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ROSEMARY ESSENTIAL OIL BY HYDRODISTILLATION: SPME-GC-MS CHARACTERIZATION

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Abstract
Rosemary (*Salvia rosmarinus)* is an aromatic herb with a multitude of uses. It is both a seasoning in various dishes, and a natural remedy for several diseases mainly due to its anti-inflammatory and anti-oxidative effects. Plus, it is also used in cosmetic industry and for improving agriculture practices and helping the environment, since it may be a natural antimicrobial. It is worth noting that this plant is also grown and commercialized in Uzbekistan, thus, if added value is given to rosemary's products, it has potential for improving living conditions and helping in the economy of local farmers and entrepreneurs. In this work, we discuss a technique for obtaining rosemary essential oil, rich in terpenes, by vacuum assisted hydrodistillation. This is a solventless environmentally friendly and safe technique that allows the obtention of an added value rosemary product where terpenes, compounds responsible for most of rosemary's properties, are the most abundant. Two samples of locally acquired rosemary were sourced, essential oil was extracted, and characterization was then performed by Solid Phase Micro Extraction (SPME) and Gas Chromatography – Mass Spectrometry (GC-MS). As a comparison, a direct injection of a dilution of one of the essential oil samples was also performed. It was concluded that samples of essential oil obtained from both sources were entirely composed of terpenes or terpene derivatives. Besides, SPME was a superior method when compared to direct injection, since a larger number of volatiles was able to be identified.
Key words: Rosemary, Hydrodistillation, Sustainability, Crops, Entrepreneurship, Food Insecurity.

Introduction

Rosemary (*Salvia rosmarinus*, formerly known as *Rosmarinus officinalis* L.) is a perennial herb native to the Mediterranean region and a member of the mint family *Lamiaceae* which is closely related to other herbs such as thyme, oregano, and lavender. The name 'Rosemary' derives from the Latin words 'ros' meaning 'dew' and 'marinus' meaning 'sea' – 'dew of the sea'. This name alludes to its natural habitat in coastal areas and is often found near the sea, especially along the sea cliffs of the Mediterranean region. Although it is native to the Mediterranean region and Western Asia, where it has grown wild for thousands of years, it was later spread by humans throughout Europe and other parts of the world, including the Americas, Asia, and Africa (Elyemni *et al*., 2019; Sasikumar, 2004; Veenstra & Johnson, 2021).

Rosemary has a strong aromatic and sweet smell thus it has a multitude of uses, both as a food additive and otherwise. In cooking, it is used as a seasoning in a variety of dishes such as soups, casseroles, salads, and stews. Its leaves, both fresh and dried, are used in traditional Mediterranean cuisine as a seasoning for meats, fish, vegetables, soups and more. Rosemary dry leaves, flowers, and twigs are used in the processing of fish products. It is added in small amounts to roast meat, roast chicken, minced meat, mushroom salads, vegetable stew and other dishes. In addition to its culinary uses, rosemary is also used for ornamental purposes. In agriculture, rosemary is used as a natural antimicrobial reducing the need for potentially harmful synthetic compounds. It is often used in landscaping, thanks to its resistance to drought, heat, pests, and diseases. Moreover, due to its hardiness and low maintenance, it's a popular plant in xeriscaping, a landscaping method that utilizes water-conserving plants (Sasikumar, 2004; Gonçalves *et al*., 2022; Khomidov & Mukumov, 2022). Rosemary has been used in traditional medicine for centuries in various forms, including as a tea, essential oil, and in extracts. In traditional medicine, stomatitis, respiratory diseases, and wounds are treated with decoction made from rosemary. A bath is made from the decoction of rosemary for eczema and other skin diseases, and children are bathed in the decoction (Khomidov & Mukumov, 2022). In folk medicine, it has also been used as a remedy for various diseases including intercostal neuralgia, headaches, migraine, insomnia, stomach-ache, epilepsy, rheumatic pain, spasms, dysmenorrhea, hysteria, depression, and nervous agitation, as well as an antispasmodic, mild analgesic, and for improving memory, physical and mental fatigue, and to cure emotional upset (Ghasemzadeh Rahbardar & Hosseinzadeh, 2020).

Several of those properties have been confirmed in modern studies since rosemary has been confirmed to improve gastrointestinal health by alleviating diseases such as inflammatory bowel disorder (IBD) and colitis. The mechanisms of action involve antioxidant and anti-inflammatory properties, as measured through inflammatory biomarkers such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, myeloperoxidase (MPO), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx), malondialdehyde (MDA), and superoxide dismutase (SOD) (Veenstra & Johnson, 2021; Gonçalves *et al*., 2022). Besides, antimicrobial activity towards *C. albicans, P. aeruginosa, E. coli, S. aureus, B. cereus, E. faecalis, V. fluvialis, V. damsel*, and *S. mutans* has been well studied (Veenstra & Johnson, 2021). In general, the major individual components found in rosemary oil, involved in rosemary health improving properties, are 1,8-cineol, α-pinene, and camphor, among other terpenes (Veenstra & Johnson, 2021; Gonçalves *et al.*, 2022). Moreover, ethnopharmacological uses including anti-anxiety, anti-spasm, anti-inflammatory, memory-boosting and analgesic effects of rosemary have been validated by neuropharmacological investigation, thus its potential as a natural treatment for diseases such as Alzheimer's, Parkinson, epilepsy, anxiety, and depression, among others (Ghasemzadeh Rahbardar & Hosseinzadeh, 2020). Another important use of rosemary involves its properties for promoting hair growth. Begum et al. carried a study where after treatment with a herbal hair lotion loaded with rosemary, mice experienced an increase in hair follicle count, hair weight, and hair length (Begum *et al*., 2023). In another study, in humans, rosemary proved effective for treating androgenetic alopecia (Panahi *et al*., 2015). Several mechanisms behind rosemary's hair growth promoting properties have been proposed. A stimulation in blood circulation would promote follicles by bringing nutrients and oxygen. Its antioxidant activity would prevent damage in hair follicles by free radicals and improve scalp health. The antimicrobial effect of terpenes within rosemary oil like camphor, cineole and borneol, which have antimicrobial effects, would help control dandruff and fungal infections that can clog follicles and inhibit growth. Besides, its anti-inflammatory effect, and its role in inhibiting 5-alpha reductase enzyme, responsible for converting testosterone to Dihydrotestosterone (DHT), especially considering that alopecia is related to high levels of DHT (Dhariwala & Ravikumar, 2019).

The economic importance of rosemary is significant. It is a valuable crop for the essential oil industry, with rosemary oil being a common ingredient in many cosmetic, medicinal, and industrial products. Besides, most of its properties may be attributed to the compounds that conform rosemary's essential oil, especially terpenes. The global market for rosemary extract, primarily used in the food and beverage industry, is also substantial and growing (Ghasemzadeh Rahbardar & Hosseinzadeh, 2020; Veenstra & Johnson, 2021; Gonçalves *et al*., 2022). In Uzbekistan, rosemary was first introduced by S. N. Kudryashov (1930-36) by growing it in the Botanical Garden of the Central Asian State University (now UzMU). Later, in 1992, Yu. M. Murdakhaev re-introduced rosemary by cultivating it in the Tashkent Botanical Garden as a valuable medical and essential oil plant (Nasriddinova, 2022). Nowadays, rosemary has been successfully grown in our country (Khomidov, Mukumov, & Rasulova, 2023; Nasriddinova, 2022), and nationally manufactured rosemary is sold in Uzbekistan 'Figure 1'.

Hydrodistillation is a relatively simple and cost-effective method of essential oil extraction, making it accessible to small-scale producers, thus it is ideal for those local farmers or entrepreneurs who may be interested in producing and commercializing an added value product.

Figure 1. Rosemary manufactured and sold in Uzbekistan.

One of the main advantages of this technique is that it is solvent free, hence no harmful solvents or harsh chemical compounds that may pose health are used; therefore, it is both a safe and an environmentally friendly extraction method compared to solvent-based extraction processes. Vacuum hydrodistillation is a gentle extraction method that, due to a reduced pressure, uses a lower temperature, consequently, it helps preserve delicate compounds in the essential oil, resulting in a high-quality product with a natural aroma (Elyemni *et al*., 2019).

Solid phase microextraction (SPME) is a sample preparation technique that involves the use of a coated fused silica fiber to extract analytes from samples. The fiber, which is coated with a polymeric stationary phase, is exposed to the headspace above or directly to the sample. The analytes get partitioned between the sample matrix and the fiber coating, reaching an equilibrium. The extracted analytes are then thermally desorbed in the injection port of a gas chromatograph (GC) or high performance liquid chromatograph (HPLC) for separation and detection (Lord & Pawliszyn, 2000). Some key advantages of SPME include being fast, simple to use, solvent-free, and applicable to a wide range of volatile and semi-volatile compounds (Arthur & Pawliszyn, 1990). It provides concentration and clean-up in a single step. SPME fibers are also robust and can be reused many times with minimal carryover between samples. The technique is sensitive, with detection limits in the parts-per-trillion (ppt) range achievable. SPME is especially suitable for on-site or in-field sampling of air, water, and soil due to its small, portable design.

In this work, we disclose a technique for obtaining rosemary essential oil, rich in terpenes, by vacuum assisted hydrodistillation, followed by SPME-GC-MS analysis, and direct injection GC-MS analysis for comparison.

Materials and Methods

Materials

Fresh rosemary was sourced from two vendors in a local market. Samples from each vendor are marked as 'A' and 'B'. There is no especial distinction among vendors or plant material, but we aimed at using two different materials, thus produce two batches of essential oil, and compare the results.

Hydrodistillation

Extraction by hydrodistillation was based on conditions outlined by Calvo-Gómez *et al.* (2004) (Calvo-Gómez, Morales-López, & López, 2004). A rotary vapor apparatus IKA RV8 with a heating device IKA HB digital (IKA, China) equipped with a recirculating water chiller YHLT-10/30 (Green Distill, China) and a diaphragm vacuum pump KNF N 035.3 AN.18 (KNF, Germany) (13 mbars abs. ultimate vacuum, according to specification sheet) were used.

2 kg of fresh rosemary leaves were ground with 2 L of water for 10 minutes. The mixture was placed in a round bottom flask, connected to the rotary evaporator, which consists of a boiling flask, a condenser, and a receiver flask. The boiling flask was then slowly heated to 85 °C to bring the water and plant material mixture to a gentle boil. The rotating motion of the flask ensures uniform heating and mixing. The rotavapor was connected to the vacuum pump, which was then turned on, what allowed the mixture to be heated to the boiling point of the essential oil components at a lower temperature due to decreased vapor pressure. Cold water at 4 °C was recirculating. Rotary evaporator speed was set at 110 rpm, and pressure at 200 mmHg.

As the mixture is heated, the volatile essential oil components are extracted from the plant material and evaporate. The vapor travels up the distillation arm and condenses in the cold collection flask. Non-volatile compounds remain in the aqueous solution in the round-bottom flask. Once the hydrodistillation is complete, in 3 hours, the rosemary essential oil can be separated from the aqueous layer. The oil contains the aromatic compounds that were isolated through the simultaneous actions of heat, reduced pressure, and fluid rotation in the rotavapor.

Analysis

Both Solid Phase Microextraction (SPME) and Gas Chromatography – Mass Spectrometry conditions were based on conditions outlined by Calvo-Gómez *et al.*, 2004 (Calvo-Gómez, Morales-López, & López, 2004) with modifications.

SPME holder and the fibers: Divinylbenzene/ Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm and PDMS (Polydimethylsiloxane) 100 μm were sourced from Sigma-Aldrich (USA).

For SPME, procedure was as follows: 10 μL of essential oil were placed in a 4 mL vial, sealed tightly with a screw-top septum-containing cap, and allowed to stand at 30 °C for 1 h. The SPME needle was then inserted through the septum, the holder was secured, and the fiber was exposed to the headspace. After 30 min of sampling at 30 °C, the fiber was retracted and immediately inserted into the inlet of a GC for thermal desorption. Injection was accomplished by desorption of the fiber for 1 min at 220 °C. Analyses were performed by triplicate.

For GC-MS, analyses were carried on an Agilent 7890/5977 GC-MS (Agilent, USA) using a capillary column HP-FFAP (30m×0.25 mm i.d., film thickness 0.25 μm; Agilent, USA). Conditions were as follows: GC injector was at 230 °C in splitless mode, column temperature was held at 50 °C for 3 min and then increased at 5 °C/min to 220 °C and held for 16 min (for cleaning the column and the fiber); helium was used as carrier gas at a linear flow of 2 mL min^{-1}. Mass spectra were obtained at 70 eV, the ion source was at 230 °C, and quadrupole of the MS operated at 150 °C. Data was recorded on SCAN mode m/z 50-550) and the identification of the analyzed compounds was accomplished by comparing their mass spectra with those of authentic compounds available from computerized spectral database (NIST/EPA/NIH).

For comparing SPME with direct injection, using similar conditions as those described above for GC-MS, a single direct injection of 'B' rosemary essential oil was also performed. In this case, essential oil was dissolved in dichloromethane (1:50), and 1 μL of this mixture was injected directly into the GC-MS.

For data processing, raw GC-MS data was processed using MSD Chemstation Software ver. F01.01.2317 (Agilent, USA), and Automated Mass-Spectral Deconvolution and Identification System (AMDIS) by Steven Stein, Oleg Toropov, and Andrei Roumiantsev, ver. 2.73, build 149.31. Further data processing and tabulation was performed using MS Excel from Microsoft 365 (Microsoft, USA).

For statistics, One-Way Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test were performed. Differences between groups were considered significant at a 95% confidence level ($p < 0.05$). Statistical analyses were performed in Minitab 21.64 (Minitab LLC, USA), and astatsa.com by Navendu Vasavada (last accessed, May 10, 2024).

Results and Discussion

Rosemary essential oil obtained by hydrodistillation is a yellowish clear liquid. After analysis by SPME-GC-MS with both fibers, DVB/CAR/PDMS 50/30 fiber had a better affinity for compounds in the essential oil than PDMS 100 fiber. 'Figure 2' is a graphic representation of the total number of compounds (8 vs 20 respectively) identified in the chromatogram of rosemary essential oil when sampled by SPME using either PDMS 100 or DVB/CAR/PDMS 50/30 fibers, as well as the total area under the curve of each chromatogram (1.1×10^8 vs 1.67×10^9 respectively). Figure 3 is a representation of overlayed chromatograms obtained using both fibers. As it is observed in

'Figures 2' and 'Figure 3', both the abundances and number of compounds is much higher with the DVB/CAR/PDMS 50/30 fiber. Therefore, results in Table 1 for SPME GC-MS analysis of rosemary essential oil are reported from analyzing chromatograms of samples extracted by SPME with the DVB/CAR/PDMS 50/30 fiber. Results are displayed in percentage of total chromatogram area, and Coefficient of Variation (C.V.) is also indicated for SPME analyses (not for direct injection because only one repetition was performed in that case).

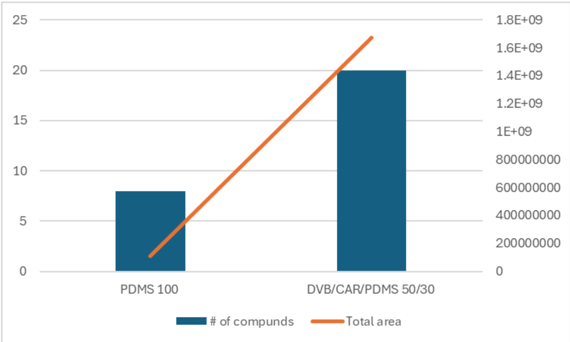

Figure 2. Comparison between PDMS 100 and DVB/CAR/PDMS 50/30 SPME fibers.

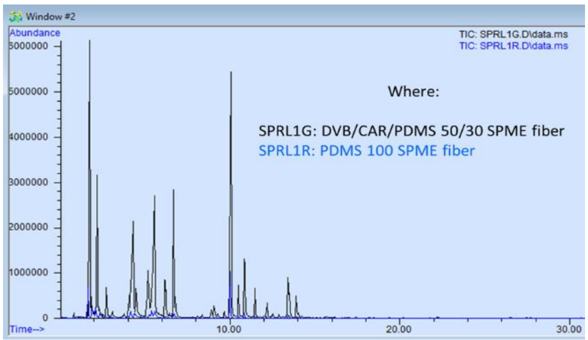

Figure 3. Overlayed chromatograms of rosemary oil by SPME-GC-MS with two different fibers.

Twenty compounds were identified in both samples. It is worth noting that all the compounds identified in rosemary essential oil are terpenes (except for endo borneol, which is an oxygenated terpene derivative). Those results are in accordance with previous studies (Al-Shaar *et al*., 2017; Elyemni *et al*., 2019; Veenstra & Johnson, 2021; Gonçalves *et al*., 2022). Besides, as it was mentioned in the introduction section, those compounds have been identified as responsible for several of the medicinal properties attributed to rosemary.

Regarding comparison between both rosemary vendors, A and B, results did not show any statistically significant difference between them since p-values were >0.05 ($p=0.9991$ in One-way ANOVA, and $p= 0.8999947$ in Tukey's HSD test). As expected, there are some differences that may be attributed to cultural practices, cultivars, or handling of herbal material. Some compounds, especially some of the most abundant such as eucalyptol, β myrcene, camphor, (+)-4-carene, α terpinolene, linalool, and D-verbenone, are in similar percentages in both samples. It is worth mentioning that only partial quantification is provided in this study, since relative amounts are based on area percentages and not in calibration curves. However, it is still possible to establish an estimate volatile profile of rosemary essential oil from both samples.

When comparing DVB/CAR/PDMS 50/30 SPME-GC-MS of the B sample vs the direct injection of the same sample, the number of compounds in SPME-GC-MS is 4 times greater than in direct injection (20 vs 5). This shows that SPME is able to detect more compounds present in a sample compared to direct injection alone. The SPME fiber acts as a concentrator and extractor, allowing it to capture more analytes from the sample that might otherwise be missed by direct injection. However, it must be taken into account that concentrations and proportions of individual compounds may be different after SPME extraction (as in the case of eucalyptol and camphor, as shown in Table 1), since affinity for the polymer in the fiber may be different (Arthur & Pawliszyn, 1990; Lord & Pawliszyn, 2000; Al-Khshemawee *et al.*, 2018). Nevertheless, SPME provides an advantage over direct injection in being able to detect and quantify a greater number of compounds in a given sample.

Table 1

Compounds in rosemary essential oil (in percentage of total chromatogram area) and C.V.

		VENDOR A		VENDOR B		
RT (min)	Compound	**SPME (%)**	C.V. SPME (%)	**SPME (%)**	C.V. SPME (%)	**Direct (%)**
1.737	α Pinene	**15.51**	3.11	**10.77**	2.73	
2.186	Camphene	**7.53**	3.34	**4.81**	3.15	
2.761	β Pinene	**3.30**	2.67	**4.51**	2.28	
4.22	cis-Sabinene	**1.46**	6.81	**4.48**	8.09	**2.02**
4.364	Beta Myrcene	**10.81**	0.99	**10.27**	0.71	
4.53	(+)-4-Carene	**1.67**	0.32	**1.62**	0.38	
5.203	D-Limonene	**4.40**	1.91	**3.47**	2.27	
5.572	Eucalyptol	**13.96**	0.44	**14.82**	0.43	**45.45**

Continuation of the Table 1

6.188	Trans β Ocimene	**1.46**	8.12	**3.71**	5.96	
6.243	γ Terpinene	**1.66**	3.42	**2.49**	3.53	
6.674	p-Cymene	**5.30**	6.37	**1.93**	8.47	**3.03**
6.794	β Terpinolene	**1.41**	1.64	**1.54**	1.76	
10.069	Camphor	**19.15**	0.67	**19.74**	0.33	**47.47**
10.501	Linalool	**1.36**	1.68	**1.59**	1.97	
10.862	Caryophyllene	**3.83**	4.30	**7.06**	4.72	
11.473	Terpinen-4-ol	**1.11**	3.36	**1.33**	2.12	
12.177	Humulene	**0.81**	0.77	**1.19**	0.41	
13.424	endo-Borneol	**2.96**	4.43	**1.67**	3.56	
13.493	β Terpineol	**1.40**	2.78	**2.14**	3.51	**2.02**
13.894	D-Verbenone	**0.93**	2.31	**0.84**	2.06	

Conclusions

1. Hydrodistillation of rosemary (*Salvia rosmarinus*) yields essential oil full of potentially pharmacologically important compounds.
2. SPME-GC-MS allowed the characterization of rosemary essential oilwhich may bring added value for farmers and entrepreneurs looking for added value in rosemary's products.
3. SPME was superior to direct injection for the analysis of rosemary essential oil volatiles.

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