THE IMPACT OF STORAGE CONDITIONS ON THE CHEMICAL & SENSORY PROPERTIES OF A FORMULATED "COLA" FLAVOUR

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For my girls

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ABSTRACT

Food flavours are complex mixtures of aroma generating compounds designed to enhance, modify, replicate or mask the taste and aroma of foods. They are nearly ubiquitous in the food and beverage industry, where flavour quality is arguably the most important contributor to the perception of overall product quality. Flavours are typically comprised of dozens to hundreds of components, encompassing many chemical families, each with its own set of physicochemical properties. However, they are usually designed around several key contributors, known as "character impact compounds". Although flavouring ingredients are highly potent and impactful, they are also potentially reactive and are subject to a phenomenon known as aging. This aging process can have serious effects on a sensory profile, as character impact components react or are lost, and off-flavours form. Despite this, there is very little information relating to the aging of flavour mixtures. Although flavour stability is a critical measure of flavour quality, very little information is publicly available – in part due to the proprietary nature of flavour mixtures. The objective of this study was to monitor the chemical changes that occur in a cola flavour formulation over the course of a 9-week storage period as affected by temperature and pH, emphasizing the major categories of flavouring ingredients (alcohols, aldehydes, esters, essential oils) and character impact compounds. An additional objective was to determine whether the observed chemical changes coincide with an appreciable sensory impact, by organizing weekly sensory evaluations with a panel of volunteers.

A model cola flavour (Flavour Mixture 1) was designed using representative members from various families of key flavouring ingredients. A second flavour (Flavour mixture 2), representing an 'all-in-one' syrup, additionally contained phosphoric acid but was otherwise identical to its counterpart. Both mixtures were stored for 9 weeks at 4, 23 and 40°C, respectively. Every week, the mixtures were analyzed by gas chromatography-mass spectrometry (GC-MS). Additionally, they were evaluated by a sensory panel comprised of 23 panellists, using tetrad and two-sided directional testing. Lastly, 13 ingredients were analyzed individually under otherwise identical conditions to those of Flavour Mixtures 1 & 2, to help identify potential degradation products. Flavour Mixtures 1 & 2 were susceptible to both chemical and sensory changes as a result of storage conditions. Chemical changes in Flavour Mixture 1 only resulted in perceptible sensory changes 5 weeks into storage, and only at a 40°C temperature condition. Conversely,

Flavour Mixture 2 was far more impacted both chemically and sensorially. This was due to extensive acid-catalyzed rearrangements of terpenes, which created both off-notes and chemical imbalances in the cola profile. Key degradation compounds that affected the sensory profile included *p*-cymene, α -terpineol, benzaldehyde propylene glycol acetal and *p*-cresol. In Flavour Mixture 1, the formation of off-notes occurred prior to a detectable loss in citrus character. In Flavour Mixture 2, however, loss of citrus character was detectable prior to formation of off-notes. Ultimately, although the strength of the overall flavour was essentially unaffected, the intensity of the citrus character was compromised while off-notes intensified. Consequently, it is not recommended that an "all-in-one" flavour model be employed for citrus-based sodas like cola.

RÉSUMÉ

Les arômes alimentaires sont des mélanges complexes de molécules volatiles conçus dans le but d'améliorer, modifier, reproduire ou masquer le goût et l'odeur des aliments. Ils sont pratiquement omniprésents dans l'industrie alimentaire, où la qualité des arômes est sans doute le facteur le plus important dans la perception de la qualité globale d'un produit. Les arômes sont généralement composés de douzaines et même de centaines de molécules englobant de nombreuses familles chimiques, chacune possédant son propre ensemble de propriétés physicochimiques. Cependant, ils sont fréquemment conçus autour de plusieurs composants clés qui donnent un caractère distinctif aux arômes. Bien que les ingrédients aromatisants soient très puissants et aient un impact important, ils sont également potentiellement réactifs et soumis à un phénomène connu sous le nom de vieillissement. Ce processus de vieillissement peut avoir des effets importants sur le profil sensoriel, car les composants clés peuvent réagir ou être éliminés, ce qui peut causer l'apparition d'arômes indésirables. Malgré tout, il existe très peu d'information sur le vieillissement des mélanges d'arômes. Bien que la stabilité d'un arôme soit essentielle à la qualité de celui-ci, le manque d'information disponible peut s'expliquer en partie à cause du droit de propriété des mélanges d'arômes. L'objectif de cette étude était d'observer les réactions chimiques qui se produisent dans un arôme de cola formulé au cours d'une période de 9 semaines en fonction de la température et du pH, en mettant l'emphase sur les principales familles d'ingrédients aromatisants (alcools, aldéhydes, esters, huiles essentielles) et sur les composants clés. Une autre partie de l'objectif était de déterminer si les réactions chimiques observées coïncidaient avec un changement au niveau de l'odeur et du goût, en organisant des évaluations sensorielles hebdomadaires avec un panel de volontaires.

Un arôme de cola typique (mélange d'arômes 1) a été conçu en utilisant quelques molécules représentatives des différentes familles d'ingrédients aromatisants. Un deuxième arôme (mélange d'arômes 2), représentant un sirop «tout-en-un», contenait de l'acide phosphorique mais était par ailleurs identique à son homologue. Les deux mélanges ont été conservés pendant 9 semaines à 4, 23 et 40 ° C, respectivement. Chaque semaine, les mélanges ont été analysés par GC-MS. En outre, ils ont été évalués par un panel sensoriel composé de 23 panélistes, à l'aide de tests tétradiques et bidirectionnels. Enfin, 13 ingrédients ont été analysés individuellement dans des conditions identiques à celles des mélanges d'arômes 1 & 2, afin d'identifier les produits de dégradation

potentiels. Les conditions d’entreposage ont entrainé des modifications chimiques et sensorielles dans les deux mélanges d’arômes. Les modifications chimiques du mélange d’arômes 1 n’ont entrainé que des changements perceptibles après 5 semaines de stockage, et uniquement à une température de 40 ° C. Inversement, le mélange d’arômes 2 a subi beaucoup plus d’impacts chimiques et sensoriels. Cela était dû à de nombreux réarrangements des terpènes catalysés par l’acide phosphorique, qui ont créé à la fois des arômes indésirables ainsi que des déséquilibres chimiques dans le profil du cola. Les composés de dégradation clés ayant affecté le profil sensoriel étaient le p-cymène, l'α-terpinéol, le benzaldéhyde, le propylène glycol acétal et le p-crésol. Dans le mélange d’arômes 1, la formation de notes indésirables s’est produite avant une perte détectable de caractère citrique. Dans le mélange aromatique 2, cependant, la perte de caractère citrique était décelable avant la formation de notes indésirables. En fin de compte, bien que la force de la saveur n’ait essentiellement pas été affectée, l’intensité de la note d’agrumes a été compromise tandis que les notes fausses se sont intensifiées. Par conséquent, il n'est pas recommandé d'utiliser un modèle d'arôme «tout-en-un» pour les sodas à base d'agrumes comme le cola.

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CONTRIBUTIONS OF AUTHORS

This thesis is written in the manuscript style so that the chapters highlighting the thesis research could be suitably edited for publication. Two authors have been mostly involved in the thesis work and their contributions to the various articles are as follows:

D'Arcy-John Sokol is the M.Sc. candidate who planned and conducted all the experiments, in consultation with his supervisor, gathered and analyzed the results and drafted the thesis and the manuscripts for scientific presentations and publications.

Dr. Varoujan Yaylayan is the thesis supervisor, under whose guidance the research was carried out, and who guided and supervised the candidate in planning and conducting the research, as well as in correcting, reviewing and editing of the thesis.

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INTRODUCTION

Food flavours are complex mixtures of aromatic compounds designed to enhance or replicate the taste and aroma of foods [1-3]. They are widespread in the pharmaceutical and cosmetic industries and are nearly ubiquitous in the food and beverage industry, where flavour quality is arguably the most important contributor to the perception of overall product quality [1,2]. Indeed, the 2017 valuation of the global flavour and systems fragrance market exceeded 28 billion USD and it is projected to grow beyond 36 billion USD by 2022 [4]. Although lucrative, the industry is fast paced, highly technical and very competitive. There is, therefore, great incentive but also great pressure for flavour houses to develop distinct flavour that best fit the needs of their customers.

Flavour mixtures are necessarily built as much for stability as they are for organoleptic quality [4]. Even the best flavour formulation becomes a liability if it rapidly deteriorates in the end-application [2, 4]. Consequently, it should be no surprise that much greater attention has recently been directed towards understanding flavour stability [1-3]. Flavouring agents undergo many well-documented chemical changes in food systems, including hydrolysis, thermal degradation, photo-oxidation and acid-catalyzed rearrangement or isomerization reactions [4-9]. However, possibly due to the proprietary nature of flavours and the secretive nature of the industry itself, very little information pertaining to the reactivity of flavour mixtures is publicly available. With thousands of ingredients at the hands of a flavour chemist, each with a distinct set of chemical properties, flavour mixtures often become highly complex systems even prior to application [1-3, 5-8]. Additionally, since flavours are typically held in storage for weeks to months (and, in more extreme cases, for a year or longer) before their intended application in a finished product, they are liable to change prior to use if not properly stored [1-2, 5-8]. This can have serious effects on the sensory profile of the finished product [1-3, 5-9].

Chemical reactivity, and therefore flavour reactivity, is influenced by a variety of internal and external factors. The structure and concentration of each flavour ingredient, for instance, may alter the reactivity of the overall mixture [1, 2, 6]. Functional groups like hydroxyl, carbonyl and carboxyl groups expose molecules to potential chemical interactions and exhibit characteristic reactivity under specific conditions [1, 2, 5, 6]. The presence and abundance of these sites plays a

large role in determining the kinetics of the system; as a reactant becomes more concentrated, a reaction becomes more likely [6]. Alcohols, aldehydes, ketones and acids are commonly employed in flavour development, due to their potency and impactful organoleptic properties [6]. However, although some of the best ingredients fall into these groups, they also tend to be the most reactive [1,6]. This is particularly true if external catalysts are involved [1, 6, 7]. Citral, for example, is a powerfully aromatic aldehyde mix that is responsible for the smell of lemon [1, 6, 9]. It adds realism and character when properly balanced but will rapidly deteriorate when exposed to warm temperatures of 40°C and beyond or a low pH of 2 to 3.5 [1, 6, 9]. Therefore, while it can effectively bolster lemon or other citrus oils, citral should not be used in acidic juices.

Environmental variables such as temperature, pH, oxygen abundance and exposure to light represent an added layer of complexity [1, 6, 7]. These parameters place extra stress upon the chemical, accelerating either the reaction or degradation of flavours [1, 6, 7]. Temperature, pH and light influence the kinetics of a chemical system [6, 7]. Heat increases the movement of molecules, promoting collision and a more rapid rate of reaction [7]. Generally, a 10°C increase in temperature doubles the reaction rate [7]. Meanwhile, acids and light may promote reactivity by serving as catalysts [7]. A common example of this is acid-catalyzed acetal formation in flavour mixtures. When propylene glycol or ethyl alcohol, two common solvents, are blended with certain aldehydes, interactions between the carbonyl and alcohol groups occur [6, 8]. This leads to a loss of both the solvent and aromatic compound as well as the formation of a conjugate. Whereas solvent loss has little impact, the loss of the flavouring agent impacts the sensory quality of the mixture. Moreover, the acetal itself often produces an off-note, compounding the issue [6, 8]. Oxygen abundance affects flavour stability by promoting oxidation, which is the major cause of chemical and sensorial deterioration of essential oil flavour components. Clearly, selection of an appropriate flavouring ingredient must involve careful consideration of other ingredients, the storage conditions of the pure flavour and its processing conditions in the final application.

Among end-users of food flavours, the beverage industry is the most notable. In 2017, beverage companies consumed approximately a third of all flavours produced that year [4]. Beverages, more than any other application, are where flavours are held under a spotlight. Most often, beverages are mixtures of sugar, water and acid – with the likely addition of colorants and emulsifiers. In many cases,, as with sparkling waters, the profile of the final product is derived

almost entirely from added flavour. In such applications, formulated flavours tend to standout and, consequently, are responsible for much of the perceived quality. As such, beverage-based flavours can become quite complex [6, 9-10]. This is well exemplified in cola beverages. Cola flavours contain representative ingredients from the major classes of flavouring materials. This includes essential oils, aldehydes, alcohols, esters, terpenoids and acids [10]. Different solvents may also be used, depending on the solubility requirements of the flavour. Additionally, cola flavours tend to be stored for months before use, to allow for the taste and aroma to equilibrate. Lastly, cola flavours are usually applied to low pH (~pH 2.5) beverages, ultimately exposing them to relatively extreme conditions [10]. As such, cola flavours are an interesting model for the study of flavour stability.

The resilience of aroma generating chemicals is fundamental to a successful flavour and the first step to preventing degradative processes is to have in-depth understanding of their interactions. Despite the importance of understanding the stability of flavours during storage, this information is notably lacking in the literature. This is likely due to the secretive nature of the flavour industry. This presents an opportunity to obtain and provide data relating to the changes observed in pure flavours over time and the perceived sensory impact on consumers.

The objectives of this research are:

I. To study the chemical changes that occur in a cola flavour formulation over the course of a 9-week storage period as affected by temperature and pH, emphasizing the major categories of flavouring ingredients (alcohols, aldehydes, esters, essential oils) and character impact compounds

II. To determine whether the observed chemical changes coincide with an appreciable sensory impact, by organizing weekly sensory evaluations with a panel of volunteers

CHAPTER 1: LITERATURE REVIEW

1.1 Flavour science

Flavour science is a field of study rooted in chemistry that also combines elements of biology, physics and art [11]. Flavour scientists are tied closely to the fields of food product development and sensory science, where their work is very important [11]. Flavour scientists may decide to focus on chromatography or other analytical analysis [11]. They may also study flavour from a physiological perspective [11]. Alternatively, a flavour scientist may specialize in the study and extraction of botanicals or other ingredients [11]. Many, however, opt to become flavourists, where they become highly skilful at the development and application of flavour mixtures [11]. Regardless of the path, successful students of flavour science are those committed to a lifetime of learning and refinement [11].

1.2 Flavour

1.2.1 Definition

A food flavour is a mixture of aroma generating chemicals designed to enhance, modify or replicate the aroma and taste of a food [11]. Flavours are usually highly concentrated and added to food products at very low dosages [11]. Flavours are considered food additives by the United States Food & Drug Administration (FDA) and Health Canada [11]. Consequently, they are regulated substances [11]. Flavours may be categorized as artificial, natural or organic and must be labeled as such on ingredient lists [11]. Although powdered flavours exist, they are less common than liquid types [11]. Therefore, this review will focus on the latter.

1.2.2 Flavour development & character impact compounds

Flavours are developed by flavourists and are customized to meet the requirements of a specific product [11]. They minimally consist of an aromatic chemical and a solvent, but are most commonly composed of several dozen to over a hundred ingredients [6, 11]. This is in stark contrast to typical foods, which drawn their taste and aroma from hundreds to thousands of different compounds [11]. The ability of a flavourist to distill the essence of a food into a small

fraction of representative chemicals is possible due to the nature of flavour recognition [11]. The core perception of a flavour profile by the human sensory system often hinges upon a small handful of compounds, known as character impact compounds [11]. In some cases, as little as a single character impact compound can successfully evoke a specific flavour profile [11]. Vanilla extract, for example, owes its richness to hundreds of flavour compounds in precise balance [11]. However, it would be totally unrecognizable without the presence of vanillin [1, 11]. The activity of this single ingredient is sufficient for the human olfactory complex to identify an aroma as being 'vanilla', though any number of additional compounds may alter the overall organoleptic profile, adding richness, depth and complexity [11]. It is therefore impossible to develop a vanilla flavour without vanillin [11]. This highlights the power of character impact compounds as ingredients, as they are not only important but also necessary to the identifiable character of a food.

Character impact compounds form the recognition skeleton of a flavour profile and upon this skeleton a single profile may take innumerable forms [11]. A vanilla flavour, for instance, can be designed to be sweet, candy, woody, smoky, spicy or leathery, to name a few [11]. Although a vanilla flavour will always be based on vanillin, there are countless ways to design a vanilla flavour [11]. Thousands of food grade chemicals are available for use by trained flavourists, each with its own set of chemical and organoleptic properties [11]. This allows for great creativity and the development of rich, unique flavours [11]. However, ingredients must be carefully selected; as a flavour increases in complexity, it can also become more liable to stability issues [1, 2].

1.2.3 Flavour stability & aging

Like any chemical mixture, a flavour is susceptible to reactivity and potential degradation [1-3, 6]. Chemical changes occur over time due to evaporative losses, isomerization and chemical reactions between ingredients [5-9, 12]. Flavour reactions can result in the loss of desirable compounds, including character impact compounds, as well as the formation of undesirable compounds [1, 5-9, 12]. The sum of these changes over time is referred to as flavour aging [6]. This aging process can have serious effects on the organoleptic profile of a flavour. Blends are carefully designed and balanced for a specific application [11]. As components are lost or others form, an imbalance is created and there may be a noticeable sensory effect [7-9, 11, 12]. The impact of these changes is most pronounced when character impact compounds are lost and when potent off-notes form [6,8]. All flavours undergo aging to varying degrees and the extent of these

changes depends on many factors [11].

Flavour aging is influenced by various internal and external conditions. Blends high in reactive functional groups, like hydroxyl, carbonyl, carboxyl and benzyl groups, tend to be less stable [1, 6-8, 11]. Common and key aroma compounds are often rich in such sites and might require over- or under-correction to account for loss or formation in a finished product [11]. Aldehydes like vanillin (vanilla), citral (lemon) and benzaldehyde (almond) are particularly reactive [1, 6-7, 12]. Similarly, external factors may affect the kinetics of a flavour system. Temperature, pH and light, for example, impact the rates of flavour reactions [6, 7]. Temperature provides energy to the system whereas pH and light act as catalysts [7]. Alcohols, esters and terpenes are particularly sensitive to environmental variables [7]. It is important to understand the processing conditions and desired shelf life of a product before a flavour can be adequately conceptualized. Moreover, optimal storage conditions must be maintained to ensure enduring quality [7, 11]. Every flavour is given a shelf life dependent upon its composition and storage conditions [11].

Flavours are purchased in their concentrated form and shipped to a customer warehouse, where they are stored for later use [11]. These mixtures are normally ordered in industrial-scale quantities of hundreds to thousands of kilograms in order to economize as well as to ensure a consistency within a given product lot [11]. Although it is ideal to use a flavour consistently, many companies warehouse a flavour for months or longer, using the ingredient incrementally over time. This increases the risk that a flavour will undergo chemical changes by introducing oxygen, removing the flavour from refrigeration for prolonged periods, and simply lending time to chemical reactions to progress. Moreover, flavours are often stored improperly in unrefrigerated conditions or, in some tropical regions for example, in warm, humid warehouses where temperatures can exceed 40°C. Such practices usually stem from a general lack of understanding of how sensitive these mixtures are to environmental variables. Therefore, the sophistication of flavour end-users plays a large role in the lasting integrity of formulated mixtures. Overall, these factors compound the issue of quality loss resulting from changes in the chemical composition of flavour mixtures.

Flavours may contain well over one hundred components, which only complicates the

overall effect of both internal and external variables [11]. Therefore, it is imperative that flavour-aging processes be characterized, understood and ultimately controlled.

1.2.4 Cola flavour: a model system

Cola flavours can serve as an important model for the chemical changes a flavour undergoes over time. Cola flavours are fairly complex mixtures both chemically and in terms of organoleptic profile. Although they vary in the number and relative abundance of their components, they typically contain members of the major flavour chemical groups, including alcohols, aldehydes, ketones, acids, terpenoids and esters. They are also rich in a variety of essential oils, and may contain significant amounts of caramel colour [10]. Some common cola ingredients include lime and lemon oils, nutmeg, cinnamon, clove and cassia oils, ginger and coriander oils, and some fairly reactive or vulnerable flavour chemicals such as benzaldehyde, vanillin, cinnamaldehyde and citral [10, 12]. Phosphoric acid and citric acid are the acidulants of choice in cola syrups [13]. However, the exact recipes for popular colas like Coca-Cola and Pepsi are notoriously well kept [10]. Only relatively broad information is available as to their constituents and those of general cola beverages [14]. Consequently, there is very little publically accessible information pertaining to cola flavour stability. This may be due to or despite the fact that carbonated beverages represent the largest segment of the global soft drink market, which was valued at approximately 840.6 billion dollars in 2013 (see **Figure 1.1**)[15]. Among these, cola-flavoured beverages are the most popular [15]. In 2015, Coca-Cola and Pepsi colas were valued at over 70 billion and 10.8 billion USD, respectively, ranking first and fourth among soft drinks whereas Diet Coca-cola was worth nearly 14 billion USD, ranking second (see **Figure 1.2**) [16]. Their unique chemistry, combined with their economic importance in the marketplace and an absence of public information, make cola flavours an interesting and opportune case study in flavour stability.

Figure 1.1. Global Soft Drink Market Size – 2013 ($840.6 billion) [15]

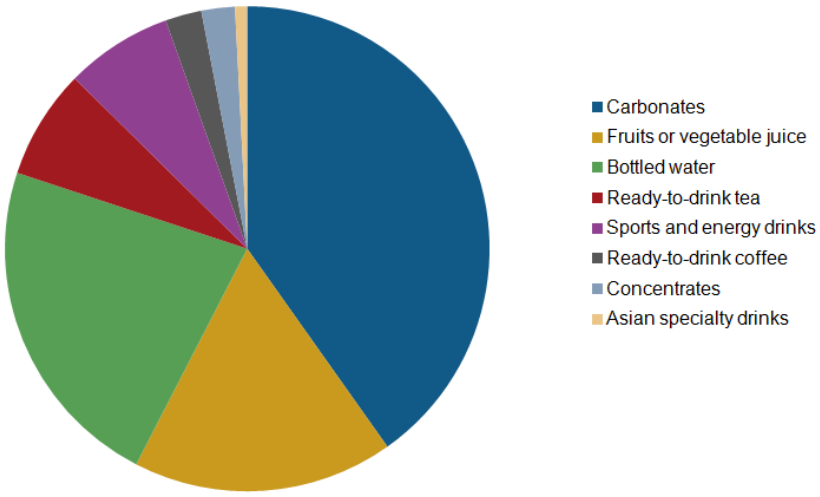


Figure 1.2. Top 15 Soft Drink Brands of 2015 [16]

	Top 15 Brands	Brand Value ($M)	Brand Value Change (%) – 2015 over 2014
1	Coca-Cola	70,042	3%
2	Diet Coke	13,799	6%
3	Red Bull	11,375	5%
4	Pepsi	10,836	16%
5	Nescafe	6,342	-5%
6	Tropicana	6.026	16%
7	Fanta	6,017	23%
8	Sprite	5,255	16%
9	Nespresso	5,224	5%
10	Gatorade	4,693	14%
11	Lipton	3,748	N/A
12	Minute Maid	2,768	11%
13	Dr. Pepper	2,697	28%
14	Mountain Dew	2,490	7%
15	Diet Pepsi	2,298	6%

1.3 Solvent & flavour stability

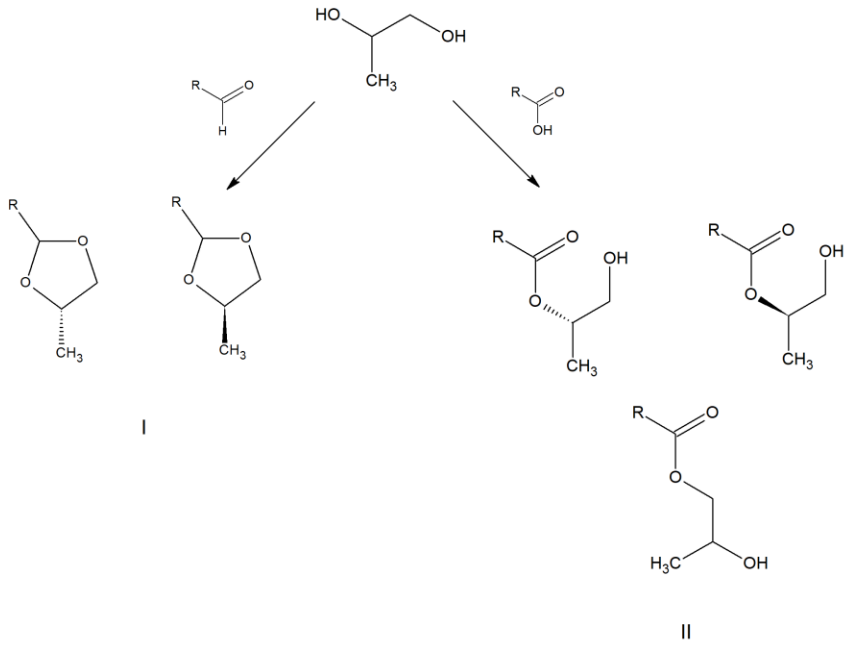
The selection of a solvent is an important step in the development of any flavour [17-21]. For liquid flavours, it serves as the main delivery system into the desired application [17]. Solvents heavily influence the stability of flavour compounds by chemically and physically altering these ingredients, and are known to physically modify food matrices as well [17, 20, 21]. Consequently, it is necessary to select a solvent not only for its ability to dissolve the necessary flavour compounds but also for its own solubility and its likely effect in the food or beverage. The most commonly used solvents in the flavour industry are propylene glycol and ethanol, with other examples including (but not limited to) triacetine, glycerine, benzyl alcohol, medium chain triglycerides, water and canola oil [1, 6, 17].

Propylene glycol (PG), also known as 1,2-propanediol, is arguably the most utilized flavour solvent, with an annual usage of approximately 7,500 metric tons by the flavour industry [17]. It is colorless, odorless, relatively non-toxic and notably inexpensive [17]. Tasted at higher concentrations, it imparts a warming sensation in the mouth along with a faint, sweet taste [17]. Though it has many desirable properties, PG is also known to interact with a number of important aromatic chemicals [17-19]. Similarly, ethanol finds widespread use in flavours. It has a more potent odor than PG but one that remains relatively inconspicuous in flavours. Moreover, it is easily miscible with a great range of flavour ingredients and, unlike PG, can dissolve essential oils [22]. Flavours are susceptible to three well-characterized reactions in alcohol solvents such as PG and ethanol, all involving the active hydroxyl groups found in the solvent system [17].

The first is a reversible reaction that involves the reactive carbonyl group of a molecule and the hydroxyl group of the solvent [17]. Depicted in **Figure 1.3** (I), this reaction is acid catalyzed and favors a movement towards equilibrium between the free aldehyde or ketone and its corresponding PG- acetal or PG-ketal [17]. Acetal formation can be divided into seven steps. Initially, the carbonyl group of the flavour chemical becomes protonated by the acid [17]. This opens up the carbonyl for nucleophilic attack by the alcohol group [17]. The conjugate is subsequently deprotonated to form a hemiacetal [17]. In step 4, the alcohol group of the hemiacetal becomes protonated [17]. The alcohol is then dehydrated, opening it up to a second nucelophilic attack by the alcohol group of the solvent [17]. Lastly, the reactant is deprotonated by water,

effectively forming the acetal [17].

Figure 1.3. Propylene Glycol Reactions with Aldehydes and Ketones [17]



Condensation of glycols results in 1,3 dioxans, five-member ringed compounds whereas glycerols generate 1,3 dioxalans [19]. Water is produced as a byproduct of this reaction and has an inhibitory effect on further acetal formation [19]. Water in a flavour also dilutes proton concentration [19]. This, by extension, slows the rate of reaction because less acid is available to protonate carbonyl groups [19]. Therefore, flavours containing water may delay acetal formation [19]. Conversely, the addition of an acid enhances the reactions. A recent study (2017) confirmed that PG and ethanol flavour mixtures form significantly more acetals when acidified with 0.03 mmol of HCl/g of mixture [6]. This is to be expected, because acetal formation is acid catalyzed [6]. In addition to water and acidity, the number of available hydroxyl groups from the solvent system and the nature of the aromatic ingredients influences acetal formation [6]. Acidified PG exhibits more extensive acetal formation than ethanol under identical conditions, as every PG molecule contains two hydroxyl groups [6]. Similarly, dicarbonyls like diacetyl show notably higher reactivity than single carbonyl-containing compounds [6]. It is important to note that this reaction is slowed – and may be entirely inhibited – by neutral or basic conditions [18]. The addition of $NaHCO_3$ to carbonyl-based flavours helps stabilize these compounds in propylene

glycol by neutralizing the acid catalyst [6, 18].

Whereas acid catalyzes the formation of acetals and ketals in the presence of PG or ethanol, acid treatment coupled with heat promotes the hydrolysis of these complexes and restoration of the full initial flavour [18]. An example of this phenomenon was demonstrated using an affected cherry flavour [18]. A carbonated beverage was prepared using a concentration of 0.5% of the flavour, which contained PG-acetal complexes, 11% sucrose and 0.8% citric acid [18]. After stirring and subsequent extraction of the flavour from the beverage using ethyl ether, gas chromatographic analysis did not detect acetals [18]. Conversely, PG-acetal complexes in products fail to hydrolyze completely, if at all, under neutral or basic solutions [18]. Additionally, these complexes are retained in solutions containing both water and oil phases [18]. This is likely due to the higher solubility of acetal in oil phases [18]. Many important aromatic compounds are susceptible to this degradation pathway, including vanillin (vanilla), benzaldehyde (cherry, almond), cinnamaldehyde (cinnamon), citral (lemon) and raspberry ketone (raspberry), to name a few [17, 18]. The resulting PG-acetal complexes, when left uncorrected, produce negative smell and taste effects reminiscent of glue, solvent and even petroleum [17]. In cherry flavours, for example, the character impact compound benzaldehyde reacts with hydroxyl groups to form benzaldehyde propylene glycol acetal, a chemical with a petroleum odor [6,18]. Therefore, the effect of acetal formation on the overall organoleptic profile of a flavour is twofold: not only does the loss of a key ingredient occur but also a potent off-note forms. Conversely, the loss of solvent is relatively benign. This is because the solvent is abundant and, in the case of PG or ethanol, contributes relatively little to the aroma [6]. Due to the common use of propylene glycol and the variety of significant compounds affected, this stands as one of the most important degradation reactions in flavour systems [6].

In addition to the formation of acetals and ketals, outlined above, there are two other significant reactions that can occur between aromatic compounds and propylene glycol: esterification and transesterification [17]. Esterification occurs when PG reacts with an acid to produce PG-acetates and butyrates [17]. Depending on the group interaction, any of four compounds may be produced. These are PG-1-monoesters, PG-2-monoesters (two stereoisomers) and PG-diesters (see **Figure 1.3-II**)[17]. If propylene glycol is in abundance, as is generally the case when it is the primary solvent, transesterification may take place [17]. During

transesterification, PG replaces the alkyl group of the ester resulting in similar byproducts as in esterification [17]. Although these reactions may have effects on the quality of the flavour over time, through the loss of positive constituents or the formation of negative ones, they also provide a form of protection to flavour companies by increasing the complexity of the flavour and, by extension, the difficulty for a competitor to match it [17].

1.4 Aroma chemical stability

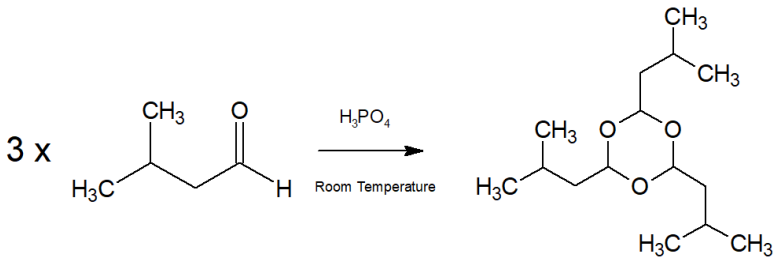
1.4.1 Aldehyde

Aldehydes are a major class of chemicals important to the development of flavours. Found widespread in nature, they are responsible for many of the rich aromas associated with foods. Often, a particular aldehyde will play a central and essential role in recreating the profile of a food type [9]. Unsurprisingly, many of the most powerful flavour ingredients belong to this family of chemicals [11]. Notable examples of aldehydes used in flavouring include vanillin, citral, benzaldehyde and cinnamaldehyde, though the list is extensive [9, 23]. Vanillin is a phenolic aldehyde responsible for vanilla flavour whereas citral is a terpenic variety key to lemon aroma [12, 24]. Benzaldehyde is a benzylic aldehyde used to recreate almond and cherry flavours while cinnamaldehyde consists of both a benzylic and simple unsaturated aldehyde [23]. Therefore, aldehydes span a diverse range of structures and organoleptic profiles. However, aldehydes are also typically reactive and affect the overall stability of flavours where they are used [9]. As observed in the presence of propylene glycol and ethanol, aldehydes readily condense with hydroxyl groups to form acetals [9, 17, 18]. Similarly, aldehydes undergo condensation reactions with carbonyl compounds, resulting in aldols [9].

Aldehyde stability is influenced by environmental conditions, as in all chemicals. Simple aldehydes are prone to trimerization under both basic and acidic conditions [25, 26]. Isobutyraldehyde, an important component of chocolate flavours, reacts to form trimers when exposed to a base catalyst. Other major flavour aldehydes also display base-catalyzed trimerization, including octanal, nonanal, decanal and undecanal. Examples of aliphatic aldehydes ranging from C1 to C18 have also been shown to undergo acid-catalyzed trimerization. For example, isovaleraldehyde – another key component of chocolate flavours - readily forms a trimer, 2,4,6-triisobutyl-1,3,5-trioxane in the presence of mineral acids like phosphoric acid (see **Figure**

1.4) [25]. Its trimer does not have the distinct chocolate character of its monomer, but rather has creamy, sweet and dairy-like properties [11]. Although this is not necessarily unpleasant, it can create disequilibrium in the profile and a loss of chocolate character recognition, which is nevertheless undesirable.

Figure 1.4 Trimerization of iso valeraldehyde to 2,4,6-triisobutyl-1,3,5-trioxane



Essential oils rich in aldehydes, such as lemon and coriander, are especially susceptible to degradation when exposed to air, light and improper temperature conditions [27, 28]. In cinnamon oil, it was shown that heat causes cinnamaldehyde to decompose into benzaldehyde and glyoxal at temperatures around 70°C. This occurs via heat-induced cleavage of the carbon-carbon bond in the presence of oxygen. This resulted not only in a loss of characteristic cinnamon flavour but the generation of a powerful off-note in the form of benzaldehyde [23]. Two important examples that demonstrate the sensitivity and reactivity of flavour aldehydes are those of vanillin and citral.

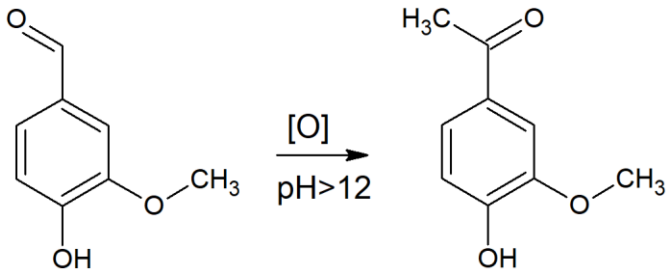
1.4.1.1 Vanillin

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most important flavouring materials in the food industry [1, 24, 29, 30]. As a pillar of all vanilla flavours, vanillin sees widespread use in a plethora of applications ranging from confections, beverages and baked goods to cosmetics, fragrances and pharmaceuticals. It is also commonly used in flavours for its ability to smoothen or balance the organoleptic profile, even in types without an obvious vanilla character. This is the case in cola flavours, for example, in which vanillin enhances the profile without ever becoming a dominant force. Vanillin is a phenolic aldehyde with an additional hydroxyl group [1, 30]. Consequently, it is capable of undergoing a variety of chemical reactions. As an aldehyde, it

is prone to condensation reactions and substitutions [8, 17, 18]. Moreover, its phenol group exposes it to esterification and etherification reactions [1]. When the hydroxyl group is protected, vanillin is also susceptible to oxidation to vanillic acid [1, 29]. The reactivity of vanillin has implications for the stability of this compound in flavours and foods.

Vanillin oxidation is dependent on pH [29, 31]. Under more basic conditions (pH >12), the rate of oxidation depends linearly on the concentrations of both vanillin and oxygen. This pathway involves both a Dakin-type reaction and a demethoxylation step, and it generates vanillic acid as a primary product [1]. The former is the reaction of p- hydroxybenzaldehyde with hydrogen peroxide [1] whereas the latter occurs via methoxy hydroquinone [1].

Figure 1.5 Oxidation of vanillin to vanillic acid



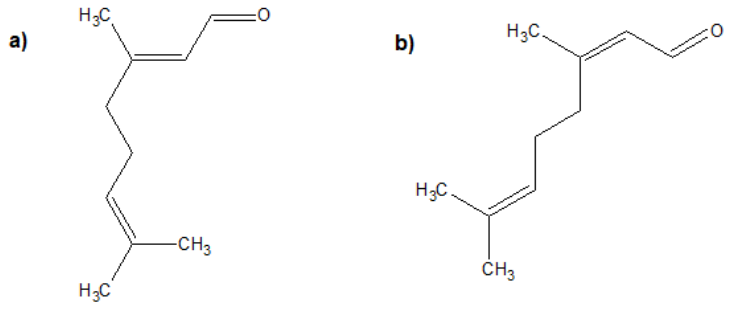
Below these levels (pH<12), however, oxidation no longer occurs directly. Instead, the reaction follows an autocatalytic oxidation mechanism that depends on the concentration of vanillin but is independent of oxygen concentration. Under these conditions, radical propagation becomes the main cause of vanillin deterioration [29]. Oxidation of vanillin becomes inhibited by more acidic conditions. A study of vanillin oxidation in dairy products showed that this process becomes almost totally inactive below pH 4. This same study demonstrated the effect of enzyme activity on vanillin stability [31]. The natural dairy enzymes xanthine oxidase and peroxidase are both, in part, responsible for vanillin oxidation [31]. Xanthine oxidase generates vanillic acid whereas peroxidase produces the vanillin dimer, divanillin [31]. Although temperature treatments like pasteurization and ultra-high temperature (UHT) pasteurization effectively inactivate these thermolabile enzymes, continuous heat also accelerates oxidation. A study of birch syrup showed that heat (upwards of 100°C) increasingly favored vanillin oxidation [32]. Although pH is the primary determinant of vanillin oxidation, other environmental factors including enzymatic acitivity and temperature also play a role.

Vanillin also displays greater instability in high moisture foods and beverages. In the presence of sufficient moisture, vanillin may form hydrogen bonds, hydrophobic interactions and electrostatic interactions with proteins [33-37]. Flavour aldehydes like vanillin may also form Schiff bases by binding protein amino groups but this is not a significant degradation pathway for this compound [36]. Because environmental conditions often interfere with direct binding between proteins and vanillin in the form of conformation changes, electrostatic interactions are typically most common [38]. Such interactions are especially problematic in applications where vanilla is the desired flavour profile because loss of vanillin directly diminishes the intensity of vanilla perception [35]. Surprisingly, there is little information pertaining to off-flavours produced by the oxidation of vanillin. This is perhaps due to the fact that vanillic acid has a creamy, milky character not necessarily out of place in typical vanilla applications, and retains some of the sweetness and vanilla flavour associated with vanillin.

1.4.1.2 Citral

Citral (3,7-dimethyl-2,6-octadienal) is a key component of citrus oils and a popular aldehydic ingredient in the flavour industry [39]. Known for its fresh, juicy aroma evoking lemon peel, this chemical is critical to the recognition of lemon flavour, thus being categorized as a character impact compound [40, 41]. In addition to its central role in lemon, citral forms part of the recognition skeleton of lime flavours and plays a lesser but significant role in other citrus flavours [41]. Consequently, citral has seen widespread use in both food and beverage applications. Both lemon and lime oils are fundamental components of cola flavours [10]. Although a powerful tool in flavour development, citral is also known to be highly unstable when stored under acidic conditions, resulting not only in a loss of fresh lemon aroma but, more detrimentally, in the formation of off- flavours [12, 39, 41, 42]. Because of its particularly important role in flavouring, the stability of citral has been the center point of many studies over the decades.

Figure 1.6 Geometric isomers of citral a) geranial and b) neral



Citral undergoes a complex and extensively studied degradation reaction under acidic conditions. Chemically, citral is a monoterpene aldehyde mixture that consists of two geometric isomers: geranial, the *cis* form and neral, the *trans* form (see **Figure 1.6**), which exist naturally at a ratio of 3:2 [39, 41]. At a pH of 2 to 3.5, geranial isomerizes to neral, a transformation that precludes cyclization and initiates the degradation process. The isomerization process occurs more rapidly than cyclization and, consequently, there is no appreciable difference between the stability of neral and that of its isomer under these conditions. Cyclization of neral produces two *p*-menthadien-8-ols (*p*-menthadien- 8-ol and *p*-menthadien-4-ol), which can then undergo either oxidation or disproportionation reactions. Both pathways lead to the formation of *p*-cymen-8-ol and generate α-terpineol as a reduction product. The *p*-cymen-8-ol molecule then becomes dehydrated and converted to more stable by-products, including α-p-dimethylstyrene, *p*-cymene, *p*-cresol and *p*-methylacetophenone. Although details remain unclear, it has been suggested that *p*-methylacetophenone is produced by the subsequent oxidation of α-*p*-dimethylstyrene. The most important contributors to off-flavour and off-odor in lemon flavour due to citral decomposition are *p*-cresol and *p*-methylacetophenone, the former having a phenolic taste and the latter giving a bitter almond taste [39].

The rate of deterioration of citral is significantly affected by both storage temperature and pH [12]. A study of by Freeburg, Mistry and Reineccius (1994) demonstrated these effects clearly [12]. Two flavour emulsions were prepared: one using untreated lemon oil and another using lemon oil treated to completely remove its natural citral content. Four simple beverages were then prepared using these emulsions: a beverage with citral- containing lemon oil adjusted to a pH of 3.3, a beverage using treated lemon oil with a pH of 3.3 and identical beverages at a pH of 2.7.

The pH was adjusted to the appropriate level using a lemon juice concentrate. Once prepared, the flavoured beverages were stored at 4, 24, 35 and 45°C. Samples were removed at varying time intervals (every 4 days for 4°C samples, every 24 hours for 25°C samples, every 16 hours for 35°C samples and every 8 hours for 45°C samples). Lemon oil was extracted from these samples and analyzed by gas chromatography to quantify citral and identify degradation products. Citral broke down most rapidly in the samples stored at 45°C; within 40 hours of storage only trace levels remained. Conversely, those beverages stored at 4°C exhibited the least amount of loss, though still considerable, with citral levels reduced to 30% of their initial concentration over a period of 20 days. It was also confirmed that citral broke down more quickly at pH 2.7 than at pH 3.3, indicating that citral degradation is also pH dependent [12].

This study also measured the resilience of other major constituents of lemon oil. The natural components limonene, α-pinene, β-pinene and α-terpinene all decreased over time whereas off-flavours such as *p*-cymene, α-terpineol and terpinen-4-ol increased. The chemical *p*-cymene, a known degradation product of citral, has also been associated with the breakdown of γ-terpinene. Consequently, these components of lemon oil both contribute to off-flavour over time. Interestingly, the results of the study showed no appreciable difference in the formation of *p*-cymene between any of the samples. In both the natural and citral-free lemon oils, γ-terpinene was initially found at about 5 times the level of citral in the natural lemon oil sample. Since *p*-cymene is the major breakdown product of γ-terpinene, an initial concentration difference that large may have shrouded the effect of citral. In both beverage types, it was shown that *p*-cymene production is pH-dependent, with greater acidic conditions favoring its formation [12]. Similar results were seen with α-terpineol, another degradation product of citral. No significant difference was found in α-terpineol concentrations after storage of regular lemon oil and citral-free beverages. However, like other breakdown products discussed above, there was greater formation of α-terpineol in the lower pH samples. Overall, this suggests that terpenes other than citral are also generating α-terpineol [12].

Although chemical transformation was shown to occur, it is important to consider the sensory effects of these changes. The organoleptic character of lemon oil was evaluated based on three categories: off-flavour, fruity/floral and throat burn. A panel of judges was trained to recognize and score these characteristics accordingly. An analysis of variance was then performed on the

results of each category. There was found to be a significant difference in fruity/floral notes between the citral-containing and citral- free beverages until about 40 hours of storage at 45°C. Until this point, the citral- containing beverage scored approximately twice as high in perceived fruity/floral character. Beyond this point, however, no notable difference was determined. With regards to off-flavour, no significant difference was observed. In both beverage types, the main off-flavour was the development of a disinfectant-like taste mostly apparent in the mouth and a turpentine/paint-like taste and aroma, linked to the formation of α- terpineol, γ-terpineol and terpinen-4-ol. In terms of throat burn, the citral-free beverage scored higher, initially. Any differences disappeared after 40 hours of storage at 45°C, coinciding with a total loss of citral. As citral levels decreased, the citral-containing beverage scored increasingly higher for a throat burn effect, indicating that citral played a masking role [12]. Overall, the citral-free beverage changed less during storage than its counterpart.

In summary, lemon oil quality loss occurs during storage as a result of both the degradation of desirable compounds and the formation of undesirable ones. Citral, a major contributor to the fruity/floral character of lemon, is susceptible to loss over time, even under refrigerated conditions. Other major constituents like limonene, α-pinene, β-pinene and α-terpinene, though less important to lemon flavour recognition, are more significantly responsible for the formation of off-flavours, largely in the form of oxidation products such as *p*-cymene, α-terpineol and terpinen-4-ol. Odor threshold plays a central role in the perception of off notes, explaining why compounds with low odor thresholds have more important effects than more prevalent constituents that have higher threshold ranges. These chemical changes are especially important in applications like cola, where lemon oil is key.

1.4.4 Aliphatic esters

Aliphatic esters are core constituents of many flavours and among the most commonly used ingredients in the industry [11, 43]. They are widespread in nature but are found particularly in fruits where they are responsible for the pleasant, fruity aroma. Whereas certain esters are inextricably linked to specific profiles, like iso-amyl acetate to banana, ethyl propionate to grape and ethyl-2-methyl butyrate to apple, others, such as ethyl acetate and ethyl butyrate, contribute a general and indistinct fruitiness [11, 40, 43]. This makes them important to an extensive range of

food applications, from simple fruit flavours to complex beverages like colas, wine and beer [44, 45]. Flavour esters are diverse but tend to be relatively volatile, hydrophilic and have short-chain structures. These characteristics are the primary cause of their powerful aroma but also result in typically lower stability in comparison to other flavour components [44]. Esters are particularly sensitive to temperature but also show a susceptibility to hydrolysis when exposed to adverse pH conditions [44, 45]. Additionally, their concentration and physicochemical properties affect their retention in flavour solutions [44].

Temperature plays a critical role in ester stability. When a flavoured product is heated during processing or prior to consumption, evaporation occurs at the surface of the food or beverage [46, 47]. This may result in steam distillation of flavours, especially those rich in high-volatile components like esters [46, 47]. Within the same chemical class, loss due to volatilization is correlated to boiling point [48]. However, Henry's law constants provide a more accurate predictor of ester loss, because they account for solubility in the food system [48]. When heated, flavour esters readily migrate through the aqueous phase of a food matrix, where they are drawn out by evaporation and lost [46, 47, 48]. Depending on the boiling points and Henry constant of the flavour components, anywhere from 10 to 95% may be lost in this way [48, 49]. This is highlighted in baking applications, where flavour is drawn out with steam [48]. Steam enhances mass transfer and readily carries esters across the product/air boundary [48]. Overall, flavour loss increases with increased moisture content whereas flavour retention increases with increased fat content [48]. Low-boiling components volatilize or 'flash off' more readily than those with higher boiling points. While the former are depleted, the latter become more concentrated, creating an imbalance in the flavour profile [47, 49]. This can be corrected by overcompensating for more sensitive constituents when formulating the flavour, though a complicated series of reformulations may be necessary to fine tune the final profile. Alternatively, compounds with lower volatility should be prioritized when applied to products that will be exposed to high heats, but this is not always possible [48]. Water vapor migration is considered the most important factor affecting flavour retention in food [47].

A 2012 study of flavour release kinetics by Khio et al. demonstrates the effect of temperature on rates of flavour loss to volatility [44]. Solutions of 10 different esters were prepared and incubated at several temperature conditions (30, 40 and 60°C) for 30 minutes. The esters

chosen represented a spectrum of polarities and volatilities. Temperatures of 30°C and 40°C were selected to represent typical storage conditions, whereas 60°C was used for greater understanding of release kinetics of higher boiling components. The volatility of these compounds increased with temperature but their release behavior varied. Larger compounds such as ethyl laurate followed zero-order kinetics at lower temperature of 30 and 40°C but followed second-order kinetics at 60°C. A similar but slightly shorter ester, ethyl decanoate, followed first-order kinetics at 30°C but second-order kinetics at higher temperatures. Conversely, mid-range esters like ethyl hexanoate, hexyl acetate and *cis*-3-hexenyl acetate followed second-order kinetics at 30°C and first-order kinetics at higher temperatures [44]. The volatility of esters depends directly on temperature but precise reaction kinetics varies.

Solvent also affects the retention of flavour components. The above esters were additionally prepared in aqueous solutions consisting of varying levels of ethanol. Four ethanol levels were chosen: 5%, 10 and 20%, and 40%, to represent the alcohol contents of beer, wine and spirits respectively. Ethanol is a common solvent for flavours, which helps to dissolve and disperse flavour molecules evenly [11, 44]. This study showed that greater amounts of alcohol improved the overall retention of the esters [44]. This protective effect may be the result of micelle or agglomerate formation, particularly with smaller esters [44]. Lower alcohol content also caused a slower release of polar flavour compounds in the headspace, such as ethyl hexanoate, hexyl acetate and *cis*-3-hexenyl acetate, but higher incidence of less polar constituents like isoamyl hexanoate, ethyl decanoate, ethyl laurate and ethyl myristate [44]. Water molecules repulse less polar, more hydrophobic molecules and trap the opposite [44]. As little as 10% of ethanol has been shown to significantly increase flavour retention [44]. By altering the diffusivity of flavour compounds in solution, ethanol stabilizes them, effectively slowing their release and loss [44].

The initial concentration of a flavour component influences its rate of loss. In general, higher levels of a constituent lead to greater volatility [44]. However, the proportionality of this effect is not the same for all compounds. Diffusion of a flavour molecule into the matrix is affected by its chemical properties, interactions with the matrix and competition with other chemicals in the mixture [44]. It is promoted by a high concentration between the component and the headspace above it, with volatility sharply increasing and then decreasing as headspace became filled [44]. Moreover, diffusivity decreases with molecular size as a result of steric crowding [44]. Similarly,

crowding and competition from other compounds may improve retention [44].

Lastly, esters also undergo hydrolysis when exposed to high temperature or adverse pH conditions. The rate of hydrolysis of ethyl esters is slower than that of acetate esters [45]. Hydrolysis of ethyl esters also increases with molecular weight [45]. This is explained by the inverse relationship between molecular weight and the activation energy required for degradation; esters with higher boiling points require less energy to react [45]. The rate of reaction is also accelerated by higher temperature and lower pH [45]. These parameters both exhibit linear relationships with ester hydrolysis [45]. Protons act as reaction catalysts and are approximately 100 times more powerful than polyprotic acids such as tartaric acid [45]. Unlike the protective effect seen on volatile retention, ethanol shows no effect on hydrolysis [45]. Hydrolysis of esters occurs when they are present in excess of equilibrium concentration [45]. The stability of esters, therefore, is influenced by temperature and pH, the solvent, and their chemical concentration therein.

1.5 Essential oil & terpene stability

1.5.1 Overview of essential oils

Essential oils are purified liquid mixtures of lipophilic aroma compounds, derived from the extraction, distillation or expression of a plant component [7, 50, 51]. Citrus and spice oils form the backbone of any cola flavour, with lemon, lime, cinnamon, nutmeg and cassia oils among the common ingredients used [10]. Typically named for the plant part from which it is obtained, an essential oil represents the distinct aromatic character of its source, also known as its essence. In many cases, different plant fractions of a single plant can be used to produce different essences. For instance, cinnamon bark oil and cinnamon leaf oil are both derived from the cinnamon tree. Although similar to spices, which are dried and ground from the same active plant parts, essential oils are much more potent and their application in food and beverage is subject to more stringent regulations [7, 50].

Essential oils are generally composed of 50 to over 100 different compounds though in some cases this can be significantly higher [7, 50]. These consist mostly of terpenes and terpenoids of molecular weights lower than 300 Da, as well as some aromatic and aliphatic constituents [7, 50]. These chemicals are found at varying proportions, even within the same species [7, 50].

Variability can be attributed to a number of factors, including country of origin, cultivar, soil conditions, season, temperature, altitude, plant part and method of isolation (solvent extraction, distillation or expression) [7, 50]. The relative abundances of essential oil components are measured via GC analysis, usually using flame ionization [7, 50]. Typically, 85-95% of the whole volume is composed of one to three main constituents, with the rest being considered minor contributors [7, 50].

In certain cases, based on the proportions of as little as one key chemical, a decision on quality may be made. Eucalyptus oil, for instance, is commercially sold for its eucalyptol content (e.g. >70% or >80%) [7, 50]. Eucalyptol is a character impact compound of eucalyptus oil and a lower eucalyptol level devalues the oil. However, it is important to note that the most abundant constituent is not necessarily the most significant for quality or sensory purposes. The aroma of an essential oil is far more dependent on the odor threshold values of its constituents, which vary with structure and volatility [39, 50]. Limonene, for instance, is by far the most abundant component of lemon oil but relatively unimportant to its sensory profile [12, 41, 42]. Conversely, citral, found at much lower levels, is considered key to lemon character recognition [12, 39, 40, 50]. In many cases, the same chemical will have a very different effect depending on its concentration and, surprisingly, may be preferred at lower or higher levels. Sotolon (3-hydroxy-4,5-dimethyl-2(5H) furanone), is a good example of this. In fenugreek, sotolon is present at desirably low levels (near-threshold levels of 0.3mg/L) where it imparts a caramel-like aroma [52]. At these levels, it is considered a character impact component of fenugreek [52]. However, if sotolon becomes increasingly concentrated, as it does in fenugreek seeds where it is present at approximately 3,000 times its threshold level, its aroma transforms to resemble that of curry, thus becoming an off-flavour in certain applications [52]. Similarly, some compounds that may be important in one essential oil might be problematic in another. The monoterpene alcohol α- terpineol exemplifies this. In lime oil, α-terpineol is crucial to the character recognition of the flavour, giving it a green and, to an extent, candy flavour [52]. For quality purposes, it must be present at a minimum concentration, particularly in distilled lime oils. Conversely, in orange oils, α-terpineol is a degradation product of limonene that gives a terpene-like, old, partially rancid flavour and is an indicator of poor quality [52]. The role of a compound as a positive or negative note is often contextual and depends on its concentration.

Although the number, nature and relative abundances of essential oil constituents influence the overall quality and richness of its organoleptic profile, certain chemicals are more important than others. Despite the potentially large number of components in an essence, character recognition can boil down to as little as one compound. The abundance of anethole in anise oil, thymol in thyme oil and carvacrol in oregano oil are but a handful of the many instances where this holds true [7, 40, 52]. Because of the commercial importance of essential oils in flavouring, fragrance, medicine and cosmetics, a significant amount of focus has been directed towards understanding the stability of these chemicals and how that stability can be improved [7]. Typical environmental stressors like temperature and pH play a relatively small role in the chemical stability of essences [7]. Ultimately, the stability of an essential oil under normal conditions becomes a question of its resilience to oxidative deterioration [7].

1.5.2 Oxidation

Oxidation is the major cause of chemical and sensorial deterioration in essential oils [7]. It is a destructive process that generates off-flavour compounds at the expense of important oil constituents, particularly terpenes [7]. Oxidation in essential oils occurs via two different mechanisms: autoxidation and photosensitized oxidation, both of which are influenced by internal and external energy conditions [7, 53]. Internally, the diversity and relative abundances of oil constituents must be considered. Structural properties are important determinants of oxidative stability and thus the nature of each chemical constituent plays a role [7]. Additionally, the presence of potential catalysts in the form of trace metal contaminants may influence the rate of oxidation [7]. External factors, including temperature, oxygen availability and exposure to light, also significantly affect reaction rates [7]. Oxidative stability is a key indicator of the quality of an essential oil and its shelf life [7].

Essential oils are susceptible to two types of oxygen: atmospheric triplet oxygen and singlet oxygen [7, 53]. Triplet oxygen, in its ground state, has two unpaired electrons in its $2p\pi$ antibonding orbitals, which produces a permanent magnetic moment [7, 53]. Consequently, it is a radical and will readily react with lipid radicals in order to conserve its angular momentum [53]. Thus, propagative autoxidation reactions are observed [53]. Photosensitized oxidation, conversely, requires light or other sensitizers and involves singlet oxygen from the atmosphere [7, 53]. Singlet oxygen has one fully occupied $2p\pi$ orbital and another that is empty [53]. Unlike triplet oxygen, it

is a non-radical [53]. Singlet oxygen instead attacks areas of high electron density and is therefore reactive with double bond sites in unsaturated molecules [53]. Both of these processes are affected by energy conditions [53]. However, photosensitized oxidation rarely occurs under normal storage conditions [7]. This is because singlet oxygen is sparse in the atmosphere due to high-energy requirements needed to excite the ground state molecule [7, 54, 55]. Sensitizer molecules like chlorophyll facilitate this process in nature but are usually eliminated from distilled essential oils [54, 55]. Consequently, the focus will be on autoxidation reactions. The influence of composition, structural properties, catalysts, temperature, and light and oxygen availability on the rate of oxidation is discussed.

1.5.3 Chemical structure & composition

Chemicals vary in their stability to oxidative breakdown. Naturally, the extent to which a molecule will react with oxygen is highly dependent on its structure. Certain chemical groups are inherently more susceptible. In essential oils, autoxidation is the most significant form of oxidative deterioration [7]. Autoxidation occurs spontaneously at room temperature in the presence of atmospheric oxygen and is known to occur more readily in unsaturated compounds containing allylic and benzylic hydrogen atoms [56, 57]. Autoxidation is a free radical chain process that is initiated by hydrogen abstraction to form an alkyl and a radical. The radical then reacts with oxygen to form a peroxyl radical that is capable of abstracting hydrogen from another molecule, effectively propagating the chain [57]. Allylic hydrogen abstraction generates radicals that are resonance-stabilized, which require a lower energy of activation [56-58]. This is why molecules rich in these groups are preferentially targeted by oxygen.

Terpenes and terpenoids possess a distinct chemical structure making them particularly susceptible to oxidation [59]. In general, terpenes consist of one or more isoprene (C_5H_8) subunits whereas terpenoids are molecules based on 5-carbon isoprene units and which have undergone a chemical modification (e.g. oxygenation)[59]. The isoprene building block permits diverse structural possibilities, with a great variety noted in nature [59]. Whereas polymerization is the fundamental categorization method for this extensive class of organic molecules, the added potential for internal carbon-carbon linkages and cyclization allows for further sub-categorization [59]. Typically, greater polymerization leads to greater susceptibility to oxygen [59]. Generally,

terpenes and terpenoids can be divided into acyclic, monocyclic, bicyclic and tricyclic compounds with further distinction given to species of similar lead compounds of that class [59]. Although composition and relative proportions may vary, terpenes make up the bulk of essential oil constituents and are arguably the most important fraction [59]. The chemical arrangement of terpenes and terpenoids is the root of their unique sensory properties but also renders them susceptible to environmental stressors [59].

A recent study (2011) of the effect of storage conditions on essential oils demonstrated the increased vulnerability of polyunsaturated monoterpene hydrocarbons to autoxidation [60]. Ocimene, β-myrcene, α-phellandrene, terpinolene, α -terpinene and γ - terpinene, all compounds rich in allylic hydrogen atoms, exhibited significant deterioration when exposed to atmospheric levels of oxygen [60]. Comparatively, α -pinene, β -pinene and ∂ -3-carene, which each contain just one less double bond, suffered notably less loss [60]. Not surprisingly, eucalyptus, pine and thyme oils, all of which contain large concentrations of polyunsaturated terpenes, underwent the greatest decomposition among the oils that were evaluated [60]. Eucalyptus oil was the most transformed; neral and geranial values decreased while carvone, a decomposition product of limonene, increased with storage time [60].

The extent of molecular autoxidation also depends on the stability of the hydroperoxides that form throughout the reaction [57]. The radical propagation step, in which radicals and oxygen interact to produce peroxides, determines the products of the oxidative chain reaction [57]. Because hydroperoxides may go on to further generate radicals, more resilient compounds tend to accumulate and react more extensively than those that rapidly degrade [55]. Therefore, more stable peroxides promote further autoxidation. Peroxide stability is correlated to the strength of the C-$OO^{\cdot}$ bond that forms and breaks during propagation, though exceptions exist [61]. It has been shown that increasing alkyl substitutions strengthens this bond [61]. Electron-donating substitutes induce and add a hyperconjugative effect, more greatly stabilizing the products [61]. Hydroxyl and methoxyl groups, for example, contribute radical stabilizing enthalpy to the C-$OO^{\cdot}$ bond in the parent peroxyl [61]. It should be noted that other substitutes may have the opposite effect by acting as electron-withdrawing groups [61]. Terpenes, especially polyunsaturated species, express the structural preconditions that favor autoxidation and generate stable radicals that promote

propagation of the reaction chain [7, 59]. Thus mixtures rich in terpenes, of which essential oils are a part, are more prone to oxidative deterioration.

The chemical stability of a compound is additionally influenced by changes occurring in other components within the mixture. It is well established that fatty acids easily undergo autoxidation. However, they also readily initiate this process in terpenes and other components by transfer of reactive oxygen [62-64]. Although essential oils do not contain polyunsaturated fatty acids, they become susceptible in applications where these compounds are present. Conversely, certain compounds may provide protection against these deteriorative processes. Several major constituents of essential oils show antioxidant properties [62, 65, 66]. Oregano oil has been shown to inhibit oxidation, which can be attributed to two of its subcomponents: carvacrol and thymol [67, 68]. Similarly, thyme oil, which contains upwards of 80% thymol, demonstrates significant resistance to autoxidation [68]. Carvacrol and thymol are examples of primary antioxidants [68]. Primary antioxidants donate hydrogen atoms or electrons to free radicals [65]. This stabilizes the radical and thereby prevents further propagation reactions, effectively terminating the chain [65]. The resulting antioxidant free radical can provide additional stability by scavenging free peroxyl radicals to form peroxy antioxidants [65, 66]. The efficacy of a primary antioxidant is greater in compounds with ethyl or butyl tertiary substitutions, as compared to basic methyl groups, as these generate more long-lived radicals [65, 66]. Although primary antioxidants are effective at relatively low levels and may become pro-oxidants at higher levels, the antioxidant activity of carvacrol and thymol increases with concentration [60, 69, 70]. Clearly, the matrix of an essential oil affects its overall stability and the stability of its constituents.

1.5.4 Transition metal contamination

Contamination of essential oils with transition metals may enhance oxidative deterioration. Trace quantities of metals are introduced inadvertently via distillation methods or through leeching from metal containers during transport and storage. Ferrous and copper ions in particular are known to increase oxidation [53]. Metals affect autoxidation by reducing the energy requirements of the initiation step to as low as 63 kJ/mol [71]. They may also favor propagation reactions by generating hydroxyl radicals from hydrogen peroxide. Copper is the most effective accelerator of hydrogen peroxide decomposition, with ferrous species approximately 50 times slower [53].

Additionally, the metals Fe^{2+}, Fe^{3+}, Cu^{+} and Cu^{2+} have been shown, in oils, to catalyze the degradation of hydroperoxyls into alkoxyl and peroxyl radicals [53]. These radicals further propagate autoxidative reactions [53]. Lastly, copper and ferrous ions can form reactive singlet oxygen from atmospheric triplet oxygen, thereby promoting coincidence of photosensitized oxidation [53].

1.5.5 Light availability

Light availability is a major influence on the rate of autoxidation. It is an accelerator of chemical oxidation, which has been demonstrated clearly by many studies [55, 72-74]. In one particular study, samples of marjoram oil were stored for 1 year in light and dark rooms, under otherwise identical conditions [73]. The oils were analyzed at 3, 6, 9 and 12-month intervals to determine any changes in the volatile composition. In samples stored in the dark, changes were relatively mild: limonene decreased by 40%, α -terpinene decreased by 10%, linalool decreased by 30% and *cis*- and *trans*-sabinene hydrate reduced by 13.9% and 3.3%, respectively [73]. In conjunction, *p*-cymene content rose from 3.7% to 6.2%, likely at the expense of α - and γ – terpinene [73]. Moreover, 4-terpineol and α -terpineol increased to 30.1% and 4.6%, respectively, over this period [73]. Comparatively, chemical changes in samples exposed to light were much more severe [73]. After 6 months, *cis*-sabinene, a core contributor to the sensory profile of marjoram, was reduced to 0.4% and was present only at trace levels after a year [73]. Similarly, *trans*-sabinene hydrate, another important component, was reduced to 0.5% by the end of the experiment [73]. After 9 months, the compounds α -phellandrene and α -terpinene totally disappeared [73]. Major constituents such as β -myrcene, limonene, β -caryophyllene and β -ocimene decreased in concentration by factors of 2.4 to 4.3 [73]. The light-exposed marjoram samples also exhibited increases in *p*-cymene (28% over 1 year), α -terpineol, geraniol, caryophyllene oxide and several other chemicals [73]. The formation of *p*-cymene was related to increased oxidation whereas α -terpineol formed through transformations of and linalool [73]. Similar patterns were observed in light-oxidation studies of laurel, fennel and lemon oils, where key components such as eugenyl acetate, estragol, *trans*-anethole, geranial, terpinolene and γ -terpinene were oxidized and oxidation products like *p*-cymene, anisic aldehyde and eugenol were formed [7, 74]. In fennel oil, light caused a twofold increase in the rate of oxidation [74]. In laurel, eugenol formation from eugenyl acetate tripled under light exposure [74]. In these three oils it was

also noted that monoterpenes exhibited comparatively sharper declines in the presence of light than other chemical groups, indicating a greater sensitivity [74]. Although essential oils stored under darkened conditions undergo oxidative degradation, those exposed to light break down more rapidly and are also subject to transformation reactions not otherwise seen in samples stored in the dark.

In addition to typical oxidation products, chemical byproducts of light exposure known as photo-artifacts have been identified in essential oils subjected to sunlight or UV exposure [7, 74-76]. Many examples of light-induced reactions in essential oils exist. Safrole, a component of many flavours used in confectionery products, is converted to 4- cyclopropyl-1,2-(methylenedioxy)-benzene when exposed to photochemical irradiation [75]. Eugenol, a core component of clove oil, is transformed into several cyclopropyl derivatives when irradiated [75]. The primary product is 4-cyclopropyl-2-methoxyphenol (12- 28%), with 2-methoxy-4-n-propylphenol (4-7%), 2-methoxy-4-(2-methoxypropyl)phenol (3-7%) and 2-methoxy-4-(1-methoxypropyl)phenol also forming [75]. Eugenol-like compounds, including methyl eugenol, ethyl eugenol and estragole form similar cyclopropyl derivatives under similar conditions [75]. In fennel oil, portions of *trans*-anethole isomerize to *cis*-anethole when exposed to sunlight and UV irradiation further promotes this process [76]. In lemon oil, irradiation of citral in the presence of oxygen produced small amounts of photocitrals, primarily photocitral A [58]. This caused a loss in fresh lemon flavour as well as the formation of a dusty, earthy note [58]. Lastly, in anise oils, exposure to light induced a cycloaddition reaction between anethole and anisic aldehyde, resulting in 4,4'-dimethoxystilbene or photoanethole [74]. In the presence of oxygen, light catalyzes cycloadditions and isomerization reactions thereby generating off-flavours or off-odors in essential oils.

The effect of light on the oxidative stability of essential oil is not uniform. Oils extracted from certain plant species exhibit greater resilience than others. This is exemplified in a 2012 study that evaluated the effect of light on oxidation of essential oils [70]. Samples of four different oils were exposed to adverse storage conditions [70]. Cool white light, imitating daylight, was cast on clear vials of samples for 24 hours/day at room temperature for up to 24 weeks [70]. This was repeated at elevated temperatures of 38°C, with samples exposed for upwards of 12 weeks instead [70]. These conditions ostensibly represented worst-case storage scenarios for light exposure [70]. In rosemary oil samples left at room temperature, the peroxide value was 4.2 times higher than the

initial value after 12 weeks and 6.2 times higher after 24 weeks [70]. Polyunsaturated hydrocarbons such as α -terpinene and α -phellandrene degraded significantly when compared to samples stored in the dark [70]. At room temperature, α -terpinene was reduced to 7.1% of its original quantity after 12 weeks whereas α -phellandrene was reduced to 12.5% [70]. This is in stark contrast to the values obtained for samples left in the dark, which contained more than double the concentration of these compounds after storage [70]. Along with dramatic increases in *p*-cymene and caryophyllene oxide over time, these signs indicate that rosemary oil is much more unstable to oxidation in the presence of light [70]. Conversely, thyme oil was shown to be very resistant to oxidative deterioration in the light [70]. Levels of its most sensitive component, α -terpinene, dropped approximately 20% [70]. This was met with only a slight increase in *p*-cymene of 2% [70]. While certain oils are strongly impacted by exposure to light, others possess qualities that provide them with greater protection.

1.5.6 Oxygen availability

It should be no surprise that oxygen availability plays a key role in the oxidation of essential oils. The concentration, location and type of oxygen in contact with the oil substrate are all factors that affect the rate of reaction [77]. At low oxygen concentrations, the rate of oxidation is dependent on the concentration of available oxygen, whereas at higher concentrations it will maximize and become independent [77]. Oil is inherently capable of absorbing more oxygen than water [77]. At 20°C, it is 5 to 10 times more soluble to oxygen and has a saturation point upwards of 11 times higher, at around 55 ppm [77]. However, the precise amount of oxygen that dissolves in the oil depends on the partial pressure of the headspace directly above it in the storage container [77]. As per Henry's Law, a greater partial pressure allows more oxygen to enter the oil thereby increasing the oxidation rate [77]. Solubility to oxygen is therefore also temperature-dependent; oxygen dissolves more readily at lower temperatures than at higher ones [77]. Conversely, a sharp loss of solubility occurs as temperature increases, causing oxygen to leave the dissolved state and enter the headspace [77]. The location of oxygen is very important. Dissolution allows for a larger area of contact with oxidizable substrate compared to oxygen trapped in the headspace [77]. However, although headspace oxygen has less surface area contact with the oil, it is significant in that it serves as a reservoir for depleted dissolved oxygen [77]. Headspace oxygen migrates into the oil either by diffusion or through agitation [77]. Oxygen will diffuse at a rate that eventually

equilibrates with the rate of oxygen consumption in the essential oil [77]. The nature of the essential oil and the design of the storage container may also influence the area of contact [77]. Certain oils have a higher surface to volume ratio, which permits more efficient oxidation [77]. Similarly, containers vary in dimensions; drums with wider diameters permit greater contact between headspace oxygen and the oil surface [77]. Additionally, the surface of a metal container itself may serve as a reduction catalyst, effectively accelerating the reaction [77]. This catalytic effect has been shown to be proportional to the surface area of the drum in contact with the oil [77].

There is a strong link between temperature and oxygen availability and both influence oxidation rates [63]. Therefore it is often difficult to extricate the effect of one from the whole [63]. It is known that higher amounts of alkylperoxyl radicals form at lower or moderate temperatures, when oxygen solubility is greater [63]. Upon initiation, oxidation occurs very rapidly and abundances of hydroperoxides are produced [63]. At these temperatures, hydroperoxides form more rapidly than they decompose [63]. Termination reaction products only tend to accumulate during the late induction period, upon depletion of available oxygen or oxidizable oil components [63]. At high temperatures the rate of oxidation is accelerated but oxygen solubility decreases [63]. The initiation reaction is favored along with an increase in the concentrations of alkyl radicals and polymeric products of alkyl-alkoxyl radical reactions [63]. At high temperatures hydroperoxides are hardly present, as they decompose more rapidly than they form [63]. Different essentials have been shown to behave differently at varying temperature and oxygen conditions [63]. Rosemary and pine oils are more susceptible to oxidation at room temperature whereas thyme and lavender oils generate high levels of peroxides at refrigerated conditions [70].

1.5.7 Temperature

Temperature plays a critical role in the advancement of oxidation. Reaction rates are typically temperature-dependent and accelerate when heat is supplied to the system. In general, as characterized by van't Hoff's law, increasing temperature by 10°C causes a twofold increase in the rate of a chemical reaction [78]. It is no surprise, then, that autoxidation and hydroperoxide degradation is enhanced by heat [53]. Moreover, abstraction of hydrogen is facilitated by the application of heat energy, effectively accelerating free radical formation [53]. At low temperatures, the induction period of autoxidation proceeds slowly [53]. By the end of this period,

hydroperoxides and polymerized compounds accumulate [53]. A 2002 study of herring oil demonstrates the effect of temperature on hydroperoxide stability [79]. In oil samples stored in the dark at 20°C, hydroperoxides formed more rapidly than they decomposed [79]. In the same samples, instead stored in the dark at 50°C, the rate of hydroperoxide decomposition was greater than the rate of formation [79]. However, the overall effect of temperature on the oxidative stability of an essential oil varies between species [7]. Rosemary oil is very resilient to oxidation at low temperatures [70]. Indeed, a 2012 study demonstrated that oxidation could be completely inhibited in rosemary oil by refrigeration conditions [70]. Comparatively, pine oil remained susceptible to oxidation at these temperatures, though the process was retarded [70]. On the other hand, lavender oil showed greater vulnerability to peroxide formation at refrigerated temperatures than at room temperature [70]. In pine and lavender oils held at 38°C, peroxides were nearly absent but an accumulation of secondary oxidation products was observed [70]. In photosensitized oxidation, temperature plays only a small role [53]. This is because the activation energy required for this reaction is very low, anywhere from 0 to 6 kcal/mole [53]. Therefore, the effect of temperature on essential oil stability is variable.

Temperature has a remarkable effect on the stability of volatile essential oil components, particularly terpenes. As temperature increases, essential oils and their terpene constituents become increasingly susceptible to thermal oxidative rearrangements. A 1999 study of four model terpenes (limonene, ∂-3-carene, α -terpinene and camphene) categorized four common oxidative reactions induced by heat in this class of compounds [80]. In the case of unsaturated monocyclic six-membered ring species, such as α -terpinene and limonene, the most common oxidation reaction was aromatization [80]. For α -terpinene, this reaction produced *p*-cymene whereas limonene formed thymol [80]. Camphene and ∂-3-carene also produced aromatic degradation products, through dehydrogenation rearrangement reactions [80]. Those models that contained double bonds were also susceptible to oxidative cleavage [80]. Camphene cleavage produced camphenilone whereas α -terpinene generated two keto aldehydes [80]. A third common reaction was epoxide formation [80]. This was observed in ∂-3-carene, limonene and α –terpinene [80]. For the first two models, 1,2-epoxy derivatives were produced [80]. For α -terpinene, 1,4 and 1,8-ethers (1,4-cineole and eucalyptol) were formed [80]. The last reaction category identified in this study was allylic oxidation [80]. The majority of limonene and ∂-3-carene molecules underwent this form of oxidation [80]. Essential oils, therefore, are thermolabile mixtures that undergo diverse

oxidative degradation pathways when exposed to temperature stresses.

A 1994 study of CO_2-extracted cardamom oil demonstrated this clearly [64]. Samples of cardamom oil were stored at freezing (0°C) and at 28°C [64]. After a storage period of 90 days, the composition of the warmer sample was compared to its counterpart [64]. In the cardamom oil, an enormous change occurred in the quantities of several major terpenes [64]. At 0°C, β -pinene, sabinene and limonene diminished by 35-50% [64]. Comparatively, at 28°C, β -pinene and sabinene dropped from 7.1% to 0.4% whereas limonene declined to 0.5% from 2.3% [64]. Eucalyptol, another important constituent, was reduced from 27% to 21.8% at freezing but plummeted to 14.7% at 28°C [64]. Lesser reductions in terpene alcohols also took place [64]. Overall losses were associated with a dramatic rise in terpenyl acetate [64]. Similar patterns were observed in oils stored at other temperature ranges [76]. A 1999 study of fennel oil samples stored at 25°C showed significant volatile losses and a marked increase in 4- anisaldehyde, compared to refrigerated samples [76]. A 2012 comparative study of four essential oils (rosemary, thyme, pine and lavender) stored at 23°C and 38°C corroborates the data, also identifying additional terpenes susceptible to heat, such as β -caryophyllene, β -myrcene and γ –terpinene [70]. Additionally, higher temperatures induce more extensive deterioration of terpenes, which in turn leads to the formation of a wider range of degradation products [80]. In myrcene, for instance, temperature increase was correlated with a greater abundance of polymerized decomposition byproducts [80]. Terpenes, therefore, are quite temperature-sensitive and undergo various degradation and transformation reactions when exposed to heat.

1.6 Acid-catalyzed reactions of terpenes

Terpenes are responsible for most of the flavour properties of essential oils, particularly citrus oils. By extension, they are also integral to the overall profile of cola flavours, which rely heavily on lemon and lime oils. Whereas terpenes are sensitive to oxidation and heat, significant research has demonstrated the sensitivity of terpenes to acidic conditions [7, 39]. This chemical group undergoes acid-catalyzed reactions in aqueous solutions of pH <6, including hydration of double bonds, dehydration, rearrangement, cyclization and hydrolysis of esters [39]. Unlike oxidation reactions, acid-catalyzed reactions in terpenes are independent of light and do not result in typical off-notes [39]. Rather, these reactions usually result in the formation of other terpenes

and thereby create imbalances in the terpene complex [39]. Consequently, the flavour may become distorted by the predominance of undesirable but not unpleasant compounds [39]. In some cases, terpenes generated by acid-catalyzed reactions undergo subsequent oxidation, resulting in off-notes. Citral is an excellent example of this as was discussed in detail previously. Because cola flavours are typically subjected to a pH of approximately 2.5, these types of chemical changes are especially likely and important. Acid-catalyzed reactions are discussed by class, with a focus on terpenes important to cola flavour [39].

1.6.1 Hydration of Unsaturated Terpenes

Two classes of terpenes readily hydrate at double-bonded sites under acidic conditions: monocyclic *p*-menthadienes and non-allylic *p*-menthenols [39]. Citrus oils are predominantly composed of the former group, which includes limonene, γ-terpinene, terpinolene, α-terpinene and α-phellandrene [39]. The hydration of limonene, which usually represents around 60-70% of the composition of lemon oil, results in the primary products α-terpineol, *trans*- and *cis*-β-terpineol and terpinolene [39]. The ratio of *trans*- to *cis*- β-terpineol 5:1 whereas the initial ratio of α-terpineol to β-terpineol is 10:1 [39]. This increases to 14:1 by 72 hours [39]. β-terpineol is more readily converted to secondary products due its more accessible exocyclic double bond, which accounts for the observed change in ratio [39]. Secondary products of β-terpineol are *trans*- and *cis*-1,8-terpin [39]. Limonene hydration is typically slow: at 75°C and pH 2.7, it exhibits a half-life of approximately 220 days [39]. Under these conditions, terpineols account for ~97% of the products formed and terpins account for ~3% [39]. At high temperatures, γ-terpinene, α-terpinene and additional terpinolene are formed from the rearrangement of an early reaction intermediate but do not form under milder conditions [39]. The hydration of limonene is accelerated by improved dispersion of the molecule [39]. The reaction can proceed at least 10 times faster in this way [39]. Limonene is mostly tasteless at levels typically found in flavour [39]. Hydration reactions result in an increased taste intensity due to the formation of odorous terpineols [39].

There is very little information pertaining to acid-catalyzed reactions of terpinolene, γ-terpinene and α-terpinene, despite their importance in essential oils and as reaction products of other acid-catalyzed reactions [39]. Terpinolene mostly generate terpinen-4-ol, with smaller yields of α-terpineol and γ-terpineol [39]. It hydrates more rapidly than limonene, at approximately double the rate, due to faster protonation of its exocyclic double bond [39]. The absence of research

into α- and γ-terpinene is likely due to their stability under acid conditions [39]. Hydration of these compounds is very slow [39]. In a 95% aqueous solution containing 0.07 N of sulfuric acid, α-terpinene formed about 6% isoterpinolene, with no other products after 45 hours at 75°C [39]. Similarly, γ-terpinene formed 6% α-terpinene and 3% isoterpinolene [39]. Additionally, γ-terpinene readily oxidizes to *p*-cymene when exposed to oxygen and this product remains stable at low pH [39].

Several non-allylic *p*-menthenols are also important to citrus oils, including α-terpineol, terpinen-4-ol, terpinen-1-ol, γ-terpineol and β-terpineol, all of which hydrate to diols under acidic aqueous conditions [39]. Terpinen-4-ol is a *p*-menthenol present in citrus oils [39]. It is also the most abundant component of nutmeg oil, an important contributor to the spice complex of cola flavours [52]. Terpinen-4-ol reacts to 1,4 cineol under acidic conditions and can further hydrate to 1,4-terpin isomers [39]. Its rate of hydration is the same as another *p*-menthenol, α-terpineol, which has a similar endocyclic double bond structure. Terpinen-4-ol can also be formed from limonene under harsh conditions but this is not the case in typical flavour storage [39].

1.6.2 Rearrangement of Bicyclic Monoterpenes

1.6.2.1 α - and β- Pinene

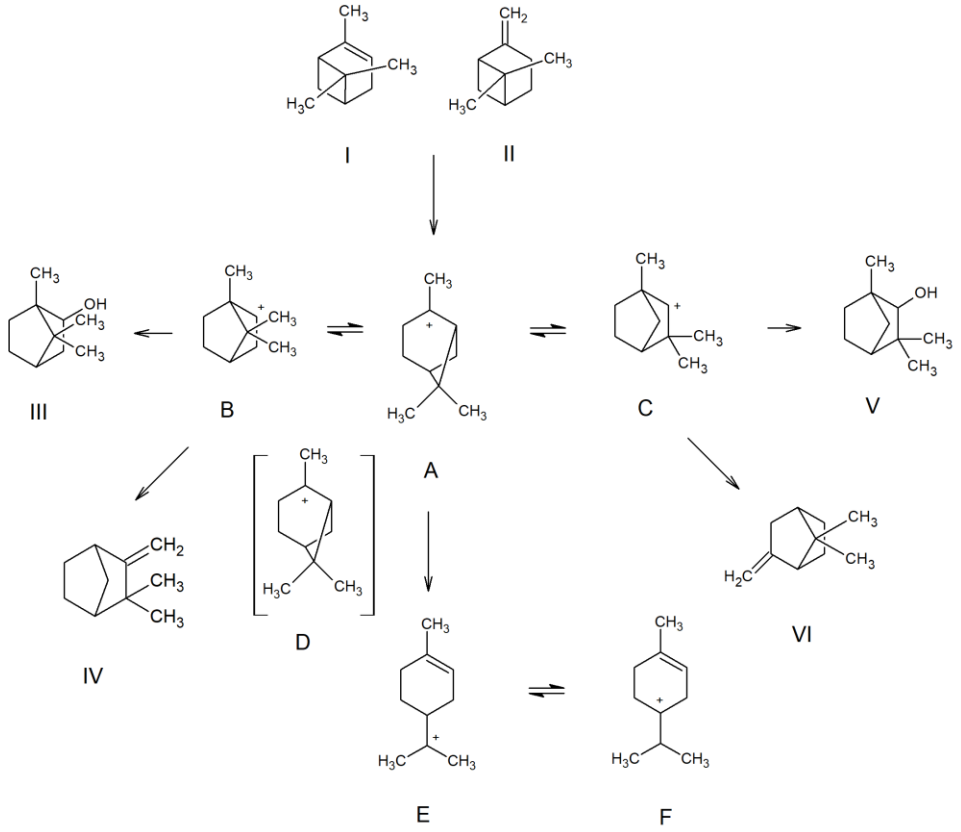
Bicyclic monoterpenes are major constituents of essential oils. α-pinene and β-pinene are important components of many citrus and spice oils that are used in cola flavours [7]. In nutmeg oil, for example, these three chemicals represent ~65% of the overall composition. In lemon and lime oils, they represent upwards of 12-15% of the mixture, with this number exceeding 20% in coastal citrus oils [39]. Although they have a significant presence in these essential oils, like limonene they contribute very little to flavour [39]. However, due to their proportions in citrus and spice oils, careful attention must be given to potential degradation reactions in this group [39]. Bicyclic monoterpenes are prone to acid-catalyzed rearrangements and subsequent hydration reactions [39]. The relative abundance of these terpenes in an oil mixture can lead to an accumulation of products with undesirable organoleptic properties, if they are exposed to low pH [39].

Pinene rearrangement has been studied in detail due to its significance as a method for terpineol synthesis [39]. The reaction is similar for both α- and β-pinene, with an overview

presented in **Figure 1.7** [39]. Rearrangement begins when the pinene (**I** or **II**) becomes protonated, forming carbocation **A**. This is considered the rate-determining step. Following protonation, the C_1-C_6 bond or the C_1-C_7 bond of **A** can migrate to form intermediary carbocations (**B** or **C**). Several different reactions may occur from this point: 1) The cation **B** undergoes a nucleophilic attack by water at C_1, generating borneol (**III**), 2) Wagner-Meerwein rearrangement takes place at $C_{10\ of}$ cation **B** and a proton is then removed, producing camphene (**IV**). The same reactions can occur with cation **C**, causing similar transformations and producing α-fenchol (**V**) or fenchene (**VI**). Additionally, carbocation **A** can open at bond **i**, to create intermediate **E**, which undergoes equilibration reactions with intermediate **F**. The major product of pinene rearrangement, α-terpineol, forms from the hydration of intermediate **D** at its C_6 bond. Removal of protons from carbocation **D** leads to the formation of other terpenes: elimination at C_8 produces limonene while elimination at C_5 leads to terpinolene. The primary products of pinene rearrangement can undergo subsequent hydration, rearrangement and cyclization reactions [39]. Hydration of camphene yields isoborneol, hydration of α-terpineol yields *trans*- and *cis*-1,8-terpin and 1,8- and 1,4-cineole likely form from these terpins [39]. The rearrangement rate of pinenes is proportional to proton concentration in the aqueous solution [39]. α-pinene reacts 16 times more rapidly than limonene and the reaction rate of β-pinene is tenfold that of α-pinene [39].

The distribution of primary reaction products varies. The most abundant product of acid-catalyzed pinene rearrangement is certainly α-terpineol but this reaction also generates bicyclic alcohols, principally borneol and α-fenchol [39]. Monocyclic hydrocarbons and additional bicyclic hydrocarbons form when conditions are intensified [39]. At lower pH levels and when less water is available, the monocyclic hydrocarbons limonene and terpinolene form [39]. The bicyclic hydrocarbons camphene and fenchene are also produced [39]. This is because the initial carbocations in pinene rearrangement reactions form primary alcohols via hydration, not by secondary protonation and subsequent hydration [39]. Water effectively captures carbocations, impeding rearrangement [39]. A greater proportion of hydrocarbons also form when higher temperatures are applied [39]. At 25°C and pH 2.4, pinene rearrangement yields ~13% hydrocarbons whereas yield increases to ~39% at 75°C and pH 1.2 [39]. Therefore, non-aqueous systems, lower pH and higher temperatures promote alternative pinene reaction pathways [39].

Figure 1.7. Acid-catalyzed rearrangements of pinenes 39]

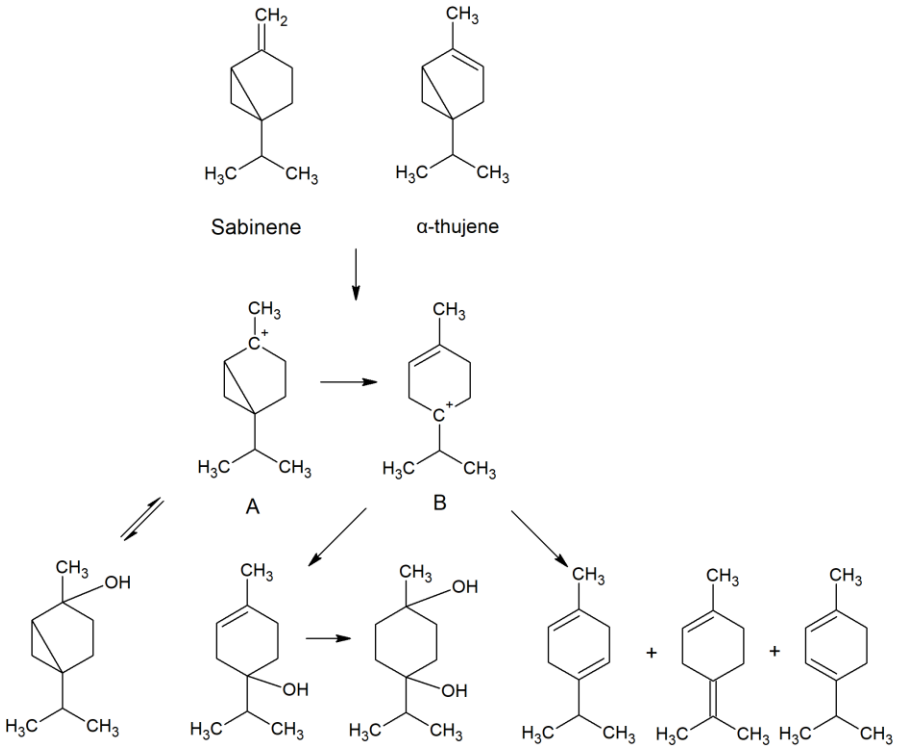


1.6.2.2 Sabinene and -thujene

Sabinene and α-thujene are bicyclic hydrocarbons present at ~3% in lemon and lime oils [39]. They are characterized by a cyclopropyl ring that results in a higher reactivity, about 40 times more rapid than β-pinene [39]. Sabinene hydrates over 2000 times more rapidly than the exocyclic double bond of limonene [39]. Similarly, it is also more reactive than thujene (~23 times) [39]. The position of its double bond results in more conducive conformation and steric effects, and a more energetic ground state level [39]. Both compounds undergo acid-catalyzed rearrangements, the chemistry of which as been well studied [39]. In the presence of water, the main products of acid-catalyzed rearrangement of sabinene and thujene are almost identical [39]. Hydration of these chemicals generates terpinen-4-ol, γ-terpinene, α-terpinene, terpinolene and sabinene hydrate [39]. The mechanism of sabinene and thujene rearrangement is well understood [39]. The initial step is similar to that of pinene rearrangement, as illustrated in **Figure 1.8**: protonation of the reactant to carbocation **A** [39]. This is followed by a rearrangement of **A** to **B**, which then leads to all the major observable products [39]. Nucleophilic attack of water at C_1 leads to terpinene-4-ol [39].

This process is stereospecific and pH dependent [39]. Terpinen-4-ol will hydrate to 1,4-terpin under dilute acidic conditions [39]. Nucleophilic attack by water at C_4, meanwhile, forms *cis* and *trans* sabinene hydrate, though this product is ultimately found in minor quantities due to its instability in acid [39]. Spontaneous ring opening at the C_2, C_6 or C_7 sites, followed by an elimination reaction, leads to γ-terpinene, terpinolene and α-terpinene, respectively [39].

Figure 1.8. Acid-catalyzed rearrangements of sabinene and α-thujene [39]



CHAPTER 2: THE IMPACT OF STORAGE CONDITIONS ON THE CHEMICAL & SENSORY PROPERTIES OF A FORMULATED "COLA" FLAVOUR

2.1 MATERIALS AND METHODS

2.1.1 Materials

Two model flavour mixtures and a cola flavour concentrate were designed for this study. Flavour mixture 1 contained representative members of the various chemical classes typically employed in flavour development, including alcohols, diols, aldehydes, esters and essential oils. It is presented in **Table 2.1**. Flavour mixture 2 represented an acidified variation of this formulation and is described in **Table 2.2**. The cola concentrate, a blend of essential oils, is outlined in **Table 2.3**. High purity samples of all aromatic chemicals were obtained from Sigma-Aldrich (acetone [CAS# 67-64-1], ethyl acetate [CAS# 141-78-6], α-pinene [CAS# 80-56-8], ethyl butyrate [CAS# 105-54-4], β-pinene [CAS# 127-91-3], D-limonene [CAS# 5989-27-5], eucalyptol [CAS# 470-82-6], γ-terpinene [CAS# 99-85-4], *p*-cymene [CAS# 99-97-6], terpinolene [CAS# 586-62-9], *p*-cymenene [CAS# 1195-32-0], benzaldehyde [CAS# 100-52-7], linalool [CAS# 78-70-6], citral [CAS# 5392-40-5], α-terpineol [CAS# 98-55-5], benzaldehyde propylene glycol acetal [CAS# 2568-25-4], cinnamaldehyde [CAS# 104-55-2] and *p*-cresol [CAS# 106-44-5]) (*Oakville*, *Canada*). The chemical structures of these compounds are presented in **Appendix A**. The essential oils used in the flavour mixtures (lemon oil, lime oil, orange oil, cassia oil, cinnamon bark oil, nutmeg oil and clove bud oil) were obtained from Cedarome (*Candiac*, *Canada*). The key compositions of these essential oils, measured by gas chromatography-flame ionization detector (GC-FID) and provided by the supplier, are detailed in **Appendix B**. Propylene glycol was obtained from Quadra Chemicals Inc. and ethyl alcohol was obtained from Alcools de Commerce Inc. Ethyl alcohol had a purity of 95% with the remaining 5% consisting of water. To properly investigate and understand flavour reactivity and deterioration in real-world scenarios, the flavours herein were developed as models that represent both a) typical product offerings and b) blends that are suspected to be particularly sensitive to storage conditions.

2.1.2 Preparation of flavour mixture

Two model flavour mixtures were designed for comparison in this experiment. They were compounded according to the formulations presented in **Table 2.1** and **Table 2.2**. During

preparation, the aroma chemical and essential oil flavour components were first added to the ethyl alcohol solvent and vortexed for 10 minutes. Subsequently, propylene glycol was added, and the mixture was again vortexed for 10 minutes. In the case of flavour mixture 2, phosphoric acid was added last and the mixture was vortexed for 10 minutes. Three hundred grams of each flavour was prepared and divided into three 100 g samples for storage.

In addition to Flavour Mixtures 1 & 2, each flavour ingredient was analyzed individually, at the same concentration as it appears in the mixtures and under the same conditions. This was done to confirm the source of observed degradation products.

TABLE 2.1: Chemical composition of model flavour mixture 1

Flavour Mixture 1	**Concentration (% weight)**
Ethyl Alcohol (95%)	65.0
Propylene Glycol	30.5
Cola Concentrate	2.0
Citral	1.0
Benzaldehyde	0.5
Linalool	0.4
Ethyl Butyrate	0.6

TABLE 2.2: Chemical composition of model flavour mixture 2

Flavour Mixture 2	**Concentration (% weight)**
Ethyl Alcohol (95%)	46.9
Propylene Glycol	28.6
Cola Concentrate	2.0
Citral	1.0
Benzaldehyde	0.5
Linalool	0.4
Ethyl Butyrate	0.6
Phosphoric Acid	20.0

TABLE 2.3: Chemical composition of cola concentrate

Cola Concentrate	Concentration (% weight)
Clove Bud Oil	0.1
Nutmeg Oil	0.3
Cinnamon Bark Oil	0.4
Cassia Oil	4.0
Orange Oil	12.0
Lemon Oil	29.20
Lime Oil	54.0

2.1.3 Storage

The freshly prepared samples of flavour mixtures 1 and 2 were sealed in air-tight 100 mL amber vials and stored at 4, 23 and 45°C. The samples were stored upright in dark incubators at their corresponding temperatures (the 4°C samples were stored in a dark refrigerator). The samples were removed at one-week intervals for analysis, over the course of 10 weeks.

2.1.4 Analysis of flavour mixtures

Headspace and Gas Chromatograph Conditions

The flavour mixtures were analyzed using an Agilent 7890B gas chromatography system via an Agilent 7697A headspace sampler (Palo Alto, CA, USA). One half gram of each storage sample was added to a 10 mL, clear, crimp top headspace vial, sealed and placed in the headspace sampler. Headspace oven, loop and transfer line temperatures were set to 70, 90 and 110°C, respectively. The vial equilibration time and injection duration were 10 minutes and 0.5 minutes, respectively. Vials were filled to a pressure of 15 psi, with a loop ramp rate of 20 psi/min, a final loop pressure of 8 psi and a loop equilibration time of 0.05 minutes.

In the GC, a 0.25 µL film thickness DB-WAX column from Agilent/J&W Scientific, 30 m x 0.25 mm i.d. was used. The column was operated at 19 psi head pressure, with analytical grade helium as a carrier gas. The column oven temperature was programmed from 70°C, with a 2 minute hold, at 2°C/min to 100°C, then 4°C/min to 155°C and then 25°C/min to 230°C, with a final 2 minute hold.

Mass Spectrometer and compound identification

An Agilent 5977B mass spectrometer detector (Palo Alto, CA, USA) equipped with MassHunter software was used as a detector. A scan range of 33 to 250 was employed, with 6.2 scans/second and a scan speed of 1,562 u/s. Results were initially cross-referenced with the NIST research library and spectral standard database. To confirm the identity of the key compounds presented herein, pure samples were injected and their retention times were compared.

Quantification

A 5-point calibration curve was constructed for each compound of interest presented in this study (see **Appendix C**). Regression lines (R^2>0.99) were then extrapolated and used to calculate the concentration (ppm) of the corresponding constituent following each analysis. The normalized peak area values for each key component and degradation product, in all instances, were calculated using the MassHunter software. Citral is presented as the sum of its isomers.

Additional Analysis

In addition to chemical analysis, and to provide greater insight into observed chemical changes, the pH of the stored samples was measured on a weekly basis. A ThermoScientific Orion VersaStar pH meter was used to determine the pH.

2.1.5 Statistical Analysis

All samples were prepared in triplicate for analysis. The average results were determined and reported herein. Standard deviation in all cases was determined using Microsoft Excel.

2.1.6 Sensory Evaluation of Flavour Samples

A sensory panel consisting of the same 23 volunteers was assembled twice a week over the course of the experiment – once to evaluate Flavour Mixture 1 and again to evaluate Flavour Mixture 2. Panellists were recruited internally from the staff of Novotaste Corporation Inc. (*Montreal*, *Canada*) and consisted only of untrained individuals, to represent average consumers.

The panel comprised men and women, aged from 23 to 62, of various ethnicities. Panellists gathered 4 to 5 at a time in a dimly lit, odour-neutral, soundproof room designed expressly for sensory evaluation. A specified tetrad test model, followed by a two-sided directional test, was chosen. For the tetrad component, panellists were presented with 4 samples (two test and two fresh samples) and were asked to group them into two groups based on similarity. For the two-sided directional test, panellists were asked which pair was a) stronger overall, b) stronger in citrus character and c) more intense in off-notes. Samples were presented in 30 mL cups, labeled with randomly generated 3-digit codes. Panellists were asked to provide their answers through a brief questionnaire consisting of 4 mandatory questions and a fifth optional question. A copy of the questionnaire can be found in **Appendix D**.

2.1.7 Statistical Analysis of Sensory Results

The data obtained from each round of testing was automatically compiled into Microsoft Excel (*Redmond*, *USA*) files using the eSurvey Creator software (*Zurich*, *Switzerland*). It was then processed and analyzed using the V-Power program (*Wilmette*, *USA*), a power tool for sensory analysis. A specified tetrad (guessing model) was chosen and standard statistical parameters were used to achieve the desired power level of 0.81 (proportion of distinguishers (pD)=0.3, α=0.05, and power=0.8). Tetrad was chosen over triangle testing because it is more consistent and sensitive to sensory differences and requires a smaller panel size [88]. The V-Power program was used to determine *p* values given these parameters. Any *p* value below 0.001 (α = 0.05) was considered significant.

Panellists were additionally given a two-sided directional test to determine whether they perceived a difference in overall flavour strength, citrus character strength and off-note intensity. The null hypothesis (H_n) in each scenario was H_n: A = B. The alternative hypothesis (H_a) was H_a: $A \neq B$. Given a limited sample size, statistical parameters of α= 0.1 and β= 0.2 were selected. There was no 'correct' answer because either outcome is of interest – hence the two-sided nature of the test. Any answer collectively chosen 16 or more times in a given week was considered significant.

2.2 RESULTS AND DISCUSSION

The goal of this study was to evaluate the effect of typical storage conditions on the chemical composition of a complex flavour. An additional goal was to determine whether any such chemical change coincided with an appreciable difference in the sensory profile of the flavour. The cola flavour used in this study was designed to include various representative ingredients from the major classes of flavour chemicals. In the spirit of presenting a real-world scenario, the flavour was developed in such a way that it could serve as a successful and marketable flavour. This approach was chosen rather than simply blending equal quantities of each ingredient. Therefore, this experimental cola flavour can be considered a balanced, albeit generic, offering.

Flavour mixtures 1 and 2 were similarly designed. Both contain the same aromatic profile but differ in their pH. The aromatic profile of these mixtures consists of several widely used flavour chemicals. Citral and benzaldehyde are common aldehydes, ethyl butyrate is a very common aliphatic ester, and linalool is a popular terpene alcohol. The cola concentrate consisted of several important essential oils, including citrus oils (lemon, lime and orange oils) and spice oils (cassia, nutmeg, cinnamon and clove oils). Clients will often request that a cola flavour be an "all-in-one", with the desired acids integrated with the aromatics. This type of offering is represented in Flavour mixture 2. Flavour mixture 2 was acidified using phosphoric acid, the main acidulant in cola beverages, to determine the effect of acidity during storage. The solvents selected for these flavours were propylene glycol and ethyl alcohol, the two most frequently used solvents in the industry. Ultimately, including resulting off-notes, 19 compounds were followed.

Both flavours were subjected to the same temperature conditions for the same period of time. Three temperature regiments were chosen: 4°C, 23°C and 40°C. These were selected to represent refrigeration, room temperature and an extreme but possible condition. Whereas refrigeration is the typically recommended storage temperature, it is common to see flavours stored at room temperature and sometimes, especially in tropical regions, significantly higher temperatures. All samples were stored in the dark, in amber vials to minimize the effect of light. While the 4°C samples were refrigerated, the other two sets were incubated at their respective temperature levels. The samples were stored for 9 weeks.

Headspace gas chromatography was employed to analyze the cola samples. Multiple factors went into this consideration. Firstly, the chemical components of the cola flavours studied in this experiment are all highly fragrant members of the aldehyde, alcohol, terpene and ester families. They are known to respond well to headspace techniques, where they are easily isolated. Secondly, headspace analysis removed the need for a solvent dilution step and allowed for the analysis of pure flavour; because of this, headspace chromatography is often the approach of choice in the flavour industry. Thirdly, and of particular importance, mixture 2 contained high levels of phosphoric acid, making it unsuitable for direct injection into the gas chromatograph. It should be noted that pre-experimental trials using solid phase micro extraction resulted in less reproducible results for this application. Samples were removed from storage for analysis on a weekly basis. Three 0.5 g of each sample were placed in headspace vials and analyzed in sequence. An initial analysis was done to establish the starting levels of all key components and samples were analyzed once a week thereafter.

2.2.1 Chemical Changes by Chemical Group

The characteristic compounds of cola flavour that were studied throughout this experiment, as well as their key degradation products, can be divided into 5 chemical groups: esters, aldehydes/ketones, acetals, alcohols and terpenes. A summary of the chemical changes that occurred in the character compounds of Flavour Mixture 1 and Flavour Mixture 2 can be found in **Table 2.4** and **Table 2.5**, respectively. The relative changes in concentration of these constituents, in relation to their starting point, are summarized in **Table 2.6** and **Table 2.7**. Changes in each compound will be discussed in detail.

2.2.1.1 Aliphatic Esters

Aliphatic fruit esters do not necessarily play a major role in the cola profile, though some may be added to impart a fruity nuance to such flavours. Nevertheless, two esters became important to the chemical and sensory profiles of the cola studied in this experiment: ethyl butyrate and ethyl acetate. The former was deliberately added to the flavour formulations prior to storage whereas the latter was an unexpected artifact that appeared at varying levels under all six treatment protocols.

Ethyl butyrate

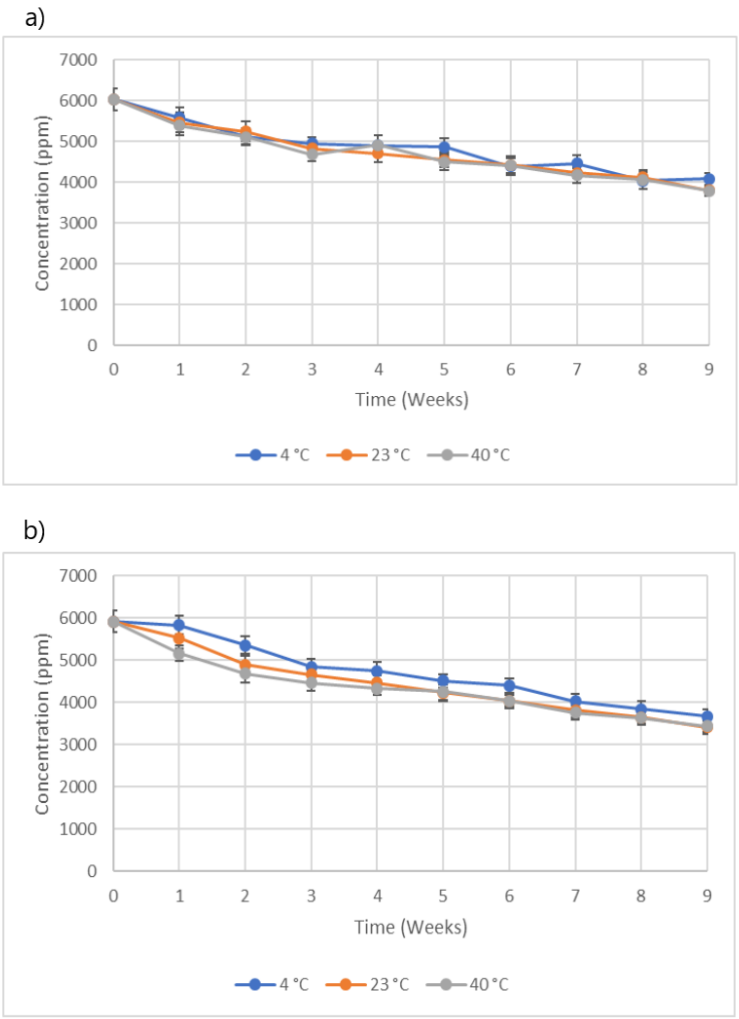
Ethyl butyrate is an ester formed by the condensation of ethyl alcohol and butyric acid. Like most esters that find widespread use in flavour mixtures, ethyl butyrate imparts a generally fruity odor, in this case an aroma most closely reminiscent of orange juice. In the cola flavour designed for this study, ethyl butyrate was chosen to accentuate the natural orange undertones of the cola concentrate and contribute to the sweetness of the flavour.

Ethyl butyrate was added at an initial concentration of 6,000 ppm. In Flavour Mixture 1, the final concentrations of this compound after storage at 4°C, 23°C and 40°C were 4,087 ppm, 3,803 ppm and 3,800 ppm, respectively (see **Table 2.4**). These represented losses of 32.3%, 37.0% and 37.0%, respectively (see **Table 2.6**). Over the course of 9 weeks, the compound declined gradually but steadily, in linear fashion (see **Figure 2.1a**). Unlike many other compounds, it did not demonstrate any sharp increases or declines. There was no indication that hydrolysis of ethyl butyrate occurred in Flavour Mixture 1; hydrolysis of this chemical produces ethyl alcohol and butyric acid, neither of which was detected at any point. Loss of this compound was lower at 4°C, which was expected. Unexpectedly, losses were essentially identical at 23°C and 40°C, but only ~5% higher than at 4°C. This can be explained by the vapour pressure of ethyl butyrate, which is very low at 4°C and only modestly higher at 23°C and 40°C [90]. Additionally, its boiling point is 120°C [90], quite higher than the conditions this flavour was subjected to. All of these factors suggest that the observed decline in ethyl butyrate in Flavour Mixture 1 was the result of volatilization alone.

In Flavour Mixture 2, a considerably steeper decline was observed. Although the initial concentration of ethyl butyrate was about the same as in Flavour 1 (an average initial concentration of 5,923 ppm was determined), after storage at 4°C, 23°C and 40°C only 3,673 ppm, 3,422 ppm and 3,440 ppm remained (see **Table 2.5**). These represented losses of 38.0%, 42.2% and 41.9%, respectively (see **Table 2.7**). Here, the same pattern can be observed as was seen in Flavour Mixture 1, only at a slightly more pronounced level: loss of ethyl butyrate was lower at 4°C but only by ~4%. At 23°C and at 40°C, losses again were almost identical. Declines were somewhat sharper during the first 3 weeks but became linear for the remainder of the storage period (see **Figure 2.1b**). Despite the possibility of hydrolysis over time, there was no evidence of this.

Hydrolysis of ethyl butyrate generates ethyl alcohol and butyric acid. Butyric acid, which imparts a powerful cheese aroma at low levels, was neither detected instrumentally or by nose. This is possibly because the hydrolytic half-life of ethyl butyrate at pH=2.8 is approximately 140 days, which amounts to 20 weeks. Another explanation is that butyric acid was quickly lost to volatilization, but this is not likely. Comparable levels of ethyl butyrate remained in both mixtures, despite the differences in acidity, which suggests that pH was not a factor.

Figure 2.1. Change in concentration (ppm) of ethyl butyrate over time in a) Flavour Mixture 1 and b) Flavour Mixture 2



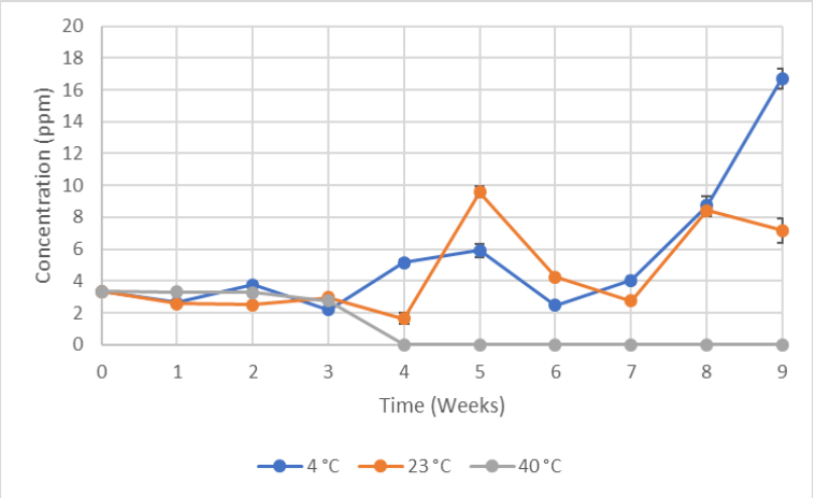
Ethyl acetate

Ethyl acetate is an ester formed by the condensation of ethyl alcohol and acetic acid. It is widely used in flavouring for its fruity, ethereal and rum-like aroma. However, under the wrong context, ethyl acetate can act as an off-note, throwing off the balance of a flavour or imparting a solvent-like taste or smell reminiscent of nail polish. Ethyl acetate was not used in the formulation of the cola flavours studied in this experiment. Rather, it became one of several unexpected formation compounds and contributors to aroma depreciation in these flavours.

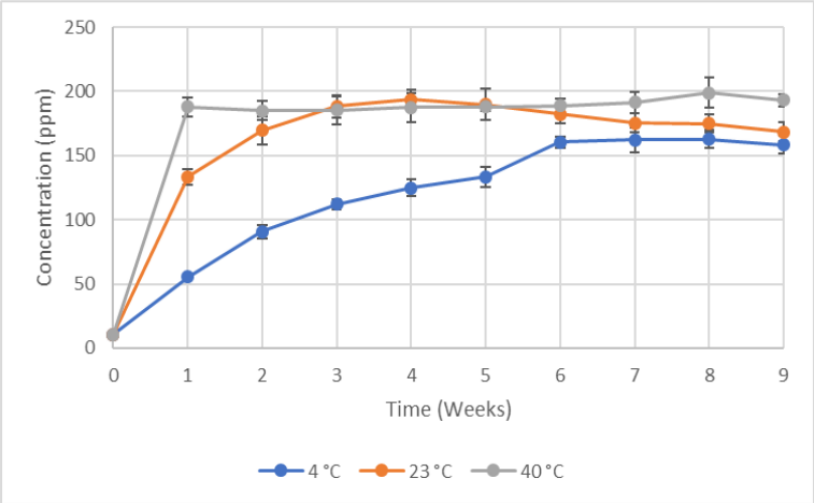
Ethyl acetate was not a component of the cola formulations. Upon initial analysis of Flavour Mixture 1, this chemical was present at 3.34 ppm. However, over the course of all three storage treatments, formation of ethyl acetate occurred. After 9 weeks, the concentrations of ethyl acetate remaining in Flavour Mixture 1 at 4°C, 23°C and 40°C were 16.7 ppm, 7.16 ppm and 0 ppm (undetected) (see **Table 2.4**). This represented an increased of 500%, 214.4% and a loss of 100%, respectively (see **Table 2.6**). The details of these changes are represented graphically in **Figure 2.2a**. At 4°C, ethyl acetate formed progressively and spiked at week 9. Similarly, at 23°C, ethyl acetate formed gradually and was at nearly its highest levels at week 9. At 40°C, however, ethyl acetate was muted. Approximately 3 ppm of this compound formed by week 2, before it faded and became undetectable by week 4. Ethyl acetate has a vapour pressure that rises rapidly with temperature [90]. At 40°C, its vapour pressure is in the realm of 26,665 Pa [90]. It also has a lower boiling point than other esters, at 77°C [90]. The effect of this can be seen in **Figure 2.2a**. At 4°C and 23°C ethyl acetate alternately spiked and dipped whereas at 40°C it remained somewhat stable at initial levels before vanishing by week 4. This resulted in the highest levels of ethyl acetate remaining at 4°C and progressively lower at higher temperatures.

Figure 2.2. Change in concentration (ppm) of ethyl acetate over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)



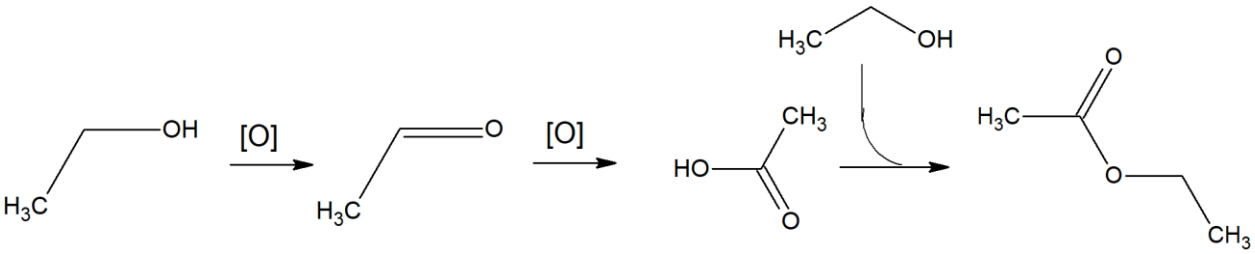
b)



In Flavour Mixture 2, significantly more ethyl acetate formed. Initially, the flavour contained 10.44 ppm and after 9 weeks of storage at 4°C, 23°C and 40°C, it contained 158 ppm, 168 ppm and 193 ppm, respectively (see **Table 2.5**). This represented vast increases of 1,519%, 1,615% and 1,856%, respectively (see **Table 2.7**). This suggests that the formation of ethyl acetate is acid-catalyzed and heat-catalyzed. As depicted in **Figure 2.2b**, the pattern of formation differed from what was observed in Flavour Mixture 1. At 4°C, a steady ramped increase was observed. At 23°C, a similar pattern was observed but with much larger initial increases. At 40°C, a large initial

spike was followed by a stabilization for the remainder of the storage period. Ethyl acetate is likely forming via oxidation of ethyl alcohol in a three-step reaction, as illustrated in **Figure 2.3**. Initially, ethyl alcohol is oxidized to acetaldehyde. Subsequently, acetaldehyde is oxidized to acetic acid. Lastly, acetic acid and ethyl alcohol esterify to ethyl acetate. Both acetaldehyde and acetic acid were detected in cola samples where ethyl acetate formed and neither was present initially, which supports this series of reactions.

Figure 2.3. Proposed mechanism for the formation of ethyl acetate



2.2.1.2 Aldehydes & Ketones

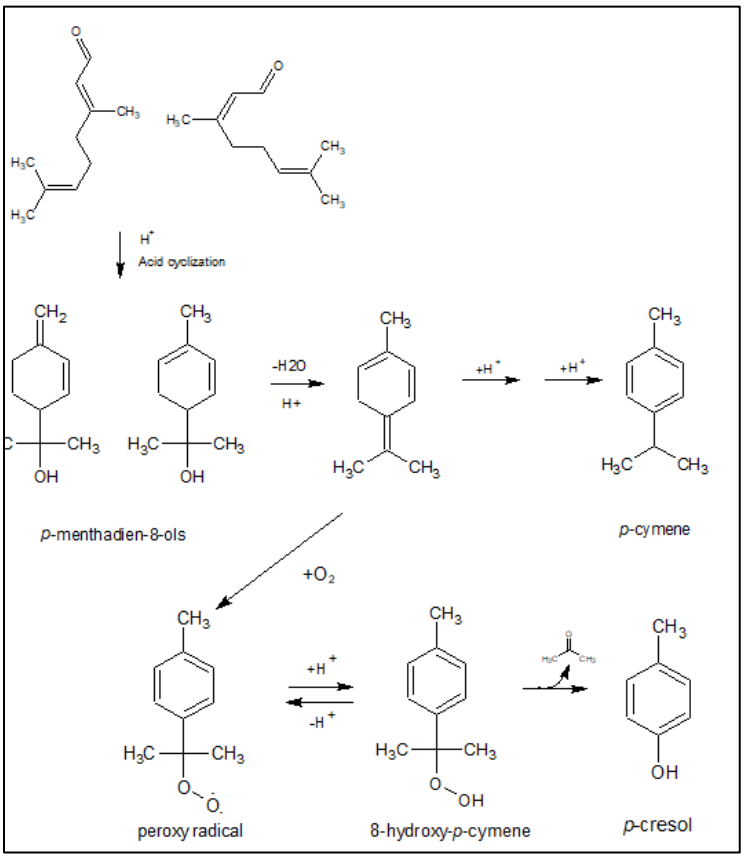
This category of flavouring agents plays a central role in the character recognition of cola flavour. Cola flavours are necessarily designed around lemon oil, lime oil and spice oils such as cinnamon. Whereas lime oil mostly derives its character from terpenes, lemon oil and cinnamon oil would be unrecognizable without their core aldehydes. For lemon oil, this aldehyde is citral whereas for cinnamon it is cinnamaldehyde. Benzaldehyde was another important aldehyde used in the formulation of the cola flavours. Although cherry-almond at its core, small amounts impart a fruitiness, complexity and depth to cola flavours. Lastly, there was an unexpected formation of acetone, a simple ketone with an ethereal, solvent-like aroma. Given the potent, chemical aroma of acetone, this compound became an important contributor to the off-notes perceived in the aged colas.

Citral, p-Cymene & p-Cresol

Citral is the fundamental contributor to lemon character recognition in lemon oil and, by extension, cola flavour. It is easily one of the most important compounds in the flavour. Citral was

added at an initial level of 11,830 ppm via lemon oil and complementary pure citral. In Flavour Mixture 1, the final concentrations of this compound after storage at 4°C, 23°C and 40°C were 6,144 ppm, 6,943 ppm and 6,440 ppm, respectively (see **Table 2.4**). These represented losses of 48.1%, 41.3% and 45.6%, respectively (see **Table 2.6**). The details of these changes are illustrated graphically in **Figure 2.5a**. Citral has a relatively low vapour pressure (0.2 mm Hg at 20°C) and a high boiling point (229°C) [90]. This would suggest that citral is less prone to evaporation than other compounds that exhibited lesser declines. The fact that the loss of citral was similar under the three different temperature conditions further suggests that another factor is at play.

Figure 2.4. Mechanism of degradation of citral under acidic conditions to *p*-cymene and *p*-cresol (1)



Two additional processed appear to be at play in Flavour Mixture 1: esterification and cyclization with dehydration, protonation and subsequent isomerization. Evidence for esterification of citral was appeared via small amounts of neryl acetate and geranyl acetate, which were detected under the three storage conditions after 9 weeks. Citral is a mixture of geometric

isomers neral and geranial. It is therefore possible that these isomers underwent hydration reactions followed by esterification, particularly given the aqueous nature of the flavour mixture. In addition to acetate esters, *p*-cymene formed under the three storage conditions. This compound is formed from citral in a three-part reaction. Initially, neral and geranial cyclize. Secondly, the intermediate is dehydrated and protonated. Lastly, there is an isomerization of the aromatic ring to give *p*-cymene. The initial level of *p*-cymene in Flavour Mixture 1 was 20.7 ppm but ultimately grew to 254 ppm, 322 ppm and 765 ppm in the 4°C, 23°C and 40°C samples, respectively (see **Table 2.4**). These represented increases of 1,227%, 1,556% and 3,696%, respectively (see **Table 2.6**). The details of this formation are illustrated graphically in **Figure 2.6a**. Brief analysis of citral alone under the same solvent, pH and temperature conditions showed that *p*-cymene was indeed forming. However, citral is not the only source of *p*-cymene in the flavour. Lastly, although oxidation was hypothesized to be a factor in Flavour Mixture 1, there was not sufficient evidence to this effect. Typical by-products of citral oxidation are *p*-cresol and *p*-methylacetophenone and typical intermediates are *p*-cymenols. None of these were detected in the non-acidified flavour.

Citral underwent much more dramatic changes in Flavour Mixture 2. After 9 weeks of storage, it was undetectable under all temperature treatments. At 4°C, citral remained for 6 weeks whereas it became absent after 2 weeks at 23°C and 40°C (see **Figure 2.5b**). This reflects the sensitivity of citral to acidity and to temperature. Paralleling these patterns, *p*-cymene rose sharply with increasing storage temperature (see **Figure 2.7**). The formation of *p*-cymene is acid-catalyzed and additionally catalyzed by heat at low pH levels, which is clearly demonstrated. The primary source of *p*-cymene in the flavour mixtures was citral, but it also formed from other terpenes like γ-terpinene, which is further discussed below. Another potent off-note that formed from citral was *p*-cresol. This degradation product forms in a series of acid-catalyzed reactions. At pH 2 to 3.5, the geranial component of the citral mixture isomerizes to neral, which initiates further breakdown. Neral cyclizes to form p-menthadien-8-ol or p-menthadien-4-ol, which subsequently undergo oxidation or disproportionation to form p-cymen-8-ol. It is important to note that the reduction product of this reaction is α-terpineol, which is among the compounds that increased in Flavour Mixture 2. The p-cymen-8-ol molecule the dehydrates to form the more stable *p*-cresol. A progressive rise in *p*-cresol occurred over the course of 9 weeks in the three samples, as illustrated in **Figure 2.8b**, with more dramatic increases observed at higher temperatures.

Figure 2.5. Change in concentration (ppm) of citral over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

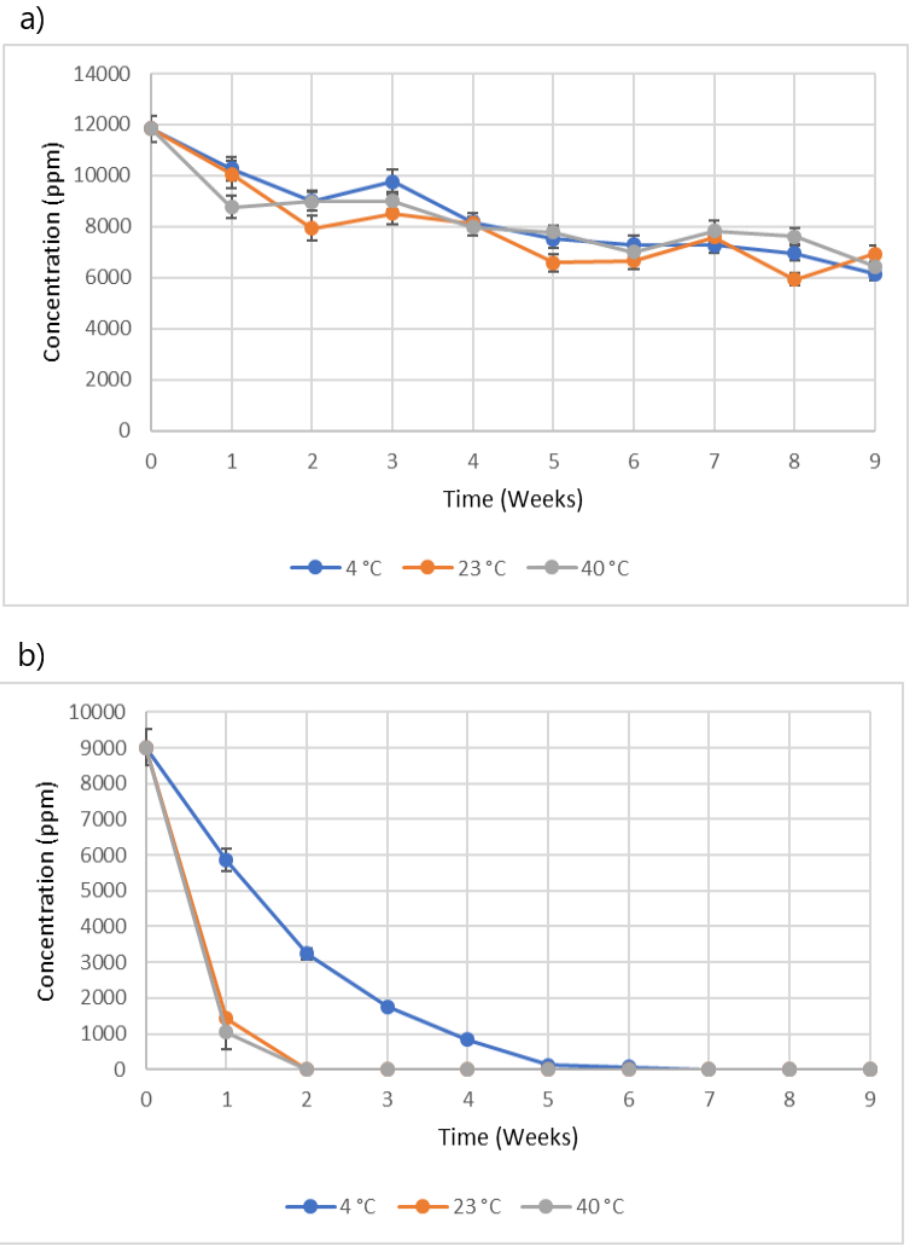
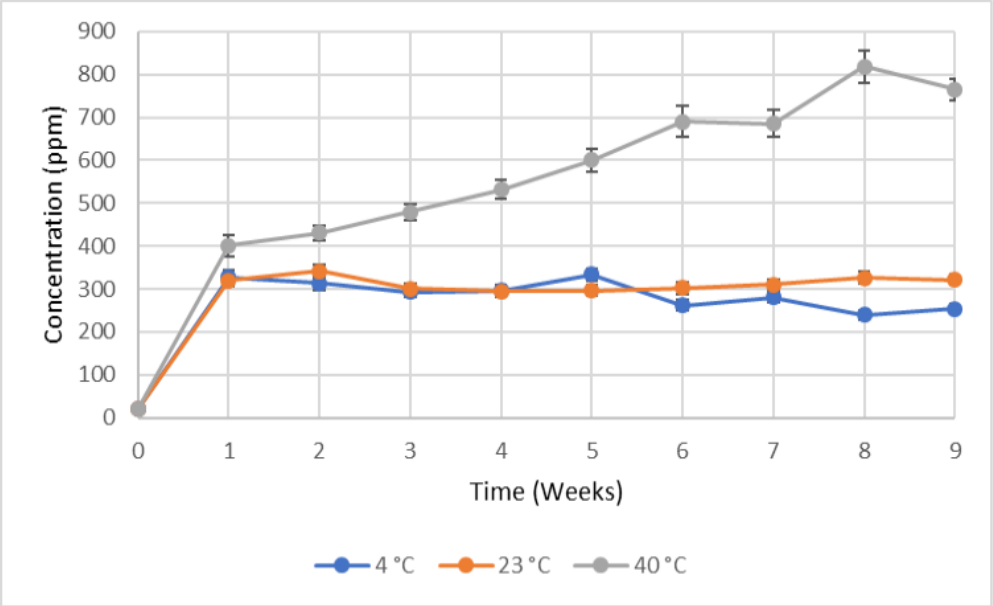


Figure 2.6. Change in concentration (ppm) of *p*-cymene over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)



b)

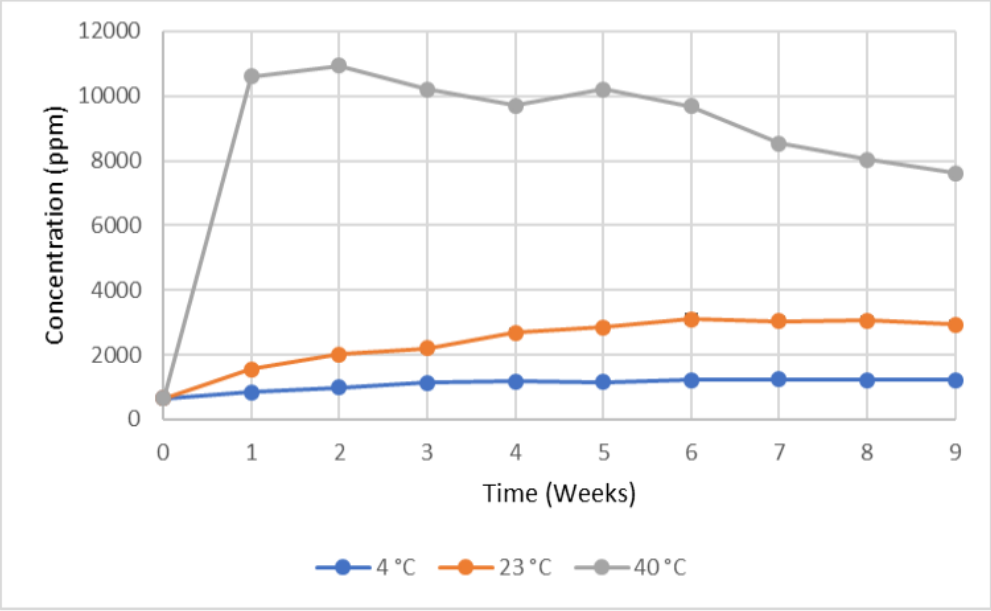
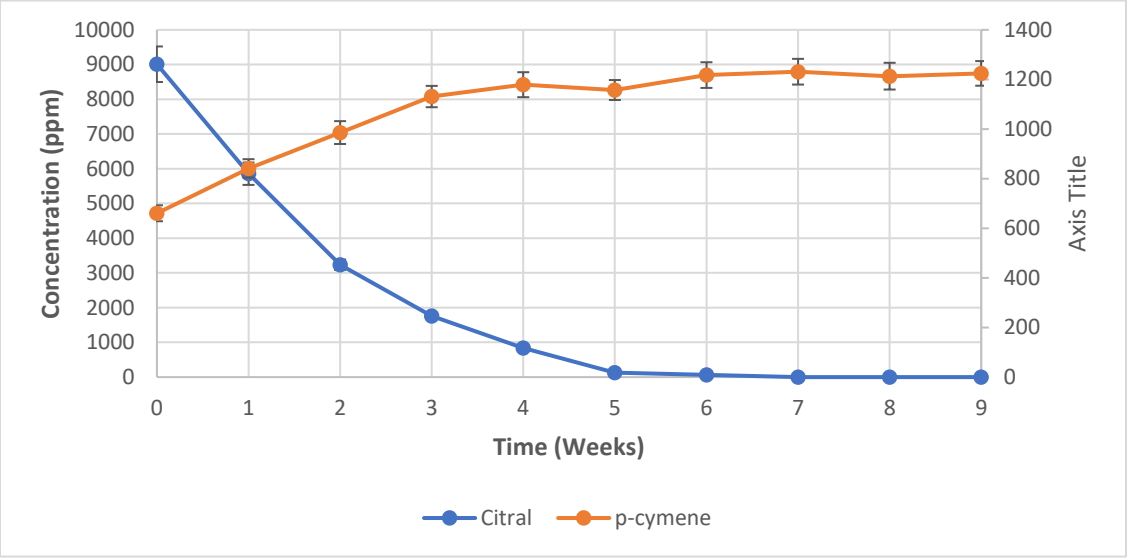


Figure 2.7. Formation of *p*-cymene and loss of citral over time in Flavour Mixture 2 at 4°C



Conversely, in Flavour Mixture 1, there was no formation of *p*-cresol, demonstrating the dependence of this reaction on pH (see **Figure 2.8a**). Initially, no *p*-cresol was present in the samples. Following the storage period, however, 411 ppm, 581 ppm and 962 ppm remained for 4°C, 23°C and 40°C, respectively (see **Table 2.5**). *P*-cresol formation paralleled citral degradation, as depicted in **Figure 2.9**, underlining its relationship with the primary source of *p*-cymene in these flavours. These results support the literature.

Figure 2.8. Change in concentration (ppm) of *p*-cresol over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

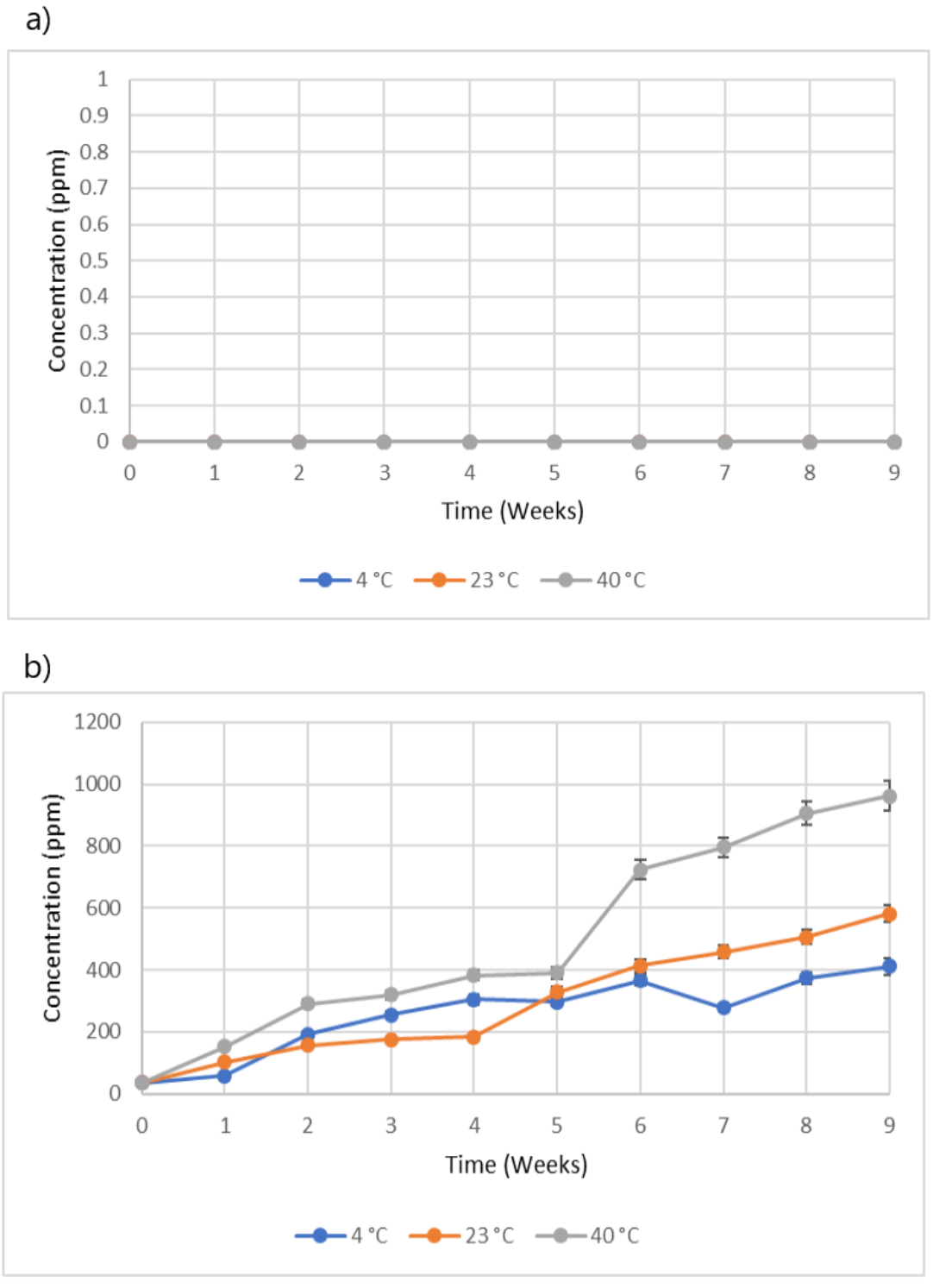
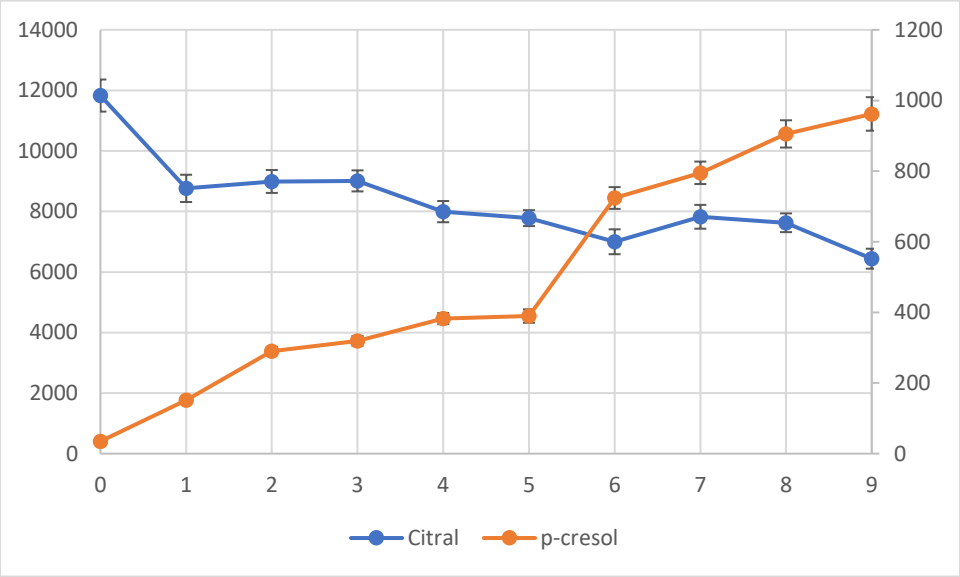


Figure 2.9. Formation of *p*-cresol and loss of citral over time in Flavour Mixture 2 at 40°C



p-cymenene

A lesser degradation product, *p*-cymenene formed over time in both flavour mixtures, but far more in Flavour Mixture 2 (see **Figure 2.10b**). In Flavour Mixture 1, it was initially identified at a level of 4 ppm and remained stable throughout the three storage treatments, as shown in **Figure 2.10a**. Conversely, in Flavour Mixture 2, it was detected at an initial level of 13.03 ppm and increased to 42.6 ppm, 97.2 ppm and 78.0 ppm after 9 weeks of storage at for 4°C, 23°C and 40°C, respectively (see **Table 2.5**). This represented an increase of 326.9%, 746.0% and 598.6%, respectively (see **Table 2.7**). *P*-cymenene likely formed from an acid-catalyzed oxidation of *p*-cymene, as illustrated in **Figure 2.11**. The fact that it did not increase in Flavour Mixture 1 suggests that it is a much slower process under more neutral pH conditions. The initial quantities in this formula likely stem from mild prior oxidation of the lemon oil used in these formulations, which contains citral, or from the pure citral itself. Although *p*-cymenene is almost certainly originating from *p*-cymene, a comparative graph would not be helpful, as *p*-cymene was produced at far higher concentrations under the same conditions and losses would not be visible.

Figure 2.10. Change in concentration (ppm) of *p*-cymenene over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

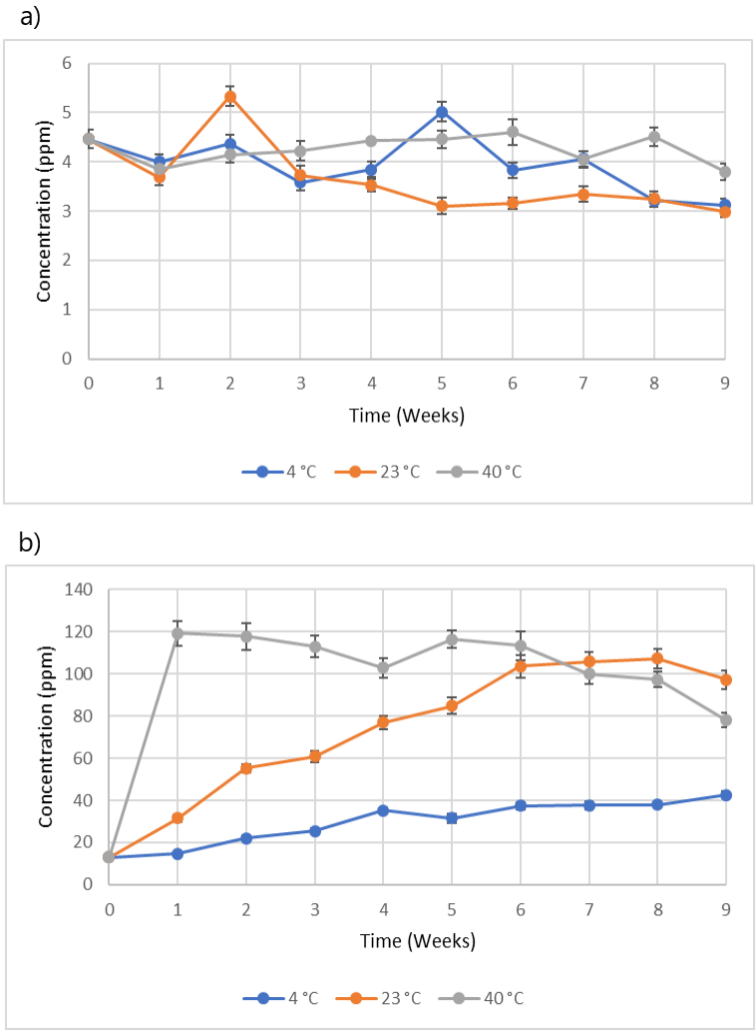
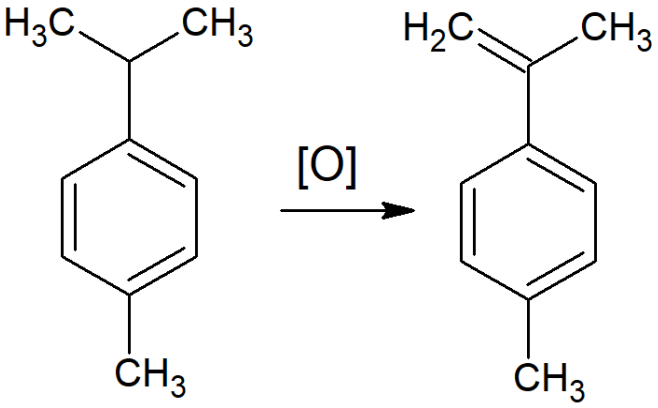


Figure 2.11. Proposed oxidation of *p*-cymene to *p*-cymenene



Benzaldehyde & Benzaldehyde Propylene Glycol Acetal

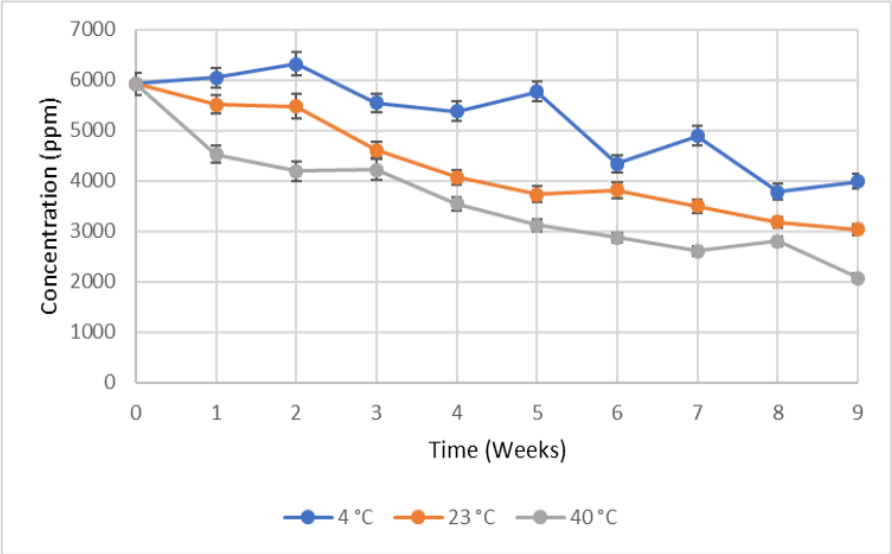
Benzaldehyde is an aromatic aldehyde and important secondary contributor to the cola flavours in this study. Benzaldehyde was added at an initial concentration of 6,000 ppm. In Flavour Mixture 1, the final concentrations of this compound after storage at 4°C, 23°C and 40°C were 3,994 ppm, 3,035 ppm and 2,078 ppm, respectively (see **Table 2.4**). These represented losses of 32.6%, 48.8% and 64.9%, respectively (see **Table 2.6**). A summary of the weekly changes in benzaldehyde over the 9-week period can be seen in **Figure 2.12**.

Benzaldehyde has been shown to undergo acid-catalyzed acetal formation in the presence of hydroxyl groups. In flavouring, propylene glycol is one of the most common solvents and was the main solvent of choice in this study. Propylene glycol has two available hydroxyl groups where the acetal reaction can occur, and it was expected that benzaldehyde would react with them to an extent. Indeed, benzaldehyde propylene glycol acetal (BPGA) was observed beginning from one week into storage. This compound formed more extensively as the storage temperature increased; after 9 weeks of storage, 286 ppm had formed at 4°C, 346 ppm had formed at 23°C and 1,808 ppm had formed at 40°C (see **Figure 2.13a**). Temperature clearly catalyzes this reaction.

Acidity appears to be the most significant catalyst for this reaction. In Flavour Mixture 2, which had a pH of approximately 2.5, BPGA had reached a level of 1,822 ppm in the brief period prior to analysis (see **Figure 2.13b**). In Flavour Mixture 1, by contrast, where the pH remained around 5.5, there was no detectable BPGA upon initial analysis. Additionally, the results obtained for BPGA support the fact that acetal formation is an equilibrium reaction. **Figure 2.14**, which depicts benzaldehyde and BPGA in Flavour Mixture 1 at 40°C, nicely demonstrates how these two compounds move towards an equilibrium with one another over time. In Flavour Mixture 1, BPGA formed most quickly and at significantly higher levels at 40°C, reaching peak concentrations by week 7 (~1,800 ppm) before stabilizing for the remainder of the storage period (see **Figure 2.13a**). As illustrated in **Figure 2.14**, this increase appears to mirror the loss of benzaldehyde. The pattern observed at 40°C in Flavour Mixture 1 occurred immediately in Flavour Mixture 2 under all temperature conditions: BPGA is present at peak concentration upon initial analysis, under all conditions, and tapers away slightly over the course of 9 weeks. Loss of BPGA over 9 weeks occurs more with increasing temperature, suggesting that this compound is also affected by heat.

Figure 2.12. Change in concentration (ppm) of benzaldehyde over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)



b)

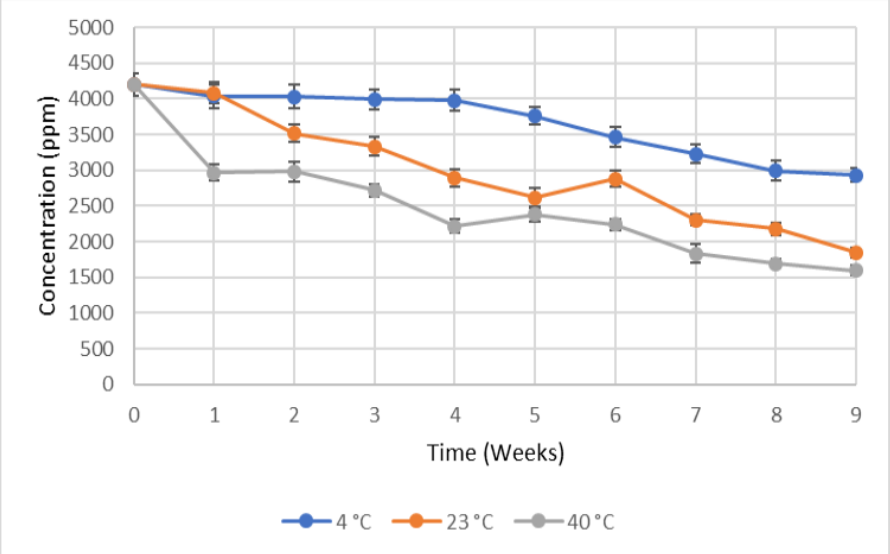
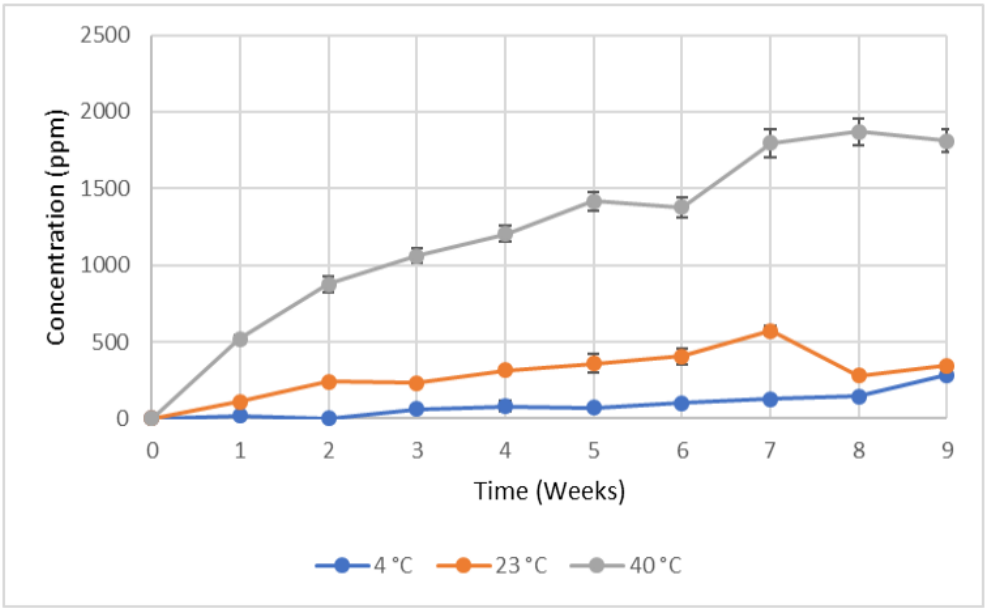


Figure 2.13. Change in concentration (ppm) of benzaldehyde propylene glycol acetal over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)



b)

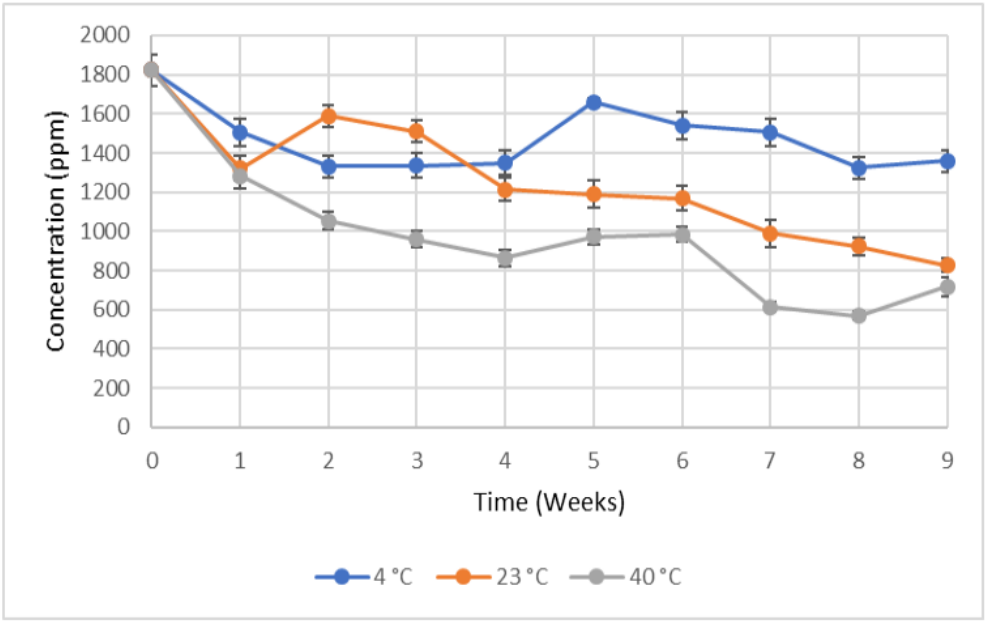
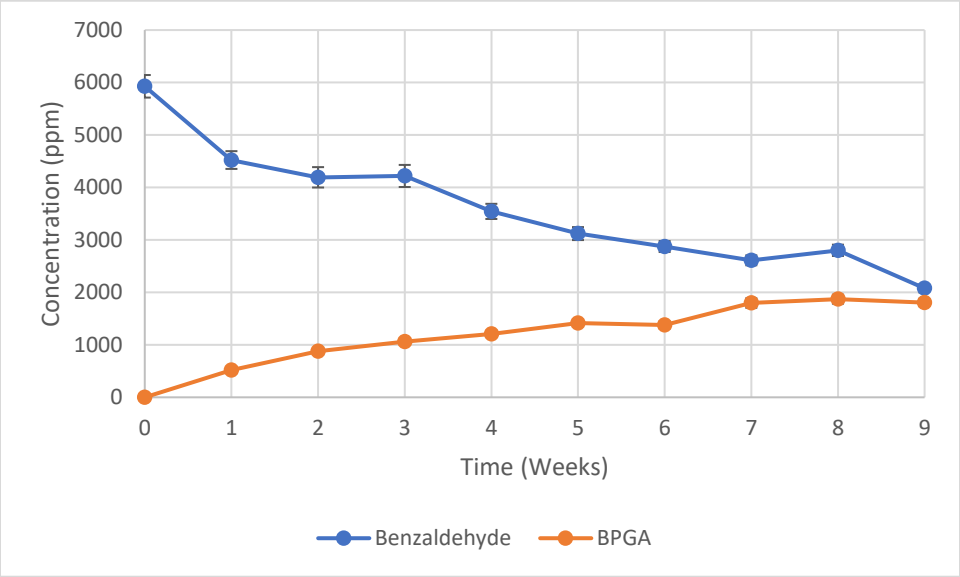


Figure 2.14. Benzaldehyde and Benzaldehyde Propylene Glycol Acetal in Flavour Mixture 1 at 40°C



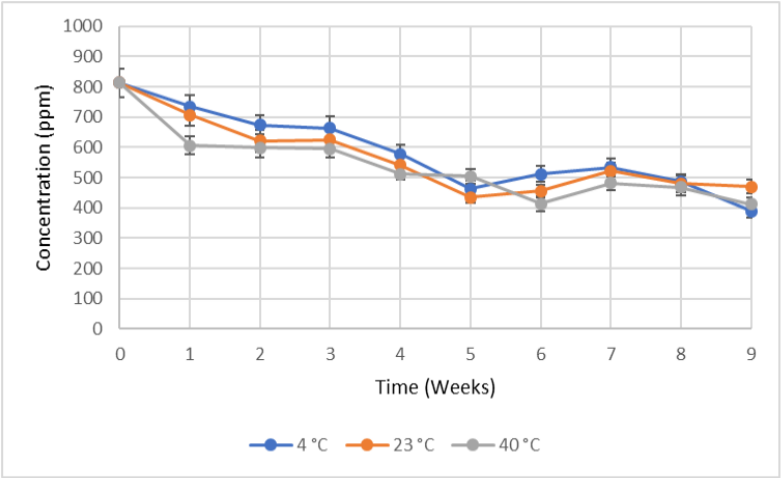
Cinnamaldehyde

Cinnamaldehyde is a flavour aldehyde responsible for the distinct aroma of cinnamon. Consequently, it is key to the cola profile, which has a characteristic spice note that derives from cinnamon oil. In Flavour Mixture 1, cinnamaldehyde was initially present at 812 ppm. After 9 weeks of storage at 4°C, 23°C and 40°C, 389 ppm, 470 ppm and 412 remained, respectively (see **Table 2.4**). These represented losses of 52.09%, 42.12% and 49.26%, respectively (see **Table 2.6**). In Flavour Mixture 1, the observed declines were very similar regardless of storage temperature (see **Figure 2.15a**). Its vapour pressure is relatively low, at 3.85 Pa [90], and there was no difference between the highest and lowest storage temperature, therefore the effects of volatilization alone should not account for these losses. There are two degradation reactions for cinnamaldehyde that could explain this. The first is the formation of a propylene glycol acetal. Like benzaldehyde, cinnamaldehyde undergoes this type of equilibrium reaction, which is catalyzed by acidity and heat. However, 4-methyl-2-[(E)-2-phenylethenyl]-1,3-dioxolane was not found in either Flavour Mixture 1 or Flavour Mixture 2, the latter of which would be most likely to produce them. It is possible that cinnamaldehyde propylene glycol acetal is not stable at high acidity and is lost. There is some evidence to support this; BPGA, at pH 2.5, was far less stable

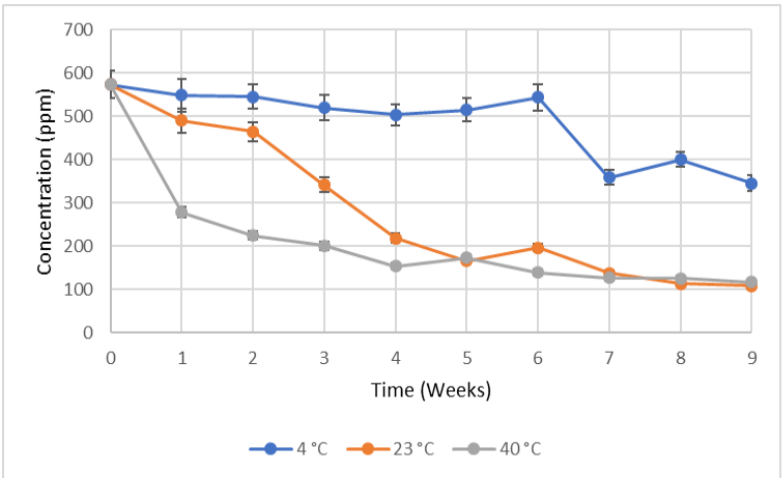
than it was in Flavour Mixture 1, despite being catalyzed by acid. Cinnamaldehyde subjected to the conditions of flavour mixture 2 was also affected significantly by acid, as depicted in **Figure 2.15b**. Although no acetals were detected, a second compound was found: ethyl cinnamate. Cinnamaldehyde was shown to form this compound when studied alone in a solvent mixture identical to Flavour Mixture 2. Ethyl cinnamate likely forms in a two-step reaction beginning with the auto-oxidation of cinnamaldehyde to cinnamic acid and followed by the esterification of cinnamic acid with ethyl alcohol (see **Figure 2.16**). Based on peak area, it appeared to form slowly and at low levels.

Figure 2.15. Change in concentration (ppm) of cinnamaldehyde over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)

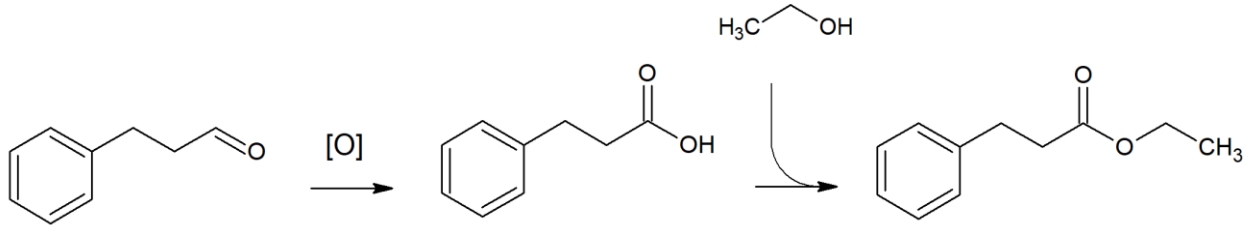


b)



Lastly, cinnamaldehyde has been shown to cleave into benzaldehyde both oxidatively and via thermal decomposition [82,83]. Presumably, any decline in cinnamaldehyde resulting from these types of reactions would result in increase benzaldehyde concentration, which was not the case. Although it is possible that benzaldehyde generated in this way would be imperceptible due to a much larger initial concentration of benzaldehyde, cinnamaldehyde did not generate benzaldehyde when studied alone. Therefore, it cannot be concluded that cinnamaldehyde is being lost other than to volatilization and ethyl cinnamate formation.

Figure 2.16. Proposed mechanism for the formation of ethyl cinnamate from cinnamaldehyde



Acetone

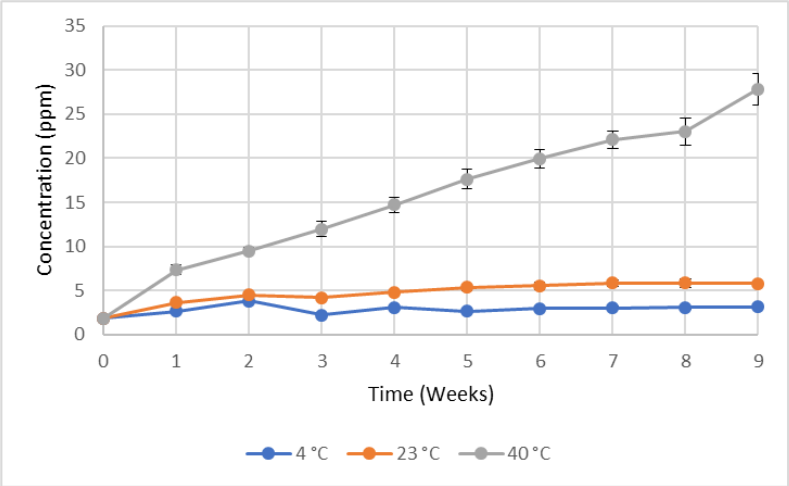
Acetone is the simplest ketone and a compound that is not deliberately added to flavours. In the flavour industry, it can be used in the analysis of flavour but is not employed to contribute a taste or aroma to aromatic mixtures. Acetone was not part of the original flavours and was present at very low levels upon initial analysis of both flavour mixtures (1.87 ppm and 3.74 ppm in Flavour Mixture 1 and Flavour Mixture 2, respectively) (see **Tables 2.4 & 2.5**). The fluctuations of this compound, in both flavour mixtures, are illustrated in **Figure 2.17**.

Acetone was an unexpected degradation product that formed in both mixtures. It formed more prominently at higher temperatures and was catalyzed by acid, as evident by the radical increase in this compound in Flavour Mixture 2 at 23°C and 40°C storage conditions, where it increased by 10,749% and 10,695%, respectively (see **Table 2.7**). An analysis of individual compounds in the cola mixtures revealed the primary source of acetone as terpinolene. Acetone was also observed in stored pure D-limonene but this was concluded to be mostly through a terpinolene intermediate – D-limonene isomerizes to terpinolene in the presence of acid. **Figure**

2.18 depicts the formation of acetone over 9 weeks versus the loss of terpinolene. The initial spike in terpinolene is due to its formation from D-limonene, a compound that was present at a much larger concentration and which degraded radically under high acid conditions (discussed further under *Terpinolene*).

Figure 2.17. Change in concentration (ppm) of acetone over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)



b)

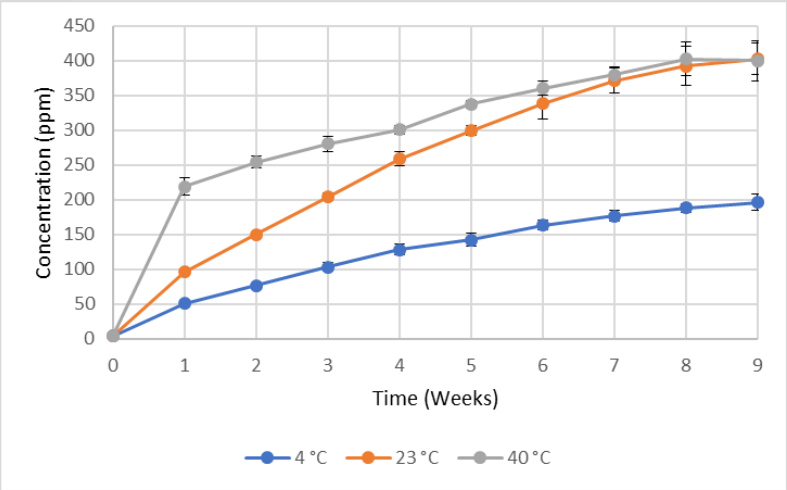
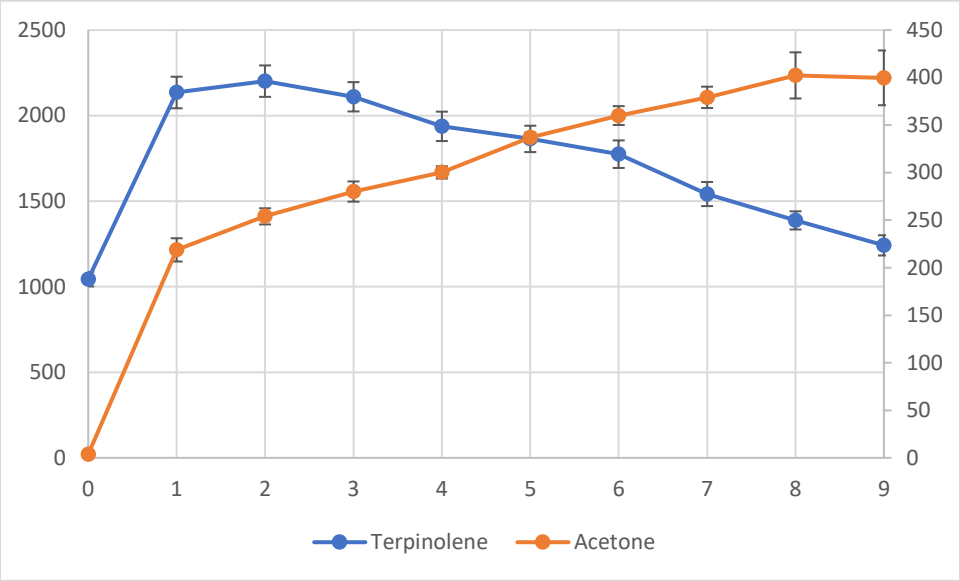


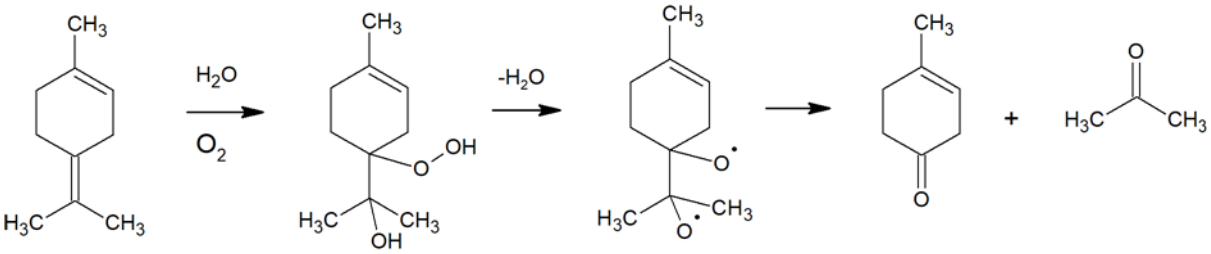
Figure 2.18. Formation of acetone and loss of terpinolene over time in Flavour Mixture 2 at 40°C



Acetone occurs in some monoterpenes via thermal oxidative pathways involving O_2 oxygen, which are accelerated by heat and available protons [84]. In terpinolene, the double bond undergoes free radical oxidative hydration to form the hydroperoxide shown in **Figure 2.19**, followed by loss of a water molecule and formation of the diradical. The diradical can quickly lose an acetone molecule through free the radical mechanism and generate 4-methyl-cyclo-3-hexenone. This mechanism was inspired from a study by Reissell et al. (1999), where a NO free radical served as initiator [84]. Both acetone and 4-methyl-cyclo-3-hexenone were detected in the samples, supporting this reaction. In the presence of catalysts, as much as 50% of acetone has been shown to form via oxidation of terpinolene. As discussed further in *Terpinolene*, a large drop in terpinolene was recorded over 9 weeks in samples where acetone formed. Other terpenes are similarly susceptible to oxidation but at significantly lower levels. D-limonene, for instance, also generates acetone via O_2 oxidation, but only at upwards of 3%. No other chemicals in these mixtures appeared to yield acetone or 4-methyl-cyclo-3-hexenone. Although samples were kept in sealed vials, they were opened for each weekly analysis. Therefore, despite limiting the potential for oxygen exposure, oxidation was well within the realm of possibility.

Acetone has a noticeably pungent, solvent-like aroma. However, it is not expected to have contributed a significant effect to the sensory profile of the cola mixtures. In the mixtures where it was most abundant, it formed in conjunction with several other compounds responsible for much more potent off-notes. These included *p*-cymene, *p*-cresol and BPGA. These compounds were typically at their highest levels at the same time as acetone and their odor descriptions best fit the notes given by panelists (e.g. petroleum & phenolic). Therefore, the impact of these two compounds on the sensory profile of the cola flavours is expected to have been minor.

Figure 2.19. Proposed mechanism for the formation of acetone and 4-methyl-3-cyclohexen-1-one from terpinolene



Most importantly, acetone is considered toxic to human health, by both inhalation and ingestion. Typically, exposure to acetone is via inhalation but information surrounding the ingestion of acetone is available [85]. The oral LD50 value, the ingested dose that is lethal to half of a test population, in adult rats is 5,800-7,138 mg/kg [85]. Symptoms resulting from persistent exposure to high levels (1,250 ppm to 20,000 ppm) of acetone in drinking water led to glycemia, cholesterolemia, hepatic fat accumulation and steatohepatitis [85]. However, acetone is not considered mutagenic or genotoxic, nor is it classified as a carcinogen by the International Agency for Research on Cancer (IARC) [85]. In adult humans, a single dose of 10-20 mL is usually not toxic but a dose exceeding 50 mL can be [86]. The observed toxic effects in adult humans tend to be relatively minor, primarily depressing the central nervous system and causing gastrointestinal disorders [86]. Symptoms of acetone poisoning may include headache, nausea, vomiting, dizziness, drowsiness and confusion [85]. Severe exposure may lead to unconsciousness or worse; in a case where 200 mL of acetone was ingested, the individual in question became comatose for 12 hours [86]. At its highest levels in the cola flavours, acetone reached above 400 ppm. This concentration in itself would not be enough to cause toxicity in humans. Considering that flavours

are applied at a fraction of their initial concentrations, the level of acetone in the final beverage would be significantly lower than this (in the realm of 0.8 ppm, if the flavour were dosed at the recommended 0.2%). Therefore, the formation of acetone should not be concerning.

2.2.1.3 Terpenes

Terpenes are the predominant chemical class, by quantity, in cola flavours. This is mainly because cola flavours are largely derived from citrus and spice essential oils. Although terpenes are not necessarily key contributors to the aroma or taste of cola, they demonstrated a susceptibility to adverse storage conditions and an inclination towards degradation. Given the high concentration of several terpenes, under certain conditions their degradation led to the formation of noticeable levels of potent off-notes, as well as important chemical imbalances.

α-Pinene & β-Pinene

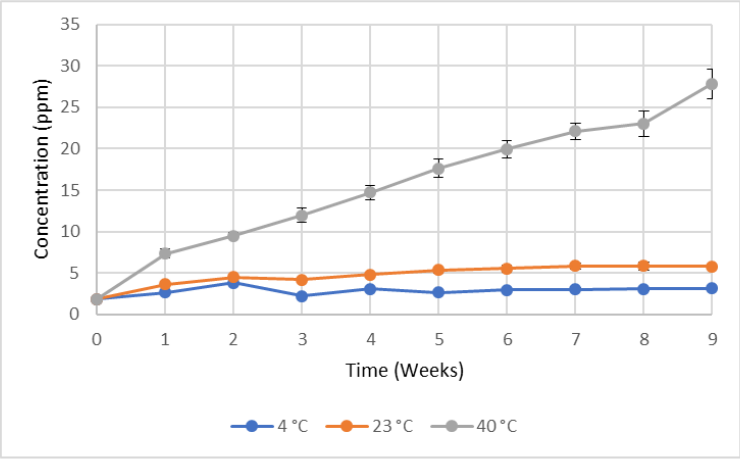
α-pinene is a monoterpene found in several essential oils important to the cola profile, including lemon, lime, cinnamon and nutmeg oils. Although it is a minor component in citrus and cinnamon oils, it is the predominant compound found in nutmeg oil. α-pinene was present at an initial concentration of 270 ppm. In Flavour Mixture 1, the final concentrations of this compound after storage at 4°C, 23°C and 40°C were 149 ppm, 137 ppm and 137 ppm, respectively (see **Table 2.4**). These represented losses of 44.8%, 49.3% and 49.3%, respectively (see **Table 2.6**). The boiling point of α-pinene is 155°C [90], therefore any losses relating to temperature would be similar from 4°C to 40°C. A similar decline both in quantity and in pattern was observed for all storage temperatures in this mixture, lending to the explanation that volatilization was the major driver of loss (see **Figure 2.20a**). Likewise, β-pinene underwent a slow, gradual decline over the course of 9 weeks under these conditions (see **Figure 2.21a**). After storage at 4°C, 23°C and 40°C, β-pinene degraded from 412 ppm to 332 ppm, 298 ppm and 286 ppm, respectively (see **Table 2.4**). These represented losses of 19.42%, 27.67% and 30.58%, respectively (see **Table 2.6**).

Other possible causes of loss than volatilization, including acid-catalyzed rearrangement and oxidation, are less likely in these cases. Flavour Mixture 1 held a pH of approximately 5.5 throughout the 9-week storage period. Pinenes are susceptible to acid-catalyzed rearrangements, even under mildly acidic conditions, which are further catalyzed by heat. However, there were only slight differences between the final values of α-pinene in Flavour Mixture 1. Although it is

expected that this was contributing to the observed losses, it was probably no more a factor than standard volatilization. Additionally, although pinenes are susceptible to oxidation, the typical indicative oxidation products for these compounds, including pinic acid and *cis*-pinonic acid were not detected.

Figure 2.20. Change in concentration (ppm) of α-pinene over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)



b)

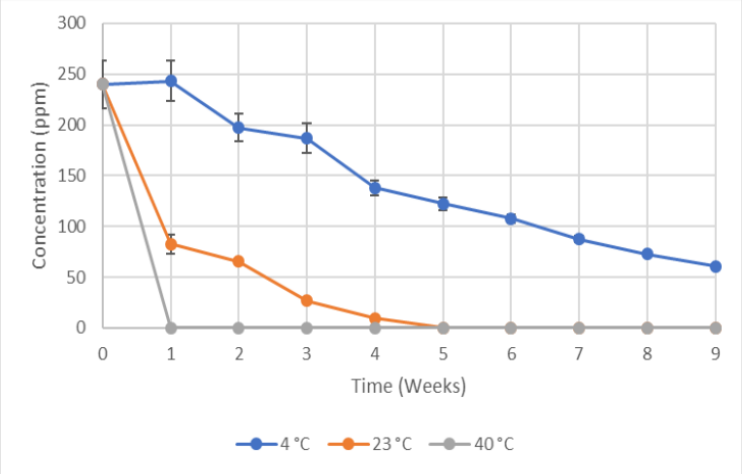
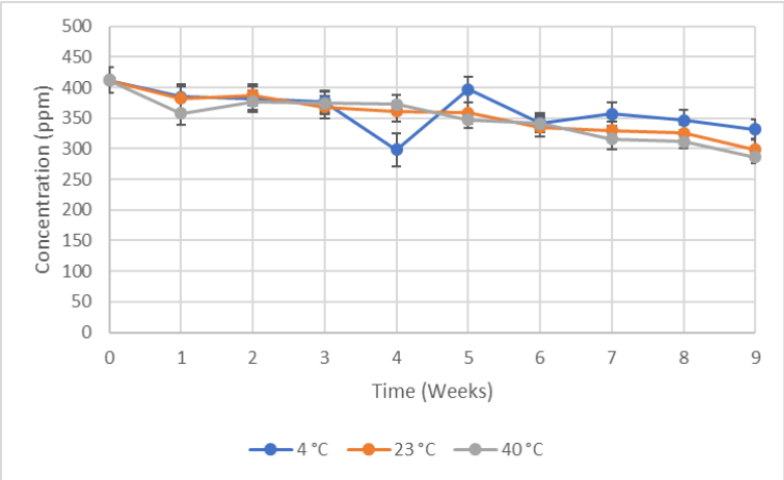
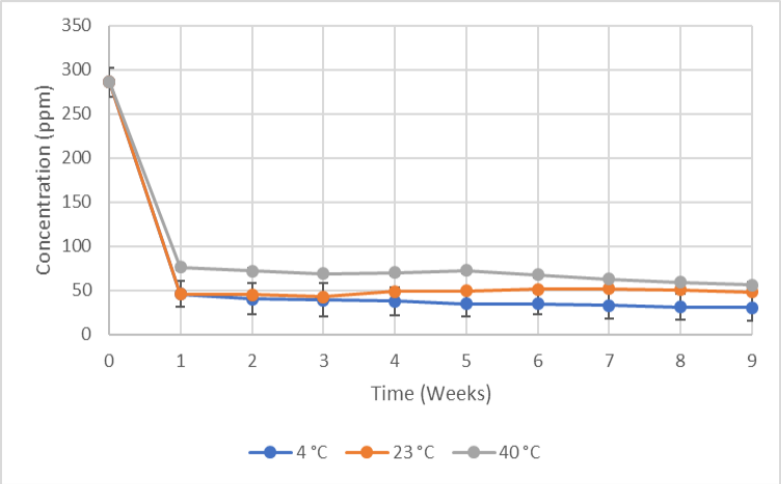


Figure 2.21. Change in concentration (ppm) of β-pinene over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)



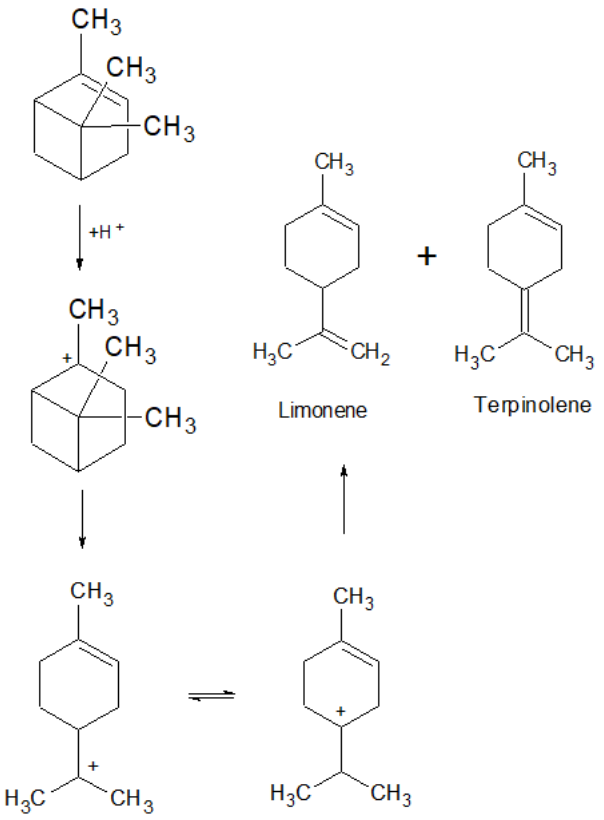
b)



Conversely, in Flavour Mixture 2, acid-catalyzed rearrangements were almost certainly the driving cause of pinene losses. A brief analysis of α-pinene, alone in an identical solvent system as Flavour Mixtures 1 & 2, showed rearrangements into numerous terpenes. In order of abundance, the major transformation compounds were D-limonene, terpinolene, camphene, terpin hydrate and fenchyl acetate. A mechanism for the formation of D-limonene and terpinolene, derived from information presented in a 2011 study [87] on pinene acid-catalyzed isomerization, is illustrated in **Figure 2.22**. At pH 2.5, all the α-pinene initially present in the analysis had vanished within 7 weeks, regardless of storage temperature. In Flavour Mixture 2, α pinene rapidly reacted at 23°C

and 40°C, having become undetectable after 5 weeks and 1 week, respectively (see **Figure 2.20b**). Flavour Mixture 2 showed initial rises in terpinolene at all storage temperature and it is expected that the coinciding drops in α-pinene contributed to this. Particularly, a total depletion of α-pinene at 40°C occurred in tandem with a sharp spike in terpinolene upon analysis after week 1, though it would not explain the extent of this increase alone. Although alone it was shown to convert more to D-limonene, any transformation of α-pinene into this compound would be mostly imperceptible in the mixture, given the initial quantity of D-limonene and the dramatic changes that it underwent throughout the experiment.

Figure 2.22. Mechanism of the formation of limonene and terpinolene from α-pinene [87]



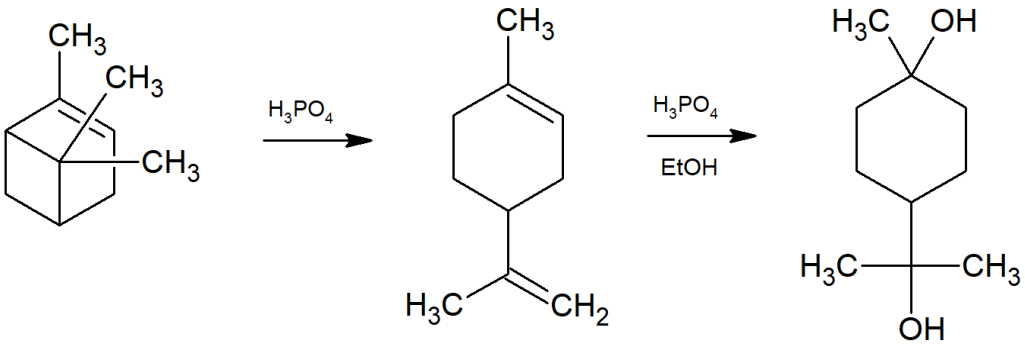
β-pinene was similarly sensitive to acidity. It broke down to a fraction of initial levels (286 ppm) within a week of storage before slowly tapering off for the remainder of the storage period (see **Figure 2.21b**). After 9 weeks of storage at 4°C, 23°C and 40°C, only 30.7 ppm, 48 ppm and 56.2 ppm, respectively (see **Table 2.6**). These represented losses of 89.25%, 83.17% and 80.35%,

respectively (see **Table 2.7**). In β-pinene, the major degradation products were terpinolene and terpin hydrate and it was therefore also a contributor to the initial spike in terpinolene observed under these conditions. Together, α- and β-pinene were the only two sources of terpin hydrate in Flavour Mixture 2.

Terpin hydrate

A compound that was initially unidentified formed over the course of storage. It became a point of interest given its unknown identity and unexpected formation. This compound remained undetected in Flavour Mixture 1, which was only mildly acidic, but formed progressively over time in Flavour Mixture 2, and more so at higher temperatures. It became clear that acid catalysis was a factor. Secondary experiments were conducted to determine the source of this compound: all the major compounds presented in this study were individually analyzed in solvent matrices corresponding to the flavour mixtures studied herein. It was found that the compound stemmed from three terpenes: α-pinene, β-pinene and D-limonene. Using this information, the compound was ultimately identified as terpin hydrate. This was confirmed on the basis of the mass spectrum and its formation was later elucidated based on the observed conditions as well as the literature. Terpin hydrate is synthesized industrially from turpentine, whose two major components are α-pinene and β-pinene. In the presence of a strong proton acid catalyst, such as phosphoric acid, which was used to acidify Flavour Mixture 2, both pinenes rearrange to terpin hydrate at relatively high levels. However, terpin hydrate requires moisture to form and although no water was deliberately added to the flavour formulations, it became evident that impurities in the ethanol were sufficient to cause hydration. A proposed mechanism for the formation of terpin hydrate from α-pinene, via a limonene intermediate, is illustrated in **Figure 2.23**.

Figure 2.23. Proposed mechanism for the formation of terpin hydrate

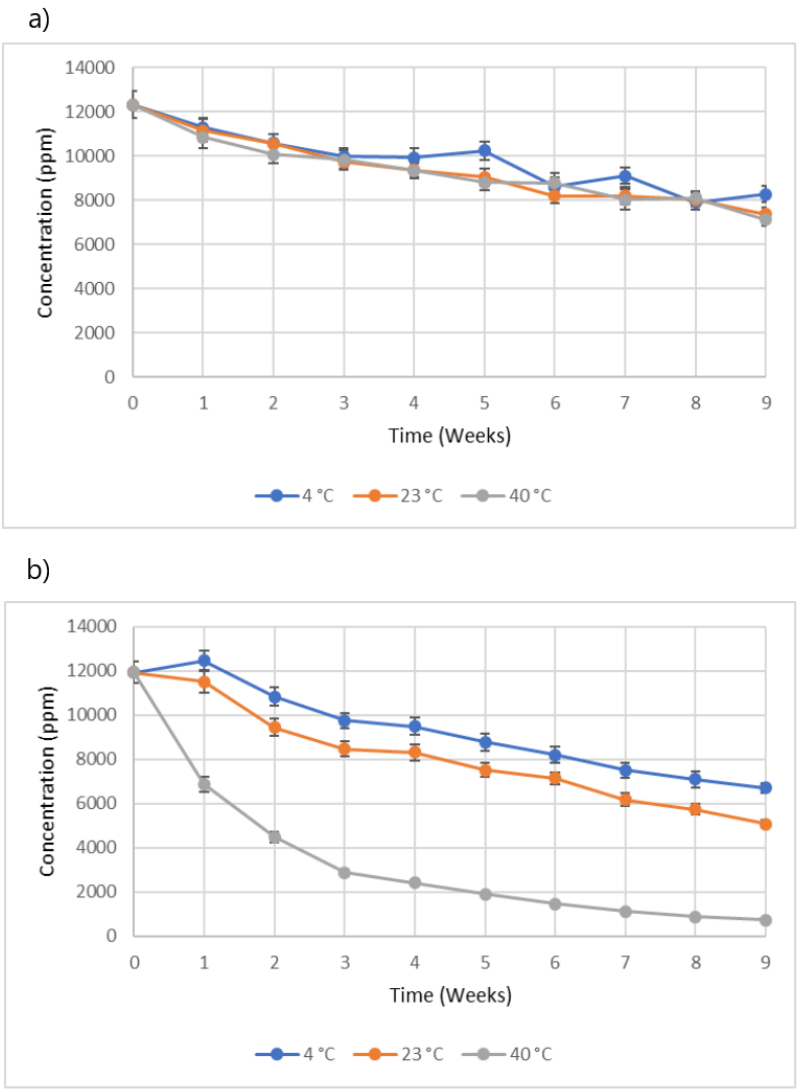


It is important to note that terpin hydrate has also been shown to form from D-limonene [39], the largest component of the cola flavour. Limonene has two unsaturated double bonds, which can be hydrated to form terpin hydrate. This is an acid-catalyzed process, which would explain why it did not occur in Flavour Mixture 1. Whereas terpin hydrate formation is far more extensive in pinenes, these precursors are found at far lower concentrations in the cola flavour. Conversely, D-limonene was present at the highest level among aromatic chemicals and small yields could be just as significant. Furthermore, pinenes form D-limonene as a predominant transformation product in the presence of organic acids. As time goes by, it is likely that D-limonene would then become hydrated by the moisture impurities in the ethanol solvent and progressively form more terpin hydrate. A simple analysis of D-limonene alone in a solvent identical to that of Flavour Mixture 2 showed formation of terpin hydrate within a week and progressive increases at two and three weeks.

D-Limonene

D-limonene is a cyclic monoterpene and the predominant aromatic compound, by concentration, in the cola flavour. D-limonene was present at an initial concentration of 12,323 ppm in Flavour Mixture 1. After 9 weeks of storage at 4°C, 23°C and 40°C, the final concentrations of this compound were 8,265 ppm, 7,375 ppm and 7,137 ppm, respectively (see **Table 2.4**). This represented a decline of 32.93%, 40.15% and 42.08%, respectively (see **Table 2.6**). The losses observed for D-limonene occurred at a similar rate at all temperatures and were both steady and gradual (see **Figure 2.24a**).

Figure 2.24. Change in concentration (ppm) of D-limonene over time in a) Flavour Mixture 1 and b) Flavour Mixture 2



In Flavour Mixture 2, loss of D-limonene was much more significant. After 9 weeks of storage at 4°C, 23°C and 40°C, the final concentrations of this compound were 6,707 ppm, 5,085 ppm and 733 ppm, respectively (see **Table 2.5**). These represented declines of 43.85%, 57.43% and 93.86%, respectively (see **Table 2.7**). The tremendous loss of this chemical in Flavour Mixture 2 compared with Flavour Mixture 1 derives from the vastly more acidic environment that it was exposed to. As a polyunsaturated terpene, D-limonene is particularly sensitive to reactions,

including acid-catalyzed rearrangements, hydrolysis, oxidation and hydration. Based on a secondary study of D-limonene, analyzed individually in a solvent matrix identical to that of Flavour Mixture 2, the major degradation product of D-limonene under these conditions was terpinolene. Terpinolene is an isomer of D-limonene, differing only in the location of its double bond in the isopropyl group. A proposed mechanism for the formation of terpinolene from limonene is presented in **Figure 2.25**. In the presence of a strong acid, the double bond migrates to the adjacent position, forming terpinolene. It is no surprise, then, that the decline in D-limonene at each temperature condition mirrors the formation of terpinolene. This was most evident in Flavour Mixture 2 at 40°C (see **Figure 2.26**).

Figure 2.25. Proposed mechanism for the acid-catalyzed isomerization of D-limonene to terpinolene and γ-terpinene

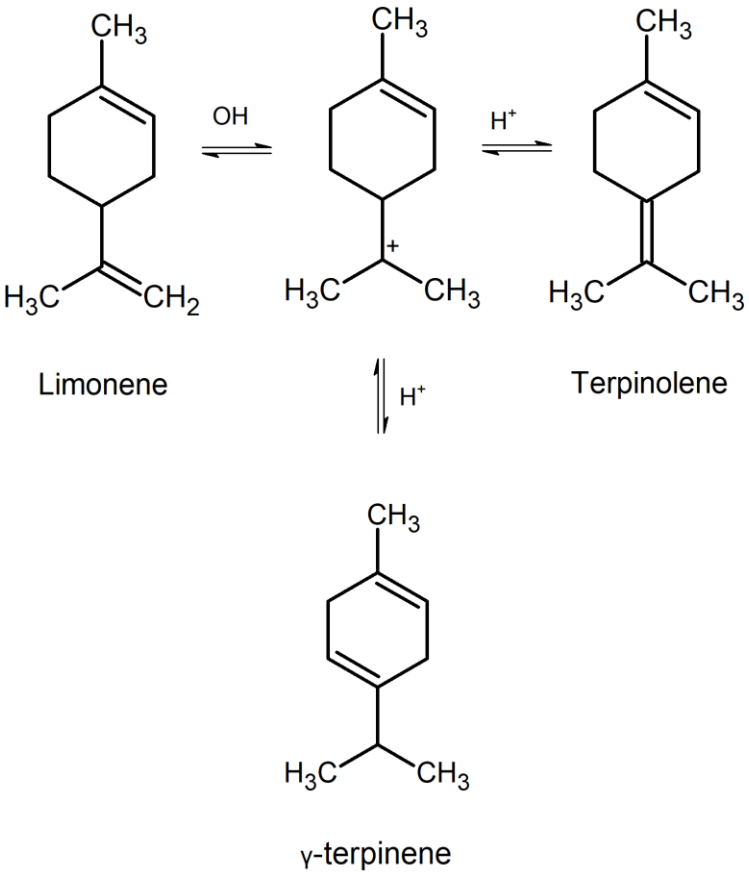
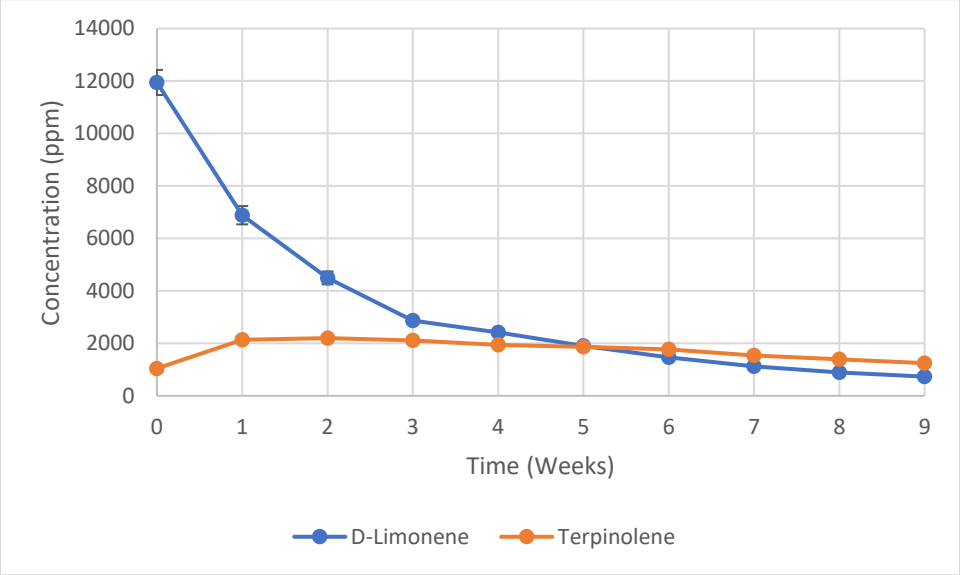


Figure 2.26. Formation of terpinolene and loss of D-limonene over time in Flavour Mixture 2 at 40°C

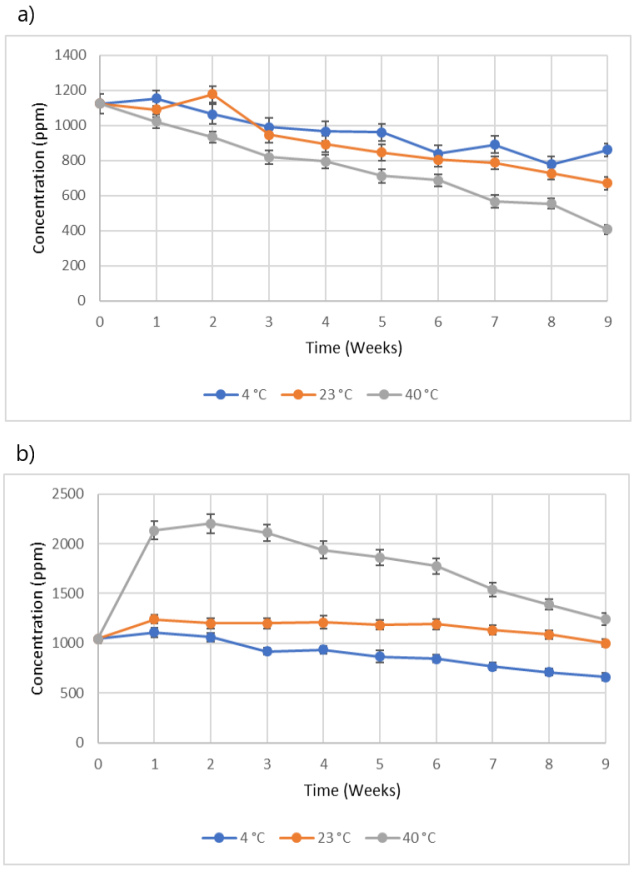


D-limonene formed several other degradation products under these conditions, albeit at lower concentrations. γ-Terpinene, another isomer of D-limonene, progressively formed over time. This chemical also differs only in the location of the double bond, which is found in the ring structure. A proposed mechanism for the acid-catalyzed isomerization of D-limonene to γ-terpinene is presented in **Figure 2.25**. Another significant formation product of D-limonene under these conditions was terpin hydrate, which was discussed in more detail in the previous subsection. D-limonene, in the presence of a strong acid like phosphoric acid, undergoes hydration at its two double bonds, effectively forming terpin hydrate, as illustrated in **Figure 2.23**. Interestingly, small amounts of acetone also appeared over time. Acetone, as discussed in greater detail in *Acetone*, likely formed following an initial isomerization of D-limonene to terpinolene, rather than directly from D-limonene.

Terpinolene

Terpinolene is a cyclic terpene found widely in essential oils. It has an odor reminiscent of wood, pine and citrus. Although it is not a major contributor to the aroma of cola, terpinolene was present at relatively high concentrations in the flavour mixtures. Like other terpenes in this study, it underwent significant degradation and lead to the formation of numerous compounds that could affect the equilibrium of the flavours. Terpinolene was initially present at 1,124 ppm in Flavour Mixture 1. After 9 weeks of storage at 4°C, 23°C and 40°C, the final concentrations recorded were 861 ppm, 670 ppm and 406 ppm, respectively (see **Table 2.4**). These represented losses of 23.4%, 40.39% and 63.88%, respectively (see **Table 2.6**). Terpinolene has a boiling point of 184°C and a vapor pressure of 66.7 Pa [90]. Terpinolene declined at a steady rate under all temperature treatments but more rapidly at higher temperatures. There were significantly higher losses at each higher storage temperature, underlining the effect of heat on this terpene (see **Figure 2.27a**).

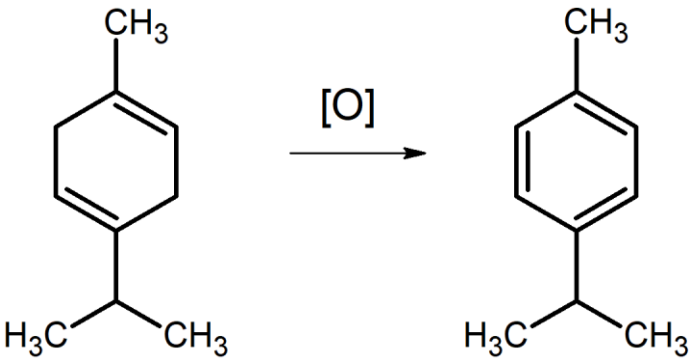
Figure 2.27. Change in concentration (ppm) of terpinolene over time in a) Flavour Mixture 1 and b) Flavour Mixture 2



In Flavour Mixture 2, terpinolene underwent additional reactions due to acid catalysis. Initially, 1,044 ppm was present in this mixture. After 9 weeks of storage at 4°C, 23°C and 40°C, the final concentrations recorded were 662 ppm, 1,003 ppm and 1,242 ppm, respectively (see **Table 2.5**). These represented losses of 36.59%, 3.93% and a gain of 19%, respectively (see **Table 2.7**). These results are in stark contrast to those of Flavour Mixture 1. Whereas losses increased with temperature in the absence of acidity, at a low pH, terpinolene remained relatively stable or increased (see **Figure 2.27b**). This can be attributed to acid-catalyzed rearrangement of D-limonene, which preferentially yields terpinolene. At 40°C, terpinolene increased dramatically at the expense of D-limonene and, despite a sensitivity to low pH and heat, remained higher in concentration even after 9 weeks.

When analyzed on its own, in an identical solvent mixture as Flavour Mixture 2, terpinolene yielded various isomers, including D-limonene, γ-terpinene and α-terpinene. Whereas isomerization from D-limonene to terpinolene was favoured (as discussed previously), it appears that isomerization in the reverse also occurred to some extent, as illustrated in **Figure 2.25**. Several degradation products also formed, including *p*-cymene and acetone. The compound *p*-cymene could be forming following an initial isomerization of terpinolene to γ-terpinene, followed by oxidation of γ-terpinene, however, as *p*-cymene appeared later (see **Figure 2.28**). Terpinolene was the major source of acetone in the mixture, with other monoterpenes producing only secondary quantities. Terpinolene readily oxidizes to acetone, especially when exposed to heat and acid. This is reflected in **Figure 2.17**, where acetone is shown to form more rapidly with greater heat and acidity. The initial spike in terpinolene in this graph corresponds with a sharp initial decline in D-limonene after 1 week of storage. Acetone, as discussed in greater detail under *Acetone*, forms from the oxidation of the double bonded site in terpinolene, followed by proton abstraction. It is an indicator of terpinolene oxidation and proves that oxygen played at least some role in the degradation of the overall flavour.

Figure 2.28. Proposed mechanism for the formation of *p*-cymene from γ-terpinene



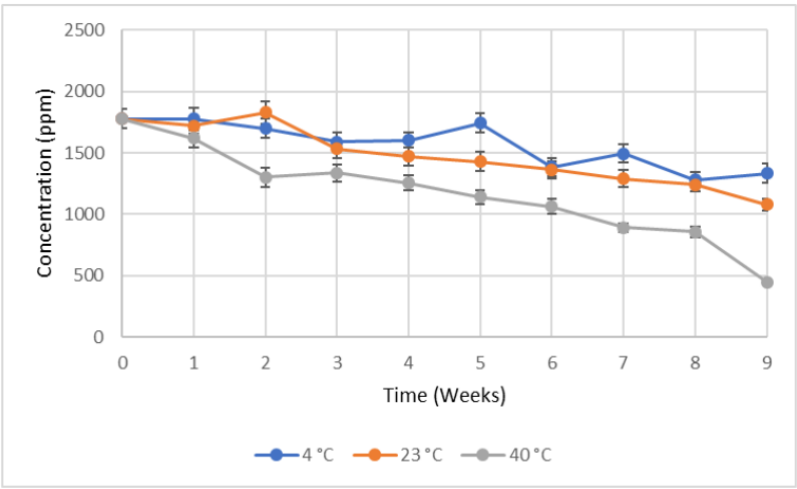
γ-*Terpinene*

γ-Terpinene is a monoterpene and an isomer of terpinolene. It is an important component of citrus essential oils; in lemon oil and lime oils, for instance, it typically represents over 7% and 11% of the overall composition. γ-Terpinene has a terpene-like, sweet, citrus aroma that often hints at lime. Therefore, it can be considered an important contributor to the sensory profile of the cola flavours.

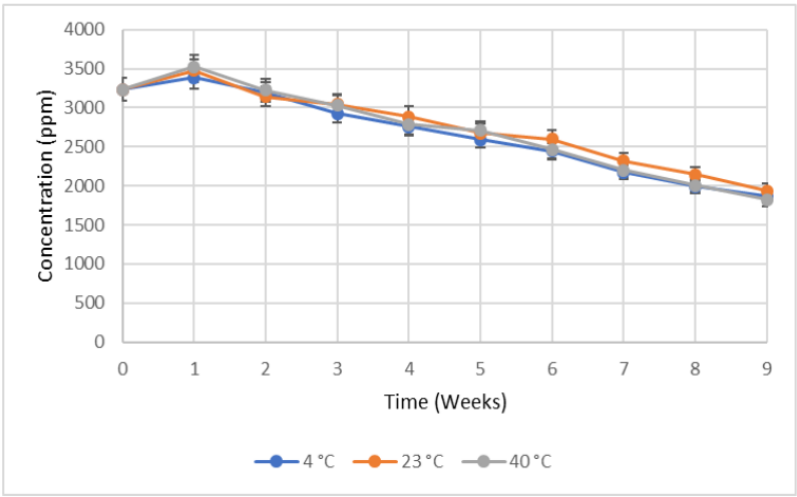
γ-Terpinene was present at an initial concentration of 1,778 ppm in Flavour Mixture 1. After 9 weeks of storage at 4°C, 23°C and 40°C, the final concentrations recorded were 1,332 ppm, 1,080 ppm and 449 ppm, respectively (see **Table 2.4**). These represented losses of 25.08%, 39.26% and 74.75%, respectively (see **Table 2.6**). Among the terpenes studied in this experiment, γ-terpinene is among the least stable to heat. This can be seen in the increasingly profound impact of higher temperature conditions (see **Figure 2.29a**). Although its boiling point is the same as its isomer terpinolene (~183°C) [90], γ-terpinene has an estimated vapour pressure of 143 Pa [90], which over twice as high. γ-Terpinene is also susceptible to oxidation, with its major oxidative by-product being *p*-cymene (see **Figure 2.28**). Taken alone, γ-terpinene produces more *p*-cymene than citral. However, citral was far more abundant and ultimately generated more of this off-note in the colas.

Figure 2.29. Change in concentration (ppm) of γ-terpinene over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)



b)



In stark contrast to its temperature-sensitivity, γ-terpinene proved resistant to acid-catalyzed rearrangements. In fact, the concentration of this compound initially rose prior to a slower, less pronounced decline than the ones observed in Flavour Mixture 1. There was very little difference in the losses of γ-terpinene at 4°C, 23°C and 40°C under acidic conditions (see **Figure 2.29b**). This was unexpected, especially considering the results of Flavour Mixture 1, which are in line with the literature. However, γ-terpinene was being produced by its isomer terpinolene, which in turn was being augmented by the degradative hydration of D-limonene, both of which were significantly more concentrated. This feeding of new γ-terpinene grew at higher

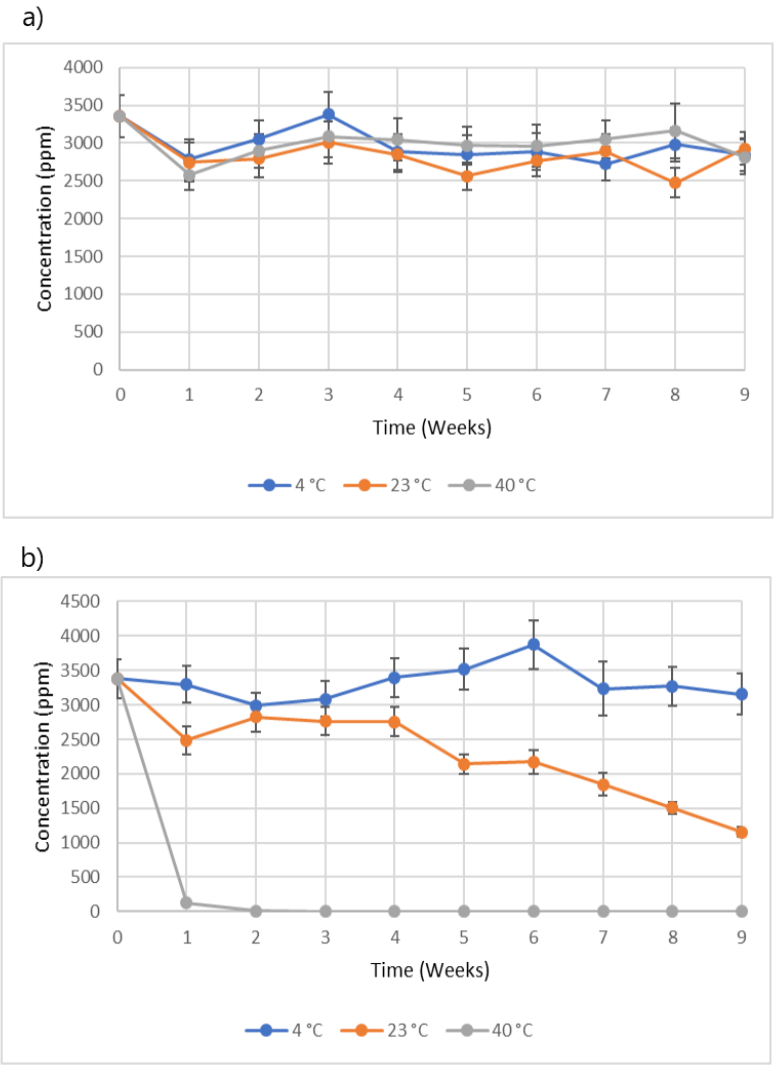
temperatures, which could explain why the losses of γ-terpinene were offset. Ultimately, γ-terpinene declined by 42.31%, 40.2% and 43.7% after storage at 4°C, 23°C and 40°C, respectively (see **Table 2.7**).

2.2.1.4 Alcohols & Ethers

Linalool

Linalool is a terpene alcohol with a potent citrus or orange-like aroma and naturally occurs in many citrus oils. It was also deliberately added to the cola flavours in its pure form to enhance these qualities. The initial concentration of this compound in Flavour Mixture 1 was 3,356 ppm. After 9 weeks of storage at 4°C, 23°C and 40°C, the final concentrations recorded were 2,844 ppm, 2,923 ppm and 2,812 ppm, respectively (see **Table 2.4**). These represented declines of 15.3%, 12.9% and 16.2%, respectively (see **Table 2.6**). When considering error, there was no significant difference in the final concentrations of these compounds. Moreover, similar patterns of change were observed throughout the 9-week storage period (see **Figure 2.30a**). Therefore, for Flavour Mixture 1, it can be concluded that differences in storage temperature did not seem to influence linalool. This makes sense given the physical properties of this compound. Linalool has a boiling point of 198°C [90] and a vapor pressure at room temperature of 21 Pa [90] and was therefore less likely to undergo extensive evaporation during storage.

Figure 2.30. Change in concentration (ppm) of linalool over time in a) Flavour Mixture 1 and b) Flavour Mixture 2



In Flavour Mixture 2, linalool underwent much more dramatic changes. After 9 weeks of storage at 4°C, 23°C and 40°C, the final concentrations of this compound were 3,158 ppm, 1,157 ppm and 0 ppm, respectively (see **Table 2.5**). This represented losses of 6.71%, 65.82% and 100%, respectively (see **Table 2.7**). Linalool, like the other terpenes in this study, is very susceptible to acid-catalyzed rearrangements. At 23°C, near total loss of linalool occurred whereas complete degradation was observed at 40°C (see **Figure 2.30b**). Analysis of linalool alone in an acidified solvent matrix identical to that of Flavour Mixture 2 showed the formation of various terpenes over time, namely terpinolene, α-terpineol and eucalyptol. α-Terpineol forms from the cyclization

of linalool (see **Figure 2.31**) and eucalyptol likely formed via isomerization of α-terpineol, as illustrated in **Figure 2.32**. Additionally, esterification appeared to have taken place, with linalyl butyrate forming. Geranyl ethyl ethers also accumulated over time. Whereas storage temperature had no apparent effect alone, when heat was coupled with a low pH these acid-catalyzed reactions were further accelerated.

Figure 2.31. Proposed mechanism for the formation of α-terpineol from linalool

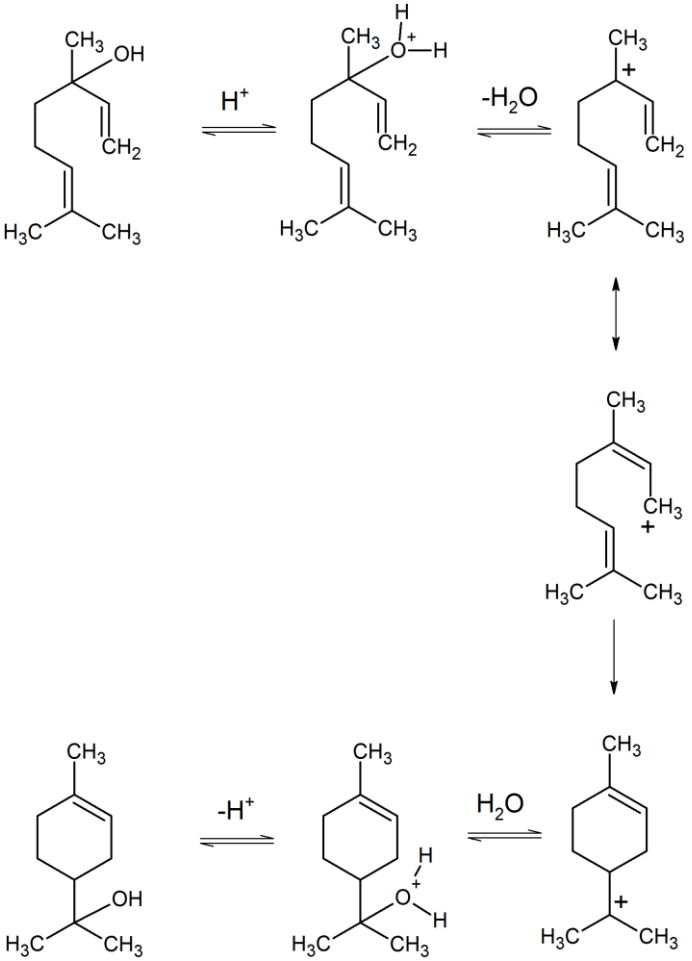
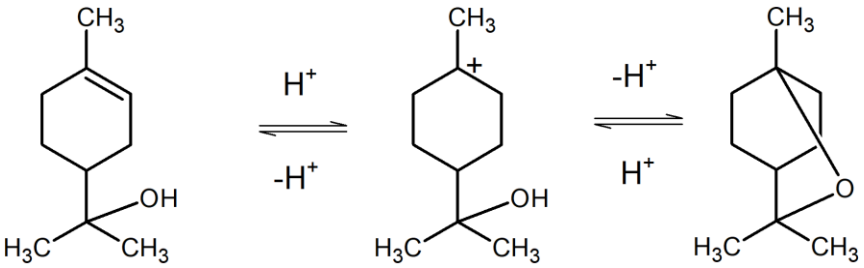
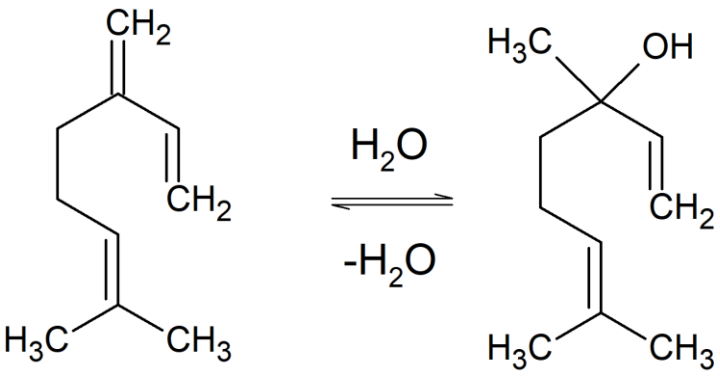


Figure 2.32. Proposed mechanism for the formation of eucalyptol from α-terpineol



The decline in linalool observed in Flavour Mixture 2 was not steady. In fact, at certain analysis points, the concentration of linalool had surprisingly increased. This may be due to reactions relating to myrcene, a terpene that is present in both lemon and lime oils but which does not have an important sensory function in cola flavours. Myrcene, like the terpenes presented herein, declined steadily over the course of storage and could yield linalool upon hydration, as illustrated in **Figure 2.33**. The ethanol used in the cola formulation had a 5% impurity consisting almost entirely of water, making this possible. Similar hydration reactions were observed in D-limonene, yielding terpin hydrate. However, none of the major terpenes presented here produced linalool.

Figure 2.33. Proposed mechanism for the formation of linalool from myrcene



α-terpineol

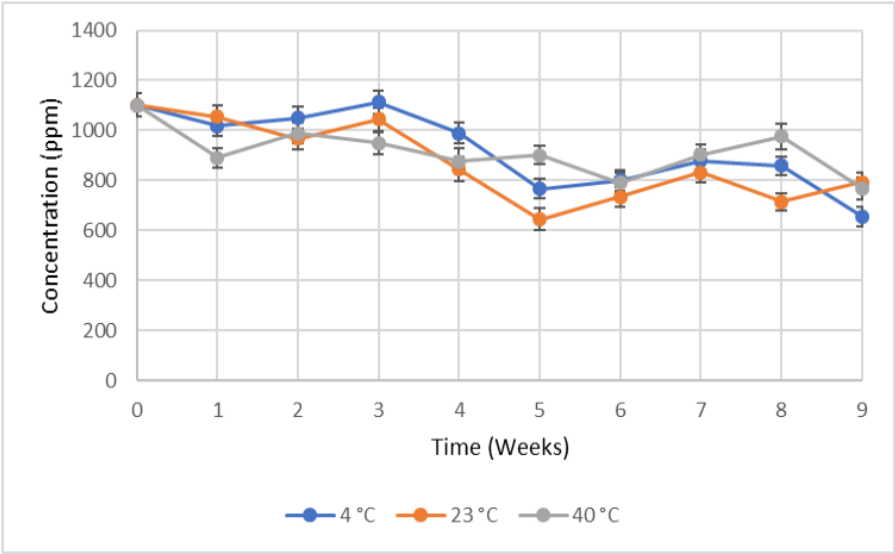
α-terpineol is a monoterpene alcohol that is an important component of several essential oils, including citrus oils. Its odor is pine-like and citrus-like, with an element of woody, terpene character. α-terpineol occurred naturally in the lime oil, lemon oil and, to a lesser extent, the cinnamon oil used in the cola flavours In Flavour Mixture 1, it was present at an initial level of 1,102 ppm and, after 9 weeks of storage at 4°C, 23°C and 40°C, 656.5 ppm, 793.2 ppm and 768.3 ppm remained, respectively (see **Table 2.4**). This represented losses of 40.47%, 28.04% and 30.31%, respectively (see **Table 2.6**). The pattern of formation is illustrated in **Figure 2.34a**. This compound was expected to decline more at higher temperatures, but this was not the case. Rather, more α-terpineol remained at 23°C and 40°C, suggesting that other compounds were degrading and generating it. Upon closer analysis of individual compounds, it was determined that α-terpineol was forming both from D-limonene and from linalool. This was confirmed after further research into the literature. When both compounds are present, α-terpineol reactions becomes increasingly complex, as it forms from two sources and degrades. D-limonene, via a hydration reaction catalyzed by both pH and heat, is directly converted to α-terpineol. Linalool, meanwhile, dehydrates, isomerizes and rehydrates to form α-terpineol in a series of reactions that are also catalyzed by heat and temperature. α-terpineol itself isomerizes to eucalyptol, particularly in the presence of protons. It can also dehydrate to D-limonene.

The influence of pH on α-terpineol formation is reflected in Flavour Mixture 2. After 9 weeks of storage at 4°C, 23°C and 40°C, α-terpineol increased from 1,143 ppm to 1,724 ppm, 3,159 ppm and 4,311 ppm, respectively (see **Table 2.5**). This represented significant increases of 150.9%, 276.4% and 377.2%, respectively (see **Table 2.7**). Conversely to Flavour Mixture 1, α-terpineol rose under at all storage conditions (see **Figure 2.34b**). Most notably, at 40°C α-terpineol rose sharply, in conjunction with a decline of D-limonene, before peaking at 6 weeks and slowly declining, only after D-limonene had been nearly depleted, as depicted in **Figure 2.35**. α - Terpineol has been shown to degrade into *cis*- and *trans*-1,8-terpins, as discussed in *Rearrangement of Bicyclic Monoterpenes:* α- *and β-Pinene*. Although these compounds were identified based on their spectral properties, standards were unfortunately unavailable, and they could not be quantified. Based on their relative proportion to other compounds, however, their

formation seemed limited. Ultimately, contrary to expectations, α-terpineol became increasingly concentrated under more acidic and under heated conditions.

Figure 2.34. Change in concentration (ppm) of α-terpineol over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

a)



b)

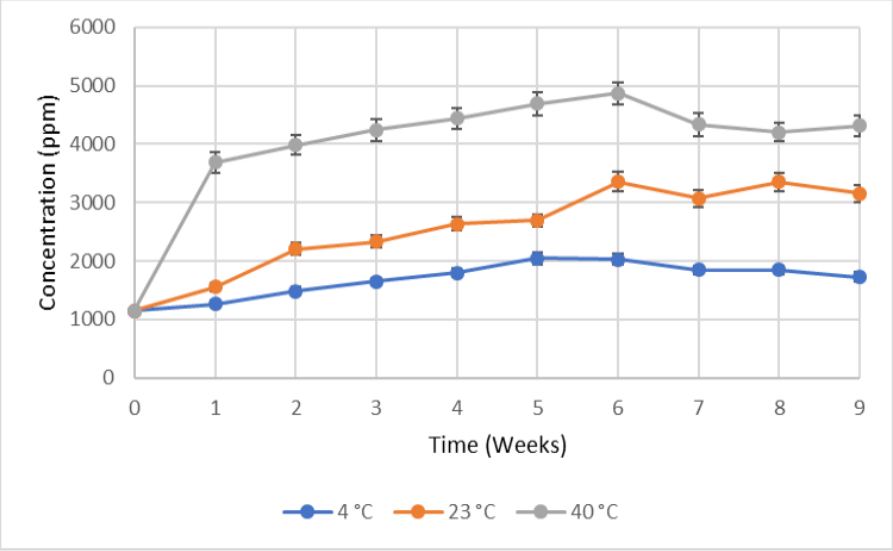
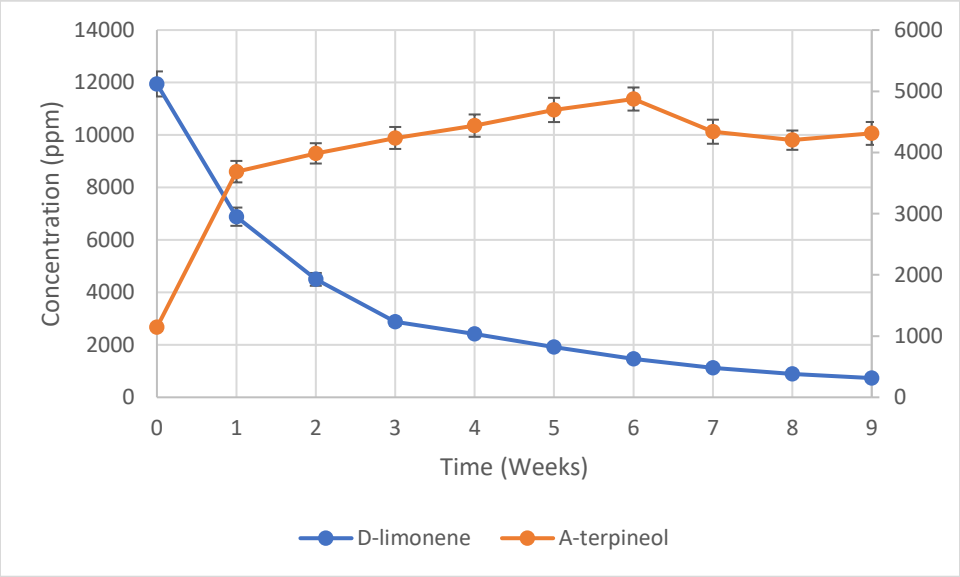


Figure 2.35. Formation of α-terpineol and loss of D-limonene over time in Flavour Mixture 2 at 40°C



Eucalyptol

Eucalyptol, AKA 1,4 cineole, is a cyclical ether monoterpenoid found in many essential oils. Because it does not contribute strongly to the sensory profile of cola flavours, it was not initially a compound of interest. Eucalyptol has a strong, camphoraceous aroma that can also be described as fresh, minty and cool. It is also a potent flavoring agent when properly employed. Eucalyptol was initially found at rather low levels in the cola flavour mixtures, mostly because it naturally occurs in lime oil. In Flavour Mixture 1, it was present at 208 ppm initially. After 9 weeks of storage at 4°C, 23°C and 40°C, eucalyptol declined to 150 ppm, 127 ppm and 125 ppm, respectively (see **Table 2.4**). These represented losses of 27.88%, 38.94% and 39.9%, respectively (see **Table 2.6**). These were likely simple losses due to volatilization; the vapour pressure of eucalyptol at 25°C is 253 Pa [90] and, correspondingly, a gradual decline over time was observed (see **Figure 2.36a**). The changes in eucalyptol were more noticeable and interesting in Flavour Mixture 2 (see **Figure 2.36b**). After 9 weeks of storage at 4°C, 23°C and 40°C, it reduced to 162 ppm, 157 ppm and increased to 326 ppm (see **Table 2.5**). This represented losses of 27.68%, 29.91% and a gain of 45.5%, respectively (see **Table 2.7**). Unexpectedly, under these conditions, more eucalyptol remained than in Flavour Mixture 1. However, this is most likely the result of eucalyptol formation from other sources, rather than any resistance to low pH conditions. At low

pH, eucalyptol forms from the isomerization of α-terpineol (see **Figure 2.32**), as demonstrated in an individual study of α-terpineol in a solvent mixture identical to Flavour Mixture 2. Since α-terpineol increased dramatically in Flavour Mixture 2, it is difficult to graphically illustrate the relatively small quantities that isomerized to eucalyptol. However, of the 19 compounds studied in this experiment, eucalyptol only formed where α-terpineol was present.

Figure 2.36. Change in concentration (ppm) of eucalyptol over time in a) Flavour Mixture 1 and b) Flavour Mixture 2

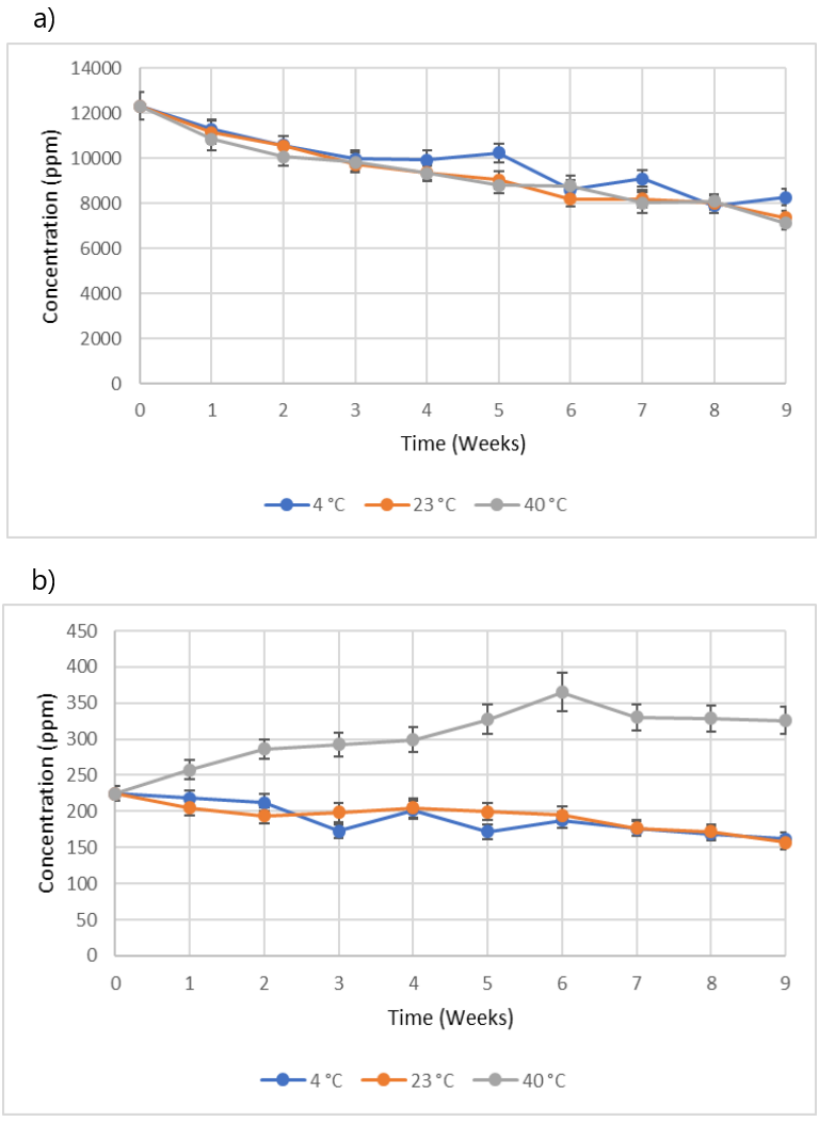


Table 2.4 Initial and final concentrations (ppm)* of characteristic compounds in Flavour Mixture 1 subjected to three temperature conditions

Compound	Retention time (t_R) (min)	Initial Concentration (ppm)	Final Concentration (ppm)		
			Storage at 4°C	Storage at 23°C	Storage at 40°C
Acetone	1.23	1.87 ±0.11	3.13 ±0.16	5.81 ±0.30	27.8 ±1.78
Ethyl Acetate	1.35	3.34 ±0.21	16.7 ±0.63	7.16 ±0.77	ND**
α-Pinene	1.90	270 ±7.37	149 ±6.09	137 ±9.17	137 ±4.78
Ethyl Butyrate	1.93	6036 ±272	4087 ±147	3803 ±111	3800 ±140
β-Pinene	2.55	412 ±20.7	332 ±15.6	298 ±16.5	286 ±10.8
D-Limonene	3.67	12323 ±620	8265 ±366	7375 ±288	7137 ±303
Eucalyptol	3.88	208 ±8.85	150 ±7.38	127 ±8.21	125 ±7.27
γ-Terpinene	4.48	1778 ±78.0	1332 ±79.4	1080 ±47.7	449 ±20.4
***p*-cymene**	4.99	20.7 ±8.36	254 ±10.7	322 ±13.4	765 ±26.0
Terpinolene	5.28	1124 ±54.7	861 ±36.6	670 ±37.7	406 ±27.8
***p*-cymenene**	9.76	4.46 ±0.18	3.12 ±0.13	2.99 ±0.12	3.80 ±0.16
Terpin Hydrate	10.4	ND	ND	ND	ND
Benzaldehyde	12.8	5928 ±215	3994 ±138	3035 ±116	2078 ±82.4
Linalool	15	3356 ±278	2844 ±212	2923 ±216	2812 ±229
Citral (Neral + Geranial)	20.8 + 23.2	11830 ±530	6144 ±242	6943 ±317	6440 ±330
α -Terpineol	21.9	1102 ±45.6	656 ±40.4	793 ±37.2	768 ±41.9
Benzaldehyde Propylene Glycol Acetal	26.9	ND	286 ±13.0	346 ±15.3	1808 ±74.5
Cinnamaldehyde	32.5	812 ±47.3	389 ±20.4	470 ±22.2	412 ±21.6
***p*-cresol**	33.65	ND	ND	ND	ND

*Average of three replicates; **ND=Non-detected

Table 2.5 Initial and final concentrations (ppm)* of characteristic compounds in Flavour Mixture 2 subjected to three temperature conditions

Compound	Retention time (t_R) (min)	Initial Concentration (ppm)	Final Concentration (ppm)		
			Storage at 4°C	Storage at 23°C	Storage at 40°C
Acetone	1.23	3.74 ±0.18	196 ±11.4	402 ±22.3	400 ±28.8
Ethyl Acetate	1.35	10.4 ±0.58	158 ±6.91	168 ±7.53	193 ±5.07
α-Pinene	1.90	240 ±12.4	60.7 ±3.60	ND**	ND
Ethyl Butyrate	1.93	5923 ±247	3673 ±169	3422 ±169	3440 ±125
β-Pinene	2.55	286 ±16.6	30.7 ±15.6	48 ±2.29	56.2 ±2.92
D-Limonene	3.67	11945 ±478	6707 ±231	5085 ±174	733 ±31
Eucalyptol	3.88	224 ±10.1	162 ±8.27	157 ±9.22	326 ±18.4
γ-Terpinene	4.48	3236 ±140	1867 ±90.0	1935 ±95.3	1822 ±81.8
***p*-cymene**	4.99	660 ±32.2	1224 ±49.6	2938 ±112	7611 ±248
Terpinolene	5.28	1044 ±42.2	662 ±38.2	1003 ±41.3	1242 ±59.1
***p*-cymenene**	9.76	13.03 ±0.79	42.6 ±1.85	97.2 ±4.32	78.0 ±3.30
Benzaldehyde	12.8	4198 ±159	2932 ±101	1846 ±76.0	1596 ±66.2
Linalool	15	3385 ±282	3158 ±292	1157 ±67.0	ND
Citral (Neral + Geranial)	20.8 + 23.2	9010 ±512	ND	ND	ND
α -Terpineol	21.9	1143 ±58.5	1725 ±80.6	3159 ±147	4311 ±187
Benzaldehyde Propylene Glycol Acetal	26.9	1822 ±80.8	1359 ±56.6	827 ±36.1	718 ±47.9
Cinnamaldehyde	32.5	574 ±32.3	345 ±18.2	108 ±6.39	117 ±5.66
***p*-cresol**	33.65	34.8 ±1.87	411 ±28.5	581 ±27.8	962 ±47.5

*Average of three replicates; **ND=Non-detected

Table 2.6 Relative changes in concentration of characteristic compounds during storage of Flavour Mixture 1 under three temperature conditions in relation to concentrations at starting point (t=0; 100%)

Compound	Retention time (t_R) (min)	Relative concentration in relation to starting point after storage (%)		
		Storage at 4°C	Storage at 23°C	Storage at 40°C
Acetone	1.23	167.4%	310.7%	1,487%
Ethyl Acetate	1.35	500.0%	214.4%	0%
α-Pinene	1.90	55.19%	50.74%	50.74%
Ethyl Butyrate	1.93	67.71%	63.01%	62.96%
β-Pinene	2.55	80.58%	72.33%	69.42%
D-Limonene	3.67	67.07%	59.85%	57.92%
Eucalyptol	3.88	72.12%	61.06%	60.10%
γ-Terpinene	4.48	74.92%	60.74%	25.25%
Terpinolene	5.28	76.60%	59.61%	36.12%
***p*-cymenene**	9.76	70.00%	67.04%	85.20%
Terpin Hydrate	10.4	N/A*	N/A	N/A
Benzaldehyde	12.8	67.38%	51.20%	35.05%
Linalool	15	84.74%	87.10%	83.79%
Citral (Neral + Geranial)	20.8 + 23.2	51.94%	58.69%	54.44%
α -Terpineol	21.9	59.53%	71.96%	69.69%
Benzaldehyde Propylene Glycol Acetal	26.9	N/A	N/A	N/A
Cinnamaldehyde	32.5	47.91%	57.88%	50.74%
***p*-cresol**	33.65	N/A	N/A	N/A

*N/A=Non-applicable (compound was initially undetected)

Table 2.7 Relative changes in concentration of characteristic compounds during storage of Flavour Mixture 2 under varying temperature conditions in relation to concentrations at starting point (t=0; 100%)

Compound	Retention time (t_R) (min)	Relative concentration in relation to starting point after storage (%)		
		Storage at 4°C	Storage at 23°C	Storage at 40°C
Acetone	1.23	5,241%	10,749%	10,695%
Ethyl Acetate	1.35	1,519%	1,615%	1,856%
α-Pinene	1.90	25.29%	0%	0%
Ethyl Butyrate	1.93	62.01%	57.78%	58.24%
β-Pinene	2.55	10.75%	16.83%	19.65%
D-Limonene	3.67	56.15%	42.57%	6.14%
Eucalyptol	3.88	72.32%	70.09%	145.5%
γ-Terpinene	4.48	57.69%	59.80%	56.30%
***p*-cymene**	4.99	185.5%	445.2%	1,153%
Terpinolene	5.28	63.41%	96.07%	119.0%
***p*-cymenene**	9.76	326.9%	746.0%	598.6%
Terpin Hydrate*	10.4	380%	2027%	4540%
Benzaldehyde	12.8	69.84%	43.97%	38.02%
Linalool	15	93.29%	34.18%	0%
Citral (Neral + Geranial)	20.8 + 23.2	0%	0%	0%
α -Terpineol	21.9	150.9%	276.4%	377.2%
Benzaldehyde Propylene Glycol Acetal	26.9	74.59%	45.39%	39.41%
Cinnamaldehyde	32.5	60.10%	18.82%	20.38%
***p*-cresol**	33.65	1181%	1,670%	2,764%

*Values for terpin hydrate represent relative changes in peak area

2.2 The Effect of Chemical Changes on Sensory Properties

To assess whether observed changes in composition resulted in perceptible sensory effects, the sensory properties of flavours 1 and 2 were evaluated on a weekly basis. The flavours were evaluated based on their strength, citrus character and the presence or absence of off-flavour. These parameters were assessed by a group of 23 untrained panelists. The goal was to determine at which point(s) the chemical aging of the flavour became perceptible to the average consumer.

In selecting a sensory test, the statistical parameters of the evaluation must be clearly defined. Typical industry standards dictate pD, α and power values of 0.3, 0.05 and 0.8, respectively and were therefore adopted [88]. To meet these conditions, the two most available sensory tests were the triangle and tetrad tests. Whereas the triangle test is more common than the tetrad test, it is less powerful and requires an approximately three times larger sample size [88]. Given the limited availability of regular participants, the triangle test would have required repeat testing, which would have significantly weakened the quality of the data [88]. The tetrad test, conversely, required 23 participants, which was a manageable group [88]. In addition to being more powerful than the triangle test, the tetrad test is also more consistent and precise at measuring sensory differences [88].

To further understand what chemicals could be resulting in statistically significant sensory changes, a two-sided directional test was additionally given. A two-sided directional test is given in scenarios where the examiner seeks to measure the characters of specific elements of a food product [89]. In this case, it was important to determine how flavour strength, citrus strength and off-note intensity varied between fresh and test samples. Although participants could have different perceptions of what constitutes strength, citrus character and off-notes, training is not a strong requirement, making it particularly suitable to represent the average consumer [89]. The null hypothesis (H_n) in each scenario was H_n: A = B. The alternative hypothesis (H_a) was H_a: A ≠ B. Given a limited sample size, statistical parameters of $\alpha = 0.1$ and $\beta = 0.2$ were selected, which was considered acceptable. Using these conditions, there is an 80% chance of detecting an effect if present [89]. There was no ‘correct’ answer because either outcome is of interest, making this test ‘two-sided’ [89]. Any answer collectively chosen 16 or more times in a given week was considered statistically significant based on the statistical parameters [89].

It was expected that Flavour Mixture 2 would be more impacted than Flavour Mixture 1 due to its more adverse storage conditions. The results of the tetrad support this. As summarized in **Table 2.8**, differences between Group A (fresh samples) and Group B (aged samples) were only observed 7 out of a potential 27 times for Flavour Mixture 1. Conversely, statistical differences were recorded 14 times for Flavour Mixture 2. In Flavour Mixture 1 the majority of these differences were seen in samples stored at 40°C, which is unsurprising. From week 5 to week 9, the final week of testing, all samples stored at 40°C were statistically different from fresh samples. The remaining two cases occurred at week 1 and week 2, in samples stored at 23°C and 40°C, respectively. **Table 2.9** summarizes the results of the two-sided directional test for Flavour Mixture 1. Again, statistical differences were observed in samples stored at 40°C from weeks 5 to 9. Differences in off-notes appeared first, in week 5. In weeks 6 & 8, differences were detected in both off-note intensity and citrus character. At week 7 & 9, there were perceived differences in all categories. In these cases, off-note intensity was always higher in aged samples, but overall strength and citrus character were stronger in fresh samples. These results suggest that off-notes formed over time at the highest temperature conditions and that the overall citrus character of the flavour declined with time, which was hypothesized.

The primary off-notes that formed in the flavours were BPGA, *p*-cymene, *p*-cresol, α-terpineol and acetone. In Flavour Mixture 1, α-terpineol fell over time whereas acetone was muted and *p*-cresol did not form at all. Conversely, BPGA formed relatively rapidly, reaching almost 50% of its final concentration within two weeks. Similarly, *p*-cymene had reached 50% of its intensity by week 2. This might explain why higher off-note intensity was statistically perceived at week 2 in the aged flavour. However, it would not explain why there were no further differences until week 5. Consistent statistical differences only began once the citrus components had fallen. By week 5, citral, the key contributor to the citrus profile, had fallen by 40-50%. This was clearly detectable by the panel, which perceived lower citrus character in the aged flavours from weeks 6 to 9. However, citral fell similarly at 4°C and 23°C as well, suggesting that a combination of citrus loss and off-note formation was necessary for differences to be observed.

In Flavour Mixture 2, statistical differences occurred earlier and under more temperature conditions. Week 1 was the only week where the flavour appeared unaffected under any condition. Samples stored at 4°C underwent the least amount of change, as expected, but also became

detectable in two cases near the end of the experiment. Surprisingly, samples stored at 23°C appeared to be more susceptible to change than at 40°C. In 7 out of 9 tests, statistical differences were observed for samples stored at 23°C. Comparatively, differences were observed in 5 out of 9 analyses for samples stored at 40°C. **Table 2.10** summarizes the results of the two-sided directional test for Flavour Mixture 2. Differences in citrus character were observed from as early as week 1. Similarly, off-note intensity was noticeable by week 3 and for the remainder of the experiment. Conversely, differences in flavour strength were only detectable in the final two weeks of testing. Based on these results, aging clearly a broad impact on both off-note intensity and citrus character in Flavour Mixture 2, with less of an effect on flavour strength.

The sensory results of Flavour Mixture 2 coincided with major declines in several key compounds, along with a progressive concentration of off-notes. In Flavour Mixture 2, more so than in Flavour Mixture 1, panellists consistently complained of a note comparable to "gasoline" or "rubber", which was likely caused by BPGA. The formation of BPGA peaked within a week at all three storage temperatures in Flavour Mixture 2. There were also complaints of a paint-like note, which can be attributed to *p*-cymene, *p*-cresol and possibly α-terpineol. *P*-cresol did not form in Flavour Mixture 1, which could be particularly impactful; it formed progressively over time in Flavour Mixture 2 and was very likely a key contributor to these off-notes. Additionally, *p*-cymene leaped from trace levels to over 10,000 ppm after one week and remained at similar levels for the remainder of the experiment. Many panellists claimed to easily be capable of distinguishing the flavours due to these potent off-notes. There is some merit to this claim; as many as 20 panellists out of 23 indicated higher off-note intensity in the aged flavours near the end of the experiment. Similarly, panellists increasingly indicated a lower citrus character in aged flavours, consistently reaching 18-21 responses out of 23 in the final weeks. This is justified, as citral had totally depleted at 4°C by week 6 and at 23°C and 40°C within 2 weeks. Similarly, D-limonene plummeted almost 50% within 1 week at 40°C; by week 9 it was almost non-existent. The loss of typical citrus notes was compounded by the formation of other terpenes, through acid-catalyzed rearrangements. Compounds like terpinolene, α-terpineol, eucalyptol and γ-terpinene became over-expressed and may have contributed to the perceived "paint" or "paint-thinner" note that panellists noted. In the end, it was this combination that made differentiation easier in Flavour Mixture 2 than it was in Flavour Mixture 1.

Table 2.8. Proportion of distinguishers (N_c) of flavour mixtures 1 and 2

	Flavour 1				**Flavour 2**			
Time	Temperature Condition	N	N_c	*p*	Temperature Condition	N	N_c	*p*
Week 1	4°C	23	6	0.17244	4°C	23	12	1.00035×10^{-4}
	23°C	23	16	**2.64×10^{-8}**	23°C	23	10	0.002286
	40°C	23	12	7.3×10^{-12}	40°C	23	10	0.002286
Week 2	4°C	23	9	0.008602	4°C	23	11	0.000518
	23°C	23	6	0.17244	23°C	23	14	**2.29×10^{-6}**
	40°C	23	15	**2.69×10^{-7}**	40°C	23	16	**2.64×10^{-8}**
Week 3	4°C	23	7	0.074917	4°C	23	11	0.000518
	23°C	23	11	0.000518	23°C	23	15	**2.69×10^{-7}**
	40°C	23	10	0.002286	40°C	23	14	**2.29×10^{-6}**
Week 4	4°C	23	10	0.002286	4°C	23	10	0.002286
	23°C	23	9	0.008602	23°C	23	13	**1.64×10^{-5}**
	40°C	23	12	7.3×10^{-12}	40°C	23	12	1.00035×10^{-4}
Week 5	4°C	23	7	0.074917	4°C	23	17	**2.14×10^{-9}**
	23°C	23	10	0.002286	23°C	23	14	**2.29×10^{-9}**
	40°C	23	14	**2.29×10^{-9}**	40°C	23	14	**2.29×10^{-9}**
Week 6	4°C	23	8	0.027549	4°C	23	11	2.64×10^{-8}
	23°C	23	10	0.002286	23°C	23	16	**2.64×10^{-8}**
	40°C	23	14	**2.29×10^{-9}**	40°C	23	16	**2.64×10^{-8}**
Week 7	4°C	23	6	0.17244	4°C	23	15	**2.69×10^{-7}**
	23°C	23	8	0.027549	23°C	23	12	1.00035×10^{-4}
	40°C	23	15	**2.69×10^{-7}**	40°C	23	11	2.64×10^{-8}
Week 8	4°C	23	10	0.002286	4°C	23	12	1.00035×10^{-4}
	23°C	23	9	0.008602	23°C	23	16	**2.64×10^{-8}**
	40°C	23	13	**1.64×10^{-5}**	40°C	23	18	**1.4×10^{-10}**
Week 9	4°C	23	6	0.17244	4°C	23	10	0.002286
	23°C	23	7	0.074917	23°C	23	13	**1.64×10^{-5}**
	40°C	23	15	**2.69×10^{-7}**	40°C	23	12	1.00035×10^{-4}

Values in bold indicate a significant difference ($p < 0.001$)

Table 2.9. Number of participants perceiving higher flavour concentration, higher citrus character and higher level of off-notes (Flavour Mixture 1) (Group A = fresh; group B = aged)

		Concentration			Citrus			Off-Notes		
		A	B	Neither	A	B	Neither	A	B	Neither
Week 1	4°C	6	6	11	10	5	8	7	12	4
	23°C	2	9	13	9	2	12	5	**17**	1
	40°C	15	7	1	2	6	15	4	8	11
Week 2	4°C	4	4	15	1	8	14	11	6	6
	23°C	4	10	9	4	13	6	3	8	12
	40°C	8	5	10	9	8	6	4	18	1
Week 3	4°C	4	4	15	2	12	9	4	11	8
	23°C	8	1	14	2	14	7	6	11	6
	40°C	8	6	9	7	2	14	8	1	14
Week 4	4°C	7	15	1	7	1	15	8	6	9
	23°C	11	12	0	10	4	9	5	15	3
	40°C	15	3	5	5	6	12	5	7	11
Week 5	4°C	6	2	15	8	9	6	6	2	15
	23°C	1	7	15	4	10	9	5	9	9
	40°C	13	3	7	2	9	12	7	**16**	1
Week 6	4°C	4	6	13	9	1	13	2	12	9
	23°C	10	2	11	3	6	14	8	10	5
	40°C	7	3	13	**16**	4	3	5	**18**	0
Week 7	4°C	8	3	12	7	5	11	1	12	10
	23°C	2	9	12	6	12	5	6	10	7
	40°C	**16**	3	4	**17**	2	4	4	**18**	1
Week 8	4°C	13	3	7	15	6	2	5	3	15
	23°C	5	12	6	14	8	1	7	10	6
	40°C	14	4	5	**16**	5	2	3	**19**	1
Week 9	4°C	5	10	8	4	5	14	6	10	7
	23°C	15	6	2	4	8	11	5	3	15
	40°C	**16**	7	0	**16**	6	1	3	**18**	2

Values in bold indicate a significant difference

Table 2.10. Number of participants perceiving higher flavour concentration, higher citrus character and higher level of off-notes (Flavour Mixture 2) (Group A = fresh; group B = aged)

		Concentration			Citrus			Off-Notes		
		A	B	Neither	A	B	Neither	A	B	Neither
Week 1	4°C	11	9	3	14	6	3	6	10	7
	23°C	8	9	6	**17**	1	5	7	9	7
	40°C	7	10	6	15	5	3	4	13	6
Week 2	4°C	12	9	2	13	6	4	8	11	4
	23°C	8	11	4	**16**	5	2	5	13	5
	40°C	7	5	11	**17**	4	2	7	13	3
Week 3	4°C	13	9	1	15	5	3	8	9	6
	23°C	6	12	5	**17**	6	0	4	14	5
	40°C	5	3	15	**19**	2	2	5	**16**	2
Week 4	4°C	14	6	3	**16**	3	4	8	11	4
	23°C	7	13	3	**16**	4	3	7	11	5
	40°C	15	1	7	**19**	3	1	4	**17**	2
Week 5	4°C	10	5	8	15	4	4	4	**16**	3
	23°C	5	13	5	**20**	2	1	5	**16**	2
	40°C	9	5	9	**19**	4	0	5	**18**	0
Week 6	4°C	13	6	4	**17**	3	3	6	14	3
	23°C	3	12	8	**17**	4	2	4	**18**	1
	40°C	15	3	5	**21**	2	0	3	**20**	0
Week 7	4 °C	14	5	4	15	5	3	6	14	3
	23°C	7	15	1	**18**	4	1	7	15	1
	40°C	15	5	3	**19**	4	0	5	**16**	2
Week 8	4°C	7	11	5	**16**	25	2	6	15	2
	23°C	5	15	3	**18**	5	0	3	**19**	1
	40°C	6	**16**	2	**21**	2	0	3	**20**	0
Week 9	4°C	6	13	4	15	4	4	7	**16**	0
	23°C	3	**17**	3	**20**	3	0	4	**19**	0
	40°C	2	**17**	4	**18**	4	1	4	**18**	1

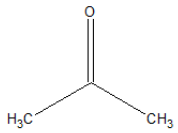
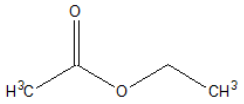
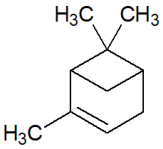
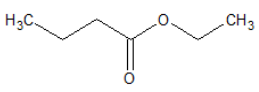
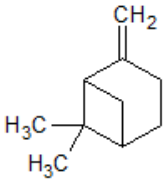
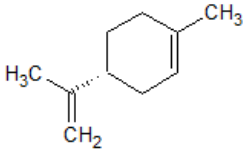
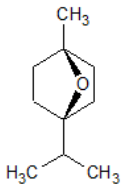
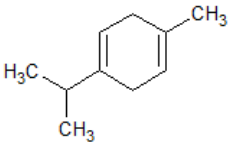
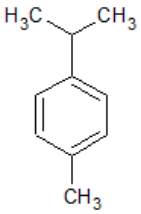
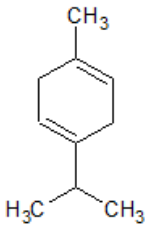
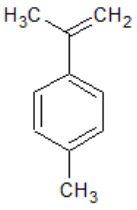
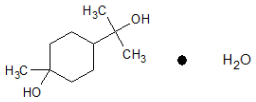
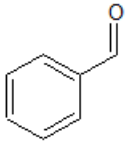
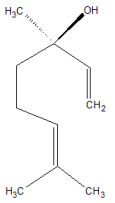
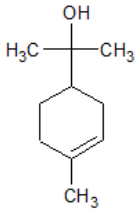
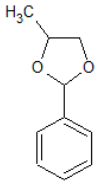
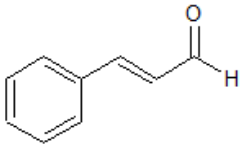
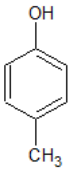
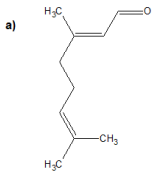
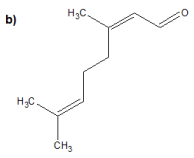
Values in bold indicate a significant difference

CHAPTER 3: GENERAL SUMMARY AND CONCLUSION

Flavour Mixtures 1 & 2 were susceptible to both chemical and sensory changes as a result of storage conditions. Chemical changes in Flavour Mixture 1 only resulted in perceptible changes 5 weeks into storage, and only at a 40°C temperature protocol. Conversely, Flavour Mixture 2 was far more impacted both chemically and sensorially. This was due to extensive acid-catalyzed rearrangements of terpenes, which created both off-notes and chemical imbalances in the cola profile. Key degradation compounds that affected the sensory profile included *p*-cymene, α-terpineol, benzaldehyde propylene glycol acetal, *p*-cymene and *p*-cresol. In Flavour Mixture 1, the formation of off-notes occurred prior to a detectable loss in citrus character. In Flavour Mixture 2, however, loss of citrus character was detectable prior to formation of off-notes. Ultimately, although the strength of the overall flavour was essentially unaffected, the intensity of the citrus character was compromised while off-notes intensified. Consequently, it is not recommended that an "all-in-one" flavour model be employed for citrus-based sodas like cola.

APPENDIX A: Chemical structures of compounds of interest

Figure A.1. Chemical structures of compounds of interest

 Acetone	 Ethyl Acetate	 α-pinene	 Ethyl Butyrate
 β-pinene	 D-limonene	 Eucalyptol	 γ-terpinene
 *p*-cymene	 Terpinolene	 *p*-cymenene	 Terpin Hydrate
 Benzaldehyde	 Linalool	 α-terpineol	 Benzaldehyde propylene glycol acetal
 Cinnamaldehyde	 *p*-cresol	 a) Geranial	 b) Neral

APPENDIX B: Essential oil compositions by GC-FID

Table B.1. Composition of key lemon oil components by GC-FID

COMPONENT	COMPOSITION (%)
D-LIMONENE	**61.83**
CITRAL	**10.46**
BETA-PINENE	**3.01**
ALPHA-TERPINEOL	**0.97**
ALPHA-PINENE	**0.64**

Table B.2. Composition of key lime oil components by GC-FID

COMPONENT	COMPOSITION (%)
D-LIMONENE	**47.72**
GAMMA TERPINENE	**10.63**
TERPINOLENE	**7.79**
ALPHA-TERPINEOL	**7.77**
BETA-PINENE	**1.93**
EUCALYPTOL	**1.85**
ALPHA-PINENE	**1.19**

Table B.3. Composition of key orange oil components by GC-FID

COMPONENT	COMPOSITION (%)
D-LIMONENE	**95.02**
ALPHA-PINENE	**0.51**
LINALOOL	**0.42**

Table B.4. Composition of key cassia oil components by GC-FID

COMPONENT	COMPOSITION (%)
CINNAMALDEHYDE	**78.3**
BENZALDEHYDE	**0.73**

Table B.5. Composition of key cinnamon bark oil components by GC-FID

COMPONENT	COMPOSITION (%)
CINNAMALDEHYDE	**75.81**
D-LIMONENE	**4.4**
LINALOOL	**2.53**
ALPHA-PINENE	**2.41**
ALPHA-TERPINEOL	**0.78**

Table B.6. Composition of key nutmeg oil components by GC-FID

COMPONENT	COMPOSITION (%)
ALPHA-PINENE	**19.30**
BETA-PINENE	**13.08**
GAMMA TERPINENE	**6.13**
D-LIMONENE	**5.44**
ALPHA TERPINENE	**4.28**

Table B.7. Composition of key clove bud oil components by GC-FID

COMPONENT	COMPOSITION (%)
EUGENOL	**75.99**
EUGENYL ACETATE	**9.86**

APPENDIX C: Calibration Curves

Figure C.1. Calibration curve - Acetone

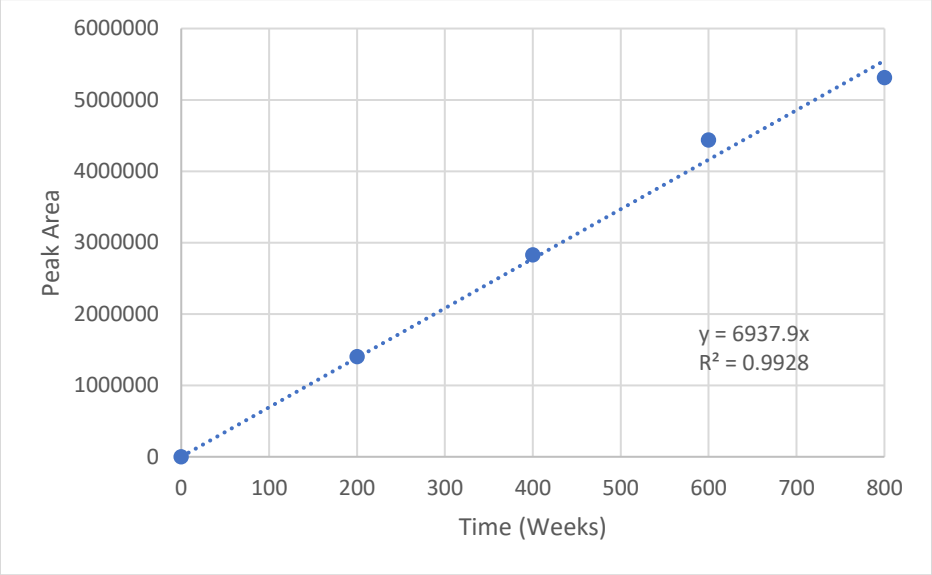


Figure C.2. Calibration curve – Ethyl Acetate

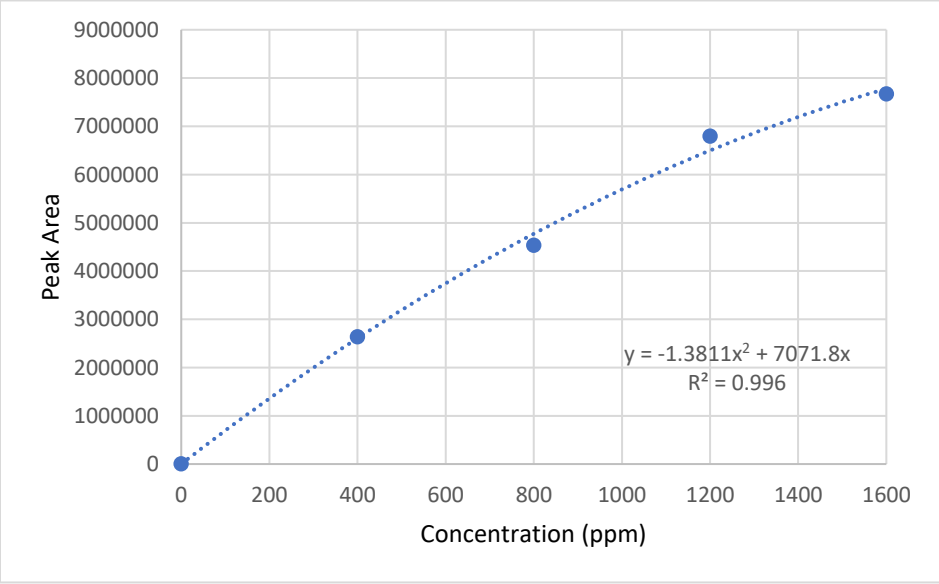


Figure C.3. Calibration curve – Alpha-Pinene

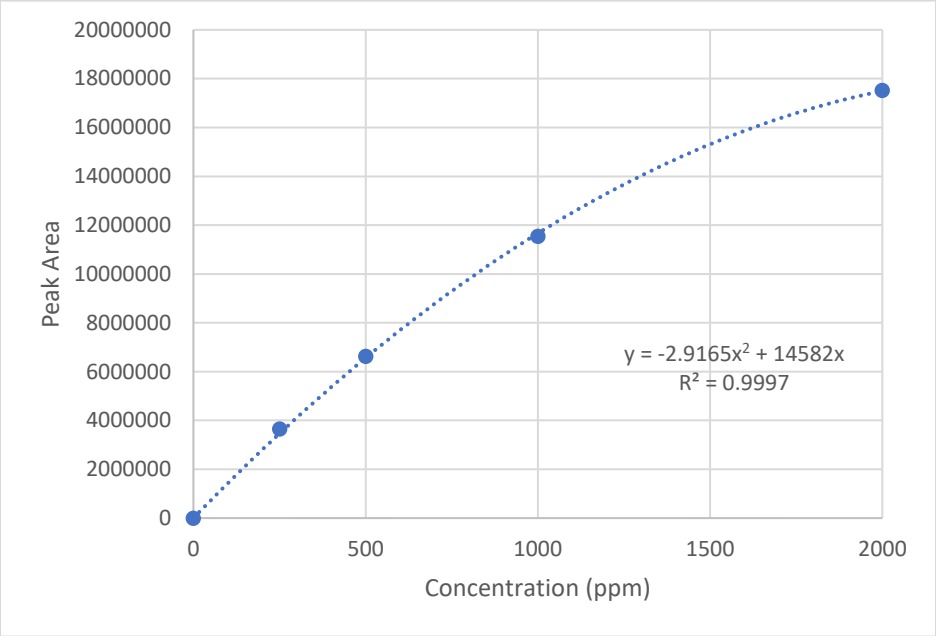


Figure C.4. Calibration curve – Ethyl Butyrate

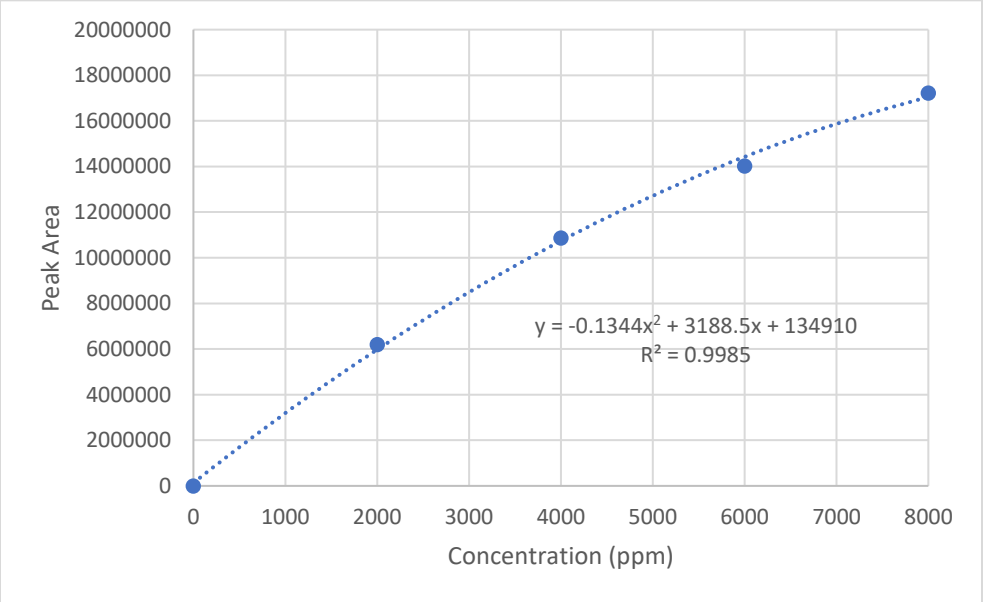


Figure C.5. Calibration curve – Beta-Pinene

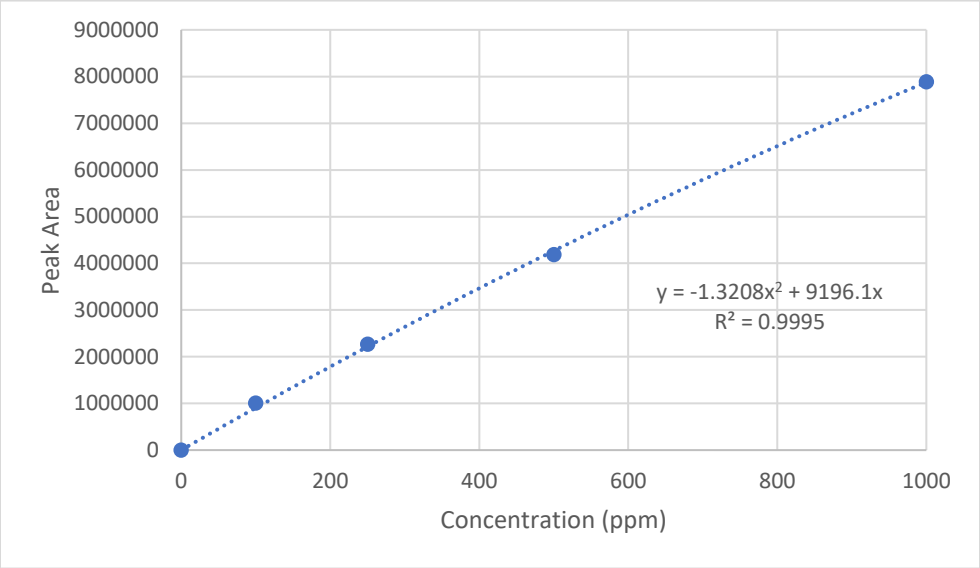


Figure C.6. Calibration curve – D-Limonene

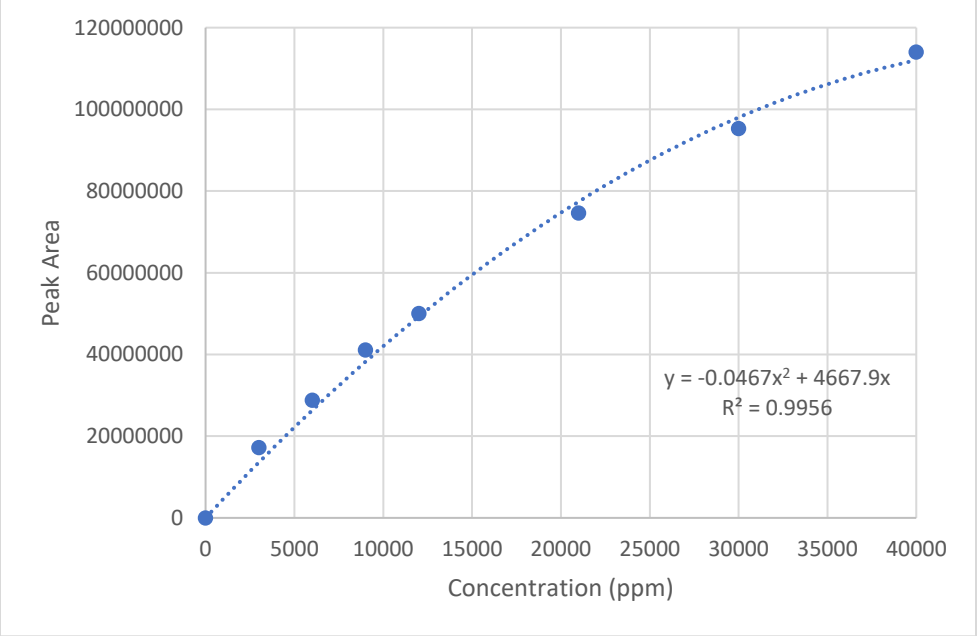


Figure C.7. Calibration curve – Eucalyptol

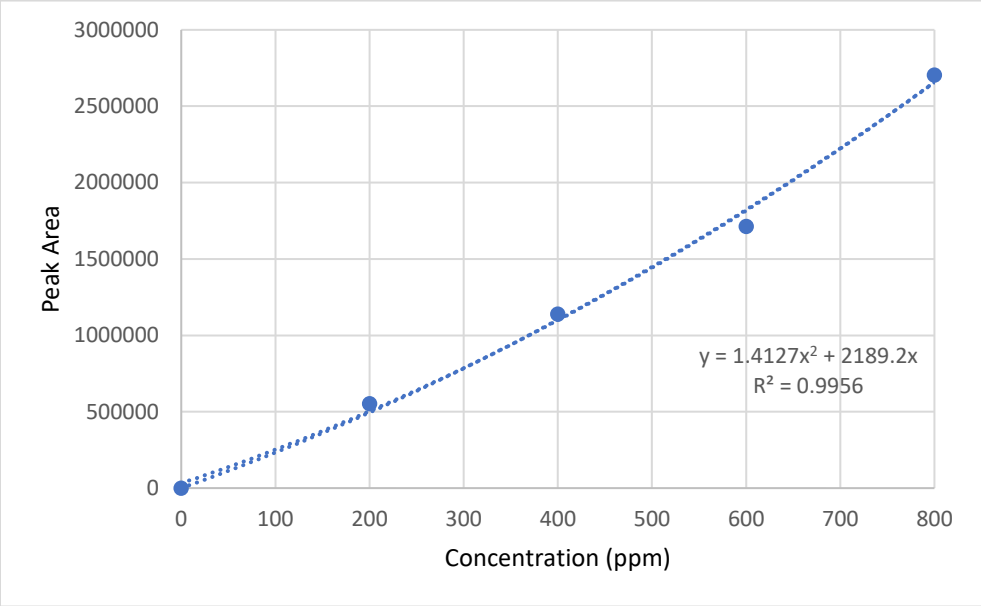


Figure C.8. Calibration curve – Gamma-Terpinene

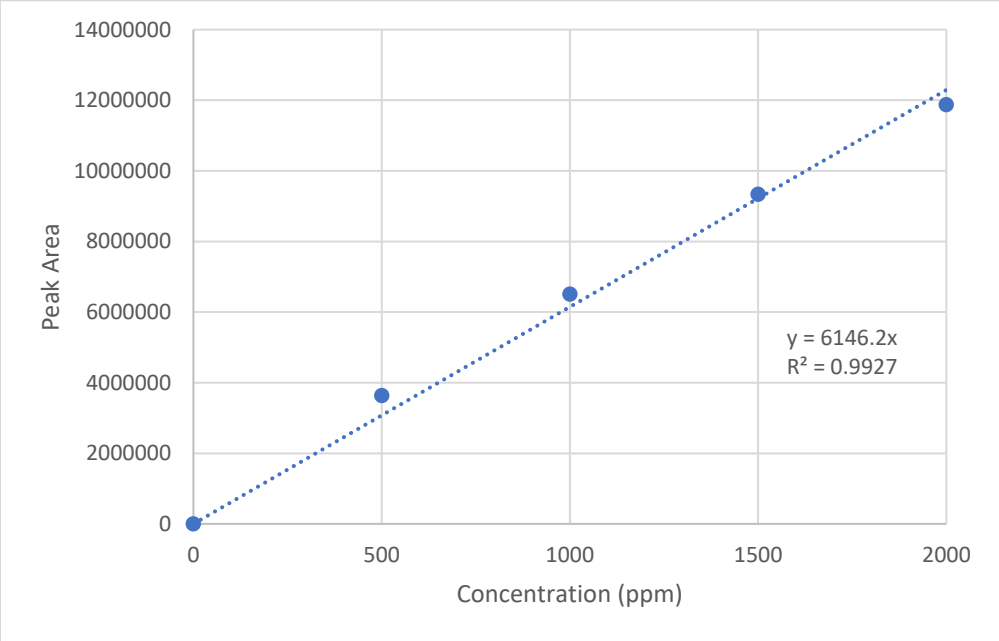


Figure C.9. Calibration curve – *p*-Cymene

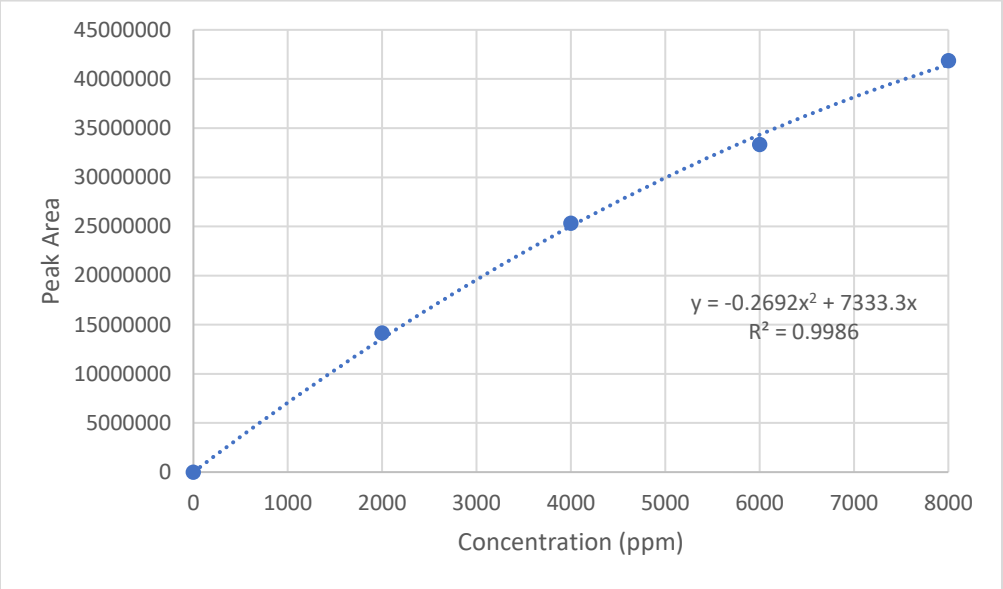


Figure C.10. Calibration curve – Terpinolene

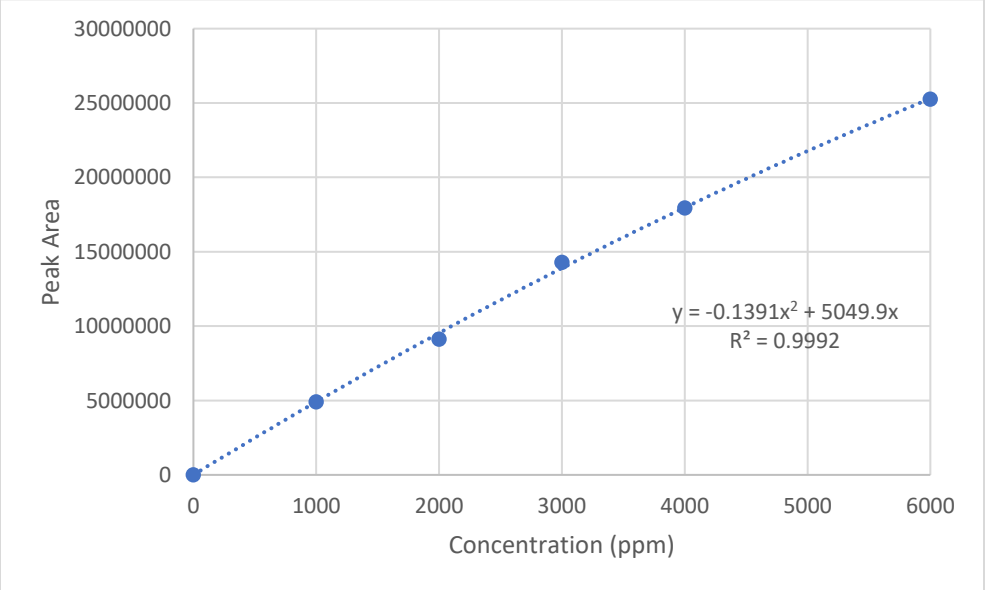


Figure C.11. Calibration curve – *p*-Cymenene

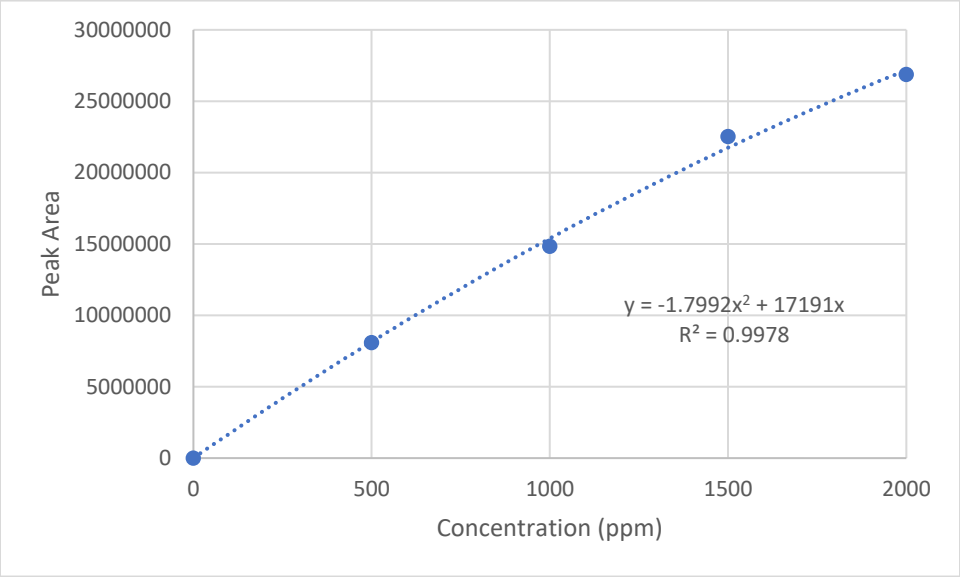


Figure C.12. Calibration curve – Benzaldehyde

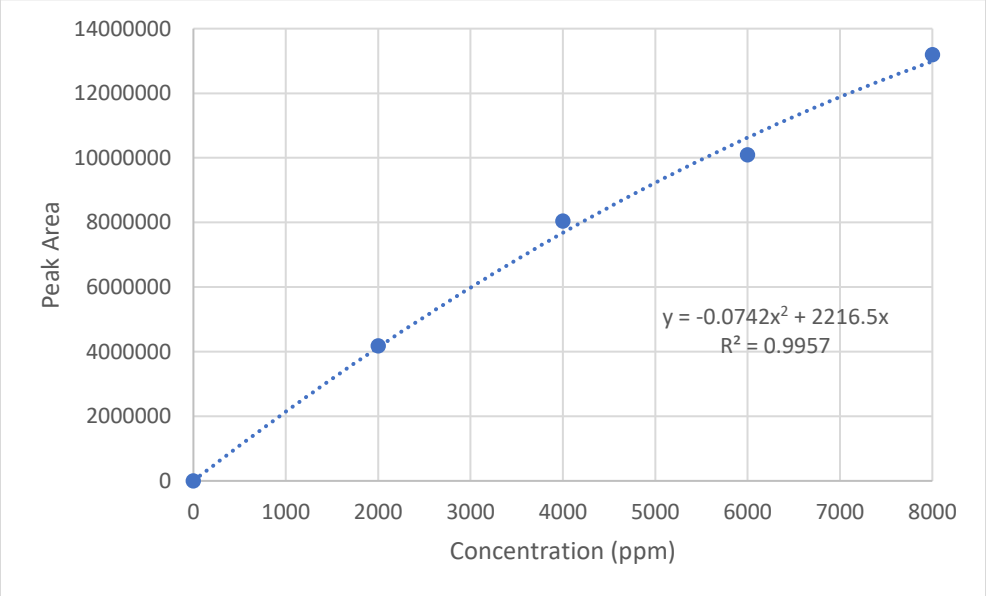


Figure C.13. Calibration curve – Linalool

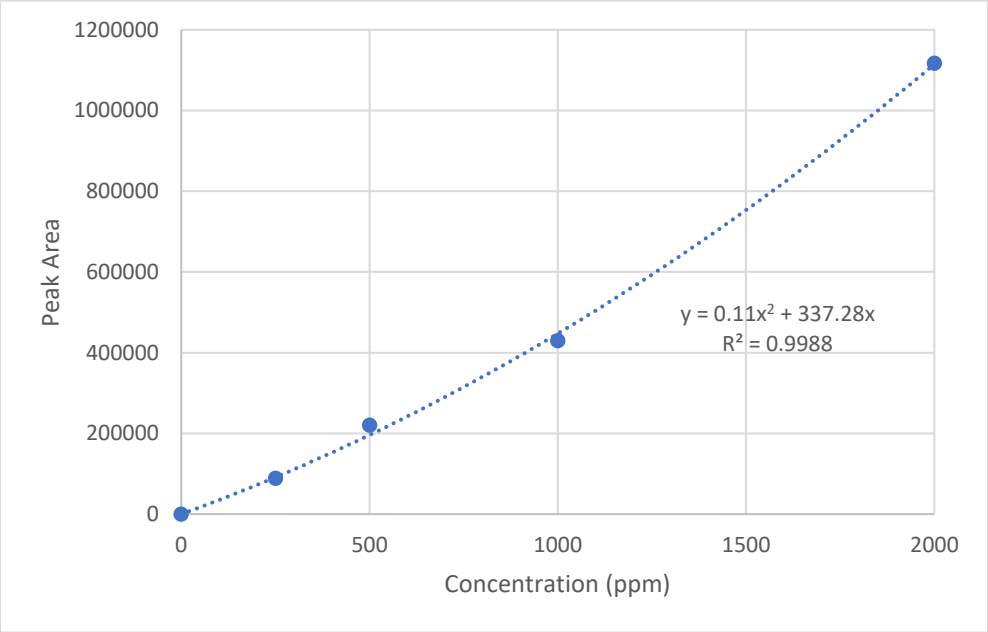


Figure C.14. Calibration curve – Citral

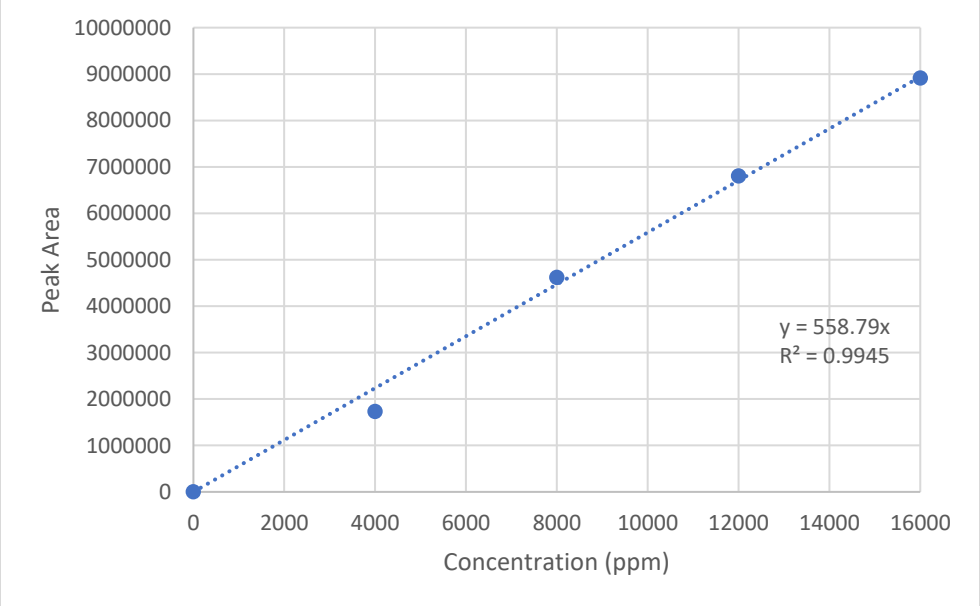


Figure C.15. Calibration curve – α-Terpineol

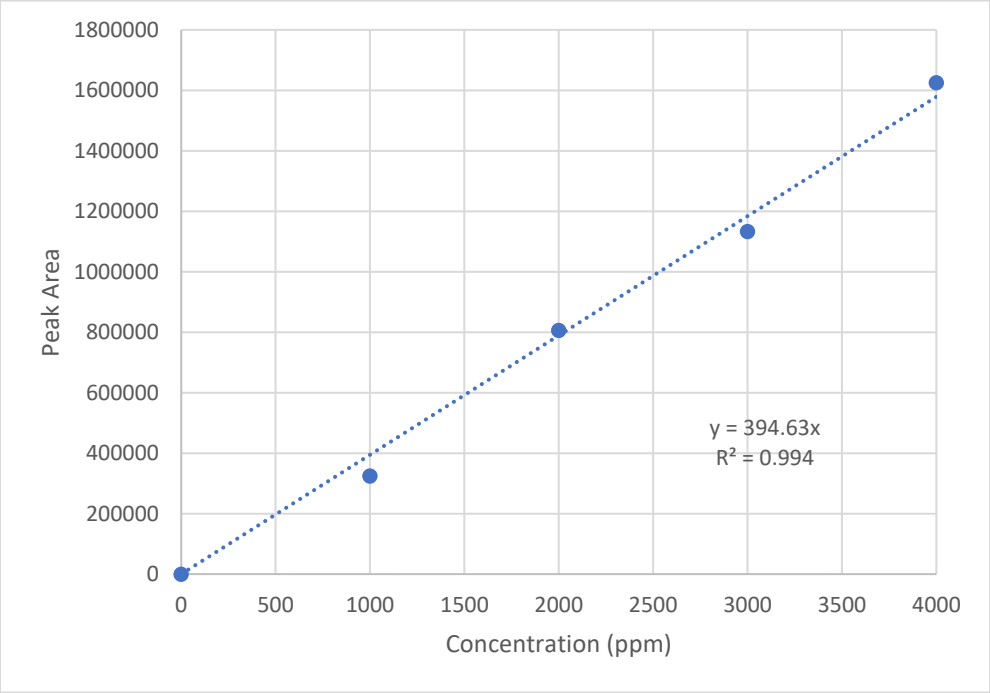


Figure C.16. Calibration curve – Benzaldehyde Propylene Glycol Acetal

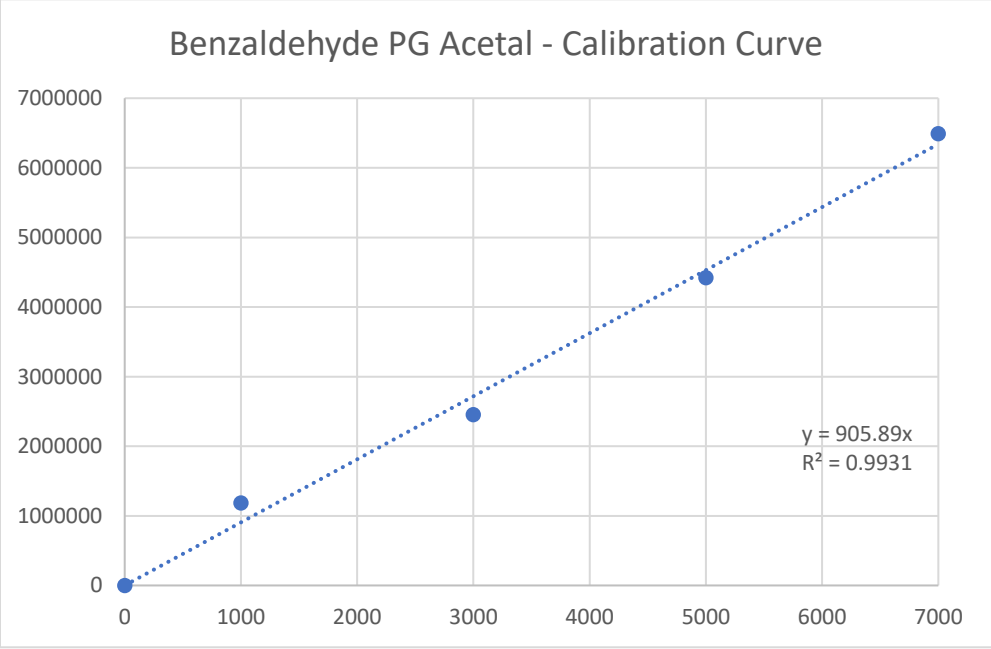


Figure C.17. Calibration curve – Cinnamaldehyde

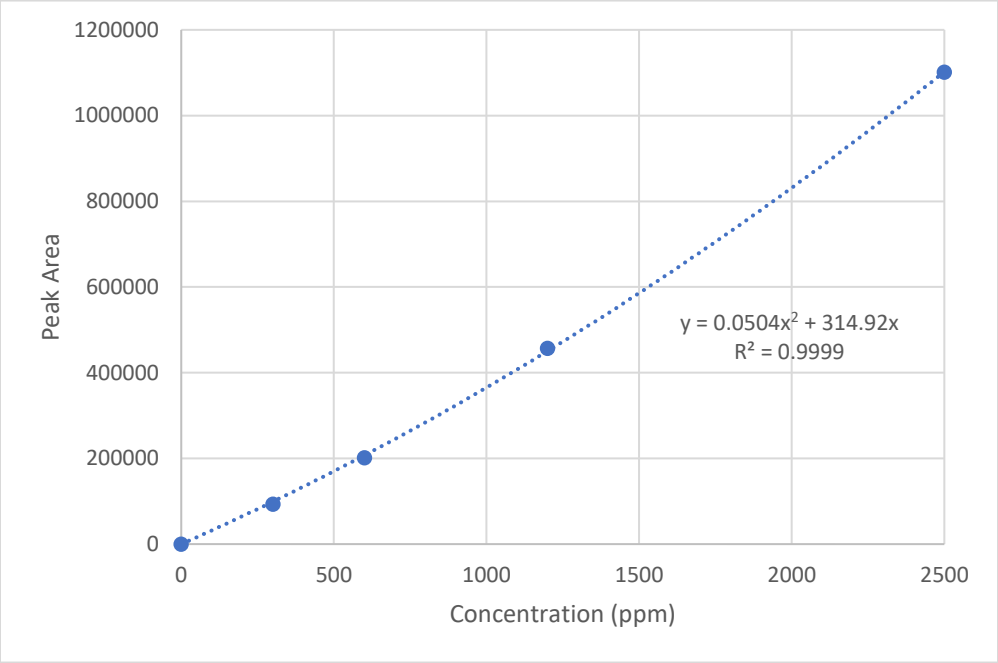
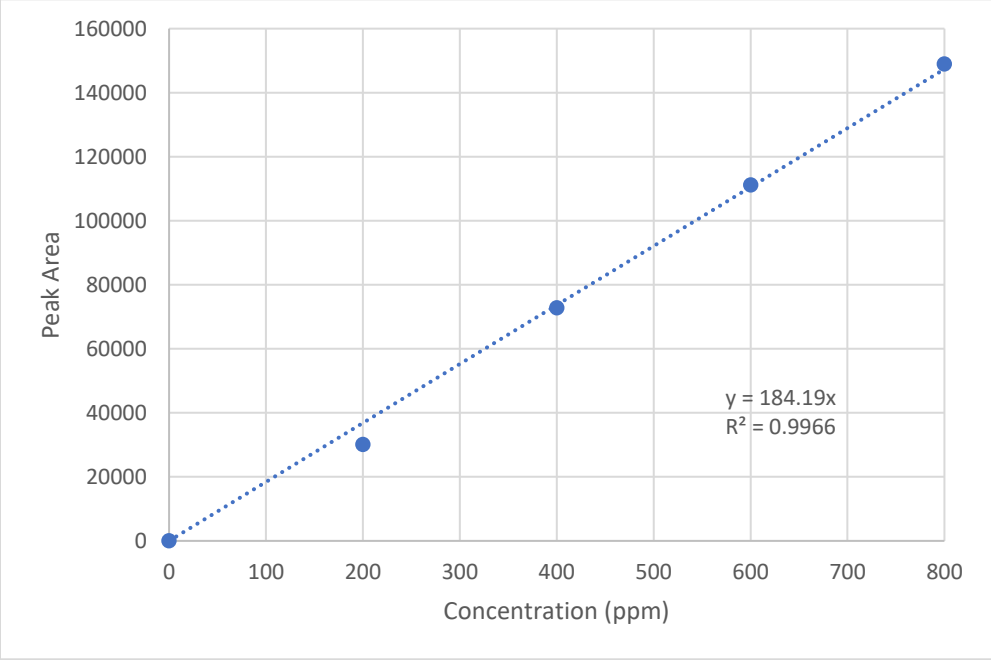


Figure C.18. Calibration curve – *p*-Cresol



APPENDIX D: Sensory Evaluation Questionnaire

Figure D.1. Sensory evaluation questionnaire (page 1/2)

NovoTaste Sensory Evaluation Test

Cola Type Natural Flavour

SAMPLE SET (internal use only): _____

NAME: _____

Instructions:

You will be presented with a set of Cola flavour samples, prepared in sweetened water. Before you taste each product, please take a sip of water and a bite of cracker so that you remove any lingering tastes in your mouth. Please drink at least half of each sample so that you can form an opinion. Once you are done with the entire questionnaire, there will be a 5-minute break and then you will go on to the next sample set. The same procedures will be followed for the second product.

PLEASE TASTE THE PRODUCT AND ANSWER THE FOLLOWING QUESTIONS. PLEASE DRINK AT LEAST ONE-HALF OF THE PRODUCT SO THAT YOU WILL BE ABLE TO FORM AN OPINION

1. Everything considered, please group the samples based on similarity into two groups: a group of two and a second group of two.

	Group A 2 Similar	Group B 2 Similar
Sample XXX	○	○
Sample XXX	○	○
Sample XXX	○	○
Sample XXX	○	○

Figure D.2. Sensory evaluation questionnaire (page 2/2)

NovoTaste Sensory Evaluation Test

2. Considering overall flavour concentration, which pair of samples tasted more concentrated?

   Group A..... ○
   Group B..... ○
   Neither..... ○

3. Considering flavour citrus notes, which pair of samples tasted more citrus?

   Group A..... ○
   Group B..... ○
   Neither..... ○

4. Did you detect any off-notes in either pair of samples?

   Yes, in Group A..... ○
   Yes, in Group B..... ○
   No..... ○

5. If you have any additional comments, you are encouraged to share them here:

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