Liquid Carbon Dioxide Extraction of Various Food Flavors: Evaluation and Analysis

Deven Lee Shinholt

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Liquid Carbon Dioxide Extraction of Various Food Flavors; Evaluation and Analysis

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Deven Lee Shinholt
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Abstract

Sub- and supercritical carbon dioxide has been utilized as an extraction solvent for a variety of natural compounds. This requires the use of specialized high-pressure vessels. It was reported recently that common laboratory apparatus (centrifuge tubes) could be utilized in liquid carbon dioxide extractions obviating the need for specialized equipment. Various herbs and spices (including orange, lemon, lime, and grapefruit zest, oregano, rosemary, sage, spearmint, nutmeg, black peppercorns, cloves, caraway seeds, and vanilla beans) were used as substrates for liquid carbon dioxide extractions by this straightforward technique. The extracted oils, containing terpenes and terpenoids, were then analyzed through GC/MS. Liquid carbon dioxide extraction by this procedure was evaluated utilizing an internal standard and GC analysis for orange zest and caraway seeds. The terpenes and terpenoids of interest were limonene for orange zest, and limonene and carvone for caraway seeds.
Chapter I: Background

The Chemistry of Flavor

There are many "categories" of flavors that can be attributed to food. There are the typical sweet, salty, sour, and bitter flavors, but these basic "tastes" are simply not enough to describe more complicated flavor combinations. There are also savory, meaty, fruity, spicy, smoky, and a host of many other flavor complexes (1). These individual flavors are analogous to musical instruments, each having a characteristic essence that adds to the overall experience of a meal.

There is more to flavor than just taste. The aroma (or odor) and mouthfeel (or trigeminal) of the food also contribute to the interpreted flavors (1,2). For instance, the esters and aldehydes that characterize the flavors of non-citrus fruits are detected by their aroma, rather than through taste. If aroma is eliminated from the flavor equation, then distinguishing among these fruits becomes much more difficult. This can even create difficulty distinguishing between an apple and an onion. The trigeminal component, however, can contribute to pungent, astringent, or even cooling sensations of flavor. This is easily noticeable when one eats chili peppers or peppermints. (1)

The essences of herbs and spices are contained within the combination of compounds as well as the concentrations of these compounds. One compound may have a particular flavor at a low concentration, but this flavor may alter significantly at higher concentrations. Fisher demonstrates this when she says,

"Since odour quality may change with concentration, a chemical such as trans-non-2-enal has more than one recognition threshold: just above its detection threshold of 0.1 ppb, trans-non-2-enal possesses a woody character. Above 8 ppb, it smells fatty, becoming unpleasant at 30 ppb,"
and, in an aqueous solution at 1000 ppb, it has a strong flavour of cucumber. This change in flavour with increasing concentration of an individual compound can perplex the flavour chemist... Another problem that can occur is interaction when compounds are mixed together. When they interfere with flavour detection, this is called antagonism. When they enhance the ability to detect the flavour, it is called synergism... To demonstrate flavor synergism, one can look at ketones at concentrations where each, individual, has no aroma in water (butan-2-one, 5 ppm; pentan-2-one, 5 ppm; hexan-2-one, 1 ppm; heptan-2-one, 0.5 ppm; octan-2-one, 0.2 ppm), but a solution containing all of them together at these specific concentrations has a definite aroma.” (3)

The sense of taste is a highly-developed sense within the human body. Fisher also states “It has been estimated that as few as eight molecules are required to trigger one human olfactory neuron and that as few as 40 molecules can produce an identifiable sensation. Such levels are below the sensitivity limits of present-day analytical techniques; thus, the human nose is a better detector than the best instruments of today!” (1,2). Several studies have been conducted to determine olfactory detection limits and mechanisms (4).

**Flavor Compounds**

Flavor compounds are often categorized by volatility. This methodology is generally used because volatility essentially determines the manner in which the flavor compounds are detected by a person. Volatile compounds are typically sensed in the nose, whereas less volatile compounds are detected by the taste buds. The analysis of volatile flavor compounds (which usually have a molecular weight below 300 amu) is often done using gas chromatography. The relative concentration of volatile compounds is often not proportional to those compounds’ flavor contributions. Fisher demonstrates the importance of this correlation when she says,

“It is now well established that many of the larger peaks on gas chromatograms of foods do not correlate to flavour. For example,
limonene is the major component by weight of citrus oils, but it has a weak aroma. It is the oxygenated terpenes present in small amounts in these oils that have the major impact on the flavour. Although hydrocarbons like limonene may not have much aroma, they do act as a solvent for the powerful odorants.” (5)

Generalizations of a chemical’s odor, and thus its flavor, can be made based upon that compound’s functional group(s). Some major types of volatile compounds include aldehydes, ketones, alcohols, carboxylic acids, esters, furans, phenols, terpenes, and terpenoids. The volatile compounds of importance in this study are mainly terpenes and terpenoids, which will be discussed in-depth later. (1)

Non-volatile flavor compounds do not have an aroma, but they can be broken down into volatiles through heat from cooking, or even enzymes. Because of their non-volatile nature, they are detected primarily by the taste buds in the mouth. They are often characteristic of the five “tastes”: sweet, sour, salty, bitter, and umami. Some major types of non-volatile flavor compounds include: amino acids (such as L-tryptophan), small peptides (such as aspartame), organic acids (such as citric acid or malic acid), sugars (as in sucrose or glucose), salts (such as sodium chloride or the amino acid salt monosodium glutamate, also known as MSG), alkaloids (such as caffeine in coffee and tea or nicotine in tobacco), phenols (such as capsaicin in chili peppers or piperine in black peppercorns), and isothiocyanates (such as allyl isothiocyanate in horseradish). (1)

Terpenes and Terpenoids

Terpenes are naturally occurring compounds which are composed of various combinations of isoprene units. Isoprene, shown in Figure 1, (also known as 2-methyl-1,3-butadiene) is a biologically important molecule.
Through various biological processes, isoprene is partially polymerized to form terpenes which contain 10, 15, 20, or more carbons (6). Shown in Figure 2 is β-carotene, a tetraterpene; β-carotene is an example of a high-molecular weight terpene.

These biological processes may also add functional groups to these compounds; thus transforming these terpenes into terpenoids. Fisher also gives a broad overview of these important compounds:

"Terpenoids are substances derived in nature from the metabolic intermediate, mevalonic acid, which provides the basic structural unit, the isoprene unit. Hemi-, mono-, sesqui-, and di-terpenoids, having one, two, three or four isoprene units, respectively, are well known, but it is the monoterpenoids that provide the character-impact flavours of many herbs, spices and citrus fruits. The terpenes (hydrocarbons) are found in the essential oils of most plants, but have little flavour of their own. Usually the oxygenated terpenes have flavour threshold values much lower than those of the hydrocarbons. The hydrocarbon terpenes may simply interact with the oxygenated terpenes as a solvent to enhance the ability of the flavor compounds to reach the organoleptic receptors." (7)
Some terpenes and terpenoids contain chiral centers and are found in enantiomeric or diastereomeric forms. An example of this is limonene, where (+)-limonene is a major component of citrus fruits, while (−)-limonene is an important component in mint leaves. These stereoisomers can also affect flavor. For instance, (+)-carvone gives caraway seeds their distinctive aroma, while (−)-carvone characterizes spearmint’s unique and refreshing flavor. (8)

Terpenes and terpenoids are very important molecules in biological and ecological processes. It has been shown that some flowers can produce terpenoids to attract various pollinating insects. Also, terpenoids emitted from some plants have been found to attract beneficial mites, which can feed on herbivorous insects that threaten that plant. It has been estimated that the total terpene and terpenoid plant emissions per year is around 500 tetragrams. As a result, the environmental effects of terpene and terpenoids and their degradation products is a topic of current study in environmental chemistry. (9)

**Extraction Methods**

Essential oils have extracted from herbs and spices for medicinal purposes since ancient times. Many traditional Chinese medicines were made by soaking, then boiling an herb, or a collection of herbs, in water. By doing this, the water would become infused with the essence of the herb (10). Today, many different types of cooking oils are made in a similar manner by soaking dried herbs and spices in cooking oil and then heating the mixture to “infuse” the oil with the herbal flavor essences.

Many methods have been developed to isolate food flavors from natural sources. Some of the more common methods include steam distillation, organic solvent extraction,
supercritical fluid extraction, sonication, and cold-press extraction (10-14). It seems that no single method is always a better choice than another, because the extraction method is heavily dependent on the substrate and preferred compounds as well as on materials, time, and energy available. For instance, ethanol extraction (often in conjunction with enzymes) is generally preferred when making natural vanilla extracts as it can easily extract the aromatic compounds that are characteristic of vanilla (13). The decaffeination of coffee beans is usually performed with supercritical carbon dioxide, as certain conditions can be modified to alter the specificity of the compound to be extracted. In this case, conditions can be adjusted to maximize the solubility of caffeine, but minimize the solubility of the precious flavor compounds (15). Also, cold-press extraction and steam distillation are both often used to extract citrus oils from citrus zests. Steam distillation is often quicker and less expensive than cold-press extraction, but produces lower quality oils due to the heat involved in the process (14).

Dr. Eng Shi Ong reviews and compares some common methods for herbal extractions (12). Some of the ones he discusses include: Sonication, Soxhlet extraction, Microwave Assisted Extraction (MAE), Supercritical Fluid Extraction (SFE), static Accelerated Solvent Extraction (ASE), dynamic Pressurized Liquid Extraction (PLE), Pressurized Hot Water Extraction (PHWE), and Surfactant assisted PHWE. MAE is an excellent, recent method using microwaves to assist in extraction. Extraction times are often short (less than an hour) and solvent consumption is generally small. PLE is similar to SFE, only at sub-critical pressures and temperatures. PLE has also been shown to have yields comparable to Soxhlet extraction. PHWE is similar to PLE, but using sub-critical water at high temperature and pressure. This option may be preferred when organic
solvents are an issue, but not when thermal stability is a factor. Surfactant assisted PHWE, as the name suggests, is PHWE with a surfactant added to increase the solubility of the less-polar compounds. Organic modifiers (such as short-chained alcohols) may also be added to increase the solubility of the less-polar compounds.

Soxhlet extraction generally has a high yield because of the continuous extraction process. The major disadvantage, however, is the often high temperature, long extraction time, and large amount of solvent use needed (10). Sonication is a method in which ultrasound is used to speed the process of extraction within a system. This is a fairly recent method that has proven itself useful in certain systems, as low frequency sonication has been shown to degrade toxic alkaloids during the extraction of certain medicinal compounds. It has been suggested that sonication is superior to Soxhlet extraction because less solvent is involved and the process is safer for compounds that are easily thermally degradable (10).

Supercritical Fluid Extraction is a newer and greener process involving supercritical solvents. By increasing the pressure and the temperature of a solvent, that solvent can be forced into the supercritical phase where it has both liquid and gaseous properties. Carbon dioxide is one of the more favored solvents, often being pressurized to 300 bar and being held at ~31 °C. A small amount of cosolvent, such as ethanol or isopropyl alcohol, is added to greatly increase the solubility of the desired polar compounds (16). The solvent strength of a supercritical fluid is easily tunable by modifying the pressure, which in turn changes the density of the fluid. This allows for a dynamic specificity, of sorts. Carbon dioxide has an advantage over most other solvents as it can reach its critical point at ~31 °C, allowing for the ease of extraction of thermally
degradable compounds. Also, the fact that carbon dioxide is a gas at room temperature and pressure allows for the ease of isolation of the extract from the solvent (17). This becomes a key advantage in the method proposed in this study.

Steam distillation is an early discovered method for the extraction of essential oils from herbs and spices. By steaming the leaves, seeds, or even the bark of a plant, volatile compounds can be released and collected with the aqueous runoff. Most steam distillates will need a liquid-liquid extraction to pull all the desired compounds from the aqueous portion. In one study, dried thyme, basil, rosemary, chamomile, lavender, and cinnamon were steam distilled at 55 °C for 3 hours at a reduced pressure of 95 mmHg (11). The resulting distillates were extracted with dichloromethane in a liquid-liquid continuous extractor for 6 hours, dried over anhydrous sodium sulfate, and concentrated using a rotary flash evaporator. The resulting yields were 1.24 % (thyme), 1.05 % (basil), 0.087 % (rosemary), 0.45 % (chamomile), 1.06 % (lavender), and 1.08 % (cinnamon) (w/w). Turkish bitter orange and lemon leaf oils were extracted in much the same way (18). Steam distillation was carried out at atmospheric pressure for 2 hours on fresh leaves. This extraction, therefore, was carried out at significantly higher pressure and temperature, possibly resulting in more terpene/terpenoid rearrangements due to side reactions. The oil was isolated, dried, and condensed to give 0.28 % yield for bitter orange leaf and 0.49 % for lemon leaf (w/w).

The comparison of liquid CO₂ extraction, ethyl ether extraction, and steam distillation was carried out using clove buds as the food substrate (19). The total yields were 15.7 % (steam distillation), 18.0 % (liquid CO₂ extraction), and 23.0 % (ethyl ether extraction). In addition to being analysis by gas chromatography, the extracts were
evaluated by a panel of eight trained judges to determine the quality of the extracts. On a nonstructured scale with 1 = not typical and 9 = very typical, the extracts were rated 2.12 (steam distillation), 8.94 (liquid CO$_2$ extraction), and 6.47 (ethyl ether extraction).

According to the literature, the liquid CO$_2$ extract was a light yellow transparent liquid, the steam distilled extract was colorless, and the ethyl ether extract was a dark green viscous liquid, which still retained the scent of ethyl ether. It was reported that the steam distillation did not produce artifacts, as all of the detected compounds in the GC chromatogram were detected in the extracts of the other two extraction methods.

Steam distillation, hexane extraction, and supercritical CO$_2$ extraction were compared using lavandin and Indian ginger root. Interestingly, “Several cooked dishes (soup, fish, poultry) were seasoned with ginger prepared by the same three methods. Tasters commented favorably on the flavor balance and fresh characteristics of food seasoned with SCCO$_2$ extracted ginger” (19). It was reported that the yields of the lavandin extracts were 3.0 % (steam distillation), 1.2 % (hexane extraction), and 3.5 % (supercritical CO$_2$).

The cold-press process is a method which is often used to extract oils from the zests of citrus fruits. This is often a superior process of extraction for these oils because the extraction is done at a temperature that is lower than steam distillation or solvent extraction. This is done to minimize the possibility of terpene rearrangements and side reactions that are typical of other extraction processes. These unfavored reactions can produce byproduct flavors and an overall lower quality product. The Citrus Research and Education Center of the University of Florida explain in an article the theory behind the cold-press process:
"Citrus oil is present in small glands contained in the flavendo, which is the colored portion of the peel of the fruit. Cold-pressed citrus peel oil is obtained commercially by a process that starts with the rupture of these glands during juice extraction. For its recovery, the oil is washed away from the peel with water forming an oil-water emulsion. It is important to maintain an excess of water to prevent the oil from being reabsorbed by the peel once it is released. A two stage centrifugation process is used to recover orange oil from the oil-water emulsion." (20)

After the oil becomes isolated, it must be chilled to sub-freezing temperatures for anywhere from a few days to a few weeks to solidify and remove the waxes present in the oil. Then, the final oil product can be stored slightly below room temperature in an inert gas atmosphere to prevent possible oxidation (14).

Hydrotropic solubilization is another method of extraction of flavor compounds. This is a process where hydrotropes, a shorter-chained type of surfactant, are used to selectively extract water soluble phytochemicals from natural products. The hydrotropes are very water soluble, but tend to lack the strong surface acting forces that cause surfactants to bubble and form micelles. Also, the shorter-chained hydrophobic portion of the molecule can be modified to selectively solubilize particular non-polar compounds. The size and properties of these hydrotropes allow for them to penetrate tough cell walls and preferentially extract certain molecules in the substrate matrix. This is a process similar to the surfactant assisted PHWE discussed earlier, only with more selective "surfactants" and a much lower temperature and pressure (around standard temperature and pressure versus 80-200 °C and 10-20 bar). In a recent report, this method was used to study the extraction of piperine from black peppercorns. The proposed theory of extraction is as follows: "The hydrotrope molecules probably adsorb on the cellulosic cell wall, disorganize its structure, and then penetrate into the cell membrane, assisting in
disordering the amphiphilic lipid bilayer and permeabilizing it to enable the easy release of piperine" (21).

**Liquid CO₂ Extraction**

The extraction method under investigation in this study is liquid CO₂ extraction. Unlike most other extraction methods, this method is intended to be a small scale operation. This method was initially conceived as a method for extraction in undergraduate laboratory experiments (22). In this experiment, a filter trap was made from a coiled copper wire with piece of filter paper. This was lowered into a 15 mL centrifuge tube and about 2.5 g finely grated orange zest placed on top of the filter. After being filled with dry ice and capped, the tube was placed into a graduated cylinder filled with hot tap water (40-45 °C) and allowed to undergo extraction. The schematics of this process are displayed in Figure 3. The entire extraction process occurs in less than five minutes.

Once the tube is lowered into the water bath, the dry ice quickly sublimes. This causes pressure to build inside the tube, allowing the gaseous carbon dioxide to condense and collect at the bottom of the tube. Once the liquid begins to form, the remaining ice rapidly melts. This all happens within a minute or two. Once the liquid has formed, it begins to boil and the gaseous carbon dioxide leaks from the top of the tube between the threading of the tube and cap. This “boiling and leaking process” lasts about five to ten
minutes on average and is accompanied with a hissing sound. Depending on the “tightness” of the cap, the liquid may boil away in as little as three minutes, or as long as twenty minutes. The filter trap holds back the food residue and allows the extracted compounds to be isolated at the bottom of the tube. The filter trap and remaining substrate can now be removed to reveal the isolate.

Liquid CO$_2$ vs. Supercritical CO$_2$

Carbon dioxide is a versatile extraction solvent as it can be used in either its liquid or supercritical forms. Both solvent phases require different conditions, and thus provide different extraction conditions which can be tailored to the specific needs of the system at hand. A study performed on essential oil extraction methods with carbon dioxide makes some distinctions on the uses of liquid and supercritical carbon dioxide:

“In food and pharmaceutical applications, carbon dioxide (CO$_2$) is by far the most used fluid since it meets most of the characteristics for an ideal processing fluid. Depending on the goal of the process, CO$_2$ can be used as a supercritical fluid (SCCO$_2$ or SLCO$_2$) or a subcritical liquid (LCO$_2$). In applications where yield and operating cost are more important than solvent selectivity, as in the case of vegetable oil extraction, SCCO$_2$ has been used. On the other hand, in deodorization and extraction of essential oils and aromas, the selectivity of the solvent is often more important than its solubilization power, and LCO$_2$ has been the preferred solvent. The extraction and/or fractionation of essential oils and oleoresins by using CO$_2$ has been investigated before, but most of the research activity in this area occurred in the last five years. At least one product (hops essence) is currently produced on a large scale by using SC fluid technology.” (23)

It has also been shown that liquid and supercritical carbon dioxide can be used to concentrate flavor compounds in essential oils, juices, and other foodstuffs (24,25). Carbon dioxide is being more frequently used in the citrus industry now than ever. The extraction of essential oils from lemon peel was performed with supercritical carbon
dioxide at a pressure of 30 MPa and a temperature of 40 °C. This produced a yield of 0.9% that was qualitatively consistent with cold pressed lemon oil (26).

Carbon dioxide and water are both simple, triatomic molecules with a variety of uses. Water is often held as “the universal solvent”, while carbon dioxide has also been shown to be a formidable solvent for extraction purposes. The phase diagrams for these two molecules are relatively similar in shape. These diagrams are compared in Figure 4.

![Phase Diagrams](image)

**Figure 4.** The comparison of the phase diagrams for (a) water and (b) carbon dioxide. These diagrams are shown for simplicity and are not to scale (27).

The main difference in these two phase diagrams is in the slope of the liquid-solid boundary. Carbon dioxide has a positive slope at this region, as most compounds do, implying that an increase in pressure is required to keep CO₂ a solid whenever it is heated above -56.4 °C. On the other hand, H₂O has a negative slope. This odd behavior is the
result of the decrease in density when water freezes. When a block of ice is pressurized, a strain is placed upon the hydrogen bonds holding the lattice together. This increase in strain will cause the ice to melt at lower temperatures. It can be seen that water can exist in all three phases at atmospheric pressure because its triple point falls well below standard pressure, at a mere 0.00603 atm. This is simply not the case with CO₂, as its triple point lies at 5.11 atm. Also, supercritical CO₂ is much more easily obtained than supercritical H₂O, with critical points of 31.1 °C / 73 atm and 374 °C / 218 atm, respectively.

Although it is unknown exactly what pressures are produced during the proposed liquid CO₂ method, it must be at least 5.11 atm. Due to observations and tube specifications, it is assumed that the temperature and pressure conditions of the liquid CO₂ extraction are near the triple point (-56.4 °C and 5.11 atm) (22). Pressures that are too much greater than this can cause the extraction tube to crack and even explode. This issue was not reported in the literature.

Significance of Project

The proposed extraction method has some distinct advantages over other commonly used extraction methods. Firstly, the extraction process is a green process, as it uses carbon dioxide as solvent, rather than potentially dangerous solvents, such as dichloromethane, hexane, toluene, or methanol. Secondly, the separation of the solvent from the extract is relatively simple, as the carbon dioxide spontaneously boils away, leaving behind a pure extract. This eliminates any steps involving a rotary evaporator, which may leave behind a trace amount of the solvent. Thirdly, the cost of an extraction
is significantly less expensive than a traditional extraction, as all that is needed is the plastic centrifuge tube, a wire, glass wool, and dry ice. A traditional extraction requires the initial investment of organic glassware, such as separatory funnels or Soxhlet extractors, as well as a continuous supply of fresh solvent. These extractions may require hundreds of milliliters of solvent for only a gram or less of isolate. All of the materials required for the proposed method of extraction are readily available and inexpensive compared to the materials and machinery required for a typical liquid or supercritical carbon dioxide extraction. Overall, this extraction process can demonstrate fundamental extraction techniques in the undergraduate laboratory.

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Chapter II: Qualitative Analysis

Sample Collection and Preparation

Convenience food samples were gathered from either grocery stores or hand-picked in the Indianapolis, IN area for analysis. These samples fall primarily into three categories: herbs, spices, and citrus zests. Depending on the sample type, the samples were prepared with a coffee grinder, a zester, or a knife. A Hamilton Beach Custom Grind™ coffee grinder (model # 80365) was used to grind dry herbs and seeds. It was found that grinding the samples for 12 seconds on the 4-cup espresso setting produced optimum extraction yields. Black peppercorns were ground for 36 seconds due to their composition. The citrus zests and fresh garlic were prepared with a fruit zester that contained circular grating slots of ~2 mm in diameter. This zester produces zests of ~1 cm in length. Fresh herbs were finely chopped into small square pieces, approximately 2 to 3 mm long, with a small knife. Only the leaves of the herbs were used. Vanilla bean pods were sliced lengthwise, the beans were scraped from the skin with a blade, and the pod was cut into sections approximately 3 to 5 mm wide. All of the pod portions were used as the sample. Samples, once prepared, were extracted immediately. The specific items tested and their preparation method is shown in Table 1.
Table 1. Summary of food substrates used and their prepared state.

<table>
<thead>
<tr>
<th>Substrate Category</th>
<th>Substrate</th>
<th>State of Substrate Tested</th>
<th>Preparation Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herbs</td>
<td>Oregano</td>
<td>Fresh (minced)</td>
<td>Knife</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dried/Fresh-frozen</td>
<td>Grinder</td>
</tr>
<tr>
<td>Rosemary</td>
<td>Fresh</td>
<td>Knife</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dried/Fresh-frozen</td>
<td></td>
<td>Grinder</td>
</tr>
<tr>
<td>Sage</td>
<td>Fresh</td>
<td>Knife</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dried/Fresh-frozen</td>
<td></td>
<td>Grinder</td>
</tr>
<tr>
<td>Spearmint</td>
<td>Fresh</td>
<td>Knife</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dried/Fresh-frozen</td>
<td></td>
<td>Grinder</td>
</tr>
<tr>
<td>Dill Weed</td>
<td>Dried</td>
<td>Grinder</td>
<td></td>
</tr>
<tr>
<td>Thyme</td>
<td>Dried</td>
<td>Grinder</td>
<td></td>
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<tr>
<td>Tea leaves (black)</td>
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<td></td>
</tr>
<tr>
<td>Tobacco leaves</td>
<td>Dried</td>
<td>Grinder</td>
<td></td>
</tr>
<tr>
<td>Spices</td>
<td>Black peppercorns</td>
<td>Whole/ Crushed/ Coarsely ground</td>
<td>Grinder</td>
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<td></td>
<td>Nutmeg</td>
<td>Coarsely ground</td>
<td>Grinder</td>
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<tr>
<td></td>
<td>Cloves</td>
<td>Coarsely ground</td>
<td>Grinder</td>
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<td>Caraway seeds</td>
<td>Whole/ Crushed/ Coarsely ground</td>
<td>Grinder</td>
</tr>
<tr>
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<td>Vanilla beans</td>
<td>Chunked/ Minced</td>
<td>Knife</td>
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<td>Sesame seeds</td>
<td>Coarsely ground</td>
<td>Grinder</td>
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<td>Safflower seeds</td>
<td>Coarsely ground</td>
<td>Grinder</td>
</tr>
<tr>
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<td>Chili peppers</td>
<td>Coarsely ground (dried)</td>
<td>Grinder</td>
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<td></td>
<td>Garlic (Fresh)</td>
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<td>Garlic (Freeze-dried)</td>
<td>Coarsely ground</td>
<td>Grinder</td>
</tr>
<tr>
<td></td>
<td>Cinnamon (cassia bark)</td>
<td>Coarsely ground</td>
<td>Grinder</td>
</tr>
<tr>
<td>Citrus Zests</td>
<td>Orange zest</td>
<td>Zested</td>
<td>Zester</td>
</tr>
<tr>
<td></td>
<td>Lemon zest</td>
<td>Zested</td>
<td>Zester</td>
</tr>
<tr>
<td></td>
<td>Lime zest</td>
<td>Zested</td>
<td>Zester</td>
</tr>
<tr>
<td></td>
<td>Grapefruit zest</td>
<td>Zested</td>
<td>Zester</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Coffee beans</td>
<td>Coarsely ground</td>
<td>Grinder</td>
</tr>
<tr>
<td></td>
<td>Instant coffee</td>
<td>Powdered</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Milk chocolate</td>
<td>Coarsely ground</td>
<td>Knife</td>
</tr>
<tr>
<td></td>
<td>Dark chocolate</td>
<td>Coarsely ground</td>
<td>Knife</td>
</tr>
</tbody>
</table>
Coarsely ground samples refer to a sample size approximating the appearance of instant coffee, while finely ground samples refer to sample grains the size of powdered sugar. Whole samples (seeds or leaves) generally did not produce an extract. This is probably due to the thick, rigid outer covering of the seeds and the thick membranes surrounding the leaf cells. It has been suggested that glycoproteins may be the primary inhibitor of solvent penetration in leaves (1). Glycoproteins are generally more abundant in leaf membranes than in seeds or fruit zests. It is possible that these glycoproteins bind tightly to the oils in the leaves, not allowing them to be easily removed through these extraction conditions. Raman and Gaikar discuss the cellular structure of black peppercorns and how this structure inhibits the extraction of piperine by hydrotropic solubilization (2). The thick cellulose walls in the pericarp, which are glucose polymers held tightly together by hydrogen bonding, may provide a thick barrier that shields the volatile compounds from the solvent.

Coarsely grinding the sample provided much better extraction results than no grinding at all. By grinding the sample, the outer membranes can be ruptured, readily exposing the volatile compounds to the extraction solvent. Grinding also greatly increases the surface area of the sample exposed to the solvent. Finely ground samples did not work. If the sample was ground into a powder, the fine particles tended to clog the filter trap, which prevented the liquid carbon dioxide from reaching the bottom of the tube. All dried herbs and spices (excluding vanilla beans) were coarsely ground for analysis.

Some herbs were able to be obtained in their fresh form: oregano, rosemary, sage, and spearmint. The fresh herbs were prepared by chopping them into small pieces. Fresh
herbs were also dried for three days to remove any interference that the residual water might have on the extraction process. Fresh herbs were also placed into 2 zip-top bags and frozen at a temperature of \(-18\, ^\circ\text{C}\) for approximately a week. These frozen herbs were then thawed and dried for three days. This process, producing “fresh-frozen” samples, ruptured the cellular structure of the leaves before the removal of residual moisture. The dried and fresh-frozen herbs were coarsely ground for 12 seconds in the grinder prior to being placed into the extraction apparatus.

The citrus fruits were zested with a small zester to remove the outermost tissue of the fruits. Prior to zesting, the skin of the fruit was wiped with hexane to remove any wax coating on the surface. If left intact, this wax coating would contaminate the isolate and interfere with the analysis. The vanilla beans were sliced lengthwise, and then chopped into small pieces to allow proper solvent penetration. The chocolate was also chopped into small pieces.

Dry ice was obtained from Pain, Inc. (Indianapolis, IN). After purchase, the dry ice was stored in a \(-60\, ^\circ\text{C}\) freezer until use. Care must be taken when handling dry ice to prevent water contamination of samples to the extent possible (removal of residual surface moisture).

Analytical Standards

Analytical standards were purchased from Sigma-Aldrich for qualitative and quantitative analysis purposes: limonene; carvone; menthol; and vanillin. Information concerning these standards is detailed in Table 2.
Table 2. Analytical standards purchased from Sigma-Aldrich.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Manufacturer</th>
<th>Grade</th>
<th>Catalog No.</th>
<th>Lot No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-(+)-Limonene</td>
<td>Fluka</td>
<td>≥ 99.0 %</td>
<td>62118</td>
<td>1360549</td>
</tr>
<tr>
<td>(+)-Carvone</td>
<td>Fluka</td>
<td>≥ 98.5 %</td>
<td>22070</td>
<td>1300739</td>
</tr>
<tr>
<td>L-Menthol</td>
<td>SAFC</td>
<td>99+ %</td>
<td>W266523</td>
<td>01304EH</td>
</tr>
<tr>
<td>Vanillin</td>
<td>SAFC</td>
<td>≥ 97 % (certified kosher)</td>
<td>W310727</td>
<td>17005DH</td>
</tr>
</tbody>
</table>

Extraction Apparatus

The method developed by McKensie, et al. was slightly altered to better suit this project (3). The main difference is the use of glass wool as a filter medium rather than filter paper. In our hands, filter paper was not porous enough to allow the liquid carbon dioxide to flow through. Many different filter options were tested, but did not provide sufficient solvent penetration: filter paper, kimwipe, tea bag filter, and cotton. A thin layer of glass wool provided sufficient solvent penetration while also allowing fine particulate matter, such as crushed herbs, to be studied. Extremely fine particulates, such as fine powders, are not suitable for this method because they clog the space between the strands of glass wool.

Glass wool was the only filter-like material that provided sufficient results for these studies. Care must be taken to achieve the desired results. If the layer of glass wool is too thick, then it loses its porosity and presents the same challenges as the other attempted filter materials. If the layer is too thin, then the gaps between fibers become too large and the substrate flows to the bottom of the tube. With practice, the proper amount of glass wool needed for an extraction can be consistently achieved. Approximately a 50 mg tuft of glass wool, or ~1 mm layer, is optimal.
Several different tubes were tested for their ability to contain and withstand the pressures necessary for an extraction. Essentially, two different brands (Corning and BD Falcon), three different tube plastics (polyethylene terephalate, polypropylene, and high density polyethylene), two different cap types (plug seal and centristar), and two different tube sizes were tested (15 mL and 50 mL). Table 3 shows the different tubes tested and their successes/failures.

Table 3. Various centrifuge tubes tested and their applicability as extraction tubes.

<table>
<thead>
<tr>
<th>Brand</th>
<th>Cat. No.</th>
<th>Size</th>
<th>Tube Comp.</th>
<th>Cap type</th>
<th>Success/Failed</th>
<th>Max RCF** (x g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corning</td>
<td>430791</td>
<td>15 mL</td>
<td>PP</td>
<td>Centristar</td>
<td>Limited success; does not seal easily</td>
<td>15,500</td>
</tr>
<tr>
<td>Corning</td>
<td>430053</td>
<td>15 mL</td>
<td>PET</td>
<td>Plug Seal</td>
<td>Failed: tube cracks after extraction</td>
<td>3,600</td>
</tr>
<tr>
<td>Corning</td>
<td>N/A**</td>
<td>15 mL</td>
<td>PP</td>
<td>Plug Seal</td>
<td>Success</td>
<td>15,500</td>
</tr>
<tr>
<td>Corning</td>
<td>N/A**</td>
<td>15 mL</td>
<td>PET</td>
<td>Centristar</td>
<td>Failed: tube cracks after extraction</td>
<td>3,600</td>
</tr>
<tr>
<td>BD Falcon</td>
<td>918-PG***</td>
<td>15 mL</td>
<td>PP</td>
<td>Dome-Seal</td>
<td>Limited success: tends to explode at a lower pressure threshold</td>
<td>8,400</td>
</tr>
<tr>
<td>BD Falcon</td>
<td>518-PG***</td>
<td>15 mL</td>
<td>PP</td>
<td>Screw Cap</td>
<td>Limited success; does not seal easily</td>
<td>12,000</td>
</tr>
<tr>
<td>Corning</td>
<td>430829</td>
<td>50 mL</td>
<td>PP</td>
<td>Centristar</td>
<td>Failed: cannot provide a sufficient seal</td>
<td>3,600</td>
</tr>
<tr>
<td>Nalgene</td>
<td>3119</td>
<td>15 mL</td>
<td>HDPE</td>
<td>Screw Cap</td>
<td>Failed: contains pressure, but opening is too narrow</td>
<td>50,000</td>
</tr>
</tbody>
</table>

*Max RCF refers to the maximum capable Relative Centrifugal Force of the centrifuge tube
**The caps were switched on the Corning # 430791 and Corning # 430053 to generate these two tube combinations
***Catalog numbers obtained from Dot Scientific Inc.

A Corning 50 mL PP was tested because it had the potential to hold more sample and produce more extract. This was desirable, as larger amounts of extract are easier to quantitate than smaller amounts. Several attempts were made, but the tube simply could not hold enough pressure before it began to leak. Parafilm and Teflon tape were used to try and seal this leak, but these attempts were unsuccessful.
A Nalgene HDPE centrifuge tube was tested for its extraction potential, but its opening was too small relative to the body of the tube. This smaller opening limited the function of the filter trap because the trap had to be made small enough to fit into the tube. The bottom of the tube was spherical and not conical, not leaving much space for the extract to reside after the extraction. The HDPE composition of the tube did allow it to maintain the proper pressure containment needed to produce liquid carbon dioxide.

Overall, the Corning 15 mL PP centrifuge tube with the plug-seal cap and the BD Falcon 15 mL PP centrifuge tube with the plug-seal cap provided the optimal conditions to produce liquid carbon dioxide. The BD Falcon tubes were selected for most of the extractions because of their greatly reduced cost compared to their Corning alternative. It was later discovered that the BD Falcon tubes had a lower tolerance for pressure than the Corning tubes. Occasionally, a BD Falcon tube would explode under the pressure produced by the rapidly subliming dry ice. The safety precautions taken are described below.

If time and resources were permitted, a customized extraction device could be built to optimize the conditions necessary for successful extractions of these substrates. Perhaps a custom built filter, similar to the stainless steal filters used in a coffee espresso maker, could be fashioned. This would eliminate many complications, but would also detract from the low cost and ease of availability of the extraction device in the first place.
Safety Precautions

Throughout this project, there have been several incidents where a centrifuge tube would explode during the extraction process. This was experienced with only the BD Falcon 15 mL PP centrifuge tubes with the plug-seal caps. Because these explosions did not occur until sometime after the tube-selection process, it is unknown whether any other centrifuge tubes might experience this same problem. It was initially assumed that the explosions were the result of “bad” batches of dry ice, but other factors may be the cause. It is possible that the BD Falcon tubes do not have the same pressure capabilities as the Corning tubes.

A secondary container was used to contain any possible explosions. A large, steel desiccator was used for the secondary containment. A smaller metal desiccator was placed inside and used as the water bath. In addition to this, the extractions were performed in a fume hood with the safety doors closed.

Extraction Procedure

First, a new extraction tube was selected and weighed. Next, the filter trap was prepared by taking a copper wire and coiling one end of it so that there are at least two full coils. The wire is bent so that the coils can be easily lowered into and pulled out of the tube. The wire gauge was thick enough to easily hold its shape, but thin enough to be easily modified. A small tuft of glass wool (~ 50 mg) is placed between the two coils, where it is held securely. This filter trap can now be placed into the extraction tube.

Next, the sample is prepared and immediately placed into the tube, on top of the filter trap. The dry ice is crushed and placed on top of the sample, filling the rest of the
tube. This is diagramed in Figure 1. Then, the tube is immediately capped and placed into a 50 °C water bath inside the secondary container. The lid of the secondary container is locked in place, but with a small opening allowing for air pressure equilibration in the event of an explosion. The fume hood doors are closed and the apparatus is left to extract for at least 20 minutes.

After the extraction, the tube is removed from the water bath and the sample and filter trap are removed from the tube. Further extractions may be necessary if very little extract is generated. The extracted residue at the bottom of the tube is dissolved in ~1 mL of dichloromethane and placed into a GC sample vial. The extract should be removed from the tube relatively quickly, as the isolated oils tend to leech the plasticizers from the tube walls. Care should also be taken with dichloromethane, as it quickly leeches plasticizers as well. Approximately 1 μL of this solution is injected into a Varian GC/MS for analysis. All observed compounds eluted from the column within ten minutes.

A GC/MS was the chosen instrument of analysis because of the volatility of most of the isolates and for the ease of identification by mass spectroscopy. This was the most commonly used instrument in the literature in the analysis of essential oil extracts.
Instrument Specifications

The Varian CP-3800 GC was equipped with an AT-5ms capillary column (30 m × 0.32 mm × 1.00 μm film thickness) with a stationary phase consisting of 5% phenyl and 95% dimethylpolysiloxane (Alltech Associates, Inc., USA). The oven temperature was programmed with an initial temperature of 50°C held for 1 min, followed by an increase of 20 °C/min to 260°C, and then held for the remainder of the analysis. The injector temperature was held constant at 260°C. Helium was used as a carrier gas at a constant column flow rate of 1.0 mL/min. The GC was also equipped with a Saturn 2000 MS/MS ion-trap mass spectrometer with a mass range of 35-550 (m/z).

Results and Discussion

Samples Analyzed with GC/MS

The following samples produced isolates that were analyzable by GC/MS: oregano; sage; rosemary; spearmint; black peppercorns; nutmeg; cloves; caraway seeds; vanilla beans; orange zest; lemon zest; lime zest; and grapefruit zests. These samples contained a significant amount of terpenes and terpenoids, which were the primary class of compounds detected with this method. Table 4 summarizes the primary components extracted from these food samples.
Table 4. Primary compounds extracted from food items

<table>
<thead>
<tr>
<th>Food Source</th>
<th>Major Components of Extract</th>
<th>Literature Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oregano</td>
<td>thymol, β-caryophyllene</td>
<td>4, 5, 6</td>
</tr>
<tr>
<td>Rosemary</td>
<td>camphene, 1,8-cineole, camphor, β-caryophyllene</td>
<td>4, 5</td>
</tr>
<tr>
<td>Sage</td>
<td>β-caryophyllene, α-humulene</td>
<td>4, 5</td>
</tr>
<tr>
<td>Spearmint</td>
<td>carvone, germacrene D</td>
<td>7, 8</td>
</tr>
<tr>
<td>Nutmeg</td>
<td>α-pinene, sabinene, β-pinene</td>
<td>9, 10</td>
</tr>
<tr>
<td>Black peppercorns</td>
<td>β-caryophyllene</td>
<td>11, 12, 13, 14</td>
</tr>
<tr>
<td>Cloves</td>
<td>eugenol, β-caryophyllene</td>
<td>15</td>
</tr>
<tr>
<td>Caraway seeds</td>
<td>limonene, carvone</td>
<td>16</td>
</tr>
<tr>
<td>Vanilla beans</td>
<td>vanillin</td>
<td>17</td>
</tr>
<tr>
<td>Orange zest</td>
<td>limonene</td>
<td>18</td>
</tr>
<tr>
<td>Lemon zest</td>
<td>β-pinene, limonene, γ-terpinene</td>
<td>18</td>
</tr>
<tr>
<td>Lime zest</td>
<td>β-pinene, limonene, γ-terpinene</td>
<td>18</td>
</tr>
<tr>
<td>Grapefruit zest</td>
<td>limonene</td>
<td>18</td>
</tr>
</tbody>
</table>
The structures for the compounds presented in Table 4 are displayed in Figure 2.

![Chemical structures](image)

**Figure 2.** Structures of major detected compounds

When analyzed by GC/MS, some isolates produced simple chromatograms, while others were relatively complex, showing 20 or more peaks. For instance, caraway seed, vanilla bean, and orange zest extracts were relatively simple, containing only a few compounds. It should be noted, however, that minor trace compounds consisting of approximately < 0.5% of the extract (as determined by signal response to the detector of the GC/MS) were disregarded to simplify the qualitative analysis. Some sample extracts, such as black peppercorns, fresh rosemary, and lime zest extracts, were very complex and contained a large number of compounds. Chromatograms for caraway seeds and black peppercorns are compared in Figure 3 to demonstrate this.
The two peaks in Figure 3a represent limonene (RT = 4.84 min) and carvone (RT = 6.56 min), respectively. The major peak in Figure 3b represents β-caryophyllene (RT = 7.83 min). Appendix A displays the chromatograms obtained for these samples and Appendix B displays the mass spectrums for the major compounds. There is also a table listing the qualitatively determined compounds, their retention times, and in which sample extracts they were detected.

There were some liquid CO₂ extracts that had a composition that was very similar to what was found in the literature. For instance, the GC chromatogram obtained for the black peppercorn extract matches very well with data from Pino/Rodriguez-Feo et al. (11). This comparison is made in Figure 4. The black peppercorn oil analyzed in the
literature was obtained through steam distillation, which generated an extract yield of 2.8 % (v/w). The retention times in reported by Pino/Rodriguez-Feo et al. (Figure 4a) are about 3.5 times longer than what was observed with our method (Figure 4b). This is probably because a temperature gradient of 4 °C/min was used on a column with a flow rate of 0.6 mL/min to obtain the data in Figure 4a, while a temperature gradient of 20 °C/min was used on a column with a flow rate of 1 mL/min to obtain the data in Figure 4b. A lower temperature gradient and gas flow rate will increase retention time.

Figure 4. GC comparison of black peppercorn extracts from (a) Pino/Rodriguez-Feo et al. and (b) our liquid CO₂ method
Poor Substrates for Our Study

The following food samples produced isolates that were unable to be analyzed by GC/MS: sesame seeds; safflower seeds; dried chili peppers; fresh garlic; coffee beans; and cinnamon sticks (cassia bark). It is assumed that the isolate primarily contained non-volatile oils, which never eluted from the GC column.

The sesame and safflower seeds contain fatty acids and their esters, such as linoleic acid and various triglycerides. These compounds were probably part of the extracted residue, but they are non-volatile and probably have molecular weights above the mass spectrum range. Capsaicin may have been extracted from the chili peppers, but it degrades before its vaporization point. Sulfur containing compounds, such as allicin and diallyl sulfide, are present in garlic. Allicin also easily degrades before it boils. Diallyl sulfide has a boiling point of 138 °C, but apparently was not isolated. It was expected that caffeine and theobromine could be extracted from coffee beans, but tannins were probably the resulting isolates. The molecular weights of these minor peaks extend near the 550 m/z upper limit. There was a large amount of noise present in the coffee extract chromatogram. Cinnamon bark contains many volatile organic compounds, such as cinnamaldehyde, cinnamyl alcohol, and cinnamate esters. Instead, various tannins inside of the bark may have been the resulting extract. The volatile compounds were probably not observed because of the bark matrix and may have been inhibited by glycoproteins. The bark may have to be more finely ground to extract these volatile compounds.
Samples Producing No Extracted Isolates

The following samples did not generate any isolate when subjected to liquid carbon dioxide extraction: dried tobacco leaves; dried black tea leaves; crushed mint leaves; dry dill weed; dry thyme; dried garlic; instant coffee; and chocolate (both milk and dark). It is assumed that either there were simply very little terpenes and terpenoids present in the samples, or that the sample matrix interfered with the extraction. The herbs may have lost these volatile compounds during their drying and storage process. The tobacco, tea leaves, coffee beans, and chocolate were chosen because of the alkaloids present, nicotine, caffeine, and theobromine. These structures are displayed in Figure 5.

![Figure 5. Structures of nicotine, caffeine, and theobromine](image)

As it is known that caffeine can be removed from coffee beans by supercritical carbon dioxide (19), it would seem that our extraction method does not achieve the temperature and pressure required for this extraction. Our method also does not appear to be effective for the extraction of any alkaloid.

Comparison with Soxhlet Extractions

Soxhlet extractions with dichloromethane were performed on orange zest, caraway seeds, and vanilla beans to determine if these two extraction methods are significantly different in the types of compounds they extract. A medium sized Soxhlet
extraction was performed with a 45/50 Soxhlet extractor. The samples were prepared as described for a liquid CO₂ extraction. The prepared samples were wrapped inside of a kimwipe and tied into a pouch with cotton thread. All extractions were performed with 150 mL of dichloromethane and were allowed to run for 18-24 hours. After extraction, the solution was gravity filtered and the majority of the dichloromethane was removed with a rotary evaporator in a 40 °C water bath. The major compounds in the extracts of both extraction methods are: limonene and carvone in caraway seeds; limonene in orange zest; and vanillin in vanilla beans. These compounds consisted of at least 98 % of the total extract from both extraction methods. Figure 6 compares the chromatograms for both extraction methods of caraway seeds; Figure 7 for vanilla beans; and Figure 8 for orange zest.

![Figure 6. (a) CO₂ and (b) Soxhlet extract chromatogram comparisons of caraway seeds](image-url)
Figure 7. (a) CO$_2$ and (b) Soxhlet extract chromatogram comparisons of vanilla beans

Figure 8. (a) CO$_2$ and (b) Soxhlet extract chromatogram comparisons of orange zest
It should be noted that the retention times of the Soxhlet chromatograms have shifted relative to the CO\textsubscript{2} chromatograms. Several months have passed between the acquisition of these two data sets. Some unaccounted instrumental tampering (software method modification, column change, etc.) may have been done during this time period to account for this shift. The peaks in each chromatogram have been match via GC to match the compounds despite their differing retention times.

Qualitatively, the caraway isolates were very similar for both extraction methods. This shows the relative affinity for the extraction of the volatile compounds between these two methods. The Soxhlet orange zest and vanilla bean extracts contained a few more minor compounds than in the CO\textsubscript{2} extracts. This may indicate that the Soxhlet method is more thorough with the extraction of volatile compounds than our method. On the other hand, it may indicate that our method is more selective, extracting only a few, particular compounds.

The Soxhlet extractions appeared to yield several times more isolate than our method. Qualitative analysis was not done with the Soxhlet extractions, but a crude weight was measured. Not all of the solvent was able to be removed from the Soxhlet extracts. This contaminates the extract, causing the extract yield to appear higher than it really is. For instance, it was shown that about 2 g of orange zest generates approximately 0.5 g of extract with the Soxhlet method, while this same amount generates approximately 20 mg of extract (in one extraction) with our liquid CO\textsubscript{2} method.

It is possible that the extract yields of a Soxhlet extraction can be achieved with multiple consecutive extractions of this substrate with our method. This is probably a factor of the substrate involved, as some substrates are much easier to extract from than others with
our method. It appears that our method may be more selective, but Soxhlet extraction may be more complete.

It should also be noted that one extraction with our method takes approximately 20 minutes, while the Soxhlet extraction requires about 18-24 hours. Our method is able to cut the extraction time significantly without the interference of potentially harmful solvents.

**Future Qualitative Work**

In the future, LC/MS instrumentation would like to be used as a means to determine the composition of the non-volatile extracts. Also, this instrument would be useful in detecting any non-volatile compounds present in the extracts that contained many volatile compounds. It is possible that terpene rearrangements may occur due to the high temperature of the gas phase of the GC during analysis. Using the LC could help to eliminate this problem. This may not be a major issue, as the compounds were identified through mass spectrometry. It appears that any rearrangements that may have occurred would have most likely been internal rearrangements, which would do little to affect analysis.

Freeze-drying may be another option of sample preparation for fresh herbs. This would be another effective way to remove residual moisture from the samples without losing volatile compounds. This may prove to be better method of drying samples prior to grinding and extraction. Some fresh herbs, such as oregano, rosemary, sage, and spearmint, should be freeze-dried prior to analysis to test the viability of this sample preparation method.
References

1. Villani, Philip; Associate Professor at Butler University, personal communication.


Chapter III: Quantitative Analysis

Experimental

Liquid carbon dioxide extractions that were performed for quantitative purposes were performed identically to the qualitative extractions. The extracts were dissolved in acetonitrile rather than dichloromethane. For multiple extractions on a single sample, the sample and filter trap were transferred to a new extraction tube for each consecutive extraction.

An Agilent GC-FID was used for quantitative analysis rather than the GC/MS. This was done because the GC-FID provided better software capabilities for integration than the GC/MS. The GC-FID software used is Agilent's GC ChemStation (Rev. A.09.01). An LC/MS/MS would have ultimately been the optimum choice, but time constraints and instrument availability proved this option unfeasible at this time. Using an LC/MS/MS would allow quantitation through the daughter ions as well as the parent ions, potentially allowing for better specificity. LC/MS/MS quantitation is desired for future work.

Instrumental Specifications

The Agilent 6890N GC was equipped with a flame ionization detector and an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm film thickness) with a stationary phase consisting of 5% phenyl methyl siloxane (Agilent Technologies, Inc.). The oven temperature was programmed with an initial temperature of 60 °C held for 3 min, followed by an increase of 20 °C/min to 250 °C, and then held for the remainder of the
analysis. The injector temperature was held constant at 250 °C. Helium was used as a carrier gas at a constant column flow rate of 1.7 mL/min at a pressure of 8.67 psi.

*Calibration Solutions*

Calibration solutions were made for the analytes limonene, carvone, and vanillin with menthol as the internal standard. Seven solutions were made with the analyte concentrations 10, 20, 50, 100, 200, 500, and 1000 ppm. These solutions each contained 54.7 ppm menthol as an internal standard. Acetonitrile was used as the solution solvent. The concentrations were measured, but the calculated concentrations are used in the data. The retention times for each analyte are compared for the GC/MS and GC-FID in Table 1. The calibration curves for these analytes are displayed in Figure 1, with the y-axis being a peak area response factor with respect to the menthol internal standard. The regression data is shown in Table 2. Internal standardization was used as the method of calibration (1).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>GC/MS</th>
<th>GC-FID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limonene</td>
<td>4.83</td>
<td>6.78</td>
</tr>
<tr>
<td>Menthol</td>
<td>6.04</td>
<td>8.38</td>
</tr>
<tr>
<td>Carvone</td>
<td>6.55</td>
<td>9.05</td>
</tr>
<tr>
<td>Vanillin</td>
<td>7.70</td>
<td>10.36</td>
</tr>
</tbody>
</table>
Figure 1. Calibration curves for limonene, carvone, and vanillin

Table 2. Regression data and figures of merit

<table>
<thead>
<tr>
<th>Regression Equation</th>
<th>Correlation Coefficient ($R^2$)</th>
<th>Instrumental Precision (%RSD)</th>
<th>Calibrated Range (ppm)</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limonene $y = 0.0313x + 0.1300$</td>
<td>0.9994</td>
<td>6.642 %</td>
<td>7.79 – 817.9</td>
<td>6.779 ± 0.001</td>
</tr>
<tr>
<td>Carvone $y = 0.0277x + 0.0845$</td>
<td>0.9978</td>
<td>6.757 %</td>
<td>8.86 – 930.2</td>
<td>9.050 ± 0.002</td>
</tr>
<tr>
<td>Vanillin $y = 0.0181x + 0.1433$</td>
<td>0.9956</td>
<td>5.524 %</td>
<td>9.35 – 981.9</td>
<td>10.357 ± 0.002</td>
</tr>
</tbody>
</table>

The instrumental precision was obtained by injecting the 100 ppm calibration solution five times. The values shown are the percent relative standard deviation of the instrumental response of each analyte in the solution.

Extraction Data

Extractions were carried out in triplicate for caraway seeds and orange zest, and vanilla beans. The data show that $1.65 \pm 1.02$ mg limonene and $3.79 \pm 2.26$ mg carvone
are extracted from caraway seeds from one extraction with our method (5.44 ± 3.28 mg total). The data also show that 21.98 ± 4.34 mg limonene is extracted from 2.0 g orange zest from one extraction with our method. This is nearly a 1.1 % (w/w) extraction yield. Approximately 240 mg of total extract was isolated from 2.6 g orange zest during a Soxhlet extraction. This is a 9.2 % extraction yield. There probably is some residual dichloromethane and water left in the extract after the rotary evaporatory process; so the actual yield is probably less. Results are inconclusive at this time for the quantitation of the extraction of vanillin from vanilla beans.

Five consecutive extractions were carried out in triplicate for 2.00 g caraway seeds. Table 3 displays the numerical data, while Figure 2 displays this data graphically.

Table 3. Masses for consecutive caraway seed extractions (in mg)

<table>
<thead>
<tr>
<th>Extraction No.</th>
<th>Limonene</th>
<th>Carvone</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>1.65 ± 1.02</td>
<td>3.79 ± 2.26</td>
<td>5.44 ± 3.28</td>
</tr>
<tr>
<td>2nd</td>
<td>0.30 ± 0.13</td>
<td>1.13 ± 0.44</td>
<td>1.43 ± 0.58</td>
</tr>
<tr>
<td>3rd</td>
<td>0.33 ± 0.28</td>
<td>1.48 ± 1.04</td>
<td>1.80 ± 1.32</td>
</tr>
<tr>
<td>4th*</td>
<td>0.06 ± 0.03</td>
<td>0.39 ± 0.24</td>
<td>0.44 ± 0.26</td>
</tr>
<tr>
<td>5th**</td>
<td>0.09</td>
<td>0.06</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*Extraction performed in duplicate
**Only single extraction performed
The extractions are somewhat consistent based on the data obtained. Although only two replicates have been performed successfully, the data is ultimately incomplete. More replicates are needed to justify claims of accuracy, precision, and replicability. It would be helpful if a method could be developed to standardize the thickness and porosity of the glass wool layer in the filter trap. This may lead to more consistent extractions which could be optimized for maximum yields.

The Caraway #3 sample data is the most consistent with what was generally expected to be seen for a typical series of extractions. This trend is also present with the Caraway #2 sample data. It can be seen that the data sets for samples #2 and #3 are fairly similar. It also can be seen that very little extract is generated after the fourth extraction, and very little more would probably be produced with subsequent extractions.
If the mass of the isolates are summed for each consecutive extraction, the total isolate masses can be calculated for each extraction. This data is tabulated in Table 4, and is displayed graphically in Figure 3.

Table 4. Summed isolate masses for consecutive caraway extractions (in mg)

<table>
<thead>
<tr>
<th>Extraction No.</th>
<th>Limonene</th>
<th>Carvone</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>1.65 ± 1.02</td>
<td>3.79 ± 2.26</td>
<td>5.44 ± 2.48</td>
</tr>
<tr>
<td>2nd</td>
<td>1.95 ± 1.11</td>
<td>4.92 ± 2.59</td>
<td>6.87 ± 2.82</td>
</tr>
<tr>
<td>3rd</td>
<td>2.28 ± 0.83</td>
<td>6.40 ± 1.55</td>
<td>8.68 ± 1.76</td>
</tr>
<tr>
<td>4th*</td>
<td>2.81 ± 0.21</td>
<td>7.68 ± 0.38</td>
<td>10.48 ± 0.44</td>
</tr>
<tr>
<td>5th**</td>
<td>3.05</td>
<td>8.00</td>
<td>11.05</td>
</tr>
</tbody>
</table>

*Extraction performed in duplicate
**Single extraction performed

As was seen in Figure 2, little extract seems to be generated beyond the forth extraction. Figure 3 shows that the Caraway # 2 sample approaches ~10 mg extract (~0.50 % w/w yield), while the Caraway # 3 sample approaches ~11 mg extract (0.55 % w/w yield) on
the fourth consecutive extraction. Approximately 190 mg of total extract was isolated from 2.00 g caraway seeds during a Soxhlet extraction. This is a 9.5 % extraction yield. As with the orange zest, there probably is some residual dichloromethane and water left in the extract after the rotary evaporatory process; so the actual yield is probably less.

**Quantitation Issues**

Several issues arose which made quantitation difficult. Mainly, the isolates tend to adhere to the glass wool, not fully collecting at the bottom of the tube. This appears to be less of an issue when a smaller amount of glass wool is used. The amount of glass wool that is used (both the thickness and the volume) for each sample is different. This affects the amount of extract collected for analysis. If a different and more uniform type of filter material can be used successfully, perhaps this issue can be avoided.

The tightness of the cap on the tube may also affect quantitation. The tighter the cap is, the longer the CO₂ remains in the liquid state and is available as in extraction solvent. If the cap can be standardized to a particular “tightness” (and thus a particular extraction time), the extraction procedure can be made more consistent. Also, tube explosions impede safety and convenience, especially during consistent extractions.

**Future Quantitative Work**

The extraction efficiency of the method needs to be evaluated. If a known amount of a terpene or terpenoid can be spiked into a sample substrate prior to extraction, then the recovered amount of the original spike can be determined. This can be studied for a variety of samples, as well as for consecutive extractions. This can be used as a
means to determine how many extractions are necessary to recover 95% of the original spike. The only issue with this method of analysis is the loss of the sample matrix effect. Spiking the sample causes the analyte to lie on the surface of the substrate, rather than within the substrate cells. In a typical extraction, the compounds are isolated by extracting the compounds from within the substrate cells. No standard reference materials have been found for terpenes or terpenoids in herbs or spices.

More extractions will also be necessary to validate the data. Consecutive extractions in triplicate for orange zest and vanilla beans will also be necessary. This study can also be expanded to include a larger variety of sample substrates.

**Conclusions**

The data seems to show that the extractions are not very consistent and generally do not generate much extract yield. It must be noted, however, that the data generated in Table 3 and Table 4 (and the resulting data in Figure 2 and Figure 3) is still preliminary. More extractions are needed to justify claims of accuracy, precision, and reproducibility. The evaluation of the extraction efficiency of our method will greatly help with making these justifications.

**Overall Conclusions**

As was shown in Chapter II, this method is very selective towards terpenes and terpenoids from a variety of different food items. In some cases, such as orange zest, caraway seeds, and vanilla beans, only a few compounds were extracted. In most cases, however, only terpenes and terpenoids were observed as extraction products.
The effectiveness of liquid carbon dioxide extraction is dependent on substrate choice. Seeds, fruit zests, nuts, and beans work well and generate significant amounts of extract. Dry leaves do not appear to be good substrates for this method, as they consistently generate little to no residue. Leaves in general did not seem to have as successful extractions as some of the other substrates. In every case, the ground substrate generated more extract than the whole substrate. Finely ground (powdered) substrate tends to clog the filter, not allowing for the compounds to be isolated at the bottom of the tube.

There are a few drawbacks to our method when compared to existing extraction methods. For instance, smaller volumes have to be used because of the size of the extraction chamber. This is not necessarily a drawback, as micro-extractions are desired in some settings. Also, a more complete extraction requires sequential loading of carbon dioxide. This is necessary to obtain the optimum extraction yield with our method. It has been shown that some brands of centrifuge tubes have a tendency to explode under the pressures generated. Care must be taken whenever these extractions are performed.

There are also several benefits to our method compared to other extraction methods. Our method takes considerably less time than many other extraction methods. For instance, Soxhlet extractions require about 18-24 hours, while our liquid CO₂ method requires only about 20 minutes for an extraction. The apparatus is easily made from inexpensive and common laboratory materials. Our method generates extracts similar to other methods. This has been demonstrated particularly for orange zest, caraway seeds, and vanilla beans with the Soxhlet extraction method. Our method tends to selectively extract terpenes and terpenoids. Other compounds, such as alkaloids, seem to be
neglected in the extraction process. Other non-volatile compounds that may have been extracted were not analyzable with the GC/MS. An LC/MS analysis may be useful in the analysis of the non-volatile compounds. There is no need of potentially harmful solvents for our extraction that must be removed. This is overall a very unique and useful approach to the extraction of essential oils from herbs and spices.

References

Appendix A

This appendix displays the GC chromatograms of the volatile extracts obtained with our liquid carbon dioxide method. All chromatograms were generated on the Varian GC/MS.

Figure 1. Fresh oregano extract.

Figure 2. Fresh rosemary extract.
Figure 3. Fresh sage extract.

Figure 4. Fresh spearmint extract.
Figure 5. Nutmeg extract.

Figure 6. Black peppercorn extract.
Figure 7. Clove extract.

Figure 8. Caraway seed extract.
Figure 9. Vanilla bean extract.

Figure 10. Orange zest extract.

Figure 11. Lemon zest extract.
Figure 12. Lime zest extract.

Figure 13. Red grapefruit zest extract.
Appendix B

This Appendix displays the mass spectra of the major compounds discussed in Table 4 of chapter II (p. 32). All mass spectra were generated on the Varian GC/MS.

Figure 1. Mass spectrum of α-pinene.

Figure 2. Mass spectrum of camphene.

Figure 3. Mass spectrum of sabinene.
Figure 4. Mass spectrum of β-pinene.

Figure 5. Mass spectrum of limonene.

Figure 6. Mass spectrum of 1,8-cineole.

Figure 7. Mass spectrum of γ-terpinene.
Figure 8. Mass spectrum of camphor.

Figure 9. Mass spectrum of carvone.

Figure 10. Mass spectrum of thymol.

Figure 11. Mass spectrum of eugenol.
Figure 12. Mass spectrum of vanillin.

Figure 13. Mass spectrum of β-caryophyllene.

Figure 14. Mass spectrum of α-humulene.

Figure 15. Mass spectrum of germacrene D.
Appendix C: Full Table of Detected Compounds

This appendix displays a table detailing all identified compounds within the volatile extracts.