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Essential Oil Analysis by Headspace Solvent Microextraction coupled with Hydro-Distillation Method (HD-HSME) of *Rosmarinus officinalis* L. from Noshahr, Iran

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ABSTRACT

The aim of this study is to provide a simple, rapid access and commodious technique used in the quantitative analysis of analytes and requires less sample handling and also lowering the cost of analysis by reducing both the amount of solvent necessary to prepare a sample and the amount of waste requiring disposal. In this headspace solvent microextraction coupled with hydrodistillation method a droplet of n-Hexadecane containing n-Octadecane as an internal standard was used for extraction. Analytes were extracted by suspending a 2 µL droplet directly from a microsyringe tip over the surface of the solution. After extraction, the droplet was retracted back into the syringe and injected directly into a GC injection port. The effect of different parameters on extraction efficiency were optimized using a one at a time optimization method, including: extraction solvent (n-hexadecane), sample mass (0.1 gr), microdrop volume (2 µL) and extraction time (6 min). On the analyte peak-to-internal standard peak ratio have been studied. The volatile components were studied both by HD-HSME and an ordinary hydrodistillation method and the main components identified were, α-pinene, 1, 8-cineol, camphene and myrcene in both methods. The results were in good correlation in comparison with the hydrodistillation method. By HD-HSME twenty compounds were identified. á-Pinene (46.5%), 1, 8-cineole (13.4%), camphene (12.9%) and myrcene (4.1%) were found to be the major constituents.

Key words: Headspace solvent microextraction coupled with hydro-distillation method (HD-HSME), microsyringe, microdrop, hydrodistillation (HD), internal standard.

INTRODUCTION

As an active research field in analytical chemistry, sample preparation techniques are a key step in an analytical procedure and it has received increasing attention in the past decade. Recently, with the trend of miniaturization and automation, micro-scale sample preparation methods have begun to generate strong interest and have undergone rapid development.

Liquid-liquid extraction (LLE) is the most time-consuming and requires large amounts of expensive high purity organic solvents, which comprise the largest source of waste in an analysis

laboratory[1]. Also hydro-distillation technique, as most conventional methods, needs about 3-4 hours and tens to hundreds grams which in some situation is problematic, especially in cases that enough plant material is not accessible.

The disadvantages of conventional extraction techniques have led to the development of new methods which use small volumes of organic solvent. In this respect, miniaturization has become an important trend in the development of sample preparation techniques[2].

Static headspace sampling is another technique for Volatile organic Compounds (VOCs) analysis. These procedures are environmentally friendlier, faster and easier to handle than conventional methods[3].

The concept of solvent microextraction (SME) can be traced back to the middle of the 1970s, when there were attempts to address the problems of high solvent consumption and poor automation in LLE. In LLE, the phase ratio is one of the critical parameters having great influence on extraction efficiency. A small amount of organic solvent was used to extract analytes from a large amount of aqueous sample to increase the phase ratio between the two phases[4].

In 1975 [5], a simple liquid extraction method based on the use of about 1 ml of organic solvent was reported. Subsequently, based on this liquid extraction method, Murray *et al.,*[6] described a method termed as solvent microextraction. Several hundred microliters of solvent was used to extract from about 1 liter of water sample. The semi-quantitative result could be improved with the possibility of injecting larger sample volumes (20-80 µl) into a GC system to increase the amount of sample and therefore method sensitivity[7, 8]. In the 1980s [9,10], the main development of solvent microextraction was flow injection extraction (FIE). FIE have the advantages of high speed, low cost and reduced solvent/sample consumption. However, the solvent consumption in FIE is still in the order of several hundred microliters per analysis and there are problems of deposition and adsorption of the particles on the optical cell windows during analysis.

In recent years, efforts have been placed on miniaturizing solvent extraction processes. Two general methods have evolved including drop-based solvent microextraction and hollow fiber combined with solvent microextraction. The developed methods are interesting alternatives to conventional LLE [11, 12]. In the former method, the extraction phase is a discrete drop of immiscible solvent suspended in an aqueous sample or its headspace.

Rosemary is a perspective plant culture in the world, it is in the middle of interest of plant breeders[13- 15]. The leaves of rosemary contain 0.5–2.5% of volatile oil. Rosemary contains a wide variety of volatile and aromatic components[16].

In this project, we were attempted to achieve an eco-friendly method for essential oil analysis of rosemary. For many different parameters that affect the extraction efficiency in an HD-HSME system, one at a time optimization method was used.

MATERIAL AND METHODS

Reagents and Materials

The aerial parts of R. ofûcinalis were collected from the Kandelus Mountain (Noshahr, Iran) in August 2010 and were dried in dark place at room temperature

Chemically reagents

Solvents such as n-tetra decane, n-pentadecane, n-hexadecane, n- heptadecane, n-octadecan and 1-phenyldecane were purchased from Merck Chemical Co.

HD–HSME of Essential Oil

The extraction and injection procedures were carried out using a 10 µl Hamilton gas-tight syringe, Model 1701N, with a ûxed bevelled-point needle. Thus, a 100 ml round-bottomed flask containing 1 g dried plant in 50 ml water was heated by a mantle. The Hamilton syringe was rinsed and primed at least seven times with the solvent/ standard solution. After uptake of 2 µl n-pentadecane containing n-octaadecane (as internal standard, 200 p.p.m.), the needle of the syringe was then inserted into the headspace of the plant sample. After extracting for a prescribed time at the top of

the boiling solution, the microdrop was retracted back into the syringe. Content of the syringe was injected into the GC for analysis. Finally, the analytical signal was calculated as the relative peak area of the analytes to the internal standard. We choose 1,8 cineole as analyte in this study.

Two methods (hot and cold) were investigated for HD–HSME. In the hot method, extraction was performed after 5 min of reûuxing at 100 °C on a mantle. In the cold method, the mixture was reûuxed for 5 min and the extraction was performed about 3 min after turning off the mantle, when the headspace temperature had reached 80 °C [17].

Hydrodistillation

For extraction of essential oils by hydrodistillation, 500 g dried plant sample was boiled in a Clevenger-type apparatus for 180 min. After cooling, the oil was collected by use of a syringe. Dry sodium sulfate was added to remove water from the oil, after vigorous shaking and filtration, the sample was transferred into a brown, capped bottle and stored in under refrigeration.

Instrumentation

GC–FID analyses of the oil were conducted using a Thermoquest-Finnigan instrument equipped with a DB-1 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 µm). Nitrogen was used as the carrier gas at the constant flow of 1.1 ml/min. The split ratio was 1:50. The oven temperature was raised from 60 °C to 250 °C at a rate of 5 °C/min. The injector and detector (FID) temperatures were kept at 250 °C and 280 °C, respectively. GC–MS analysis was carried out on a Thermoquest- Finnigan Trace GC–MS instrument equipped with the same column and temperature programming as described for GC. The transfer line temperature was 250 °C. Helium was used as the carrier gas at a ûow rate of 1.1 ml/min with a split ratio of 1:50. The constituents of the volatile oils were identiûed by calculation of their retention indices under temperature-programmed conditions for n-alkanes (C6–C24) and the oil on a DB-1 column under the same conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library (Wiley 7.0) or with authentic compounds, and confirmed by comparison of their retention indices with authentic compounds or with those reported in the literature[18-27]. Semi-quantitative data was obtained from FID area percentages without the use of correction factors.

RESULTS AND DISCUSSION

Choosing the most suitable extraction condition is very important for achieving good selectivity of target compound. For this purpose several parameters such as solvent type, time of extraction, time of condensation, sample weight and drop volume were optimized.

For the first step hydrodistillation instrumental part with headspace solvent microextraction (HD-HSME) part was combined. Two pathways were defined for this method: Hot & Cold. Experimental conditions for Hot include: solvent type, n-pentadecane; droplet volume, 2 µL; extraction time, 5 min; plant weight, 0.5 g and for cold pathway, condensation time 3 min. The components identified by hydrodistillation, together with cold HD–HSME methods (optimized flow), are presented in Table 2, compounds are listed in order of their elution from the DB-1 column. The results are comparable in hydrodistillation and the cold method. So as shown in Fig.1., the cold method was investigated and different parameters, such as extraction solvent, sample mass, microdrop volume, extraction and condensation time for this method, were optimized.

The ûnal choice of solvent was based on comparison between selectivity, extraction efficiency, incidence of drop loss and level of toxicity of solvents. The solvent should have an appropriate polarity to dissolve a variety of species. It should not evaporate substantially during the extraction procedure, and should have an appropriate retention time on the GC column to not interfere with the sample components. To reduce the risk of

Table 1: RSD value

Compound	Mean	RSD(%)
1,8-cineole	8.9	1.1

evaporation and avoid overlapping of the solvent with analyte peaks, only solvents with high boiling points were selected. Five solvents, n-teteradecane, n-pentadecane, n-hexadecane,

n-heptadecane, 1- phenyl decane containing n-octadecane as internal standard (200 ppm) were tested to find the solvent of choice for the extraction of essential oil of R. ofûcinalis using this technique (Figure 2). n-heptadecane gave the best extraction efficiency among other solvents and 1- phenyldecane showed low extraction efficiency and n-Octadecane was used as the internal standard (IS) to correct the variation in injection volumes. Peak area ratio of 1,8- cineole to IS was calculated as the analytical response.

The HD-HSME is not an exhaustive extraction method and complete equilibrium is not needed for accurate and precise analysis. But only when sufficient mass is transferred into the microdrop in an exact reproducible extraction time is adequate. Figure 3 shows an increase in extraction with sampling time in the range of 2-5 min, and decreasing after 5 min. This decrease can be attributed to the solvent evaporation and to the back-extraction from the microdrop into the headspace[25-27] .

The best time for essential oil constituents diffusion in to the drop were investigate with stop of heating and leaving the drop on the top of the sample. As shown in fig.4. an decrease from 5- 10

Table 2: Results of GC/MS analyses in HD and HD-HSME methods

Row	Compound	Index	HDArea %	HD-HSME Area %
1	Tricylene	927	0.6	-
2	α-pinene	940	46.5	47.9
3	camphene	950	13.7	12.9
4	3-octanene	963	1.7	1.7
5	β-pinene	975	2.0	2.3
6	myrcene	981	4.9	4.1
7	α-phellandrene	999	0.3	-
8	α-terpinene	1011	0.8	0.9
9	ρ-cymene	1014	0.5	0.8
10	1,8-cineole	1026	14.1	13.4
11	β-ocimene[2]	1035	0.1	-
12	γ-terpinene	1050	0.9	1.1
13	terpinolene	1081	1.8	1.3
14	linalool	1083	0.2	1.0
15	camphor	1128	2.7	3.1
16	verbenol	1132	0.5	-
17	neoisopulegol	1134	0.2	0.4
18	pinocamphene	1142	0.5	
19	borneol	1155	0.3	-
20	isopinocamphen	1157	0.3	-
21	terpinen-4-ol	1166	0.2	0.2
22	α-terpineol	1178	0.2	0.2
23	verbenone	1190	2.1	3.5
24	pulegone	1222	-	0.1
25	lilanylacetate	1240	0.1	-
26	bornylacetate	1276	1.9	2.2
27	geranylacetate	1350	0.1	-
28	β-caryophyllene	1429	0.2	0.4

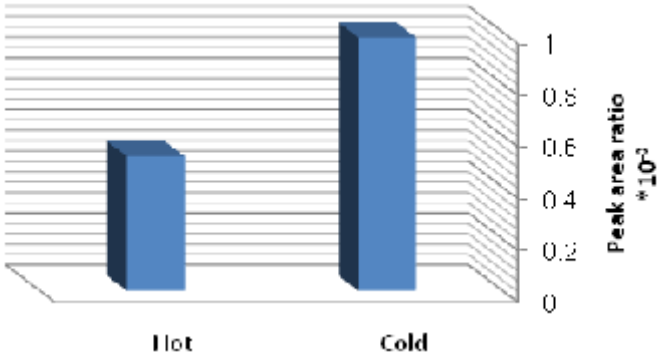

Fig. 1: Comparison of the relative peak heights of one pathway from Hot and Cold Experimental conditions: solvent, n-pentadecane; droplet volume, 2 µL; extraction time, 5 min; plant weight, 0.5 g; condesation time 3 min

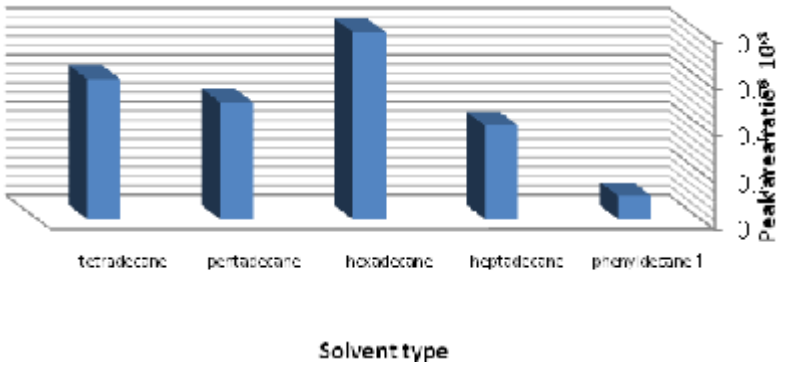

Fig. 2: Comparison of the relative peak heights of one major volatile component of rosemary. using different solvents for microextraction. Experimental conditions: droplet volume, 2 µL; extraction time, 5 min; plant weight, 0.5 g; conditioning time 5 min

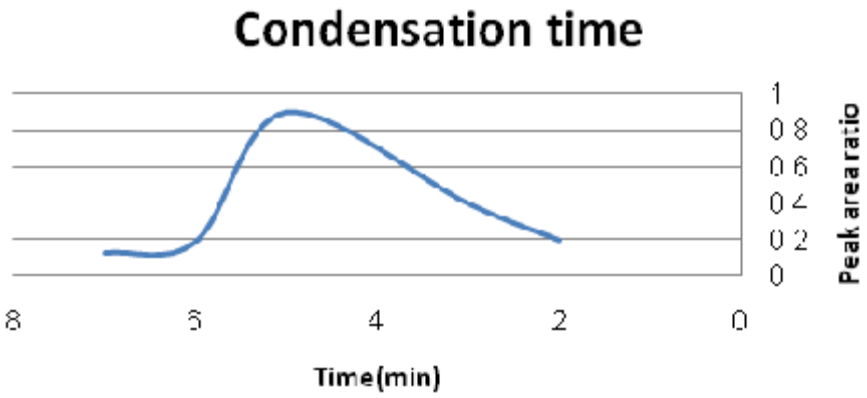

Fig. 3: Comparison of the relative peak heights of different time for condensation. Experimental conditions: solvent, n-hexane; droplet volume, 2 ìL; extraction time, 5 min; plant weight, 0.5 g;

min were seen, that probably because of condensation of volatile oils and coming back to liquid phase and also most increase peak area ratio was seen in 5 min.

The influence of organic solvent drop volume on HD-HSME optimization was investigated in the range of 1-3 µL under the following conditions: solvent, n-hexane; condensation time, 5 min; extraction time, 6 min; plant weight, 0.5 g.

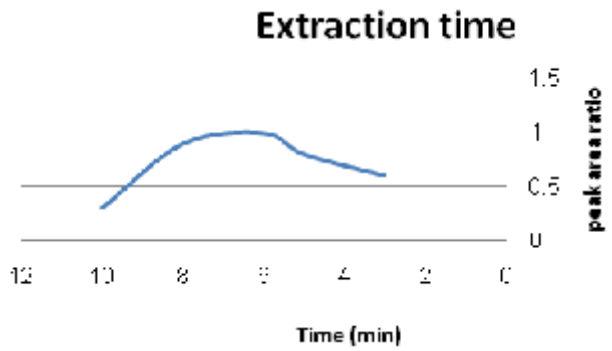

Fig. 4: Comparison of the relative peak heights of different time for extraction. Experimental conditions: solvent, n-hexane; droplet volume, 2 µL; condensation time, 5 min; plant weight, 0.5 g

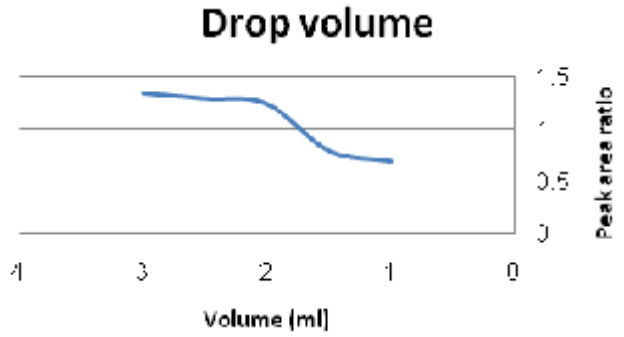

Fig. 5: Comparison of the relative peak heights of different volume for drop. Experimental conditions: solvent, n-hexane; condensation time, 5 min; plant weight, 0.5 g; extraction time, 6min

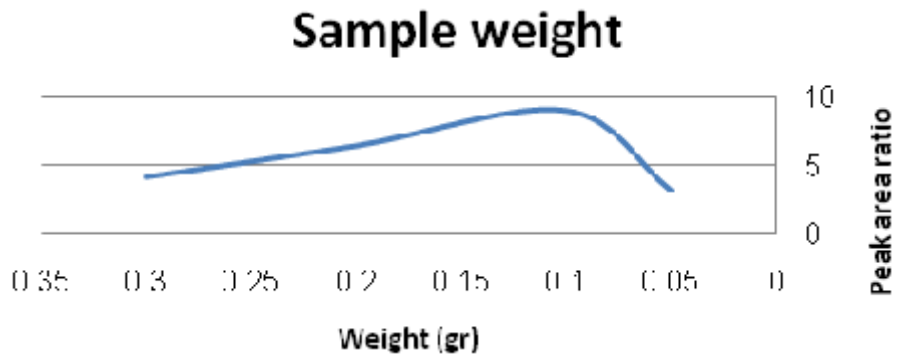

Fig. 6: Comparison of the relative peak heights of different sample weight. Experimental conditions: solvent, n-hexane; condensation time, 5 min; extraction time, 6min; drop volume, 2 µL

As shown in Figure 5, the peak areas for 1, 8-cineole in different drop volume, increased in the range of 1-3 µL. most increase belongs to 3 µL but we choose 2 µL for continue. The reason for this choice was probability of overweight and drop falling in further process.

The inûuence of sample weight is shown in Figure 6. The extracted amounts of 1,8-cineole increased continuously with increasing sample weight up to 0.1 g, then showed a variation after that. This observation can be explained by the fact that a microdrop has already been saturated with volatile compounds in the presence of 0.1 g of sample. After that, increasing the sample weight only enhances the volatile compounds concentration in the headspace, while the transference of mass into the microdrop remains constant and sometimes back-equilibrium was accrued. Hence the optimum sample weight was chosen at 0.1 g.

The experiment was repeated at optimization conditions and relative standard deviation was determined. The results are shown in table 1. As shown the RSD value is acceptable.

The extracted volatile oils by both methods were injected to GC/MS instrument and identiûcation of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library (Wiley 7.0) or with authentic compounds, and conûrmed by comparison of their retention indices with authentic compounds or with those reported in the literature.

The volatile components were studied both by HD-HSME and an ordinary hydrodistillation method and the main identified components (as shown in table 2.) were, α-pinene, 1,8-cineol, camphene and myrcene in both methods. The results were in good correlation in comparison with the hydrodistillation method. By HD-HSME twenty compounds were identified. α-Pinene (46.5%), 1, 8-cineole (13.4%), camphene (12.9%) and myrcene (4.1%) were found to be the major constituents.

CONCLUSION

In comparison with the conventional method and solvent microextraction methods, the main drawback of the proposed method is the limitation on the selection of extraction solvent because of overlapping of solvent peak with some analytes peaks. However, many solvents are available that have suitable melting points and could be used in this method. In addition, the use of selective detection systems such as GC/MS can decrease this limitation. However, the extraction of essential oils still is a problem from the point of view of duration and also amount of plant material needed. The most common method of extraction, hydro-distillation needs about 3-4 hours and tens to hundreds grams which in some situation is problematic, especially in cases that enough plant material is not accessible.

HD-HSME technique minimizes sample size and solvent usage, thereby reducing the supply costs, health and safety issues, and waste generated. Hanging drop based method is a simple, fast and easy sample enrichment technique. It can be concluded that HD-HSME is a novel sample preparation technique, which offers an attractive alternative to traditional and recently developed extraction techniques for the analysis of natural aromas.

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