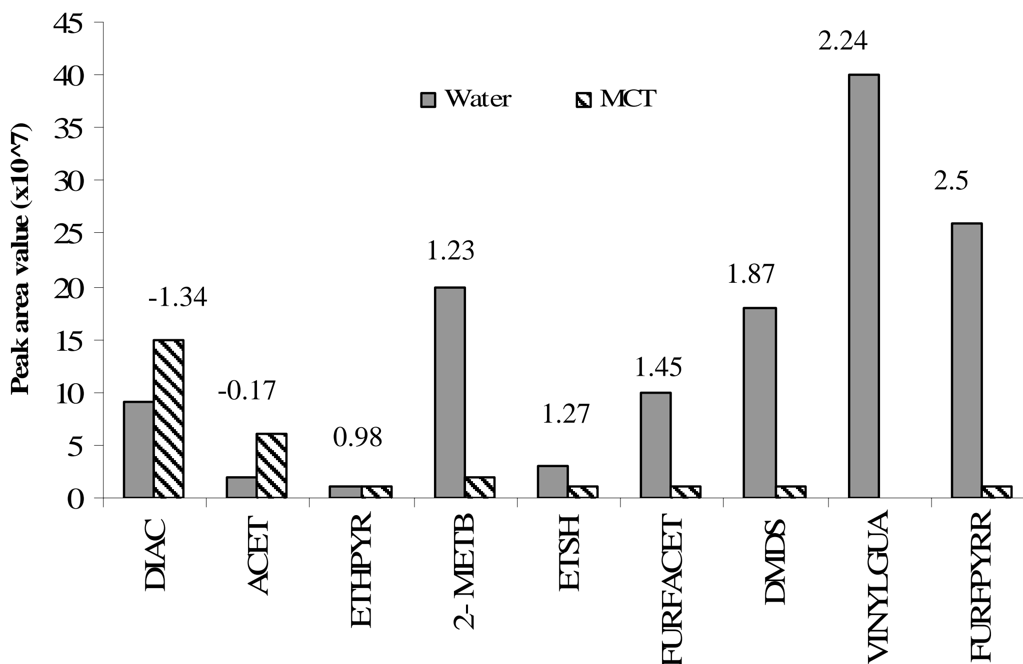

Figure 4-1: Peak areas for model compounds in water and MCT (air environment) at initial time. (Log P values (10) are inserted above compound)

4.3. Stability of model compounds during storage

The effects of two parameters (type of matrix and presence or absence of oxygen) were studied in this work. The effects of each of these parameters on volatile stability are presented and discussed below.

4.3.1. Water vs. oil systems (modeled by MCT)

The first comparison of volatile stability is in an oil system (MCT) vs. a water system. Since it is impossible due to space limitations to present plots of percent loss vs. time for all of the compounds included in this study under all storage conditions, only selected data are plotted to illustrate a range in behaviors. Loss

rates for all compounds across all storage conditions are presented in later figures. In **Figure 4-2** one can see that Furfuryl acetate is much more stable during storage in MCT than in water. Furfuryl acetate concentration in the water matrix dropped below the detection limits in only two weeks while ca. 82 % remained after 12 weeks storage in MCT (**Figure 4-2**). While 2-methylbutanal is also more stable in MCT than water, the difference between matrices is much less pronounced.

The rate of losses for each compound obtained from the linearized (log-transformed) percentage data for samples stored in MCT and water matrices (and air) is presented in **Figure 4-3**. Overall, the rate of volatile loss was less in water than in MCT (stored in air), the extent of volatile loss depending on the individual compounds (although losses in the water system were in some cases low). For the water matrix stability decreased in the order of acetaldehyde < 2,3–butanedione ≈ 2-ethylpyrazine ≈ 2-methylbutanal < ethanethiol ≈ dimethyldisulfide < 4-vinylguaiacol < furfuryl pyrrole < furfuryl acetate. Those results are in good agreement with the results obtained by Chen et al. (7) when studying flavor stability in methylcellulose and fat systems. In their study, sulfur compounds degraded to a large extent in the absence of oil and to a lesser extent when the matrix contained 5% oil. In addition, the authors also found that the effect of matrix on storage stability was significantly compound dependent.

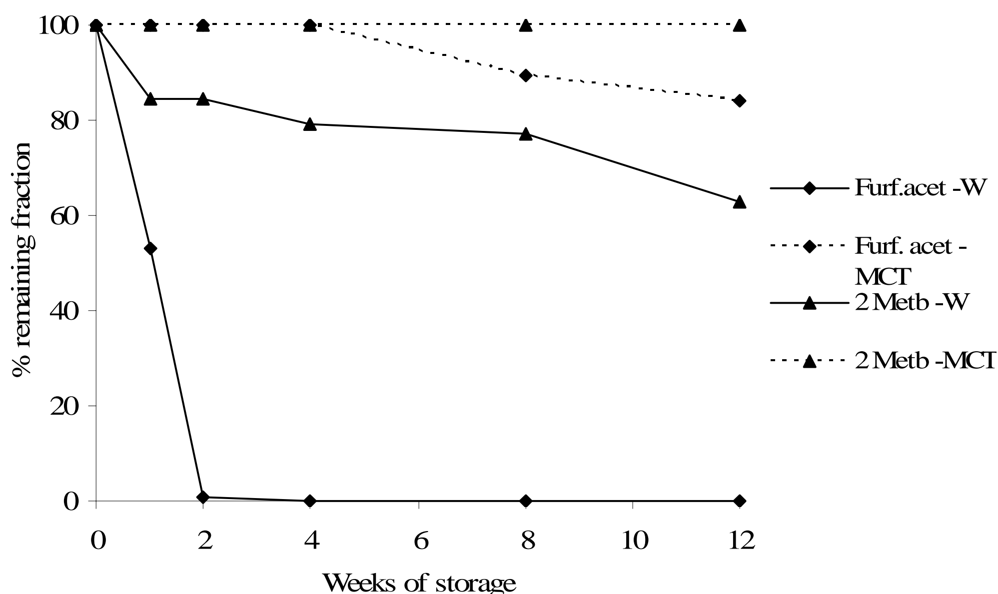

Figure 4-2: Relative amount of furfuryl acetate and 2-methylbutanal upon storage in water (=W) and MCT (=Medium Chain Triglycerides) in air atmosphere.

While a detailed investigation of the degradation mechanisms for the individual compounds was not the subject of the current study, the differences in compound stability point to very different pathways and parameters that govern their stability. Water as a aroma carrier may be a reactant in aroma degradation itself, like in the hydrolysis of esters (furfuryl acetate) or act as catalyst in protonic reactions (e.g. condensation of pyrroles with aldehydes). Oil as aroma carrier, particularly a shelf-stable fully saturated triglyceride like MCT, is not directly involved in such degradation reactions. (12)

As noted above, the model aroma compounds were quite stable in the MCT matrix. Acetaldehyde, 2-methylbutanal and furfuryl pyrrole did not degrade to any measurable amount over the entire storage period. The rate of loss increased in the following order: diacetyl ≈ dimethyldisulfide < furfuryl acetate < ethylpyrazine ≈ ethanethiol < vinyl guaiacol.

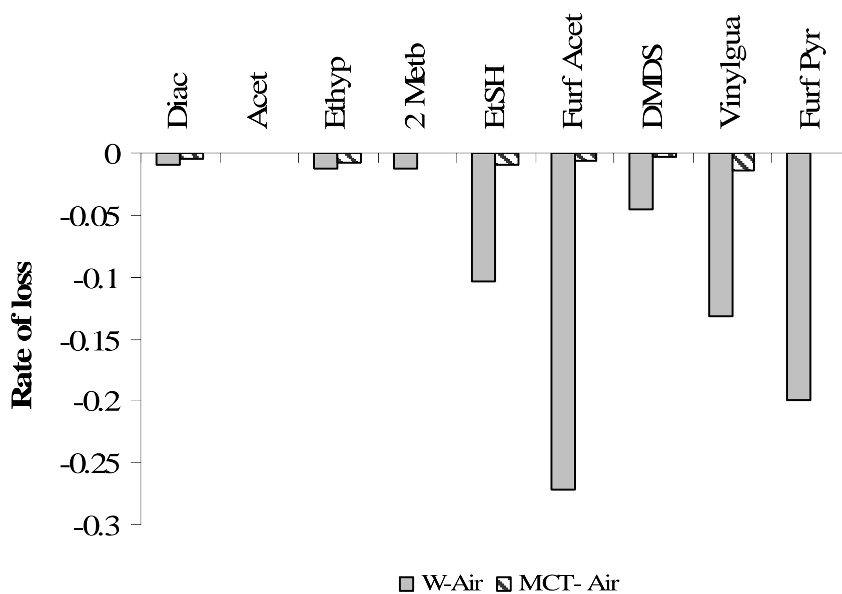

Figure 4-3: Rates of loss after 12 weeks of storage of individual model volatiles stored in water (W) and MCT in ambient oxygen level environment.
Y-axis units are rates of loss in log (%)/ week, calculated from linearization of log (% remaining fraction).

4.3.2. Influence of the presence of oxygen

The influence of oxygen on the losses of selected compounds (ethanethiol, furfuryl pyrrole, and acetaldehyde) during storage is presented in **Figure 4-4**.
These compounds where chosen to illustrate their very different behaviors in the two atmospheres. Samples labeled “air” have been stored in an air environment while those labeled as being stored in “argon” have been flushed with argon and have less than 0.5% residual oxygen (**Table 4-2**).

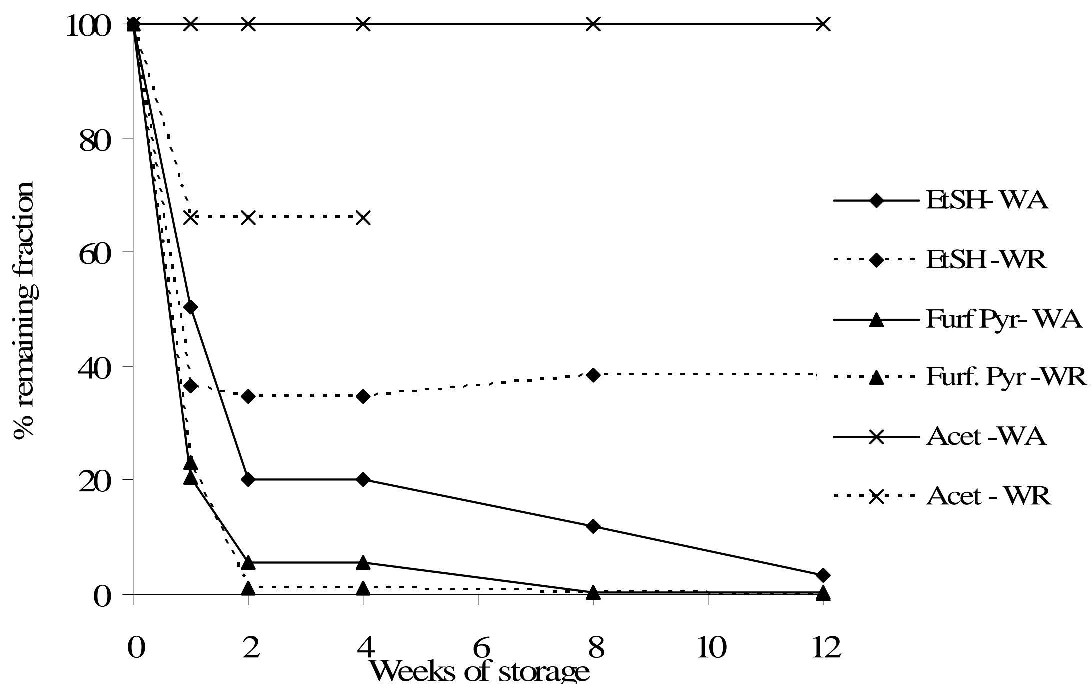

Figure 4-4: Influence of storage atmosphere on the percent of Ethanethiol (ETSH), Furfuryl pyrrole (FURFPYR) and Acetaldehyde (ACET) remaining in sample headspace during storage (W=water; R= argon; A= air).

Table 4-2: Average Level of oxygen determined in pouches and vials.

Sample	% Argon	% Nitrogen	Maximum Calculated O2 %
Pouch	98.3 ± 0.7	1.3 ± 0.4	0.41
Vial	98.2 ± 0.8	1.5 ± 0.4	0.46

Values presented are averages of 20 pouches and 40 vials. (Average ± standard deviation)

The absence of oxygen resulted in a minor reduction in the loss of ethanethiol (60% lost in reduced oxygen environment after 2 weeks compared to 80% lost in ambient air after 2 weeks). It is clear that oxygen reduction alone is not sufficient to prevent the degradation of sulfur compounds (in our model systems). Furfuryl

pyrrole was lost very quickly in the water matrix (> 90% within 2 weeks), and the change of atmosphere showed no observable difference on the rate of loss. A similar pattern was observed for furfuryl acetate. To the contrary, acetaldehyde was lost much more quickly from the sample headspace when stored in a low oxygen environment (30% lost after 1 week in low oxygen environment vs. no loss in ambient air environment). While this may not seem rational, there may be reasons for this result since it is likely that different reactions take place in the presence or absence of oxygen. Storage under a low oxygen environment may produce degradation products from other volatiles that react with the acetaldehyde resulting in its loss. These three aroma compounds illustrate the diversity of influences of the presence of oxygen on the storage stability of flavor compounds.

A more global view of the influence of oxygen level on volatile stability during storage is presented in **Figure 4-5**. In this figure, the rates of loss are presented for all compounds in both the water and MCT matrices. This data overview supports the conclusion that volatiles are typically less stable in an ambient oxygen level environment than a low oxygen environment, regardless of the matrix system they are diluted in. If a reduction of loss is observed for a compound in water + low oxygen environment compared to water + ambient air, it is mostly the case as well when diluted in oil, and vice versa.

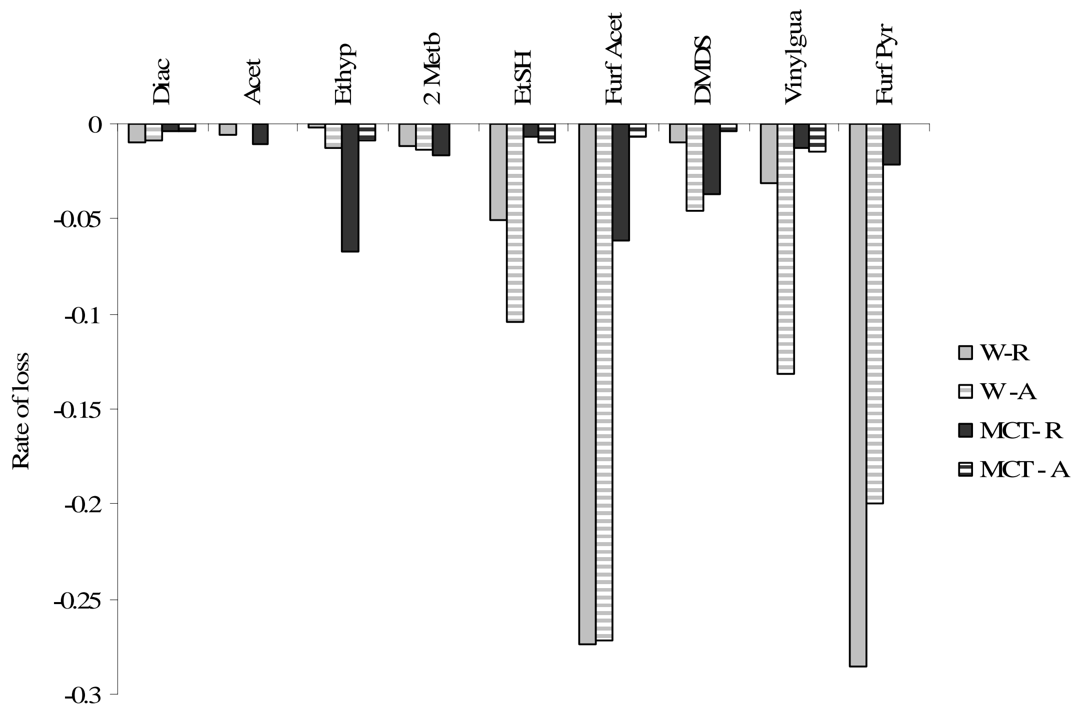

Figure 4-5: Rates of loss of individual model volatiles during storage (W=water; MCT=Medium Chain Triglycerides; R= argon; A= air)
Y-axis units are rates of loss in log (%)/ week, calculated from linearization of log (% remaining fraction).

An additional observation is that some volatiles are equally stable in both environments: their degradation is unaffected by the presence or absence of oxygen as is the case for furfuryl acetate which is likely lost via hydrolytic reactions in the water system. However, the anticipated corresponding end product of this hydrolysis, furfuryl alcohol, was not detected in the stored systems. No degradation mechanisms could be proposed for the water or the oil system based on our data since no degradation products were detected by GC-MS in full scan mode.

4.4. Statistical analysis

A statistical 2-way ANOVA was conducted. The results showed that the simple factors have an influence on the rate of compound loss at a 5% significance level. A 2-way interaction was not found to have a significant effect on the rate of degradation according to this analysis. Additional ANOVAs were conducted on the data set to focus on individual compounds and on individual factors to detail their relative influence. Results are grouped in **Tables 3 and 4**.

Statistical analysis confirms the trends observed in the study and presented in the previous graphs. They show in addition that the effects of each of the studied parameters differ in importance for given compounds, reinforcing the idea that aroma compounds vary in stability depending upon the matrix they are dispersed in.

Table 4-3: Results of ANOVAs conducted for each of the individual compounds. '**' represents a significant effect of the source of variation at a 1% level, '*' at a 5% level and '{blank}' represents no significant influence of the source of variation at these levels.

Compound name	Type of Matrix	Type of Atmosphere
Acetaldehyde	*	*
Ethanethiol		*
2-methylbutanal	*	
Diacetyl		*
Dimethyldisulfide		
Ethylpyrazine		
Furfuryl acetate	**	
Furfuryl pyrrole	**	
4-vinylguaiacol		*

Table 4-4: Results of ANOVAs conducted for each of the type of oil. '**' represents a significant effect of the source of variation at a 1% level, '*' at a 5% level and '{blank}' represents no significant influence of the source of variation at these levels.

Type of matrix	Type of Compound	Type of Atmosphere
Water	**	*
MCT		

Modified atmosphere packaging is typically done using N2 with or without CO2. Product environment (headspace gas) has been used to extend shelf-life from a microbiological point of view, as it slows down the growth of spoilage organisms (e.g. meat products). It is also frequently used to limit lipid oxidation in numerous food products (13) thereby reducing the formation of off-flavors, e.g. in citrus beverages, meat products, snack foods, etc. However, most of this past work has focused mainly on the appearance of defects such as color or off-flavors during storage rather than the stability of the desirable aroma components (14-16). Our study suggests that storage under a normal oxygen environment may be detrimental to the flavor of a food due to the enhanced loss of some desirable flavor notes and even reduced oxygen environment may only retard the degradation of some of these notes or may have no impact at all (e.g. esters, pyrroles). Therefore, in order to design an effective flavor protection approach for a food product, the desirable key aroma compounds that need to be preserved as well as their predominant degradation pathways need to be known. Strategies to further protect some of the aroma compounds from degradation require more insight into the mechanisms as well as research on further stabilization. The effect of antioxidants on aroma stability needs to be investigated as it has been observed that a low oxygen atmosphere generally provided more stability. It is our opinion that the protection of desirable, characterizing aroma compounds could be as important in extending shelf- life as the inhibition of off-flavors.

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Chapter 5: Effect of Type of Oil and Addition of Delta-Tocopherol on Model Flavor Compound Stability during Storage

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1. Abstract

The objective of this study was to investigate approaches to protect selected flavor compounds from deterioration when stored in an oil matrix. An aroma compound model mixture was prepared in a medium chain triglyceride (MCT) or sunflower oil (SfO) matrix, and stored under either an ambient air or argon atmosphere containing respectively ca. 20% and <0.5% residual oxygen as controls, or containing a natural antioxidant, δ- tocopherol (0.01%). Samples were analyzed by static headspace - GC/FID to determine the stability over time of the compounds in mixture. We found that the type of oil had the greatest effect ($P< 0.01$) on overall compound stability. A low oxygen atmosphere also had a significant ($P< 0.05$) protective effect on the aroma compounds in both oils. The addition of δ-tocopherol generally offered little additional protection. No significant relationship could be determined between the oxidation of the lipid matrix and the loss of oxidation sensitive thiol compounds.

2. Introduction

One of the major causes of quality deterioration in food products during storage is oxidation. The effects of oxygen and oxidation have been intensively studied for their influence on quality of meat products (1, 2), fats and oils (3, 4) and citrus beverages (5). Oxidation of a food product most commonly implies the appearance of off-flavors. However, it is most probable that the loss of flavor quality is caused not only by the formation of undesirable sensory notes but also by the disappearance of desirable ones.

The loss of desirable flavorings due to oxidation has been extensively studied in citrus oils, particularly for limonene, mostly in dry flavorings (5-8). In the area of wines several investigations have provided evidence of the formation of phenolic polymers as a result of oxidation. The polymeric phenolics alter mouthfeel and the flavor profile of wine (9). These studies document that oxidation of desirable flavor components produces undesirable changes in flavor profile. Recently we have shown (10) that the presence of oxygen can be detrimental to the stability of flavor compounds also in various oil matrixes. A faster degradation was found for of ethanethiol, diacetyl and acetaldehyde in an air vs. a low oxygen environment both in water and oil model systems. These results are concordant with the findings of Williams *et al.* (11) which showed the increased loss of desirable flavors over time for peanut flavor when stored in an oxygen-containing environment.

In order to slow the negative effects of oxygen on food quality, antioxidants can be employed. A variety of food antioxidants have been studied and classified according to their action mechanism or their origin (12, 13). The main category belongs to “chain-breaking compounds” or primary antioxidants. In lipid oxidation reactions they end the free-radical chain reaction by donating either an electron or a hydrogen radical to the fatty acid free radicals. A diversity of antioxidants belong to this group, including naturally occurring compounds such as tocopherols, flavonoids and vanillin, and synthetic antioxidants such as BHA, BHT, and TBHQ. These antioxidants are extensively used in the food industry

(12, 14). Vitamin E (tocopherols) can function as an oxidation inhibitor either by donating their hydrogen from hydroxyl group to the radical or by scavenging singlet oxygen molecules (14). Various tocopherol isomers have been used in the food industry to stabilize essential oils in encapsulated materials (15) and in beverages (16, 17). Alpha tocopherol is used typically as supplementation in vitamin E for food products and as antioxidant, whereas the other isomers (β-, γ-, δ- tocopherol) are used exclusively as antioxidants. Abundant literature is available on the specific activity of tocopherol isomers in vegetable or animal oils, in bulk oil or in emulsion (18, 19). Delta- tocopherol is considered having a high antioxidant activity in bulk oil, and showing a prooxidant activity only at very high concentration (20, 21), therefore easy to incorporate into food products (19).

The ability of antioxidants to slow lipid oxidation has been well documented. Despite a lack of literature demonstrating that flavor compounds are protected by antioxidants, we assumed that antioxidants could also slow their oxidation. Thus, the first objective of the work presented here was to investigate the use of a natural antioxidant (δ-tocopherol) to better preserve flavor compounds in oil systems. The second objective was to determine if the type of oil matrix in which the aroma compounds were diluted influenced aroma compound stability. Lastly, it was of interest to investigate if the oxidation of the compounds was correlated to the oxidation of the lipid matrix they were present in.

3. Material and Methods

3.1. Aroma compounds

A mixture of 10 flavor compounds was prepared using acetaldehyde, dimethyl sulfide, propane thiol, butane -2thiol, diacetyl, N-methypyrrole, 2-ethyl pyrazine, furfuryl mercaptan, 3- mercapto 3-methylethyl formate and furfuryl acetate. The following will focus on data collected for 4 of these compounds, representative of the variety of degradation pattern observed for the different compounds: diacetyl,

dimethyl sulfide, N-methyl pyrrole and furfuryl mercaptan. All chemicals were purchased from Aldrich Chemicals at the highest purity available and used at equal molar concentration (7.5 x 10-3 mol/L). The 4 compounds were selected as representing several different chemical classes and differing in loss mechanism. Furfuryl mercaptan has been shown to be very reactive, degrading rapidly in an oxidative environment such as Fenton-type conditions (22). Dimethyl sulfide has also been reported to degrade due to oxidative reactions (10). Diacetyl on the other hand was equally stable in these two systems. Lastly, N-methyl pyrrole was found to polymerize under mild or strong oxidative conditions and can be used as biopolymer (23, 24).

3.2. Oils used for dispersion of the model aroma compounds

In our primary study two oils were selected as flavor solvents: medium chain triglycerides (MCT, Delios V, Cognis / Grünau; fully saturated, initial peroxide value = 0.5 meq/ kg) and sunflower oil (SfO; high oleic content, Nutriswiss, Morges, initial peroxide value = 0.5 m_{eq}/ kg). A third oil, soybean oil (Crisco® Pure Vegetable Oil), was used in a secondary short study to determine if the oxidation state of the oil was an important factor influencing the stability of our model flavor compounds during storage.

3.3. Preparation of oxidized oil

To investigate the effects of lipid oxidation on our model flavor compounds, our volatiles were diluted individually in both fresh and oxidized soybean oil (polyunsaturated, SbO) and then stored. The “fresh” oil was purchased from a local grocery store. The oxidized oil was prepared from the fresh oil by placing 150 ml of the oil in a 200 ml flask and heating it in a water bath (75 ˚C) for 24 hr while bubbling compressed air through the oil at a flow rate of 50 ml. min^{-1}. Samples were stored only under ambient O_2 levels. Oxidation state of the oils was monitored via peroxide value (PV, AOCS method, as cited by (25)). In this analysis, five ml of stored sample was analyzed each time. Each storage time

was analyzed in duplicate and results averaged and presented in m_{eq}/ kg. PV was determined for all oils.

3.4. Antioxidants used

A natural antioxidant, δ-tocopherol (Aldrich Chemical, DTOC), was added to aliquots of both MCT and SfO at 100 parts per million (ppm) by weight. The δ-tocopherol isomer was chosen as the antioxidant for study based on published literature (19, 20, 21) suggesting a high antioxidant capacity. Results and conclusions reported are specific to this isomer.

The controls used in the experiment consisted of a group of positive controls (matrix and flavor mix only, low oxygen environment) and a group of negative controls (matrix and flavor mix only, ambient air environment). A summary of the samples prepared is given in **Table 5-1**.

Table 5-1: Summary of samples prepared for storage, each containing a mixture of 10 compounds at equi-molar concentration

		Air atmosphere	Low Oxygen atmosphere	Addition of δ-tocopherol (air atmosphere)
Medium Chain Triglyceride (MCT)		X	X	X
Sunflower oil (SfO)		X	X	X
Soybean oil (SbO)	Fresh	X		
	Oxidized	X		

3.5. Sample packaging for storage and analysis

Five ml of each aromatized oil matrix was dispensed in 20 ml GC headspace sampling vials. Vials containing sample to be stored in ambient air were immediately closed with septa previously baked to avoid any odor contamination, and then caps. Vials containing sample to be stored in a low oxygen environment were taken quickly to an anaerobic glove box for gas flushing and similar closure, as described in (10).

3.6. Storage of vials

Samples were stored standing upright in an incubator at 30 ˚C. Sampling times were 0, 1, 2, 4 and 8 weeks of storage. At each sampling time, samples were transferred to a -46 ˚C freezer until analysis. Samples were prepared in triplicate and analyzed once each.

In the lipid oxidation experiment, aromatized fresh and oxidized soybean oil samples were stored at higher temperature (50 ˚C) to accelerate degradation reactions; sampling times were 0, 3, 5, 7, 10, 14 and 20 days.

3.7. Analytical method for volatile analysis

3.7.1. Extraction method

A static headspace autosampler (Agilent 7694) with a 3 ml loop was used in this study. The extraction parameters were as follows: 45 min equilibration of the sample at 55 ˚C; 0.75 min pressurization of the vial at 4 psi; loop filling time 1.5 min; injection time 1.5 min.

3.7.2. Separation and identification

A Hewlett Packard gas chromatograph (HP- 5890) equipped with a DB-Wax column 20 m x 0.1 mm x 0.2 µm (J&W Scientific, Folsom, CA, USA) was used. The following instrument operating parameters were used: column head pressure 45 psi; 3 ml min^{-1} carrier flow (helium) coming from the autosampler, 50 $ml.min^{-1}$ total split flow; split-less mode for 5 min; oven program 45 ˚C/1 min/10 ˚C min^{-1} /125 ˚C/ 5 ˚$C.min^{-1}$/160 ˚C/20 ˚$C.min^{-1}$ /190 ˚C/ 5 min. A flame ionization detector (FID) was used and HP ChemStation software for data collection.

To identify possible degradation products, sample headspace was analyzed by GC/MS in full scan mode (ions 29-450). The gas chromatograph (HP Model 6890) was equipped with the same column and used with similar operating parameters as above. A mass spectrometer (Hewlett Packard Model 5972 mass selective detector) was used in compound identification coupled with Hewlett-Packard ChemStation software. The parameters were set with 0.5 min solvent delay and 1.84 $scan.s^{-1}$. A Wiley library was used for tentative identification.

3.7.3. Quantification

Calibration curves were prepared to ensure aroma compound concentrations were within the method linear range for detection and quantification. Quantification was based on the peak area at an elution time corresponding to that of the pure reference compound. The losses of model volatiles were expressed in terms of percentage of remaining fraction compared to the initial peak area value of week 0, after averaging the triplicate values obtained for each week point.

3.8. Method for gas analysis

The gas analysis in pouches and vials was performed using a gas chromatograph (GC, HP- 5890) equipped with thermal conductivity detector (TCD) and a HP-Molesieve column 30 m x 0.53 mm x 50 µm (J&W Scientific, Folsom, CA, USA). The GC operating parameters were: injection port 150˚C,

isothermal run at 40 ˚C, detector 175 ˚C, column head pressure 5 psi, and column flow of 5 ml. min^{-1}. Ten µl gas samples were taken with a gas tight syringe (Hamilton, Switzerland) from either pouches or vial headspace.

3.9. Data analysis

Statistical analyses were performed with the R software (R 2.0.1 Software, www.r-project.org) on the fraction remaining after 8 weeks in the different systems. T-tests were performed for each compound to evaluate: i) the effect of atmosphere type, i.e. between the two controls; ii) the effect of type of oil matrix; and iii) the effect of δ-tocopherol in a given oil ($P < 0.05$). For each compound, an analysis of variance (Anova) was performed with the R package to determine the combined effects of oil, presence of δ-tocopherol and one-way interaction ($P < 0.05$).

4. Results and Discussion

In the primary study we have monitored the retention of selected flavor compounds during storage when diluted in a "stable", highly saturated oil (MCT) and a potentially oxidizable oil (unsaturated) sunflower oil (SfO). The study involved adding the model flavor system to each oil and then storing the oils under different conditions including ambient and low oxygen samples, as well as a sample sets containing an antioxidant (δ- tocopherol, DTOC) as shown in Table 1. The results of this first study showed that the flavor compounds were less stable in the SfO than the MCT. We hypothesized that the SfO may have become oxidized and free radicals formed may have contributed to the degradation of the model flavor system. Therefore, we intentionally oxidized a second oil and added our model flavor system to this fresh and oxidized oil. We stored both oils at an elevated temperature to determine if the oxidation state of

the oil had an influence on the stability of out model flavor system. For reporting purposes, the fractions of each model compound remaining in each sample treatment during storage are presented (**Figures 5-1 to 5-10**). The results presented are organized by model flavor compound.

4.1. Diacetyl

Stored under ambient O2 levels Diacetyl was very unstable in SfO (Ctl) with only 10% remaining after two weeks of storage (**Figure 5-1**). A reduced O_2 environment improved the retention of diacetyl: 40% remained after 2 weeks storage and 20% after 8 weeks of storage. While diacetyl was not very stable in any of the SfO systems, the absence of oxygen slowed its degradation. The presence of DTOC in SfO did not significantly improve ($P > 0.05$) the stability of diacetyl compared to the Ctl.

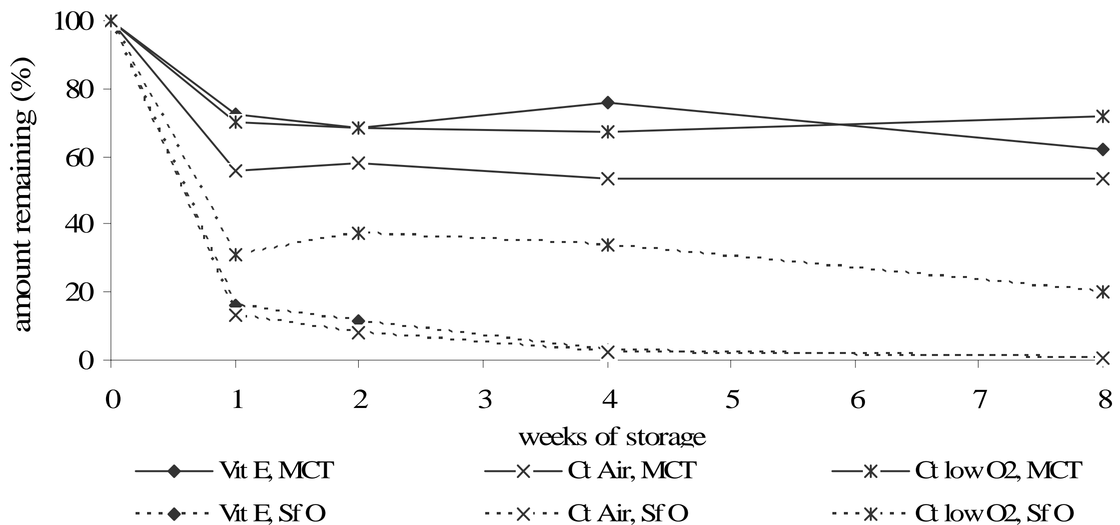

Figure 5-1: Amount remaining (%) of Diacetyl during storage (30 ˚C) when diluted in presence of DTOC or controls, in MCT or SfO.

Diacetyl was substantially more stable in MCT: About 50% of it remained in the Ctl samples after 8 weeks of storage, and about 70% remained in low O_2 sample.

In these two systems, the major loss occurred within the first week of storage and then losses stabilized. This is in contrast to the loss profile in SfO where losses continued throughout the whole storage period. Reducing O_2 or adding DTOC significantly improved the retention of diacetyl in MCT ($P < 0.05$).

Based on above data it would appear that diacetyl may be at least partially lost via oxidation, being more stable in MCT than in SfO. Considering the data presented in **Figure 5-2**, we see that diacetyl losses occurred in the SfO and the oil clearly was oxidizing (increasing PV with time). However, the PV of the SfO at low O2 (**Figure 5-3**) was virtually unchanged during storage, suggesting no significant oxidation of the SfO occurred and yet substantial amounts of diacetyl was lost. This suggests that diacetyl losses appear to be independent of oil oxidation. This was also observed in SbO when losses of diacetyl could not be correlated with changes in PV (data not presented). In addition, substantially different degradation patterns could be observed in the three oils, but could not be related to the saturation level of the oil matrix (**Figure 5-4**, data presented over 4 weeks). It is possible that diacetyl interacted with other components in the systems which resulted in apparent losses.

4.2. Furfuryl mercaptan (FM)

Similar degradation patterns as for diacetyl were observed for FM (**Figure 5-5**): ca. 17% of FM remained after only 2 weeks of storage in SfO Ctl compared to about 33% in low O_2. However after 8 weeks, the final loss in the two systems was similar. The addition of DTOC did not significantly reduce losses of FM in SfO.

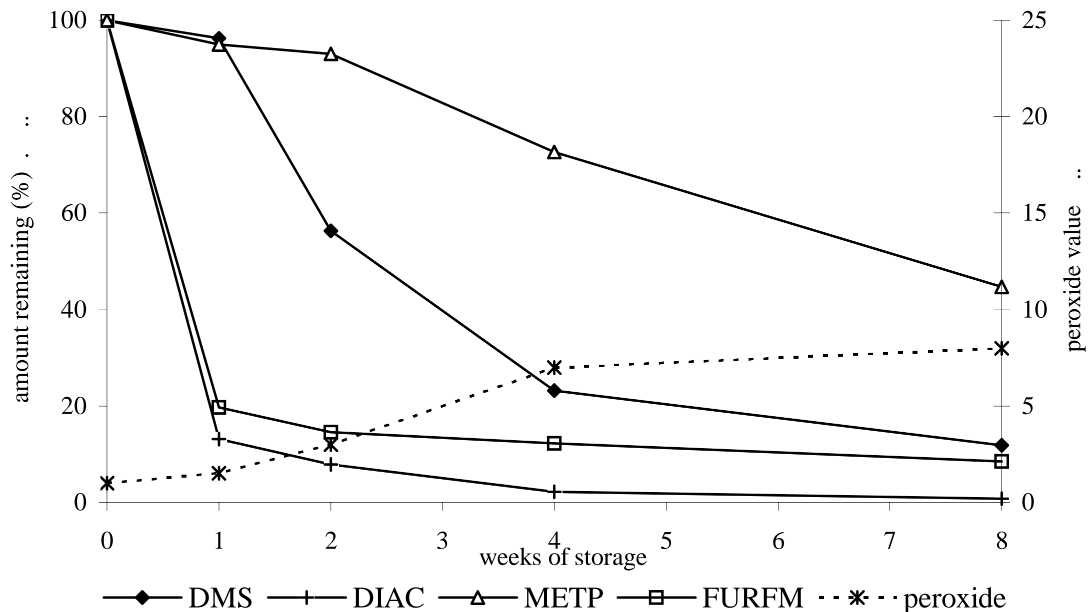

Figure 5-2: Amount remaining (%) of model compounds during storage (30 ˚C) when diluted in SfO in ambient air environment and corresponding peroxide value of the oil matrix (m_{eq}/kg).

Again similar to diacetyl, FM degrades less quickly and to a lower extent when dissolved in MCT compared to SfO (37% left after 8 weeks in MCT Vs. 8% left in SfO [ambient O_2]).The effect of oil type was found to be highly significant ($P < 0.01$) after the total storage period. Low O_2 vs. Ctl provided a significant benefit ($P < 0.05$) for FM in MCT. While the addition of DTOC in this oil may have slowed early losses of FM, it did not provide significant protection during continued storage. Our findings are in agreement with those of Blank *et al.* (22) that oxidative conditions induce rapid and complete degradation of FM. The low O_2 environment or added tocopherol showed limited protection for FM and were not efficient in avoiding its losses.

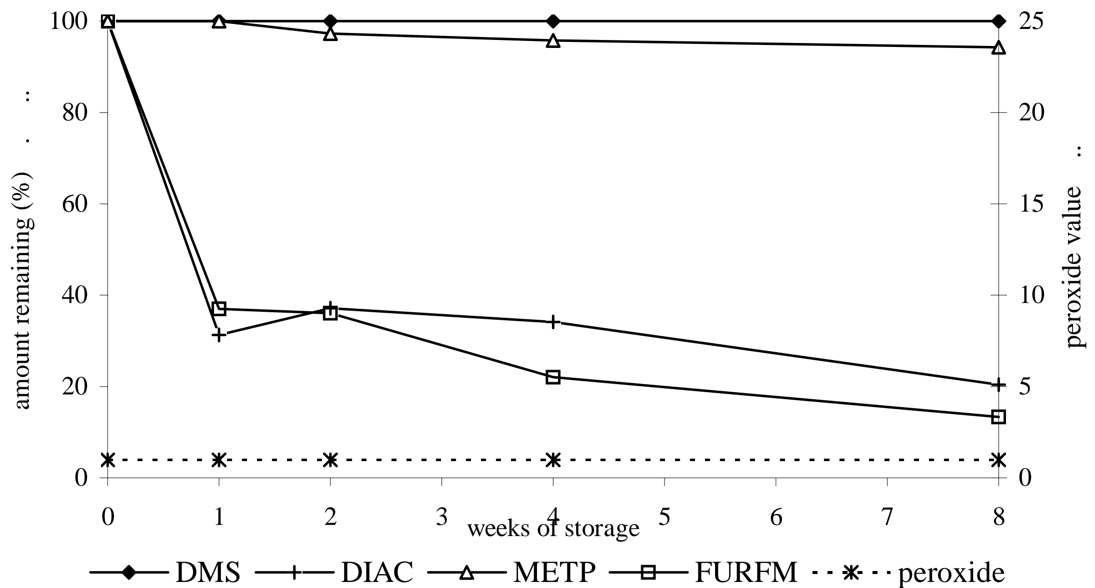

Figure 5-3: Amount remaining (%) of model compounds during storage (30 ˚C) when diluted in SfO in low oxygen environment and corresponding peroxide value of the oil matrix (m_{eq}/kg).

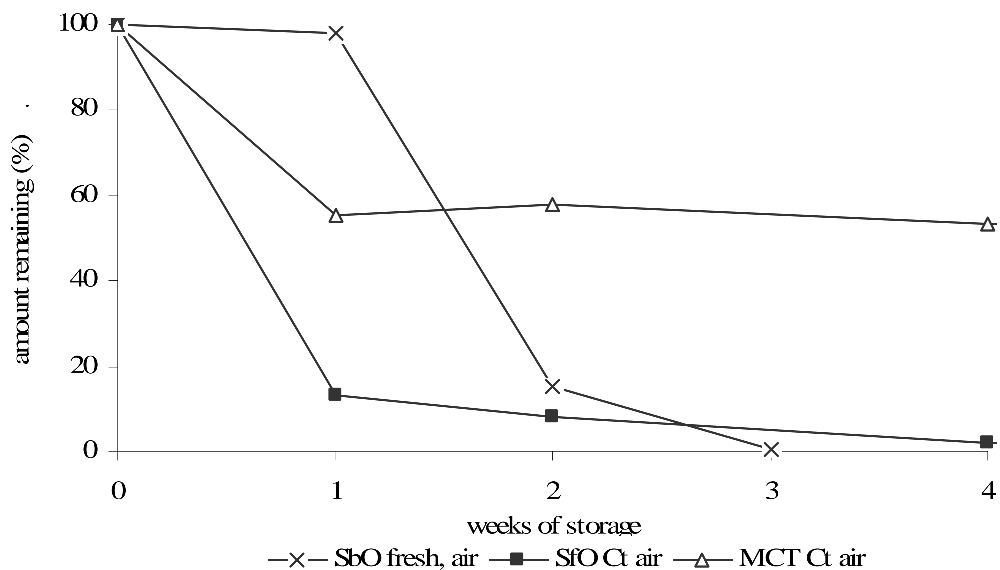

Figure 5-4: Amount remaining (%) of Diacetyl during storage when diluted in MCT (air, 30˚C), SfO (air, 30˚C) and SbO fresh (air, 50˚C).

Overall, the oil type has a greater effect on compound stability than added antioxidant or reduced O_2 storage. According to data collected on FM losses in fresh soybean oil (**Figure 5-6**), it seems that the more unsaturated the oil matrix, the more FM degraded during storage, at least on a long term basis (> 2 weeks of storage). Additional work would confirm this observation.

Similar to diacetyl losses, FM losses occurred whether the SfO was undergoing oxidation or was stable to oxidation (**Figures 5-2 and 5-3**, respectively). Poor stability of FM was found when stored under low O_2 even though the PV of the SfO was stable over the storage period.

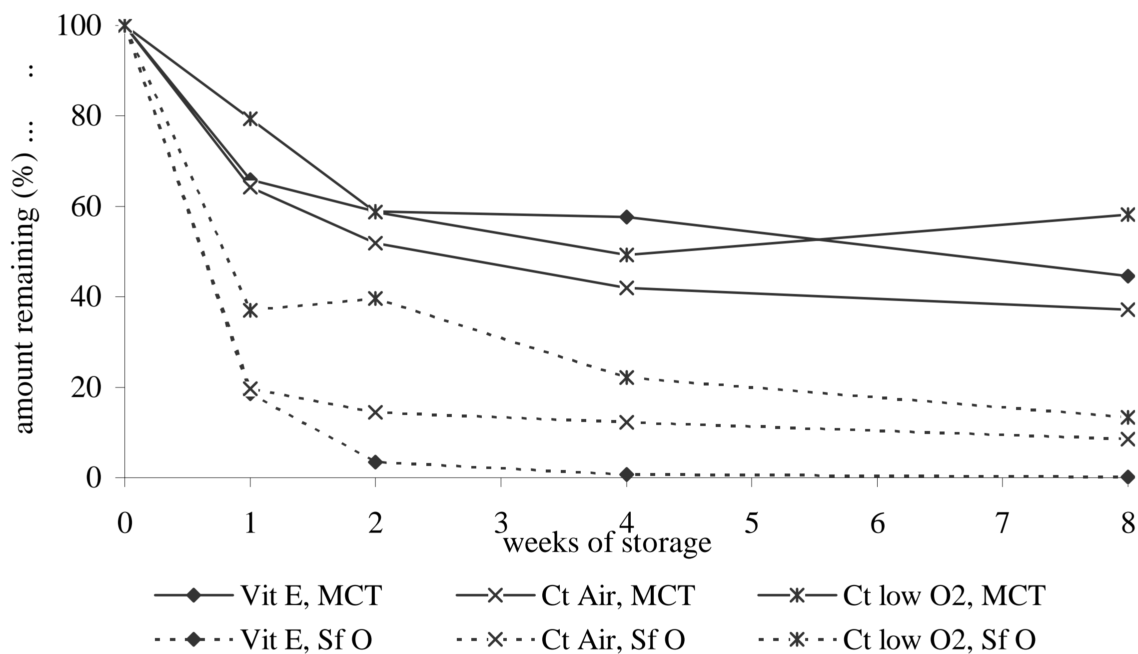

Figure 5-5: Amount remaining (%) of furfuryl mercaptan (FM) during storage (30 ˚C) when diluted in presence of DTOC or controls, in MCT or SfO.

4.3. Dimethylsulfide (DMS)

This study included a second sulfur–containing compound, dimethylsulfide (DMS, **Figure 5-7**). The retention profiles of DMS are very different than observed for diacetyl and FM. DMS losses from SfO were substantial (70% remaining after 2 weeks storage with antioxidant; 54% remaining in ambient O_2 samples) except for the sample stored in the reduced O_2 environment which had no detectable losses even after up to 8 weeks storage). While the presence of DTOC may have initially slowed DMS losses, the difference between the treatment sample and the control was not significant after 8 weeks storage (12 % remaining in Ctl Vs. 17% remaining with DTOC). DMS was quite stable when added to MCT (**Figure 5-7**). While it appears that DMS was lost to a greater extent in the low O_2 sample, this difference was not statistically significant. Despite this lack of statistical significance, this result is consistent with a previous study (10) where DMS was found to undergo equal or greater loss when stored at a reduced O_2 environment than ambient O_2 levels in MCT.

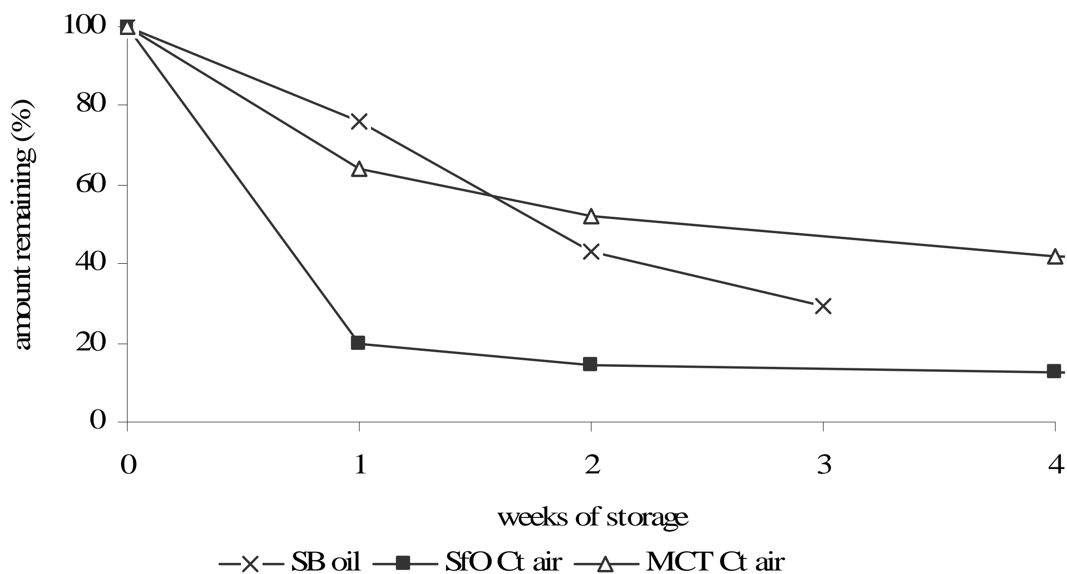

Figure 5-6: Amount remaining (%) of Furfuryl Mercaptan during storage when diluted in MCT (air, 30˚C), SfO (air, 30˚C) and SbO fresh (air, 50˚C).

As can be seen in **Figures 5-2 and 5-3**, DMS tended to be inversely related to the change in PV of the samples: DMS levels in the sample undergoing oxidation (increased PV value) decreased while there was no DMS loss in the sample with a stable PV value. This suggests a relationship between oxidation of the oil (free radical formation or the formation of reactive oxidative products?) and DMS stability. When DMS was added to fresh and oxidized SbO and stored (**Figure 5-8**), the initial losses of DMS were higher in the oxidized oil than in fresh oil. Losses stabilized in the oxidized oil but continued at a slow rate in the fresh oil sample to reach similar overall loss after 8 weeks of storage. It appears that losses increased when PV> ≈15. However, this trend could not be confirmed in the two other oils.

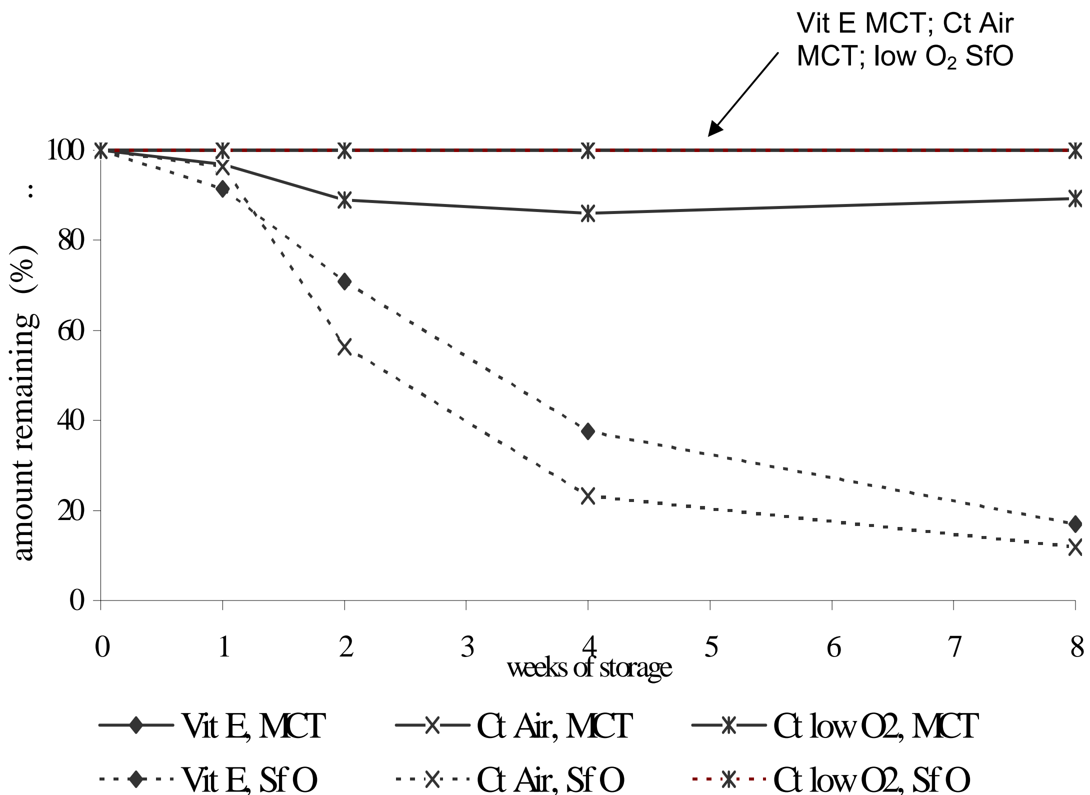

Figure 5-7: Amount remaining (%) of dimethyl sulfide (DMS) during storage (30 ˚C) when diluted in presence of DTOC or controls, in MCT or SfO.

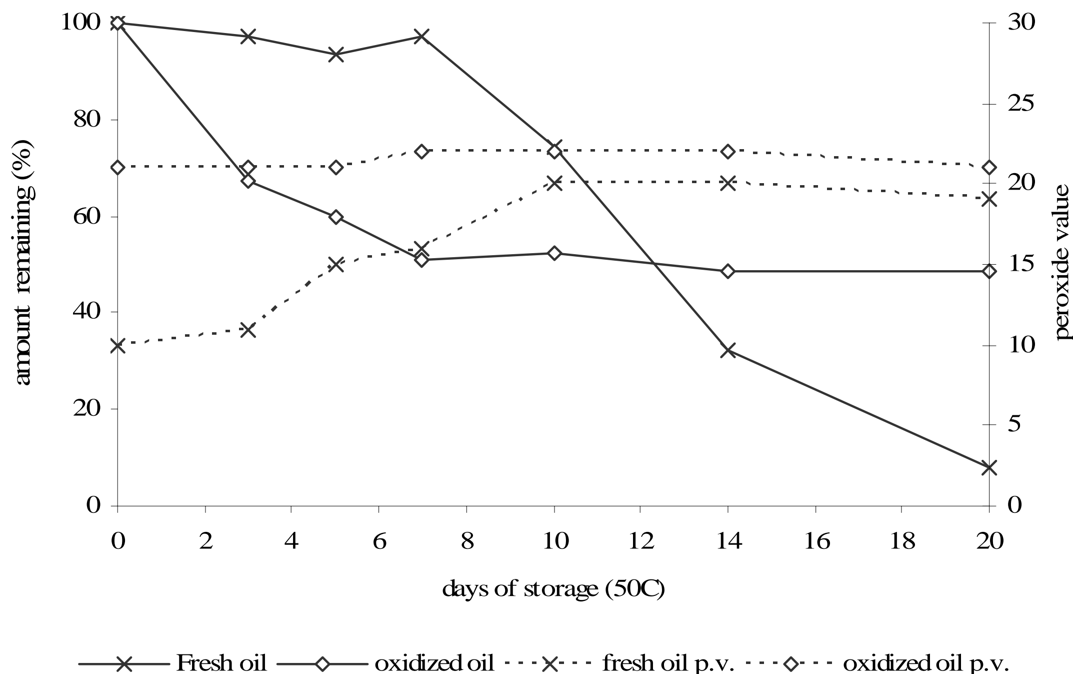

Figure 5-8: Amount remaining (%) of Dimethyl Sulfide (DMS) during storage when diluted in SbO fresh and SbO oxidized (air, 50˚C) and corresponding peroxide value of the oil matrix (meq/kg).

Similar to FM and diacetyl, DMS stability was significantly influenced by oil type ($P < 0.01$) which led us to conclude here again that in these experimental conditions, the effect of the matrix (MCT Vs. SfO) is greater than the effect of adding vitamin E antioxidant.

4.4. N-methyl pyrrole (NMP)

NMP degraded substantially in SfO Ctl (45% remaining after 8 weeks of storage) while no loss was detectable in low O_2 in the same matrix (**Figure 5-9**). This suggests that oxygen may be involved in NMP loss. Similar trends and relative differences were found for NMP in MCT (75% remaining after 8 weeks in Ctl Vs. 88% in low O_2). NMP losses were significantly lower in the low O_2 systems compared to Ctl systems ($P < 0.01$) in the two oils. In addition, losses were

reduced in SfO when DTOC was added to the system compared to Ctl (60% vs. 45% in Ctl) but there was no effect of DTOC when added to the MCT system. The observation that losses were reduced by lowering the O_2 but not as effectively by the addition of antioxidant suggests that molecular oxygen might be involved in the degradation process.

Data on NMP losses when added to fresh and oxidized SbO (**Figure 5-10**) also support this hypothesis since same degradation pattern and losses were observed regardless of the oxidation state of the oil. The fact that NMP is degraded under oxidative conditions is in agreement with the use of oxidation to polymerize this compound to form conducting biopolymers in biotechnologies (23). Since we used a headspace method in this study, we would not detect polymerized NMP. It would be interesting to conduct further studies using C-14 labeled compounds to help in determining loss mechanisms.

Similar to the other model compounds, the type of oil had a highly significant effect ($P < 0.01$) on NMP stability after 8 weeks of storage. This effect was found to surpass the effects of oxygen or antioxidant in terms of long term stability.

Although the presence of DTOC was found to be significant for the stability of some compounds in a given matrix, as well as the type of matrix, no significant

In addition to measuring peroxide values, sample headspace was analyzed by GC/MS in full scan mode (ions 29-450) to identify possible degradation products. Hexanal, heptanal and octanal could be detected and identified in SfO Ctl after 8 weeks of storage while none of these aldehydes could be detected in SfO DTOC or low O2 control, nor in any of the MCT systems. These findings are in agreement with the PVs in the various systems (Ctl Vs. low O_2 vs. DTOC). interaction could be determined statistically (ANOVA) for any of the four model compounds studied.

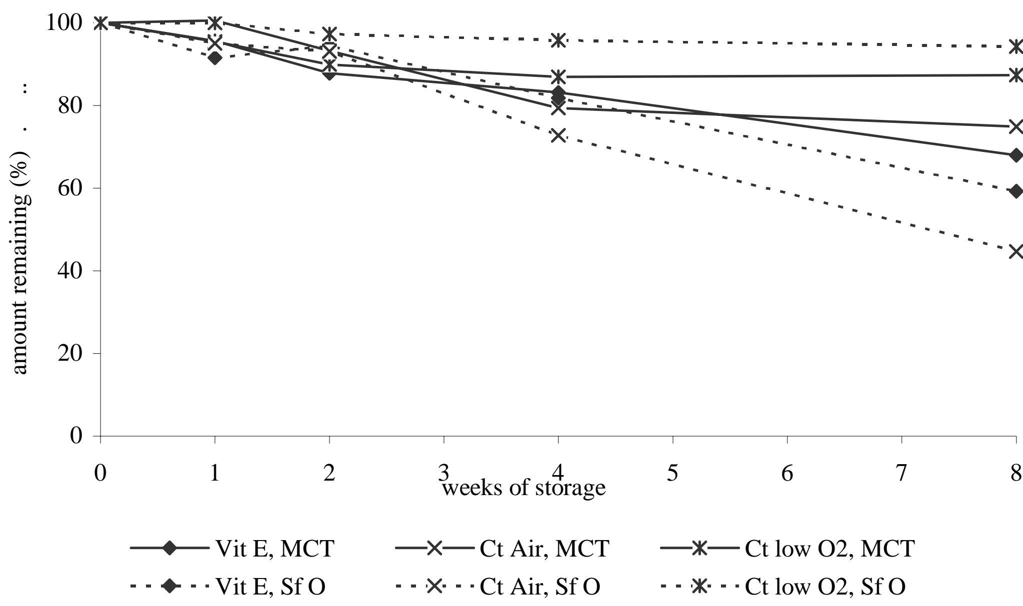

Figure 5-9: Amount remaining (%) of N-methyl pyrrole (NMP) during storage (30 ˚C) when diluted in presence of DTOC or controls, in MCT or SfO.

The addition of antioxidant or reduced O_2 levels effectively limited SfO oxidation. Assuming oxidation is a loss mechanism for some of our model compounds, it appears that DTOC does not protect all oxidizable compounds equally, i.e. it might preferentially target the fatty acids rather than the flavor compounds. Hras *et al.* (24) found a pro-oxidant activity of α-tocopherol when added to sunflower oil (0.01 %). In the present study, δ-tocopherol was added at 100 ppm (0.01%) and an opposite effect was observed. This contradiction may be explained by the work of Huang *et al.* (18) who studied the influence of α- and γ- tocopherol on lipid oxidation in corn oil emulsions. They found different optimal levels for each type of tocopherol.

Since lowering the O_2 in the sample environment often lead to reduced losses of our model volatiles, we expected that the addition of DTOC might also offer similar protection. However, of our model compounds only diacetyl in MCT benefited from added DTOC under the conditions of our experiments

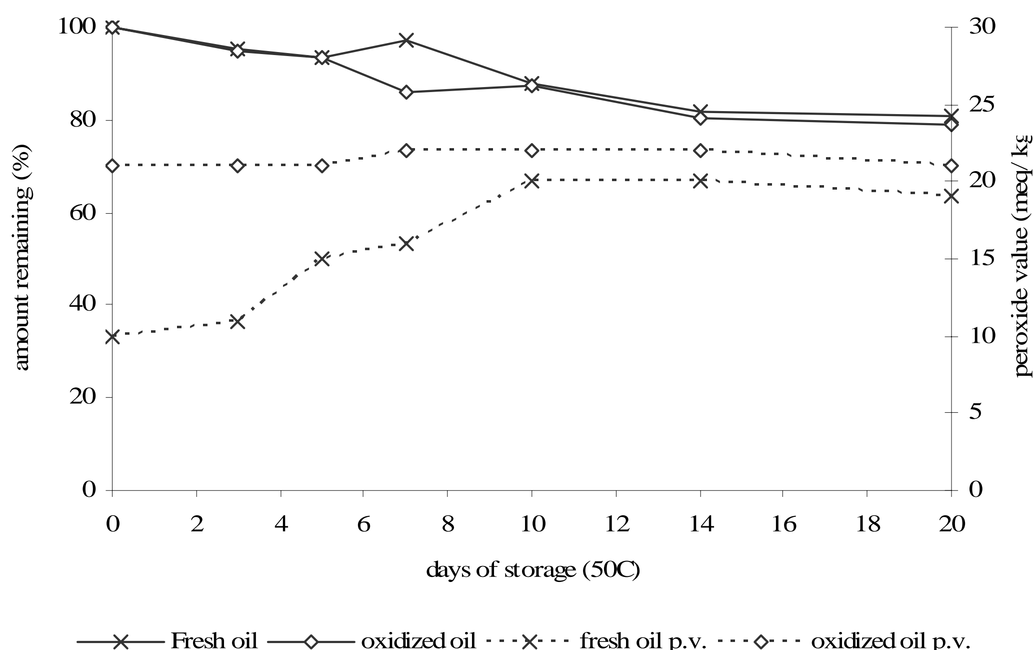

Figure 5-10: Amount remaining (%) of N-methyl pyrrole (NMP) during storage when diluted in SbO fresh and SbO oxidized (air, 50˚C) and corresponding peroxide value of the oil matrix (m_{eq}/kg).

Overall this study showed that DTOC provided little protection to the model volatiles studied. In one case the addition of DTOC (NMP in MCT) had a detrimental effect on stability (over 8 weeks). Reduced oxygen in the sample atmosphere generally provided a better protection. This suggests that oxidation is involved in the loss of our model volatiles but that a free radical acceptor such as DTOC may not be the right choice of antioxidant. The major factor influencing the stability of our model compounds over time was the type of oil matrix used as flavor solvent. Overall, MCT offered substantially greater stability to the volatile compounds than SfO or SbO. However, the data gathered could not provide sufficient evidence that either the saturation level or the oxidation level of the oil matrices were the key factors. The influence of other oil characteristics such as

fatty acid composition or presence of trace components would need to be investigated so as to make definitive conclusions.

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Chapter 6: Comparison of Antioxidants to Prevent Oxidation of Sulfur Flavor Compound in Sunflower Oil

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Leclercq, Reineccius and Milo.

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1. Abstract

The objective of the work was to compare the effectiveness of various antioxidants in slowing sulfur flavor compound oxidation in oil matrix. A model aroma compound mixture was prepared in sunflower oil and stored for eight weeks. Six systems were studied, where samples contained 0.01% of δ-tocopherol, 0.05% of rosemary extract or a mixture of 0.02% (ratio 1:1) of δ-tocopherol and ascorbyl palmitate, or of butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), and lastly no antioxidant added with low oxygen level or with ambient air oxygen level. Aroma volatiles were analyzed by static headspace- GC/ FID to determine stability over time. The effect of each antioxidant system on their stability was generally compound dependent: BHA/BHT and systems containing tocopherol did not provide any significant protection ($P > 0.05$) to Furfuryl Mercaptan, Dimethylsufide and Propanethiol, but did protect 2-Butanethiol over 8 weeks of storage. On the contrary, adding rosemary extract did not improve the stability of 2-butanethiol but protected substantially the 3 other compounds. Overall, less reactive sulfur compounds were better protected by the presence of antioxidants. While all antioxidants systems reduced oxidation of the oil matrix over time, they were not as effective in reducing the losses of flavor compounds.

2. Introduction

One of the major causes of quality deterioration in food products is oxidation. The effects of oxidation have been extensively studied for a number of food products notably meats, fats and oils and beverages (citrus, beer) (1-6). Oxidation of a food product commonly implies the occurrence of off-flavors. However, it is most probable that the loss of flavor quality comes not only from the appearance of undesirable notes but also from the disappearance of desirable ones. A broad finding from these studies is that oxidation generally produces noticeable changes in flavor perception to consumers.

Sulfur containing compounds are important components for the flavor of food produced after thermal processing (e.g. baked goods, roasted products such as chocolate, coffee…). These compounds have been identified, quantified and their role assessed in numerous studies, as presented in the review by Vermeulen et al. (7) However, their stability has been the focus of a limited number of studies, and mostly in complex food systems (8, 9). These studies showed great instability of thiols in presence of other food constituents. To the authors' knowledge, only few studies have reported on the stability of these compounds in model studies, i.e. limiting possible interactions with other constituents (10, 11).

As detailed in previous works, it has been shown that flavor compounds may degrade over time when present in an oxidative environment (11-13). Previous work pointed out faster degradation of several of the volatiles in model systems stored in the presence of oxygen, namely furfuryl mercaptan, diacetyl and acetaldehyde (12).

In order to slow the negative effects of oxygen on food quality, antioxidants are commonly employed. A variety of food antioxidants have been studied and classified according to their action mechanism or to their origin (14, 15). The mechanism of action of the main category, called "chain- breaking compounds" also referred to as primary antioxidants, has been described primarily for lipid oxidation, where these antioxidants terminate the free-radical chain reaction by

donating either an electron or hydrogen to the free radicals. A diversity of compounds belong to that group, including naturally occurring compounds such as tocopherols, flavonoids and vanillin, and synthetic antioxidants such as hindered phenols, e.g. BHA, BHT, TBHQ. These antioxidants have been extensively used in the food industry, to delay lipid oxidation for nutritional and sensory purposes (14, 16, 17). Vitamin E (tocopherols) has been used in patented applications to stabilize essential oils in encapsulated materials and in beverages (13, 18, 19). Several studies pointed to a synergetic effect between tocopherol and ascorbic acid to limit lipid oxidation in solution, in emulsions, or in vivo (16, 20-22) Studies have found that adding BHA and BHT into diets in the meat industry stabilized meat products (23). They also have been found to be efficient over a wide range of concentrations as opposed to other antioxidants which might become pro-oxidants above a certain level (24).

Lastly, natural antioxidants have become of greater interest recently for label and marketing purposes. While numerous natural products are available, rosemary extract is considered one of the most effective antioxidants in various oils and emulsions (16, 21, 25, 26). However, most of these studies have focused on the effectiveness of these antioxidants in slowing lipid oxidation. Little literature is available evaluating these antioxidants to limit the oxidation of flavor compounds.

The underlying hypothesis of the work presented here was that oxidation of flavor compounds in a given oil matrix could be limited by the addition of antioxidants. Since antioxidants have several mechanisms of action, the objective was thus to investigate the addition of individual antioxidants in a model oil system to preserve desirable flavor compounds so as to determine which antioxidant system is most effective.

3. Material and Methods

3.1. Aroma compounds

A model flavor system consisting of dimethyl sulfide, propane thiol, butane -2thiol, diacetyl, N-methypyrrole, 2-ethyl pyrazine, furfuryl mercaptan, 3- mercapto 3-methylethyl formate and furfuryl acetate was prepared. These compounds were selected as representing several different chemical classes. They were purchased from Aldrich Chemical at the highest purity available. They were prepared at equal molar concentration (7.5×10^{-3} mol.L^{-1}) in sunflower oil. The following discussion will focus on for 4 of these compounds, the sulfur- containing compounds: dimethyl sulfide, propanethiol, 2-butanethiol and furfuryl mercaptan. 3-mercapto-3-methylethyl-formate was not included in the discussion due to poor chromatographic results.

3.2. Matrix used for dispersion of the model aroma compounds

As noted, compounds were diluted in sunflower oil (SfO; high oleic content, Nutriswiss, Morges, initial peroxide value = 0.5 m_{eq}. kg^{-1}). Previous work showed an effect of the oil type on the stability of aroma compounds, and higher losses during storage when diluted in SfO compared to Medium Chain Triglycerides (MCT) (12). The matrix was selected so as to be representative of edible oil prone to oxidation. We expect that if the antioxidant systems provide protection, it would be better detected in an initially unstable system (SfO) than in a rather stable system (MCT).

3.3. Antioxidants used

Four antioxidant systems were studied. The first system consisted of 100 ppm of each of the phenolic antioxidants (butylated hydroxy anisole [BHA, Aldrich Chemical] and the related compound butylated hydroxy toluene [BHT, Aldrich Chemical], BHA/BHT). The second and third antioxidant systems were a hindered phenol (δ-tocopherol [Vit. E]) used alone (100 ppm, Aldrich Chemical),

or in combination with vitamin C (ascorbyl palmitate, 100 ppm, Aldrich Chemical, [Vit. E/Vit. C]). The last antioxidant was a rosemary extract (500 ppm in solution, HERBOR O 25, Robertet S.A., [Rosm]). The antioxidants and their usage levels were selected based on available literature showing their effectiveness in food systems.

Two types of controls were used in this study: first a group of samples with low oxygen environment (matrix and flavor mix only) and a second group with ambient air environment (matrix and flavor mix only).

3.4. Sample packaging for storage and analysis

Five ml of oil solution with the appropriate antioxidant system (or not) was added to 20 ml headspace GC sampling vials. Vials were capped and sealed with Teflon septa to avoid volatile losses during storage. Control samples stored under an argon atmosphere were prepared with the same solutions, in anaerobic chamber containing less than 0.5 % oxygen.

3.5. Method for gas analysis

Gas analysis was performed using a gas chromatograph (HP- 5890) equipped with thermal conductivity detector (TCD) and a HP-Molesieve column (J&W Scientific, Folsom, CA, USA), as described in previous works (12). The control samples stored under argon contained < 0.5 % oxygen.

3.6. Sample storage

Samples were stored standing upright in an incubator at 30 ˚C. Sampling times were 0, 1, 2, 4 and 8 weeks of storage. At each sampling time, samples were transferred to a -40 ˚C freezer until analysis.

3.7. Analytical method for quantification of volatiles

Volatiles were analyzed using a static headspace Autosampler (Agilent 7694) coupled to a GC (HP- 5890) - Flame ionization detector using a DB - Wax column (20 m x 0.1 mm x 0.2 µm , J&W Scientific, Folsom, CA, USA). The following instrument operating parameters were used: column head pressure 45 psi; 3 $ml.min^{-1}$ carrier flow (helium) coming from the autosampler, 50 $ml.min^{-1}$ total split flow; split-less mode for 5 min; oven program 45 °C / 1 min / 10 $°C.min^{-1}$ / 125 °C / 5 $°C.min^{-1}$ / 160 °C / 20 $°C.min^{-1}$ / 190 °C / 5 min. HP ChemStation software was used for data collection. Identification of degradation products is a lengthy and tedious process. Since determining degradation mechanisms of the model compounds was not the objective of this study, no identification by mass spectrometry was carried on.

3.8. Peroxide value

To determine the oxidation of the oil system, primary oxidation products (peroxides) were measured by iodine titration using the peroxide value method (AOCS Method Cd 8-53; 8b- 90). Results are presented in $m_{eq}.kg^{-1}$, and are an average of duplicate analysis (one analysis per sample, two samples of each type analyzed).

3.9. Data analysis

Data on the degradation of model volatiles is expressed in terms of percentage remaining compared that present at time 0 (GC peak areas). All samples were prepared and analyzed in triplicate initially and at each time point during storage. These percentage remaining values were then plotted against time of storage for data presentation.

Statistical analyses were performed with the R software (R 2.0.1 package) on the percentage remaining after 8 weeks in the different systems. T-tests were performed for each compound to evaluate the effect of storage atmosphere, based on triplicate data points obtained.

A single factor analysis of variance (Anova) was performed with the R package to determine the effect of the presence of antioxidant at 8 weeks ($P < 0.05$). Then a Dunnett's test was carried out for each antioxidant system against each control ($\alpha = 0.05$) and an $\alpha = 0.05$ LSD was calculated for the mean at 8 weeks so as to determine difference and similarities between the six systems (based on the triplicate data points obtained for each compound in each system).

4. Results and Discussion

4.1. Effect of oxidative environment

We will initially present the data obtained from the controls: the samples stored under ambient oxygen levels (abbr. Ct air) and reduced O_2 levels (abbr. low O_2).

The results presented in **Figure 6-1** are average percentage lost per week of storage for individual compounds in each of the two systems. From this figure, it is clear that the degradation of the four sulfur compounds (Dimethyl sulfide [DMS], Propanethiol [1-PSH], 2-Butanethiol [2-BSH] and Furfuryl Mercaptan [FMCP]) was significantly reduced when stored in a low O_2 environment as opposed to ambient oxygen (air) storage. These results support the rationale and hypothesis underlying the work presented below, that the degradation of sulfur compounds is increased in the presence of an oxidative environment.

Slightly different observations have been reported for DMS in MCT, i.e. the presence of oxygen did not result in significantly greater losses. As concluded in this previous work, the type of oil matrix in which compounds are diluted significantly affects the degradation patterns (12).

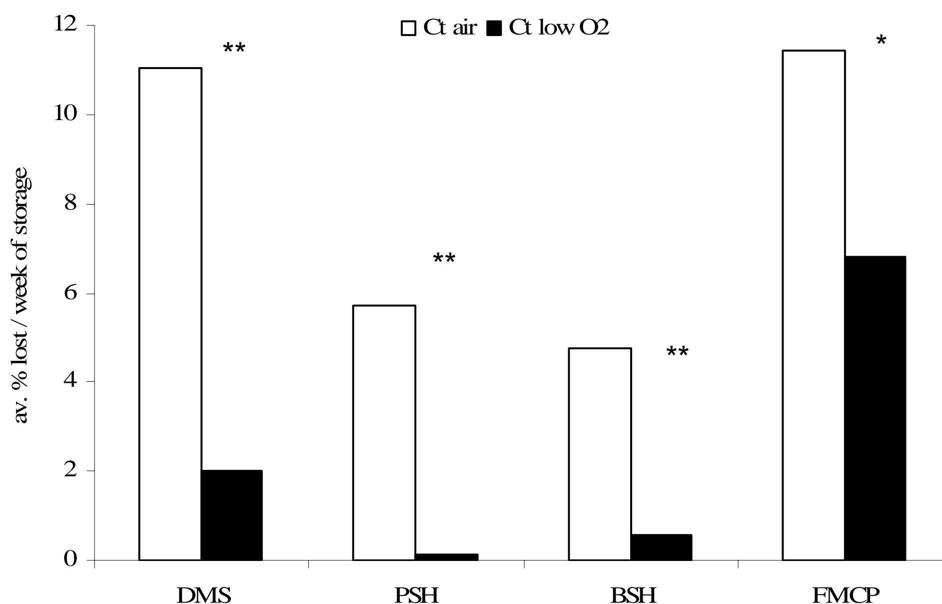

Figure 6-1: Average fraction lost per week over 8 weeks of storage, for each compound in the two control systems, ambient air environment (Ct air) and low oxygen environment (Ct low O_2). A "*" represent a statistically significant difference ($P < 0.05$) between Ct air and low O2 and "**" at $P < 0.01$.

4.2. Effectiveness of antioxidants to protect Dimethylsulfide (DMS)

From the data plotted for DMS in SfO (**Figure 6-2**), one can note different trends in DMS loss across systems. Two systems, those containing Rosm and stored in low oxygen, show most of the loss during the first week of storage and then minor losses thereafter. The four other systems (Ct air, Vit E, Vit E/ Vit E, BHA/BHT) exhibited minor losses over the first week (0 -10 %) but then substantial losses during the next 7 weeks of storage (up to 80 % losses). These 3 systems with antioxidants provided no protection compared to Ct air on long term storage ($P < 0.05$), whereas Rosm provided substantial protection to DMS compared to Ct air. Of all the treatments, low O_2 offered the best protection to DMS diluted in SfO, with only about 15 % losses over 8 weeks. According to the literature, rosemary extract has been reported to be a free radical terminator (due to phenolic compounds in the extract), a metal chelator and a superoxide radical

scavenger (27). Since the other antioxidants studied here also contain a phenolic (BHA/BHT have 1 phenol group; rosmarinic acid has 4 phenol) functional group, and the oil matrix contained no detectable metals (according to supplier's information), the action of rosemary extract as a superoxide radical scavenger may be the important mechanism protecting DMS. A study by Frankel *et al.* (21) on the antioxidative components of rosemary extract reported on several compounds, each providing different effectiveness in reducing lipid oxidation when added in either bulk oil or in emulsion form. They explained their results by the presence of different structures and functional groups in carnosol, carnosic acid and rosmarinic acid which account for about 98% of commercially available rosemary extract (14, 21, 28).

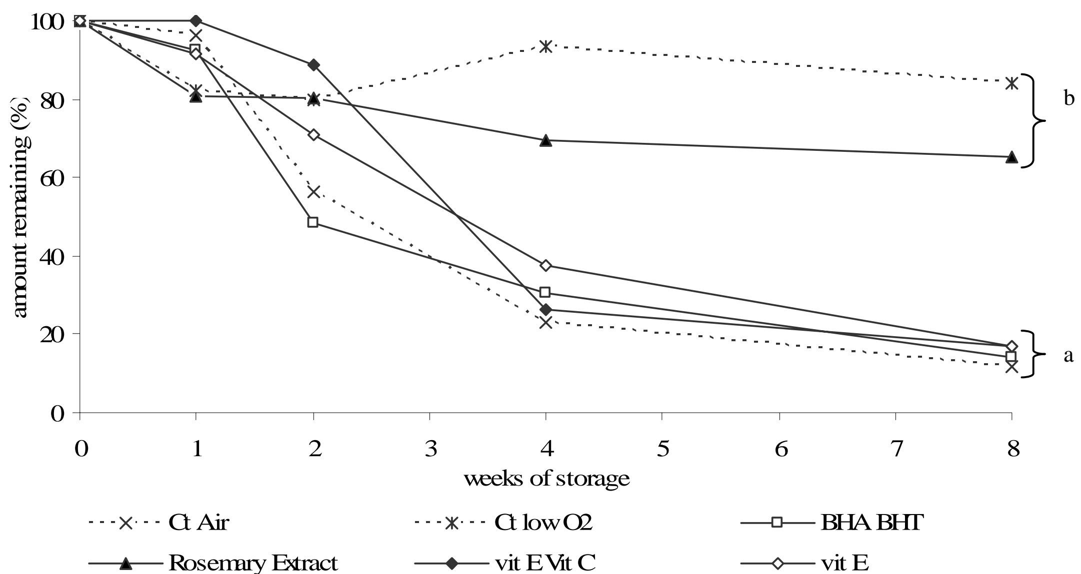

Figure 6-2: Amount remaining (%) of Dimethyl sulfide during storage (30 ˚C) in the presence of antioxidants and controls. Letters indicate statistical groups resulting from comparison to control (air) by Dunnett's LSD procedure. The rosemary and low O2 systems could not be separated statistically due to large standard deviation in peak areas at 8 weeks.

Since Rosm offered good protection to DMS during storage and the other antioxidants did not offer any protection, it was of interest to check if the other antioxidants were added at their optimal level, i.e. that they indeed worked as antioxidants in the various systems. For this reason, peroxide values of the oil (SfO) were measured during storage.

4.3. Peroxide values (PV) of matrix systems

Consistent with the data trends for DMS loss, the low O_2 and Rosm (no change from initial value week 0 to week 8, PV m_{eq} = 1) offered the greatest stability to the SfO (**Figure 6-3**). All systems containing an antioxidant were determined to have PVs below the control air system. The profiles of the PVs show that while some oxidation of SfO was detected over time, lipid oxidation was probably still in the induction phase (at weeks 8 max PV= 8 m_{eq}). In addition, these data show that the antioxidants concentrations were likely within their antioxidant limits, i.e. not at pro-oxidant levels. These data suggest that the protective effects of antioxidants for lipids are not necessarily linked to the protection of other compounds, i.e. flavor molecules, subject to oxidation.

Higher PVs in SfO were consistent with higher losses of DMS. As presented in previous work (12), the oxidation level of the oil might have an effect on the stability of DMS. Reaching higher level of SfO oxidation during storage would provide additional insight as to a possible correlation between the presence of fatty acid peroxides and the loss of DMS.

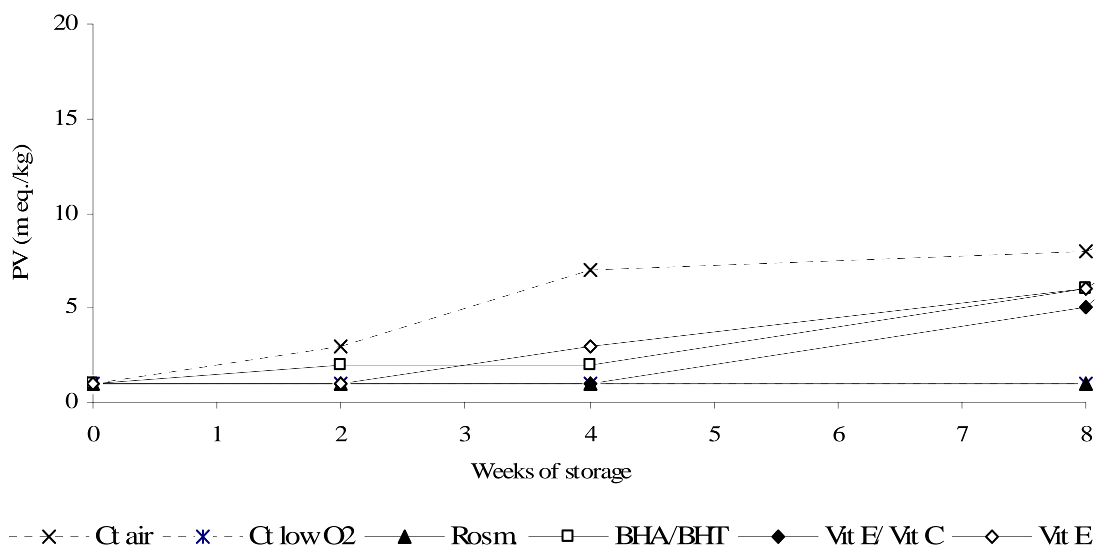

Figure 6-3: Peroxide values measured of SfO in the various systems studied: controls (Ct air and low O2) and in presence of each antioxidant (Rosm, BHA/ BHT, Vit. E, Vit. E/Vit C) during the 8 weeks of storage (30 ˚C).

4.4. Effectiveness of antioxidants to protect furfuryl mercaptan (FMCP)

While a significant difference was found in the percentage of FMCP lost between the Ct low O_2 system (45 % after 1 week) and Ct air (80 % after 1 week) during storage, greater losses of FMCP vs. DMS occurred in the low oxygen system (**Figures 6-1 and 6-4**). No significant difference could be found between any of the treatments and Ct air, nor within the treatments ($P > 0.05$). The addition of antioxidants to SfO did not provide any protection to FMCP on long- term storage. Based on the PVs found in the various systems (**Figure 6-3**), one would expect the most free radicals in samples stored in air followed by the antioxidant treatments with similar losses for Rosm. and Ct low O_2. However, significant differences were observed for FMCP between Ct low O_2 and Rosm samples. This suggests that the FMCP degradation observed are not linked to PVs. For this reason, it can be hypothesized that other degradation pathways that might involve the antioxidants may be taking place, leading to FMCP degradation.

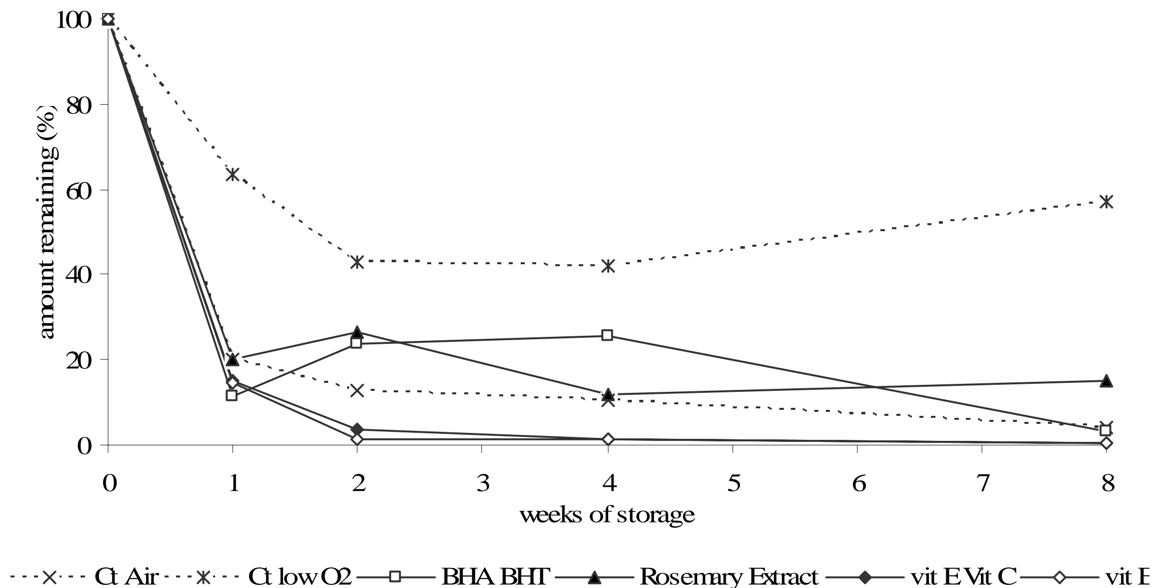

Figure 6-4: Amount remaining (%) of Furfuryl Mercaptan (FMCP) during storage (30 ˚C) when in the presence of antioxidants or controls, in SfO. Letters indicate statistical groups resulting from comparison to control (air) by Dunnett's LSD procedure.

Recently Müller *et al.* (8) suggested the formation of conjuguates between thiols and phenols, such as hydroxyhydroquinone, in coffee brew during storage, may lead to a rapid decrease (hours) in FMCP. Since our system also contained phenols from the antioxidants added, i.e. BHA, BHT (each one phenolic group), tocopherol (one phenolic group), rosmarinic acid (4 phenolic groups), we cannot exclude that some similar reactions may occur. The thiol-phenol addition products would not be detected by the headspace sampling technique we used in our experiments. However, our losses continued past a week of storage, suggesting slower apparent reaction kinetics compared to what has been reported by Müller *et al.* It is therefore reasonable to expect that several reaction pathways might take place possibly including reacting with antioxidants leading

to the apparent loss of the highly reactive FMCP. However data collected here did not allow us to propose any reaction mechanism. To conclude, the addition of an antioxidant is not an effective way to reduce degradation of FMCP in oil system. Further research needs to be conducted to provide a stabilizing environment for this compound.

4.5. Effectiveness of antioxidants to protect 1-propanethiol (1-PSH)

As shown in **Figures 6-1 and 6-5**, 1-PSH was degraded to a greater extent when stored in the presence of oxygen (Ct air) than in its absence (low O_2). After 8 weeks of storage no significant loss was detected in low O_2 system compared to initial value, while up to 45% loss was found in the Ct air sample. Due to data variation (13 to 15 %), no significant statistical difference could be determined by ANOVA between the treatments ($P > 0.05$). Still, one can note a trend that BHA/BHT did not provide any protection compared to Ct air, while the three other antioxidant systems appeared to lead to reduced losses after 8 weeks of storage. The systems containing Vit. E, alone or in combination with Vit C, were effective in stabilizing 1-PSH for up to 4 weeks of storage at 30 ˚C. The onset of degradation occurred however at prolonged storage (data collected up to 26 weeks of storage showed similar trends, data not shown).

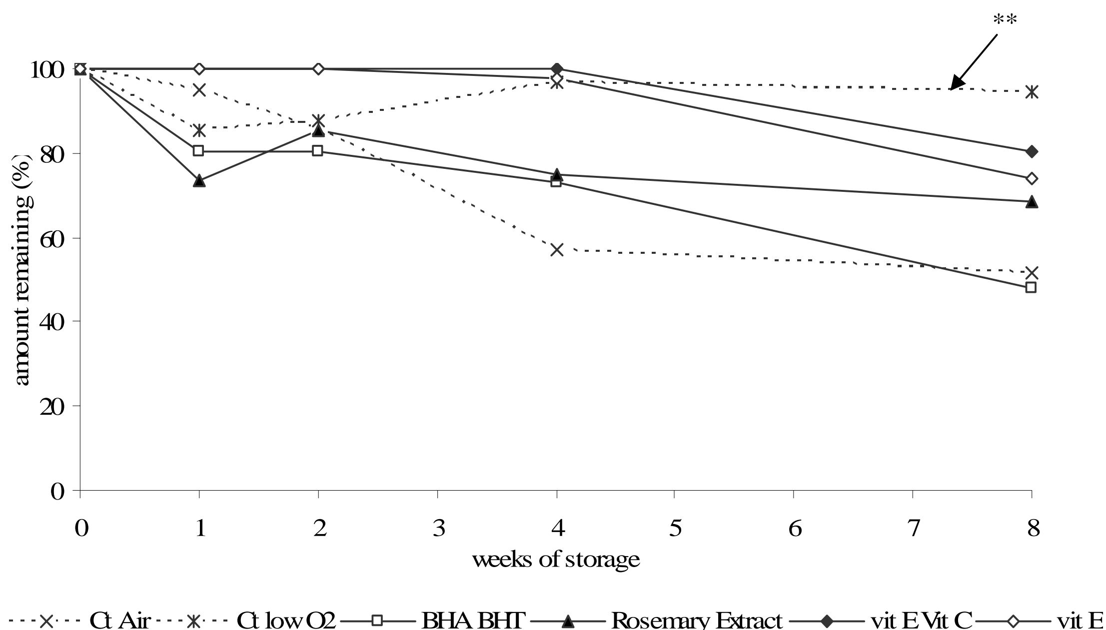

Figure 6-5: Amount remaining (%) of 1-Propanethiol (1-PSH) during storage (30 ˚C) in the presence of antioxidants or controls, in MCT. No significant difference was found between antioxidant treatments and control air by Dunnett's LSD procedure, due to large variations. "**" indicates a statistical difference compared to Ct air.

None of the degradation patterns for 1-PSH could be linked to PV (**Figure 6-3**). Therefore it seems that the degradation mechanisms of this thiol-flavor compound and the SfO occurred independently. The addition reaction of FMCP with phenolic compounds of the catechol type as discussed above does not seem to occur for 1-PSH to a similar extend since losses of this compound in systems containing phenols were not greater than losses in control air. This may be due to the lower reactivity of primary thiols compared to FMCP.

4.6. Effectiveness of antioxidants to protect 2-butane thiol (2-BSH)

As with the other sulfur compounds discussed earlier, degradation was significantly minimized when 2-BSH was stored under low oxygen environment (10 % loss after 8 weeks, **Figures 6-1 and 6-6**) compared to ambient air conditions (Ct air, 50 % loss after 8 weeks). The addition of antioxidants to the

system showed a similar impact on the degradation patterns as observed for 1-PSH. The addition of tocopherol (with or without Vit C) protected 2-BSH to the same extend as the low O_2 environment. While the addition of BHA/BHT and Rosm did not provide substantial protection, showing substantial losses (20 to 25 %) after 2 weeks of storage, the addition of tocopherol did bring protection in the short term as well as long term basis. Here again, losses could not be related to the PV of the matrix (**Figure 6-3**). For this compound, the hypothesis of reactions with phenols cannot be supported by the data, since losses were greater in Ct air than in presence of phenolic antioxidants.

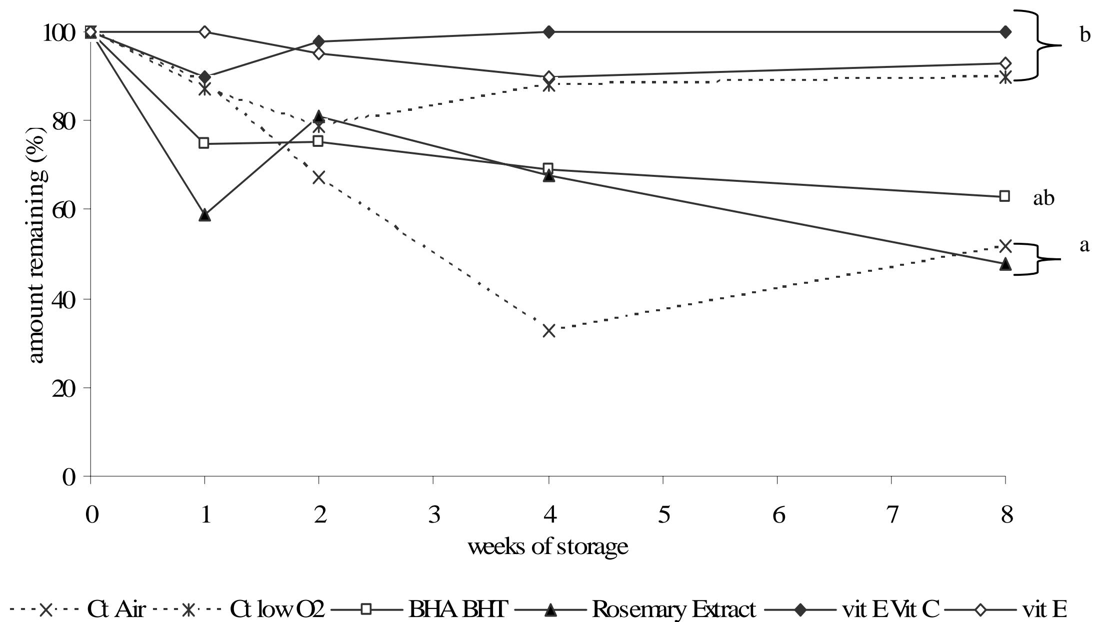

Figure 6-6: Percentage remaining of 2-Butanethiol (2-BSH) during storage (30 ˚C) in the presence of antioxidants or controls, in SfO. Letters indicate statistical groups resulting from comparison to control (air) by Dunnett's LSD procedure (large variations were observed for some treatments, therefore not statistically different).

4.7. Comparison of the various patterns for Sulfur-containing compounds

The degradation patterns of 2-BSH are somewhat different from what was observed with the other compounds, particularly DMS and FMCP. For FMCP, this difference can be explained by the higher reactivity of the benzylic thiol group in comparison with an aliphatic one. 1-PSH and 2-BSH are similarly reactive in Ct air (≈ 50 % loss after 8 weeks). Additionally, Vit E containing antioxidant systems had stabilizing impact on them with a better protection of the secondary thiol. Overall the reactivity of sulfur aroma compounds in SfO was: FMCP > DMS>1-PSH- 2-BSH and the antioxidants were mainly effective for thiols with lower reactivity.

4.8. Comparison of antioxidants

The antioxidants used in this work have been extensively studied for their effectiveness in controlling lipid oxidation but have not been evaluated for their ability to protect S-containing aroma compounds. Most studies show different efficacy in protecting fats and oils from lipid oxidation across these antioxidants. For example, Rosemary extract provided better protection than BHA/BHT and Vitamin E in forced oxidation conditions of SfO (16, 29). Peroxide Values measured in SfO during storage in our experiments (**Figure 6-3**) were consistent with these findings. However, effectiveness in slowing oxidation of sulfur aroma compounds could not be correlated with their effectiveness against lipid oxidation.

Several studies reported synergetic effects between ascorbic acid (ascorbyl palmitate) and tocopherol in slowing lipid oxidation (20, 22, 25, 30). However such a synergy was not found in protecting aroma compounds.

4.9. Comparison of other compounds present in the solutions (data not shown)

In addition to the compounds which have been discussed, five additional aroma compounds were present in the solutions used in this study. The behavior of these additional compounds was very similar to some compounds already

discussed or the effects were very minor and thus, only a brief discussion of these additional compounds follows.

Overall, N-methyl pyrrole had similar degradation patterns to these of 1-PSH, except in presence of Rosm for which it was most similar to this of 2-BSH. Degradation patterns of 3-mercapto-3-methyl-ethyl-formate were similar to these of DMS, except in presence of Vit. E Vit. C where large losses, similar to those of FMCP, were observed. Some variability (up to 20 %) was observed in the data collected on 3-mercapto-3-methyl-ethyl-formate due to low chromatographic resolution. Diacetyl could not be protected in any system, similar to FMCP. The stability of furfuryl acetate was improved compared to Ct air in systems containing Vit. E, Rosm. and low O_2. In the presence of the other antioxidants increased losses occurred unlike any of the other sulfur containing- compounds. 2-ethyl pyrazine was protected in the presence of Vit. E compared to Ct air. In presence of BHA/ BHT and low O_2, degradation patterns were most similar to those of 2-BSH. Losses were large in presence of Vit E/ Vit C, similar to losses observed with FMCP. Overall, patterns presented for sulfur compounds were representative of patterns observed for other aroma compounds. However, it is probable that degradation mechanisms are different.

4.10. Summary

We have found that reduced oxygen storage offered substantial protection to the sulfur-containing aroma compounds studied. While some antioxidants offered substantial protection to a specific model aroma compound(s), none of the antioxidants studied individually provided protection to all of our model aroma compounds. For example, Rosm offered the best protection to DMS, and systems containing Vitamin E protected 1-PSH and 2- BSH, while none of the antioxidants protected FMCP.

Our study was limited to nine model compounds and, therefore, would not reflect the diversity found in a real food system. However, we know that some compounds would be better protected than others and as a result potentially modify the flavor balance and lead to flavor defects. However, the impact of such

an imbalance would depend upon which compounds are most important in making an impact on sensory perception. If an antioxidant protects a group of key impact components, its presence could be quite important to flavor stability. To the contrary, if the antioxidant protects an unimportant group of flavor components, the impact would be nil.

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General Conclusions

The major findings of this thesis project are highlighted below:

Part 1. Coacervate materials

- All manufacturing parameters involved in making coacervates (type of colloid, ratios, pH, temperature, stirring speeds) are interrelated;

- The physical state of the core (i.e. liquid vs. solid) does not affect the manufacturing of coacervates (gelatin and gum acacia walls);

- Microcapsules made by complex coacervation offer greater protection against oxidation than spray dried capsules (45 ˚C storage temperature);

- There is no significant effect of cross-linking, size distribution and encapsulant type on the release mechanism for volatiles (release in hot water);

- Volatile release from microcapsules is significantly affected by testing temperature;

- There is a significant effect of aroma compound volatility and solubility on their real-time release from capsules but they are not predictive parameters;

- The major benefits of using coacervation as encapsulation methods are to protect sensitive materials from oxidation and to provide a slow, constant release at room temperature.

Part 2. Aroma Storage Stability

- The presence of oxygen is detrimental to the oxidative stability all model compounds;

- The type of dilution matrix has a significant effect on volatile stability: the presence of water is detrimental for some classes of compounds (aldehydes, esters, and pyrroles) compared to oil;

- The type of oil matrix has a significant effect on volatile stability, and on the protection provided by the addition of antioxidant;

- The addition of δ-tocopherol (0.01%) as antioxidant has little effect in protecting volatile compounds stored in sunflower oil or medium-chain-triglycerides;

- The oxidation level of the oil matrix itself does not correlate significantly with, or predict the degradation of volatile sulfur compounds during storage; and

- The protective effect of added antioxidants is compound and matrix dependent.

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