THE DETERMINATION OF THREE TERPENES IN ROSEMARY BY GC, USING TWO EXTRACTION METHODS, MICROWAVE ASSISTED EXTRACTION AND ULTRASONIC EXTRACTION, WITH ISO OCTANE AS SOLVENT.

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Abstract

Three different terpenes, alpha-pinene, camphor and borneol, were extracted and quantified using gas chromatography with flame ionization detector (GC-FID) from the plant Rosemary. The Extraction procedure was done using two different methods, microwave assisted extraction (MAE) and ultrasonic treatment, with iso octane as solvent. Microwave assisted extraction procedure was developed and optimized, to get optimal extraction of the three compounds from Rosemary. The optimal operation conditions were found to be 120°C, for 15min and with rosemary to solvent ratio of 1:40. Carvon was used as internal standard.

The ultrasonic treatment, which is considered the standard procedure, was used as a reference to compare the results from microwave assisted extraction. When comparing the two methods with two-way ANOVA, it was shown that the interaction was significant. Meaning, that the three terpenes behaved differently depending on the extraction method. The highest extracted concentration with MAE was given by alpha-pinene followed by camphor. For the ultrasonic extraction the highest extracted concentration was from camphor, followed by alpha-pinene. Borneol was hard to evaluate due to resolution problems.

Supervisor: Jean Pettersson
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1 Abbreviations

ANOVA - analyses of variance
GC-FID - Gas chromatography coupled with flame ionization detector
MAE - microwave assisted extraction
Rs - resolution
RSD - relative standard deviation
2 Introduction

To extract different compounds from plants, there is a wide variety of different extraction methods. The best known and most widely used extraction methods include heat reflux extraction [1][2], ultrasonic extraction [2] and Soxhlet extraction [2], to name a few. Even though all of these methods work rather well, they do have drawbacks. Microwave assisted extraction (MAE) is therefore studied to potentially reduce the total time of the extraction, improve the amount of analyte extracted and to reduce the amount of solvent used. Because MAE uses on average less solvent than most other extraction methods, it is considered to be the more environmentally friendly alternative. [1][2][3]

Even though MAE is a known extraction method, it can still be relatively difficult to find information about closed system MAE, when extracting compounds from plants. According to literature, it is more common to use MAE with a condensing tube [2], or a reflux tube [2]. But because the analytes investigated in this experiment (and the solvent) are volatile, closed system MAE was preferred for safety reasons. The closed system also minimizes the risk for losses of analyte due to volatility. [2]

The aim of this study is to develop and optimize a procedure for microwave assisted extraction for three terpenes (alpha-pinene, camphor, borneol) from rosemary and to compare the results with the results from ultrasonic extraction. The results are analyzed using gas chromatography with flame ionization detector (GC-FID) and the data is compared using two-way analyses of variance (ANOVA).
3 Theory

3.1 Sample

3.1.1 Rosemary

Rosemary belongs to the family of Lamiaceae, of the genus, Rosmarinus. Rosemary’s botanical name is Rosmarinus officinalis. This plant originated from the Mediterranean region, but is now commonly used all over the world. Rosemary is commonly used in cooking. Rosemary also contains relatively high amount of iron. The oils contained in rosemary, are known to have beneficial effect for the health. The oil distilled from Rosemary is known to have antibacterial, antifungal, insecticide, antioxidant and hepatoprotective properties. [4][5]

3.1.2 The three terpenes (alpha-pinen, camphor and borneol)

The three compounds found in Rosemary that are studied in this paper are alpha-pinen, camphor and borneol, and they can be seen in Figure 1. These three terpenes help to make up the essential oils in rosemary. Pinen and camphene are dicyclic terpenes, their oxygen containing derivatives are camphor and borneol. Pinen and borneol are found also in the oils of multiple other plants, such as pine and lavender for example. Camphor is the oxidized form of borneol and it too can be found in many essential oils. All of these three compounds are volatile. [6]

Figure 1; The structures of borneol, camphor and alpha-pinen.
3.2 Methods

3.2.1 Microwave assisted extraction (MAE)

Microwave assisted extraction, or MAE, is in most cases a fast extraction method for extracting organic compounds from complex sample matrixes, especially from plants. Closed system microwave assisted extraction is used in this case, because of the volatility of the used solvent and the extracted terpenes. The closed system decreases the hazard risk. [1][2][3]

Microwave assisted extraction works by causing changes in cells with electromagnetic waves. This promotes active diffusion of the solvent through the cell walls, this is what makes the extraction process work. The solvent diffuses into the sample cells, where the analytes are solubilized by the solvent and then the solute diffuses back out of the cell to the bulk solution. The total extraction process occurs in three general phases. The first is an equilibrium phase, where the analytes are first removed from the sample surface into the solvent. This happens at a rather constant rate. The intermediary transition phase is the second phase, there occurs diffusion and convection to the cell. In the last phase, called diffusion, the analytes are solubilized by the solvent and extracted from the cell. This last phase is also the limiting step of the process, it happens rather slow and is an irreversible process. [7][8][9]

MAE is different from other extraction methods, because with MAE the mass and heat gradients work in the same direction. In other conventional techniques the heat transfer works from out to in and the mass transfer works from in to out. With MAE the heat is divided more equally throughout. When the microwave energy interacts with the sample (and solvent) molecules, molecular interactions occur, that transfers the electromagnetic energy into thermal energy and thereby heating the sample (and solvent). During the heating, energy transfer is guided mainly by two mechanisms: ionic conduction and dipole rotation. [7][8][9]

In order to optimize the microwave extraction process, there are multiple variables to be considered, which will affect the yield of the analyte from the sample matrix. The first and arguably the most important is the choice of the solvent, the solvent sample interaction will strongly affect the outcome of the extraction. The solvent choice is dependent on the solubility of the sample, how it interacts with the sample matrix, and its ability to interact with microwave energy, also its ability to affect the sample matrix.[10] [11][12]

Another very important factor for achieving good extraction is the ratio of solvent to the sample. Too little solvent can lead to low yields due to potentially uneven heating. On the other hand, the use of too much solvent will lead to more diluted solute, which is simply unnecessary and also makes the entire extraction process less environmentally friendly. Temperature and time are also important factors to consider during microwave assisted extraction. If the temperature is too low, then the extraction will be incomplete. If it is too
high, then the analytes might start to degrade. The duration of the extraction affects also the yield due to the three main extraction phases described above. If the extraction time is too short then the yield will be low, because the extraction has probably not gone through all the three phases completely. If the time is too long, then it is simply unnecessary (because of the limiting step) and also it increases the risk for heat related degradation of the sample and analytes. [2][12][13]

3.2.2 Ultrasonic extraction

Ultrasonic extraction, is a commonly used method in extracting organic compounds from plants. This is a relatively low cost and simple method to use in extraction. Ultrasonic extraction is usually done with an ultrasonic bath. The extraction with ultrasonic irradiation works by affecting the two physical phenomena that occur during extraction from a plant. It facilitates diffusion through the cell wall and washes out the cell, once the cell walls have been broken. Ultrasonic extraction does this by oscillating solvent. [14][15][16]

The oscillation is produced by a generator. The generator consists of an electrode on each side, and a crystal in the middle. Ultrasonic waves are transmitted through the liquid when voltage is applied to the electrodes. The voltage applied to the electrodes is alternating voltage, and the voltage causes the crystal to oscillate and this oscillation of the crystal is what causes the vibrations, ultrasonic waves. This vibration causes the formation of cavities in the liquid. The cavities are small microbubbles. During the oscillation enormous amount of bubbles are created. The bubbles implode in incredibly short time after being born. The rush of liquid (solvent) caused by this implosion near or at the surface of the sample matrix causes the cell walls to break and the analytes to be “washed out” into the solvent. [14][15][16]

3.2.3 GC-FID

Gas chromatography, also known as GC, works by heating up the sample, so that the analytes in the sample go over to gas phase. That gas is then eluted through a stationary phase (column) with the help of the mobile phase (carries gas). The retention time is different for different compounds, depending on their ability to interact with the stationary phase. [17]

The retention time is dependent on the polarity of the column. In a nonpolar stationary phase the compounds are eluted for the most in the order of their boiling points, from lower to higher. On the other hand, in a strongly polar stationary phase the order at which the compounds elute through the column is dependent on their polarity. In this case the rule that “like dissolves like” applies. In the strongly polar stationary phase, the weakly polar
compounds are eluted first and strongly polar compounds are eluted last. [17] Retention time is also dependent on the volatility of the different analytes. Also, by increasing the temperature, the retention time for all the compounds in the sample is decreased. But this also leads to worse peak separation, making it harder to differentiate between the different compounds. [17]

The flame ionization detector, also known as FID, is used to measure the eluted analytes and to determine their amount. In a FID the elute is burned in a H₂ and air mixture. During the burning of the analytes, ions and electrons are produced. The number is proportional to the suspected amount of hydro carbons (-CH) passing through the flame. This produces a measurable current; this current is converted into voltage and then converted into a signal. FID is a fairly sensitive detector and responds to most hydrocarbons. [18]

To increase the likelihood of accurate results even further, then an on-column injector is also used. The on-column injector is used for samples that are thermally unstable and tend to decompose above their boiling point. This method of injection is often used for quantitative measurements. With this injection system the solutes are directly injected into the column (stationary phase), keeping the sample temperature lower, compared to if it had gone through a hot injector. By keeping the sample at a lower temperature, prior to starting the separation by heating the GC, the likelihood of losses of the sample is reduced. [19]
4 Experimental

4.1 Instrumentation

GC used was HP5890 Series II. The GC was equipped with a flame ionization detector (FID) and the detector temperature was 290°C, gases: air (100kPa), H₂ (80kPa). A cold on-column injector was used. The injection volume was 1-2µl by manual injection with a syringe. The carrier gas (mobile phase) used was N₂, 70kPa. Open tubular fused silica column coated with a J&W DB-5 stationary phase was used, d=0.25µM. The column size was 30m x 0.25mm i.d. The results were analyzed using a data handling program Borwin version 1.5. The temperature program used was with initial temperature 60°C, ramp: 8 °C/min, end temperature: 150°C, hold time: 1min, second ramp: 25 °C/min and final temperature 280 °C.

The ultrasonic bath used for ultrasonic extraction was Transsonic T 780/H (Elma), 720watt of connected power. No temperature regulation was used.

The centrifuge used was Labex company instrument, Sigma Laboratory Centrifuges 4-15. The centrifuge was used, for samples, at 3000rpm for 10min.

Microwave used for microwave extraction was a PerkinElmer Microwave Sample Preparation System Titan MPS. The operating conditions were set to be, pressure (bar) to 20, ramp 5, power 60% and temperature and time were varied during the experiments. The microwave containers were 75ml teflon containers.

4.2 Reagents and solutions

The solvent used was analytical grade Isooctane (density 692 mg/ml [20]), 99% pure, from Merck KGaA, produced in Germany. The internal standard substances were: Analytical grade 1-octanol, 99% pure, from BDH Chemicals Ltd, produced in England. Analytical grade Carvon, 99% pure, from MERCK-Schuchardt, produced in Munich. Standard substances: Analytical grade Camphor, 98% pure, from Sigma-Aldrich Chemie GmbH, produced in Germany. Analytical grade Borneol, 99% pure, from Aldrich Chemical Company Inc, produced in USA. Analytical grade alphalpha-pinene, 98% pure, from Sigma-Aldrich Chemie GmbH, produced in Germany.

All solutions were prepared by weighing. Stock solutions of internal standards and standard substances with concentration 6mg/g were prepared individually. From the stock solutions standard solutions were done containing all three standard substances, with concentrations from 40µg/g to 160µg/g. Each of the standard solutions also contained 80µg/g of both of the internal standards. These standard solutions were used to construct internal standard calibration curves for each of the analytes. The concentrations in samples were evaluated from these.
4.3 Sample preparation procedures

4.3.1 Solid sample

Dried rosemary from Santa Maria AB, Mölndal, Sweden was used. Dried rosemary was crushed into small bits using a pestle and mortar and then sieved through a 0.75mm sieve, to ensure a uniformed particle size.

4.3.2 Ultrasonic extraction

The ultrasonic extraction procedure was taken from a description of a course experiment [20], but the procedure was modified according to the amount of internal standard and sample used. Instead of 0.3g of sample, 0.4g of sample was weighed in. The amount of each of the internal standards weighed in, from the 6mg/g stock solutions, was reduced from 0.2g to 0.09g, and the amount of solvent added remained at 10ml. The added solvent was weighed.

The sample was then placed in ultrasonic bath for 60 minutes. After that the sample was centrifuged at 3000rpm for 10min. The sample was then filtered through 00H grade filter paper manufactured by Munktell Filter AB, in Sweden. All the samples were measured with GC-FID the same day they were prepared.

Repeatability for the ultrasonic extraction was studied, by preparing 6 samples as describes above and measuring the results with GC-FID. Relative standard deviation (RSD %) was calculated. Instrumental precision was also studied for GC-FID. Only this was done by measuring one sample, prepared by ultrasonic extraction, 6 times and calculating the RSD value.

4.3.3 The choice of internal standard

During this experiment two internal standards were used, 1-octanol and carvon. To determine which of these was more accurate, resolution (Rs) was obtained from chromatograms containing both of them.

A spike test was performed by first preparing a sample with ultrasonic extraction. For the spiked sample, about 0.045g of the stock solution for each of the three terpenes were weighed and diluted to 10ml with the ultrasonic sample, the amount of sample weighed. For the non-spiked sample, an equivalent amount (as the stock solutions for each of the three terpenes that were weighed in for the spiked sample) of isooctane was weighed in and diluted to 10ml, in the same way, with the same ultrasonic extracted sample. The spiked and un-spiked samples were measured and the concentrations evaluated. The results were compared with the spiked amount for each analyte.


4.3.4 Microwave assisted extraction (MAE)

The solvent and the internal standards were mixed by weighing from both of the internal standard 6µg/g solutions and then diluting that mix to a concentration of 80µg/g with the solvent isooctane. The amount of rosemary weighed was 0.47g, the rosemary was weighed into a Celia tea filter, medium size, produced by Melitta Group in the EU. All the tea filters packed with rosemary were folded the same way and placed in the bottom of the microwave container. To the rosemary 20ml of the solvent/internal standard mixture was added, by weighing. The ratio of sample to solvent was 1:30. With this sample preparation different temperatures and times were studied. The microwave containers were always left to cool, in cold water bath to room temperature before opening them. All the samples were measured with GC-FID the same day they were prepared.

The different temperatures studied were 100, 110, 120 and 130°C. For the temperatures 110, 120 and 130°C relative standard deviation (RSD %) was calculated and also mean values were evaluated. The number of replicates was 4. The time set for these extractions was 10min.

The different extraction times studied were 10, 15 and 20min. The number of replicates was 4. During the extraction the temperature was 120°C. One-way ANOVA was used for each analyte to analyze the results.

Different ratios of rosemary to solvent were also studied. The different ratios studied were 1:20, 1:30, 1:40 and 1:50. The number of replicates was 3. The extraction time was 15min and the temperature was 120°C. The results were analyzed with one-way ANOVA for each analyte.

4.4 Comparison of Ultrasonic extraction and Microwave assisted extraction

Three samples were prepared with ultrasonic extraction, as described above. Also three samples were prepared with the optimized conditions for microwave assisted extraction. The samples were measured with GC-FID and results were compared using a two-way ANOVA. Two-way ANOVA is used to see if two different factors (in my case substance and extraction method) influence the results and with replicate measurements one can also investigate if there are interactions between the factors (changing method influences the substances differently). When calculating the two-way ANOVA, there was no interest in the absolute concentration of the three analytes, instead the interesting factor was the extraction efficiency. Therefore, instead of analyzing the extracted concentrations, each of the extracted concentration replicate for each analyte was divided by the mean value for all the replicates for both of the methods for that specific analyte.
5 Results and discussion

5.1 Calibration curves

The calibration curves for the three terpenes, according to both 1-octanol and carvon, can be seen in Figures 2, 3 and 4. All the calibration curves are shown to be linear up till the highest measured concentration of 160µg/g.

Figure 2; Alpha-pinene calibration curve using both 1-octanol and carvon as internal standards.

Figure 3; Camphor calibration curve using both 1-octanol and carvon as internal standards.
Figure 4: Borneol calibration curve using both 1-octanol and carvon as internal standards.

5.2 Repeatability of Ultrasonic extraction and GC-FID

The RSD values for the ultrasonic extraction are represented in Table 8. The relative standard deviation is acceptable, because it is less than 10%. The values show that ultrasonic extraction has good repeatability.

Table 8; Relative standard deviation (%) for ultrasonic extraction.

<table>
<thead>
<tr>
<th></th>
<th>alpha-pinene</th>
<th>camphor</th>
<th>borneol</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSD %</td>
<td>6.4</td>
<td>4.4</td>
<td>6.7</td>
</tr>
</tbody>
</table>

The RSD values for GC-FID are represented in Table 9. The Relative standard deviation shows that GC-FID has very good repeatability. The results are considered very good, if they are 1% or less. This means that there are no repeatability problems with GC.

Table 9; Relative standard deviation (%) for GC-FID.

<table>
<thead>
<tr>
<th></th>
<th>alpha-pinene</th>
<th>camphor</th>
<th>borneol</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSD %</td>
<td>1.3</td>
<td>0.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>
5.3 The choice of internal standard

5.3.1 Elution order

A typical GC-FID chromatogram of rosemary, with the internal standards 1-octanol and carvon added, can be seen in Figure 5.

![Rosemary chromatogram](image)

Figure 5: A typical GC-FID chromatogram of rosemary, with 1-octanol and carvon added. 1: isooctane (solvent), 2: alpha-pinene, 3: 1-octanol (internal standard), 4: camphor, 5: borneol, 6: carvon (internal standard).

5.3.2 Resolution (Rs)

5.1.2.1 Internal standard resolution

The calculations showed that resolution is very good for both of the internal standards. The results are as followed: mean Rs value for 1-octanol was 2.09 and the range was 2.11-2.07. Mean Rs value for carvon was 2.65 and the range was 2.70-2.58. This means that regarding resolution/peak separation both can be used.

Though a problem did arise with the 1-octanol peak, while studying different ratios with microwave assisted extraction. For the rather high ratios of 1:40 and 1:50 there did appear a second peak at the same location as the 1-octanol peak, making it difficult to correctly evaluate the 1-octanol peak area. The peak appeared most likely because, with these specific conditions (120°C 15min, ratio 1:40 and 1:50), a previously unseen substance in
rosemary started to extract. Because there was nothing seen at 1-octanol location on any of the earlier tests that could potentially interfere with the 1-octanol peak resolution. Therefore 1-octanol was shown not to always be reliable.

5.3.2.2 Borneol resolution

Another peak separation issue that did arise was that the peak for borneol did not separate properly. The borneol peak did seem to overlap with something else. With concentrations of borneol up till 80µg/g in the solution the interfering peak was visible, but after the concentration rose above 80µg/g the two peaks seemed to melt into one. Also with very low concentrations, from around 10µg/g borneol and lower, the borneol peak became too small to be evaluated accurately.

5.3.3 Spike test

The preformed spike test showed that the more accurate internal standard was 1-octanol. The results can be seen in Table 1.

For both alpha-pinene and camphor, more accurate results were obtained for the spike by evaluating them with 1-octanol as internal standard. For borneol the more accurate internal standard seems to be carvon, but even with carvon there is a rather big difference between the measured spike and spike concentrations. This discrepancy could be explained by the fact that the borneol peak had bad resolution, due to an overlapping peak.

Table 1; Measured spike for each of the analytes and the spiked concentration.

<table>
<thead>
<tr>
<th>Measured spike (µg/g)</th>
<th>Spike (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>According to 1-octanol</td>
</tr>
<tr>
<td>alpha-pinene</td>
<td>44.09</td>
</tr>
<tr>
<td>camphor</td>
<td>38.73</td>
</tr>
<tr>
<td>borneol</td>
<td>47.10</td>
</tr>
</tbody>
</table>

Even though 1-octanol was concluded to be more accurate according to spike test, then carvon is the recommended internal standard, due to unreliability with 1-octanol at rather high ratios of solvent to sample with microwave assisted extraction. Carvon's resolution remained good and undisturbed throughout all different tests.
5.4 Optimization of the microwave extraction procedure

5.4.1 Temperature

The four different temperatures studied showed a trend of increasing extracted concentration from rosemary with increasing temperature. The studied repeatability was acceptable for 110 and 120°C, meaning that RSD was below 10%, but very bad for 130°C. The results can be seen in Table 2 and Table 3.

<table>
<thead>
<tr>
<th>RSD (%)</th>
<th>At 110°C</th>
<th>At 120°C</th>
<th>At 130°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-pinene</td>
<td>4.9</td>
<td>9.2</td>
<td>26</td>
</tr>
<tr>
<td>camphor</td>
<td>4.3</td>
<td>6.1</td>
<td>25</td>
</tr>
<tr>
<td>borneol</td>
<td>5.6</td>
<td>5.9</td>
<td>13</td>
</tr>
</tbody>
</table>

From Table 2 it can be seen that the RSD value increases with temperature, meaning that with increasing temperature the repeatability gets worse. This can be explained with three possible effects. The first factor is that all the three terpenes and also the solvent are volatile. This means that with increasing temperature more and more of the analytes (and solvent) goes to gas phase. This can lead to losses when opening the container, even though precautions were taken to minimize this risk.

The second factor affecting the results is, that the microwave seems not to heat the samples uniformly, meaning that there is a slight temperature difference between the different samples in the microwave. The measured temperatures in different containers, and the variation between them, during a microwave extraction can be seen in Figure 6. In Figure 6, there is a clear variation in temperature between the different containers. This also results in different extracted concentrations. The containers showing lower temperature during microwave extraction also showed lower extracted concentration. This variation in temperature seemed to be a rather significant factor in explaining the varying RSD values.
The most likely reason for the increasing RSD values is the combination of the above named two factors. Because there is a small temperature difference between the samples, this increases the risk that different amounts of the analytes and solvent are in the gas phase when the container is opened, increasing the relative standard deviation for the samples. Also the small variations in temperature mean that the extraction is not equally efficient in all the containers, at set temperature.

Also it is likely that at 130°C the solvent starts boiling (isooctane $t_b=99°C$ at 1bar, pressure in the container 20bar). This combined with the small variations in the temperature could explain why the RSD value is that bad at 130°C. When the solvent starts boiling the extraction becomes even more efficient because of convection. So when the temperature is not the same in all containers, some containers could be boiling longer/sooner than others, resulting in even bigger RSD value compared with lower temperatures such as 120°C or less, when there is no boiling.

A third factor could possible also be that the analytes are starting to break down at higher temperatures. So, even though higher amount is extracted with higher temperature, also a higher amount is broken down with higher temperature. This combined with the small variations in temperature when heating and the volatility of the analytes can lead to an increasing RSD value with increasing temperature.

Table 3; Mean values in mg/g for the three different terpenes with microwave extraction at 110, 120 and 130°C.

<table>
<thead>
<tr>
<th>Mean (mg/g)</th>
<th>At 110°C</th>
<th>At 120°C</th>
<th>At 130°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-pinene</td>
<td>1.419</td>
<td>1.951</td>
<td>3.069</td>
</tr>
<tr>
<td>camphor</td>
<td>1.670</td>
<td>2.043</td>
<td>3.531</td>
</tr>
<tr>
<td>borneol</td>
<td>0.692</td>
<td>0.801</td>
<td>0.789</td>
</tr>
</tbody>
</table>
When studying the mean values for the extracted concentrations, a trend of increasing mean concentration with increasing temperature could be seen. This can be observed in Table 3. This makes sense, because the extraction does become more efficient with increasing temperature and even more efficient when the solvent starts boiling.

When evaluating which temperature to choose as the “optimal” temperature, then RSD also has to be taken into account, not only the mean extracted concentration. The best mean concentration, with still acceptable RSD value (below 10%) for the three terpenes, was obtained at 120°C. Therefore the optimal temperature was chosen to be 120°C.

5.4.2 Time

The one-way ANOVA showed that time was not a significant factor for camphor nor borneol, but was a significant factor for alpha-pinene, at 5% significance level. For alpha-pinene with one-way ANOVA, P=0.014.

The average concentrations alpha-pinene extracted for the different extraction times are shown in Table 4. As it can be seen from Table 4, there does not really seem to be a trend. This could be explained with some of the same factors that were affecting the temperature study, or maybe 15min is the optimal extraction time for alpha-pinene. To determine this for sure, further studies are needed.

Table 4; Average concentrations alpha-pinene extracted for the different extraction times.

<table>
<thead>
<tr>
<th>Extraction Time</th>
<th>Average (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10min</td>
<td>1.975</td>
</tr>
<tr>
<td>15min</td>
<td>2.537</td>
</tr>
<tr>
<td>20min</td>
<td>2.336</td>
</tr>
</tbody>
</table>

The optimal extraction time for microwave assisted extraction was chosen to be 15min, because at this extraction time, the extracted mean concentration was highest for alpha-pinene. For camphor and borneol the extraction time was not a significant factor.

5.4.3 Ratio

The choice of ratio was shown by one-way ANOVA not to be significant for alpha-pinene, and to be significant for camphor and borneol, at 5 % significance level. The results of the one-way ANOVA can be seen in Table 5.
Table 5; Results of the one way ANOVA when comparing different ratios of sample to solvent.

<table>
<thead>
<tr>
<th>Substance</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-pinen</td>
<td>4.07</td>
<td>0.050</td>
<td>4.07</td>
</tr>
<tr>
<td>camphor</td>
<td>7.94</td>
<td>0.009</td>
<td>4.07</td>
</tr>
<tr>
<td>borneol</td>
<td>116</td>
<td>6.23E-07</td>
<td>4.07</td>
</tr>
</tbody>
</table>

The mean extracted concentrations for each of the studied ratios for all the substances can be seen in Table 6. From Table 6 there can be seen a rather consistent trend of increasing extracted concentration with increasing ratio, for alpha-pinen and camphor. For borneol on the other hand, there does not seem to be any trend. The decreasing extracted concentration of borneol with increasing ratio can be explained with the resolution issues of borneol, described in section “4.1.2.2 Borneol resolution”.

Table 6, shows the mean extracted concentrations for each of the studied ratios for the three terpenes.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>alpha-pinen</th>
<th>camphor</th>
<th>borneol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:20</td>
<td>2.236</td>
<td>2.118</td>
<td>0.511</td>
</tr>
<tr>
<td>1:30</td>
<td>2.390</td>
<td>2.072</td>
<td>0.825</td>
</tr>
<tr>
<td>1:40</td>
<td>2.584</td>
<td>2.479</td>
<td>0.241</td>
</tr>
<tr>
<td>1:50</td>
<td>2.644</td>
<td>2.900</td>
<td>0.220</td>
</tr>
</tbody>
</table>

In Table 7 can be seen the relative standard deviation for the replicates at different ratios for the three terpenes. As it can be seen from Table 7, the RSD value for the ratio 1:50 is rather much worse than for the other ratios, for all of the three terpenes. Why this is so for borneol can be explained with borneol having bad resolution at such low concentrations and therefore making it difficult to evaluate the extracted concentration correctly. For alpha-pinen and camphor, the large increase in RSD at 1:50 ratio is unclear. This could be a result of the microwave not heating sample containers evenly. Or that there is some unknown mechanism playing a role, that results in increased RSD at 1:50 ratio.

Table 7; Relative standard division (%) for the replicates at different ratios for the three terpenes.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>alpha-pinen</th>
<th>camphor</th>
<th>borneol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:20</td>
<td>3.3</td>
<td>3.5</td>
<td>1.2</td>
</tr>
<tr>
<td>1:30</td>
<td>7.8</td>
<td>1.7</td>
<td>9.4</td>
</tr>
<tr>
<td>1:40</td>
<td>0.7</td>
<td>0.8</td>
<td>14</td>
</tr>
<tr>
<td>1:50</td>
<td>9.4</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

For further tests the optimized ratio was chosen to be 1:40. This is because the RSD value for 1:50 ratio was rather large, even though the mean extracted concentration was also
larger. The mean extracted concentration for borneol also seemed to get lower with higher ratios. The ratio of 1:40 had rather small RSD value and a rather large mean extracted concentration for all the three terpenes.

5.5 Comparison of Ultrasonic extraction and Microwave assisted extraction

The microwave assisted extraction was done at 120°C, time set at 15min and the rosemary to solvent isoctane ratio being 1:40. Internal standard used was carvon.

The two-way ANOVA showed that the interaction was significant. Meaning that the three terpenes did behave differently depending on the extraction method. The mean values for the three terpenes extracted concentration with both the methods are shown in Table 10. The highest extracted concentration with MAE was given by alpha-pinene followed by camphor. For the ultrasonic extraction the highest extracted concentration was from camphor, followed by alpha-pinene. In Table 10 it can also be seen that the extracted borneol concentration is lower with MAE compared with ultrasonic extraction. This can be explained with the bad resolution of borneol at the given ratio (1:40). For alpha-pinene and camphor the extracted concentration is higher with MAE.

Table 10; Mean values for the three terpenes extracted concentration with both methods.

<table>
<thead>
<tr>
<th></th>
<th>MAE (mg/g)</th>
<th>Ultrasonic. (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-pinene</td>
<td>2.584</td>
<td>1.478</td>
</tr>
<tr>
<td>camphor</td>
<td>2.479</td>
<td>1.523</td>
</tr>
<tr>
<td>borneol</td>
<td>0.241</td>
<td>0.385</td>
</tr>
</tbody>
</table>
6 Conclusions

The optimized conditions for microwave assisted extraction were found to be 120°C, time set at 15min and the rosemary to solvent isooctane ratio being 1:40. The internal standard used was carvon. Though, because of the constant reproducibility problems, due to the microwave heating unevenly and the volatility of the analytes, it is hard to determine if these conditions are the most optimal ones. It was also shown, that with increased temperature and ratio, better results can be obtained (higher extracted concentration), but with rather bad relative standard deviation. Repeatability was shown not to be a problem when using ultrasonic extraction.

When comparing the two methods with two-way ANOVA it was shown that the interaction was significant. Meaning, that the three terpens did behave differently depending on the extraction method.

It was not demonstrated that MAE is more environmentally friendly at this scale. The ratio of solvent to sample for MAE was 1:40, whiles the ratio was approximately 1:20 for ultrasonic extraction. Maybe the “optimal” ratio for ultrasonic extraction is even higher compared with MAE, but for this experiment, ultrasonic extraction used less solvent. Also the MAE equipment used is a lot more expensive and consumes more energy than the equipment for ultrasonic extraction. Furthermore, the total extraction time was longer for MAE, due to the need to cool the microwave vessels. Therefore, in this scale, ultrasonic extraction is cheaper, less time consuming and more environmentally friendly. But the total extracted concentration was rather much lower with ultrasonic extraction.

7 For further study

It would be interesting to study how the two methods compare after also the ultrasonic extraction method has been optimized. We now know that according to two-way ANOVA analysis the three terpens did behave differently depending on the extraction method. But it is difficult to compare the total extracted concentrations; because ultrasonic extraction is clearly at a disadvantage, by not having been optimized.

Another things that could be tested, is a more polar solvent during a microwave extraction. The infrared sensors that measure the temperature in the microwave contains during an extraction are more sensitive to a polar solvent. This will give a better picture of what’s happening during the extraction.

A second spike test should also be done. This time the sample should be spiked before extraction, to see better the accuracy of the extraction methods.
8 References


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