The Effects of natural Antioxidants on the Degradation of Volatile Components and the Production of Components leading to Off-Flavors in Orange Drinks Containing a Percentage of Natural Orange Juice.

By
Marc West
Supervisor
Doug Williams Ph. D.
Kalsec Inc.

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Preface

For my Senior Individualized project I performed research at Kalsec Inc. in Kalamazoo MI. I worked there under the guidance of Dr. Doug Williams. Our research involved working with natural and synthetic antioxidants in hopes of finding a natural antioxidant that would be both effective and cost efficient for use in orange beverages. This research was done in partial fulfillment for a bachelors degree from Kalamazoo College, it was also an invaluable experience in furthering my laboratory skills and scientific writing skills for future career plans.

I would like to acknowledge many people for all of their help with this research. First and foremost I would like to thank Dr. Doug Williams of Kalsec Inc.. Dr. Williams was my SIP mentor, and an invaluable source of information and direction. His assistance with the research, lab work, and editing made this project possible. I would also like to thank Kevin Meyle, Louis Burroughs, and David Bolliet of Kalsec Inc. for their daily assistance making the project an enjoyable and interesting learning experience. For information and for supplies for the research I would like to thank Joe Thorner and Randall Mennett of Kalsec Inc., Eric Dowd of AM Todd, Steve Fowler of SunPure and Carl Holmgren of Brooklyn by Perfetti. I would also like to thank Dr. Laura Furge of Kalamazoo College and Kathleen Anderson for their help with editing the final paper.
The Effects of natural Antioxidants on the Degradation of Volatile Components and the Production of Components leading to Off-Flavors in Orange Drinks Containing a Percentage of Natural Orange Juice.

The effects of three natural antioxidants, two rosemary extracts, Herbalox HT-O® and Herbalox WM-4®, and Tocopherol GT 2, were compared to the synthetic antioxidant BHA. The antioxidants were tested on single fold orange oil, the oxidation levels were monitored using a GC/MS profile and a peroxide value measurement. A second test was done on an orange drink containing 25% natural orange juice from concentrate. The oxidation levels of the second study were monitored using Solid Phase Microextraction (SPME).
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Orange juice is the most popular fruit juice in the United States and worldwide. Florida alone produces nearly 250 million 90 lb. boxes of oranges annually. Nearly 95% of this produce is processed into orange juice, which makes about 1.5 billion gallons of orange juice annually. There are four main types of orange drinks: fresh squeezed juice, chilled juice, juice concentrate, and orange drinks containing a percent of orange juice from industrial concentrate. Fresh squeezed orange juice is produced two ways, pasteurized and unpasteurized. Pure, unpasteurized orange juice is the highest quality and most expensive orange beverage. Chilled juices are either pasteurized fresh squeezed orange juice or orange juice reconstituted from an industrial concentrate, these are sold ready to serve. Frozen consumer concentrates are very popular due to their easy storage and relative inexpensive price. The final product category includes orange drinks such as Hi-C® or Sunny Delight. Here a drink blend is made with 1% - 70% orange juice from industrial concentrate with 10% being the most popular concentration. The popularity of orange flavored beverages and the lower costs of concentrates and orange drinks create a market for the large variety of orange drinks available today.

Today there is a growing demand for juices that are 100% juice and unpasteurized. With pure orange juice the only variance in taste comes from the different kinds of oranges used or the amount of pulp kept in the juice. There are a variety of oranges that can be used, the two most popular being Valencia and Mandarin oranges. The major problem with a pure unpasteurized fresh squeezed juice is that it has a relatively short shelf life. At 2.0°C unpasteurized
orange juice keeps a good flavor for less than two weeks and a fair flavor for less than three weeks. Chilled juices are the closest alternative to fresh squeezed juice. All pasteurized orange juice is sold as a chilled juice. The pasteurization process heats the orange juice intensely for a short period of time and then the juice is cooled for storage and transportation. This process kills most of the microbiological organisms in the orange juice thus lengthening its shelf life. Chilled juices from pasteurized orange juice have a longer shelf life of up to 60 days, but they can lose some of their desirable flavors during the pasteurization process.

The majority of the chilled juice comes from reconstituted frozen industrial concentrate. In this process the desired flavor and color is achieved by adding peel and essence oils as well as pulp and water back to the frozen concentrate. This juice is 100% natural as the substances added back to the concentrate are taken from the fresh squeezed juice before it is frozen as a concentrate. The frozen concentrate can be stored for up to one year before it is used in juice production. This process lengthens the product’s shelf life but at the expense of some of the taste and aroma of the fresh squeezed juice.

Fresh and chilled juices are also the most expensive types of juice to package and transport. The proper packaging is essential for optimal shelf life as manufactures can not add any preservatives, natural or synthetic, to the to lengthen its shelf life. The package must remain cool and in the dark during transportation, for when exposed to heat or light the decomposition process is accelerated. With the relatively large volume of orange juice the cost of distribution and storage in temperature controlled units is very high. By law,
anything that is added to these juices to extend their shelf life must be clearly labeled. Producers then lose the right to label and price their product as 100% juice. These expenses drive up the cost of 100% orange juices and opens the market for consumer ready orange juice concentrates and orange drinks that contain less than 100% juice.

Consumer ready orange juice concentrate provides an inexpensive 100% juice alternative to fresh squeezed orange juice and chilled juices. Consumer concentrate differs from the industrial concentrate used in producing chilled juices. The process for consumer ready frozen concentrate requires that the orange juice first be filtered and centrifuged to gain the desired amount of pulp. It is then put through an evaporator taking out water, an aroma water phase and an essence oil phase. This concentrate is then frozen, packaged, and shipped. Concentrate has a relatively long shelf life. It is also easy to store, allowing the consumer to purchase it in large quantities. The reduced volume of concentrate results in lower cost of packaging, transportation, and cold storage in comparison to fresh squeezed and chilled juices. These factors make juice from concentrate an inexpensive alternative to fresh squeezed juice and the combination of price and convenience make concentrates a strong competitor of the fresh squeezed juices.

The popularity of the orange flavor has brought a large market for a variety of orange beverages that contain some fraction of orange juice or orange flavoring. These drinks include such drinks as the Hi-C® Orange Drink, Sunkist Orange Soda, Sunny Delight and many others. These orange beverages are
generally made from concentrate and contain natural and artificial flavors. The natural flavors used come from orange oil and citric acid, the same compounds that bring the flavor to orange juice. The disadvantage of this addition is that the beverage becomes susceptible to the formation of off flavors just as orange juice is. However, these beverages do not carry the label of 100% juice so they can add preservatives to extend their shelf life. The use of preservatives allows manufacturers to produce a product with a long shelf life that is easily transported and displayed. These drinks do not need the same quality of packaging and do not need to be transported or stored at a low temperature. These factors lower production costs and allow this type of drink to compete with 100% juices. Manufacturers of these types of beverages are seeking an "all natural" label. As with all drinks, manufacturers must fully list the preservatives, flavorings, and colors that are added to their drinks. With today's increasingly informed consumer, manufacturers believe that an all natural drink, labeled as such, will be a more appealing than drinks with synthetic preservatives.

There are many factors which affect orange juice shelf life, including temperature and exposure to light or air. After a certain amount of time the volatile components in orange juices and orange drinks start to degrade and the formation of off flavors begins. These components are found in the cold pressed peel oils and essence oils that make up the taste and aroma of orange juice and orange drinks. This degradation occurs in beverages that contain any amount of natural orange juice or natural orange flavor. The volatile components of orange oil consist of terpenes, aldehydes, alcohols. This group accounts for many of the
marker components that must be monitored when studying the aging process of orange drinks. The terpene compounds form the largest group of volatile compounds in orange drinks. There are three terpene structures found in orange oil; acyclic, cyclic, and bicyclic. The major acyclic compound in orange oil is myrcene. Cyclic compounds include \(d\)-limonene, alpha-terpinene, and terpinolene. The main bicyclic compound is alpha-pinene.\(^7\) These compounds are all very susceptible to both oxidation and hydration reactions, which lead to off flavors.

The major component in these orange oils is \(d\)-limonene, while limonene is not a major contributor to flavor or aroma it does degrade and react with other compounds to form many of the most potent off-flavors.

The Oxidation Degradation of Limonene.

\[
\begin{align*}
&\text{Limonene Oxides} \\
&\text{Cis} \quad \text{Trans} \\
&\text{Para Cymene} \\
&\text{Carvone} \\
&\text{Carveol Cis and Trans}
\end{align*}
\]

Figure 1: The Oxidation Degradation of Limonene
Limonene can oxidize into many different compounds such as carvone, carveol cis and trans, limonene oxides and para cymene. Para cymene has a small flavor threshold at 80 ppm and can create a significant off flavor in orange juice.\(^7\) In this reaction limonene is oxidized and forms an aromatic structure losing two hydrogen atoms. Limonene can also be hydrated to form degradation compounds such as alpha-terpineol. Alpha-terpineol is formed from limonene and linalool in a non-oxidative, acid-catalyzed reaction. Its odor threshold is near 1 ppm and it gives orange juice an old and rancid odor.\(^7\)

![Chemical structure of limonene and alpha terpineol](image)

**Figure 2: Alpha Terpineol Formation.**\(^7\)

The degradation of ascorbic acid and sugars found in orange juice and orange drinks produces many off flavors and a browning affect in the beverages. Ferulic acid, found in orange juice reacts through a decarboxylation process to form the most significant off flavor, p-vinyl guaiacol or PVG.\(^10\)

![Chemical structure of PVG](image)

**Figure 3: PVG Structure**
PVG has an extremely small flavor threshold of 50 ppb and gives an old fruit or rotten flavor to the juice.\textsuperscript{10} Other sugars and amino acids produce furaneol through Maillard reactions. 5-hydroxymethyl furfural and 2,5dimethyl 3(2h)-furanone are the two main off flavors formed from acid degradation. These are commonly called 5-HMF and DMHF respectively.\textsuperscript{7} The degradation of these acids is the main cause of browning in orange juice. This is a major concern with orange juice due to the importance of its color to consumers. Ascorbic acid also degrades to produce furfural. Furfural itself is not a major cause of off flavors but does clearly show that acid degradation is occurring.\textsuperscript{7} These degradation products make up the rest of the marker components that must be monitored while studying orange beverages.

Oxidation is the cause of many of the off flavors formed in foods and beverages. Oxidation occurs in two main processes: autoxidation and photooxidation. Autoxidation is a three-step process: initiation, propagation, and termination.\textsuperscript{11}

\begin{align*}
\text{Initiation} & \quad \text{LH} \quad \rightarrow \quad \text{L}^\cdot \\
L^\cdot + O_2 & \quad \rightarrow \quad \text{LOO}^\cdot \\
\text{Propagation} & \quad \text{LOO}^\cdot + \text{L}'H \quad \rightarrow \quad \text{LOOH} + \text{L}'^\cdot \\
\text{Termination} & \quad \text{LOO}^\cdot + \text{L}'^\cdot \quad \rightarrow \quad \text{LOOL}' \\
& \quad \text{LOO}^\cdot + \text{L}'OO^\cdot \quad \rightarrow \quad \text{LOOL}' + O_2 \\
& \quad \text{L}^\cdot + \text{L}'^\cdot \quad \rightarrow \quad \text{LL}'
\end{align*}

\textbf{Figure 4: Oxidation Process}\textsuperscript{11}

The initiation step is started with the fatty acid (LH) forming a free radical (L^\cdot). The
free radical then reacts with oxygen to form the peroxy radical (LOO•). The propagation step is the continuation of the chain reaction. In this stage the peroxy radical can gain a hydrogen atom from another fatty acid producing more radicals. This step greatly accelerates the oxidation process. The termination stage occurs when the free radicals begin to react with each other and form nonradical species. The hydroperoxides formed (LOOH) can also decompose into alcohols, aldehydes, acids, and ketones.\textsuperscript{11}

Photo-oxidation also occurs in foods and beverages.

\[
\begin{align*}
1^1S + \text{hv} &\rightarrow 1^1S^* \rightarrow 3^3S^* \\
3^3S^* + 3^3O_2 &\rightarrow 1^1O_2 + 1^1S \\
1^1O_2 + LH &\rightarrow \text{LOO}• \rightarrow \text{LOOH}
\end{align*}
\]

\textbf{Figure 5: Photo-oxidation process.}\textsuperscript{11}

This mechanism shows \((1^1S)\) absorbing ultraviolet light and moving to the higher energy level of \((3^3S^*)\). Here the excited sensitizer can transfer its energy to the lowest vibrational energy state of oxygen \((3^3O_2)\), causing the oxygen to the higher energy state, the singlet \((1^1O_2)\). The singlet oxygen can then attack the fatty acid forming the peroxy radical, which gains a hydrogen, to become the hydroperoxide. The photo-oxidation reaction is much faster than the autoxidation reaction due to the high reactivity of the singlet oxygen.\textsuperscript{9} To combat the formation of off flavors through these oxidation processes antioxidants are added to foods and beverages.

The use of antioxidants in foods and beverages is constantly growing.

Antioxidants are defined as "substances that when added at a low concentration on comparison to the oxidizable substrate, significantly retards the oxidation of that
In order to be used in foods antioxidants must have the following characteristics; they must be safe for human consumption; not impart a color, odor, or off flavor; be effective at low concentrations; be able to survive processing; be stable in the finished product; be fat soluble; be readily available at low cost; and due to a growing consumer desire today, be natural antioxidants that look natural on the label. Synthetic antioxidants are widely used today, butylated hydroxyanisole (BHA) and butylated hydroxytolulene (BHT) are two of these synthetic antioxidants used in foods and beverages. There is a growing concern on the effects of synthetic antioxidants on the human body, there are also concerns that synthetic antioxidants may be carcinogens. Due to these concerns manufacturers are turning to natural antioxidants for use in foods and beverages. These natural antioxidants come mainly from plant extracts that have a phenolic nature. Tocopherol and rosemary extracts are two forms of natural antioxidants that show high antioxidation activity.

Tocopherol comes in two forms, tocols and tocotrienols. Tocopherol works as an antioxidant in one of two primary mechanisms. The first is a chain breaking electron donor and acceptor. Here the tocopherol competes with the unsaturated fatty acid (LH) for the lipid peroxy radical (LOO•). This reduces the formation of the lipid radical (L•) and slows the propagation step of autoxidation. Tocopherol can compete with the LH by rapidly transferring a hydrogen atom to the peroxy radical (LOO•) forming the more stable hydroperoxide (LOOH). The stable end product for this reaction is tocohyeryl quinone. This compound can also react with (L•) to produce (LH), again slowing the propagation step.11

Tocopherol can also react with a singlet oxygen (1O2) to hinder the oxidation
process. There are two main methods by which tocopherol acts as an oxygen scavenger. The first is a singlet oxygen quenching and the second is an irreversible reaction with the singlet oxygen to form a variety of products. The quenching method is predominant but even the most accepted method explaining the reaction and the end products formed is not clear or totally agreed on. The relative antioxidant activity of tocopherol depends on several variables such as temperature, lipid composition, and tocopherol concentration. Studies on lard triacylglycerols using tocopherol at 2 ppm showed that it was more effective as temperature was increased. These studies also showed that as the concentration of tocopherol was increased the rate of substrate oxidation also increased. Similar results were found in studies on soybean oil and corn oil. These characteristics of tocopherol show that it has great potential for being a natural antioxidant in foods and beverages.

Rosemary extracts have shown to be an effective antioxidant in many food systems. The active antioxidant compounds found in rosemary extracts are carnosol, carnosic acid, and rosmarinic acid. In Rancimat tests, where fats and oils were oxidized under elevated temperature carnosol, carnosic acid, and rosmarinic acid showed stronger antioxidant activity than the synthetic antioxidants BHA and BHT. Carnosol and carnosic acid have been shown to be scavengers of peroxy radicals (CCL$_3$O$_2$•), (HO•) radicals. Carnosic acid has also been shown to be a scavenger of H$_2$O$_2$. These results show the potential of rosemary extracts as antioxidants in foods and beverages. Ursolic acid, oleanolic acid, and betulinic acid are also major components in rosemary extracts. These components do not show high antioxidant activity but are believed to contribute to the anticarcinogenic properties of rosemary.
This is where our research began; we intended to test the natural antioxidants on an orange drink containing a percentage of natural orange juice from industrial concentrate. The natural antioxidants tested were a tocopherol mixture and two rosemary extracts. The natural antioxidants will be tested against a control and samples containing the synthetic antioxidant BHA. The goal of the research is to find a natural antioxidant that will be as effective as the synthetic BHA.

**METHODS**

**The Orange Oil Study**

The first study of the project was on pure orange oils. This was done to identify the volatile components in orange juice without having all of the additives. A method using a GC/MS was used to identify and monitor components in orange oil. The goal for GC/MS testing was to watch the degradation of the volatile components and the formation of components that are markers for off flavors. A pure single fold orange oil was obtained from SunPure and tested using the GC/MS. Manufactures put oils through a "folding" process to decrease the concentration of the substances in the orange oil that are more susceptible to oxidation. A five fold oil contains less components that are susceptible to oxidation than a single fold oil. Five fold oils also contain more of the components that add to the flavor and aroma of the oil. The single fold oil was tested because it is expected to be more sensitive to oxidation than the more commonly used five and ten fold orange oils. To increase the rate of decomposition the orange oils were stored at 50°C in a Schaal oven. Six samples
were made, a control and five that contained antioxidants. The antioxidants used were tocopherol, ascorbic acid, rosemary extracts Herbalox HT-O®, Herbalox WM-4® and the synthetic antioxidant BHA. The GC/MS gave a high level of sensitivity for all of the major components in the orange oils. The second test ran on the orange oils was a peroxide value measurement. An electrochemical peroxide value measurement was performed on the orange oils on a weekly basis. This goal of test was to show the relative oxidation levels of the orange oils.

**The Orange Drink Study**

The second study of the project was on a prepared orange drink. The goal of this study was to observe the relative concentrations of the components that degrade to form off flavors and the components that produce the off flavors. A method for solid phase micro extraction (SPME) was performed on the prepared juice. Past experiments showed that a SPME test would provide a high level of sensitivity for the volatile components in the orange beverage. Before the final juice study was started test runs were done on Hi-C®. Hi-C® contains 10% orange juice from concentrate, which is a common amount in commercial orange drinks. The method used a Supelco divinylbenzene / carboxen / PDMS composite fiber to absorb the volatile components from the head space above the orange drink. To insure that the method would yield the best results possible variables throughout the procedure were tested. Sample preparation was the first variable to be tested. Samples were prepared for a large scale and a small scale test, both methods included a sample with just Hi-C® and a sample with NaCl added to the Hi-C®. NaCl is added to a sample to create a more polar atmosphere that aids the
absorption levels and gives the SPME fiber a higher sensitivity.\textsuperscript{6} The parameters on the GC/MS were also tested and runs using different temperatures and lengths of time for equilibration were made. The results of these test runs gave the parameters for the solid phase micro extraction that would be performed in the final juice study.

Static head space analysis includes directly injecting the volatile fraction from a closed vial containing orange juice into the GC/MS. This goal of this method was to look at the volatile components relative concentrations. To receive high sensitivity using a static headspace method it is necessary to have a high concentration of the analyte in the headspace of the sample. To achieve a high concentration of analyte in the headspace variables such as pressure and temperature were tested to produce a method that could give high sensitivity for the final drink study.

A solid phase extraction method (SPE) was also tested. The goal of this method was isolate and quantify PVG and furaneol. Four SPE cartridges were used in tested on the Hi-C\textsuperscript{®}. The results were tested using the GC/MS. The four cartridges gave a polar and a non-polar atmosphere for the extractions. Methanol and acetone were tested as extracting solutions.

A liquid – liquid extraction method condensed the orange drink 200 X through washings with liquids that would dissolve the volatile components of the orange drink. This concentrated liquid was then tested using the GC/MS for identification of components.
EXPERIMENTAL

Preparation of Orange Oil for Stability Testing

Five orange oil samples and one control were prepared for the stability testing. All samples were prepared using a single fold cold pressed orange oil obtained from SunPure (Lakeland, Florida). The oil was used as received.

Table 1: Antioxidants Used in the Stability Studies and the SPME Tests

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic Acid</td>
<td>Pfizer Inc. (New York, NY)</td>
<td>USP Grade purity</td>
</tr>
<tr>
<td>Tenox BHA Flake</td>
<td>Eastman Chemical (Kingsport, TN)</td>
<td>Mixture of 2- and 3-t-butyl-4-hydroxyanisole Synthetic antioxidant</td>
</tr>
<tr>
<td>HERBOLOX HT-O® Rosemary extract</td>
<td>Kalsec® (Kalamazoo MI)</td>
<td>Oil soluble, 4.2% carrosic acid and carnosol.</td>
</tr>
<tr>
<td>Tenox GT-2</td>
<td>Eastman Chemical (Kingsport, TN)</td>
<td>70% mixed natural tocopherols</td>
</tr>
<tr>
<td>HERBOLOX WM-4® Rosemary extract</td>
<td>Kalsec® (Kalamazoo MI)</td>
<td>Water soluable, 3.2% rorarinic acid.</td>
</tr>
</tbody>
</table>

Oil samples (400 mL) were mixed separately with 250 ppm ascorbic acid, 667 ppm Tenox GT-2, 1000 ppm BHA, or 1000 ppm rosemary extract. These and a control (200mL) were stored at 50°C in a Schall oven. Oils were sampled weekly for PV and GC/MS analysis.

Instrumental

GC/MS

All GC/MS analysis were performed on a Varian 3800 gas chromatograph
with a 1079 split/split-less injector with electronic flow control. A Saturn 2000 Ion Trap Mass Spectrometer and a Varian 8200 model auto sampler. The GC column was a DB5 type capillary (30m x 0.25mm) 0.25μL film (Supelco). The orange oil samples were prepared for injection at 3% oil in acetone (HPLC grade acetone from Fisher Chemicals). The GC/MS column was programmed with an initial temperature of 60°C, the temperature was then ramped by 3°C per minute up to 240°C. The pressure was set at 16psi and the injection was split at 50:1. The samples were tested weekly.

**PEROXIDE VALUE:**

Peroxide values (PV) of the orange oils were determined by Official Method Cd 8-53 of the American Oil Chemists Society (AOCS), with modification for coulometric detection rather than titration of iodine. Measurements were made in an EG&G Princeton Applied Research model 377A coulometric cell with Pt basket working electrode, standard calomel reference electrode (SCE), Pt flag counter electrode and a synchronous motor shaft stirrer. A EG&G/PAR model 263A potentiostat was used for controlled potential electrolysis. A 2 mm diameter Pt disk electrode was used to check the reference potential of the iodine/iodide redox couple prior to each session of measurements. The potentiostat was controlled with EG&G model 250/270 software. The reference electrode and counter electrode cells were filled with 0.5 M sulfuric acid and isolated from the working electrode compartment with Vycor glass frits. The working electrode was preconditioned by cycling it between -0.2 and +1.3 V vs. SCE (100mV/s, 10 cycles) in 30 mL of 0.5 M sulfuric with continuous
stirring. This was followed by addition of 10μL of saturated potassium iodide solution (approx. 4M) and holding the electrode at 0.0 V vs SCE for 10 min. with stirring.

A clean dry cell was loaded with 2.5 g of orange oil sample, in the ascorbic acid tests the orange oil was filtered prior to testing, and 15 mL of PV solvent (3:2 glacial acetic acid: metylene chloride, v/v). The working electrode and frits were rinsed with water and then dipped in PV Solvent to remove residual water. The sample cell was attached to the cell. A 0.250 mL aliquot of saturated potassium iodide solution was added to the sample with stirring. After 60 s, 15 mL of 0.2 M KH₂PO₄ was added to quench the conversion of peroxide into iodine. A voltage of 0.0 V vs. SCE was applied to the working electrode with stirring and the total charge needed to reduce the iodine back to iodide was recorded until the current fell to a baseline level of 100 μA. The measurement was usually complete within 10-15 min. Background charge due to iodine in the saturated potassium iodide solution was measured in a blank determination (i.e., no sample) and subtracted from the sample charge before calculation of the PV.¹ ⁴

The PV was calculated in meq/KG oil as:

\[
P V = \frac{(Q_{\text{sample}} - Q_{\text{background}})}{F} \left( \frac{1000 \text{ g kg}^{-1}}{W} \right)
\]

\[Q = \text{integrated charge from } I \text{ vs. } t \text{ plot (in mC)}\]

\[F = 96485 \text{ mC/meq}\]

\[W = \text{sample (oil) mass in grams}\]
Materials for the Orange Drink Study

Hi-C® Orange Drink (Coca-Cola Co. Houston, TX)

The Hi-C® orange drink was used for method development. It was purchased at a local grocery store. Hi-C® contains 10% orange juice from concentrate, this is a common amount in orange drinks.

Orange Drink:

The orange drink used in stability tests was prepared with 25% orange juice from concentrate. One liter of finished beverage was taken from the large scale prepared beverage made as follows. Mix 5406.00 g of mineral enhanced waster (Gordons water Kalamazoo MI) with 4.8 g sodium hexametaphosphate (Fluka Chemika), 2.7 g sodium benzoate (Aldrich Chemical CO.), and 3.3 g potassium sorbate (Supleco INC.). These were mixed thoroughly until all the preservatives were dissolved. Then 552.00 g of sugar (store bought), 288.9 g of orange juice concentrate 64 brix. (SunPure), 1.32 g of sodium citrate (J.T. Baker Chemical Co.), and 13.2 g of citric acid (Haarmann and Reimer Co.) were added. One sixth of the beverage and one sixth of the concentrate were added to make the orange drink. All antioxidants were added to the orange juice concentrate prior to the addition to the beverage. Four orange beverage mixtures containing antioxidants and one control were prepared for the final orange drink study.

Internal Standard

Phenyl ethyl alcohol

Phenyl ethyl alcohol was used as the internal standard in the SPME tests and the Static head space test. The standard was prepared a 400 ppm, with 40 mg of phenyl ethyl alcohol (Kalsec) being diluted by 100 mL of HPLC grade water (Fisher Chemical). The
internal standard was added to the SPME samples at 40 ppm and to the static head space samples at 200 ppm.

**Solid Phase Micro Extraction with GC/MS:**

Head space samples were collected by solid phase micro extraction (SPME) on a divinylbenzene / Carboxen / PDMS composite fiber (Supelco). Samples were prepared in large scale and in small scale. The small scale preparation used 0.8 g of Hi-C® of 0.512 g of Hi-C® mixed with 0.288 g of NaCl (36% NaCl). The large scale was prepared in a 22 mL vial with a Teflon seal crimp top cap using 15 g of HI-C® or 9.6 g of Hi-C® and 5.4 g of NaCl (36% NaCl). The large scale samples were heated to 40°C. The small scale samples were kept at 25°C. The SPME fiber was injected into the head-space of the Hi-C® and exposed for 20 minutes, before injection into the GC/MS. The fiber desorption time in the injector (0.8 mm open tube liner at 250°C) was set for three minutes under split-less conditions. The carrier gas was set at 1.0 mL per minute in a constant low mode. The column's temperature started at 35°C and was ramped to 140°C at 3°C per minute, then to 200°C at 10°C per minute. All samples were run in duplicate. Neither the size of the sample, the addition of salt, nor did the addition of heat produce significantly better results. The procedure for the small sample kept at room temperature with no addition of NaCl was chosen for the final orange drink study. In tests using the final orange drink a five minute head space absorption time gave similar peak area to those from the 20 minute absorption tests. The five minute absorption time was used for sampling in the orange drink stability tests. In the final orange drink analysis an internal standard of phenyl ethyl alcohol was added to the drink at 40 ppm for semi – quantitative purposes.12
Static Head Space GC/MS Method:

The static head space GC/MS method was adapted from the literature and tested with the Hi-C® to find the best method for this research. The carrier gas was helium and the head space pressure was set at 15 psi. The samples for method testing were prepared with two mL of Hi-C® in a 10 mL vial with a Teflon crimp top cap. The Vial was heated to 80°C for 15 minutes, followed by a 0.5 minutes pressurization time, a 0.5 minutes injection time, and a 0.2 minute withdraw time. The needle and the transfer line were set at 150°C. The GC column was set at 10 psi under back - pressure control. The GC/MS injector was kept in the split-less mode for 1 minute to capture the injection. There was a two - minute pause between the injection time and the start time for the data collection and the temperature ramp in the GC. The column temperature was ramped from 40°C to 200°C at 5°C per minute. The carrier gas was helium with a linear velocity of 81 cm/s. The column pressure in the GC was set at 15 psi. Phenyl ethyl alcohol was used at 200 ppm as an internal standard for semi – quantitative purposes. ³

Solid Phase Extraction

Solid phase extraction of the Hi-C® was performed with four different cartridges

<table>
<thead>
<tr>
<th>Cartridge</th>
<th>Conditioning</th>
<th>Orange drink used (mL)</th>
<th>Eluent used (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nexus</td>
<td>None</td>
<td>5 mL of Hi-C®</td>
<td>0.5 mL of methanol</td>
</tr>
<tr>
<td>Empore (C18, 3M)</td>
<td>1 mL methanol wash.</td>
<td>5 mL of Hi-C® Followed by a 1 mL water wash.</td>
<td>0.5 mL of acetone</td>
</tr>
<tr>
<td></td>
<td>2 mL water wash.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agilent (C18, 100 mg)</td>
<td>1 mL methanol wash.</td>
<td>5 mL of Hi-C® Followed by a 1 mL water wash.</td>
<td>0.5 mL of acetone</td>
</tr>
<tr>
<td></td>
<td>2 mL water wash.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Varian (C18, 500 mg)</td>
<td>2 mL methanol wash.</td>
<td>10 mL of Hi-C® Followed by a 2 mL water wsh.</td>
<td>1 mL of acetone</td>
</tr>
<tr>
<td></td>
<td>4 mL water wash.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
All of the cartridges tested were tested in duplicate. The setup includes a vacuum pump to pull the solvents through the cartridges. A 2 mL amber vial was used to collect the final wash containing the volatile components. All of the solvents used in this procedure were HPLC grade from Fisher Chemical. All of the Hi-C® for these tests was centrifuged for 15 minutes prior to the tests. The vacuum could be adjusted to control the flow rate through the cartridges. During the test runs the flow was kept at a slow to moderate pace of about 1 mL per 10 seconds. The final wash collected in the 2 mL amber vial was tested using the GC/MS. The GC/MS method that was used to test the orange oil samples was also used to test these samples.9

**Liquid – Liquid Extraction with GC/MS:**

Liquid – Liquid extraction of the Hi-C® was carried out with two solvents, pentane and methylchloride. 50 mL of Hi-C® was vigorously mixed with 10 mL of solvent. The solvent was separated from the juice and collected. 10 mL of solvent was again added and mixed with the juice and separated. The two 10 mL washes were combined and one gram of Na2SO4 was added to dry the extract. The dried extract was separated from the solvent and evaporated to 250μL under N2 yielding a 200 X dilution.6
Results

Effect of Antioxidants on the Stability of Orange Peel Oil.

Two of the volatile components of orange oil that are most susceptible to oxidation processes are limonene and myrcene. Both of these components are known to produce off flavors in orange oil. Through the 52-day study area percents of these components were kept using a GC/MS system. Limonene was the main volatile component in the orange oil with an area percent of near 90% throughout the study. However, the GC/MS system was to sensitive to limonene to give precise results on the area percents of limonene from week to week. Therefore, instead looking directly at limonene we will look at the data of the oxidation products of limonene that produce off flavors in orange oil.

The first oxidation products detected were limonene oxides, cis and trans. These components are primary oxidation products of limonene and their relative area percents are shown in Figures 6 and 7.

![Figure 6: The relative area percent of limonene oxide cis vs time.](image)

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Figure 7: The relative area percent of limonene oxide trans vs time.

Figures 6 and 7 show an increase of these oxidation products through time in the control orange oil. The area percents of the limonene oxides in the orange oils that contain antioxidants all stay very low throughout the study. The natural antioxidants, the tocopherol and the Rosemary extracts, HT-O and WM-4, are all as effective in the suppression of the limonene oxides as the synthetic antioxidant, BHA.

Carveol cis and trans are also degradation components produced through the oxidation of limonene. Figures 8 and 9 show the relative area percents of these two components.

Figure 8: The relative area percent of carveol cis vs time.
Figure 9: The relative area percent of carveol trans vs time.

The results for carveol cis and trans are very similar to the results from the limonene oxides. The control orange oil shows a significantly higher area percent of carveol cis and trans than the orange oils containing the antioxidants. Again the natural antioxidants are equally as affective as the synthetic antioxidant.

Alpha terpineol and carvone, degradation products of limonene, are identified as off flavors in orange oil. These two components also show increases in area percent in the control but remain relatively steady in the substances containing the antioxidants. Again there is not a significant difference in the affect of the natural antioxidants compared to the synthetic antioxidant.
Figure 10: The relative area percent of alpha terpineol vs time

Figure 11: The relative area percent of carvone vs time.
The degradation of myrcene could be seen by directly looking at the relative area percents of myrcene from week to week. As illustrated in figure 12, the area percent of myrcene becomes significantly lower in the orange oil not containing an antioxidant. The natural antioxidants are again as effective as the synthetic antioxidant maintaining the level of myrcene in the orange oil.

![Graph showing myrcene area percent over time]

**Figure 12: The relative area percents of Myrcene vs time.**

The results from the orange oil study show that all orange oils treated with an antioxidant did maintain a much lower oxidation level than the control. This result can been seen in both the absence of oxidation products from limonene and from the lack of oxidation degradation of myrcene in the orange oils that contained the antioxidants. The results of this study also show that the natural antioxidants are as effective on the orange oils as the synthetic antioxidant BHA. The overall oxidation level of the oils could also be seen in the results from the peroxide value measurements, as shown in figure 13.
Figure 13: Peroxide value results.

There were four methods tested on the Hi-C® in the method development work. The SPME procedure was chosen for the final orange drink study due to its high sensitivity for many of the target components. The solid phase extraction (SPE) was performed using a variety of cartridges, with all of the results showing a very poor sensitivity. The liquid-liquid extraction did identify some on the target compounds but the sensitivity was too poor to give accurate results. Figure 14 gives an example of the GC/MS readings on the headspace resulting from a liquid-liquid extraction in two different solvents.
Figure 14: Sensitivity graph of the liquid-liquid extraction method.

The Static headspace method identified many of the target components but the sensitivity of the method was not nearly as high as the sensitivity of the SPME method as shown in figure 15.
Figure 15: Sensitivity comparison between the static headspace method and the SPME method.

Effects of Antioxidants on the Stability of an Orange Drink.

The components that were followed in the orange oil study were also followed in the orange drink study. A solid phase micro extraction (SPME) procedure was used to
study the orange drink due to its increased sensitivity. The SPME procedure also gave data on significant oxidation products such furfural. Furfural itself is not a strong off flavor but is known to be a marker compound for numerous other off flavors. Figure 16 shows that the relative level of furfural remains relatively consistent throughout the study in both the drinks with an antioxidant added and the control.

![Furfural Graph]

**Figure 16:** The relative area percents of furfural vs time.

Carvone production was suppressed by the antioxidants in the orange oil study but the off flavor is not suppressed in the orange drink study. Its area percents of the orange drinks containing antioxidants and the control remain relatively equal as seen in figure 17.

![Carvone Graph]

**Figure 17:** The relative area percents of carvone vs time.
Many of the most potent off flavors in orange drinks come from the production of terpenes. In the orange drink study the production of terpenes was not significantly affected by the antioxidants. Alpha terpinene, gamma terpinene, and terpinolene all show that the antioxidants had little affect on their productions.

**Figure 18:** The relative area percent of alpha terpinene vs time.

**Figure 19:** The relative area percent of gamma terpinene vs time.
Figure 20: The relative area percent of terpinolene vs time.

The components in the orange drink that are most susceptible to oxidation also were not affected significantly by the antioxidants. Three components which could be measured precisely through the SPME method, alpha pinene, nonanal, and octanal, are susceptible to oxidation. All three of these showed that the antioxidants had no affect on their relative area percents throughout the study.

Figure 21: The relative area percent of alpha pinene vs time.
The data from the Solid Phase Micro extraction study shows that the use of antioxidants had little effect on the degradation of volatile components or on the production of off flavors. The results for many of the same components that were affected by the antioxidants in the orange oil study show no significant effect from the antioxidants in the orange drink study. A note on the data in the results for the orange drink study, the day 7 data is consistently low for all area percents of all components.
This inconsistency may be due to human error during experimentation or due to the high sensitivity of the SPME fiber to limonene.

**Discussion**

The results from the orange oil study showed that the orange oil that was treated with the natural antioxidants maintained an oxidation level similar to that of the orange oil containing the synthetic antioxidant BHA. All orange oils treated did maintain a much lower oxidation level than the control as shown in the PV measurements. The results from the GC/MS also show that the natural antioxidants inhibited the formation of known off flavors produced through oxidation processes as well as BHA did. These results meet the goals of the research, the natural antioxidants were as effective as the synthetic antioxidant BHA. Further research should be performed to determine the amount of the natural antioxidant that will give adequate antioxidation activity.

The orange drink study did not give any significant data to show that the antioxidants used were having an effect on the orange drink. Due to the success of the antioxidants in the orange oil study it is possible that the antioxidants are effective and that the production of the off flavors is through isomerization and or hydration processes. This study needs to be followed up with a series of methods that will isolate and more accurately measure the relative amounts of the components of the orange drink.

Suggestions for further research include using a solid phase extraction to get an accurate measurement of PVG and furfural\(^9\) and the use of a static headspace analysis to accurately measure the levels of para cymene.\(^6\) These components are all known degradation products of orange drinks that the solid phase micro extraction fails to
identify accurately identify and measure.

The transition to natural antioxidants in foods and beverages from synthetic antioxidants is a healthy and necessary transition. In this specific example of the use of natural antioxidants in orange drinks they proved to be as effective as the synthetic antioxidants. Further research is required to better test the effectiveness of natural antioxidants on orange drinks. This research should include the use of several methods to give accurate readings on the relative components levels in orange drinks. Once the effectiveness of a natural antioxidant is proven, a process to produce it at a low price must be found so that manufacturers will use the natural product.
Resources

2. Gray Of The American Oil Chemist’s Society 1978, 55, 539-546