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GC–MS Analysis of A *Helichrysum italicum* Hydrosol: Sensitivity, Repeatability and Reliability of Solvent Extraction versus Direct Hydrosol Analysis

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Abstract: Hydrosols have been increasingly used in the food industries as drinks and as ingredients for seasoning and flavoring. Research has shown they have antimicrobial, antioxidative and anti-inflammatory effects and therefore have potential as food preservative agents and sanitizing agents for tools used in the food industry. The composition analysis of hydrosols is commonly conducted using extraction with organic solvents. However, some hydrophilic compounds are lost with this procedure, leading to issues in identifying native composition and potential adulteration. In this paper, different methods for hydrosol analysis are studied. The methods differ in sample preparation, number of detected compounds, repeatability and reliability. Direct hydrosol analysis and 1:1 extraction with organic solvent were determined to be the most appropriate in our experiments. However, the method implementation depends on the application. Direct hydrosol analysis is simple and does not change ratios among compounds; its drawback is poor repeatability. Using 1:1 extraction has good repeatability; however, some hydrophilic compounds are lost, and adulterations with cosolvents and the addition of solubilizers cannot be discovered.

Keywords: hydrosol; GC-MS analysis; *Helichrysum italicum*; volatile compounds

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1. Introduction

Hydrosols or hydrolats have been used in the Mediterranean countries in refreshing drinks and as agents for seasoning and flavoring sweet and savory dishes. They have also been known as traditional remedies against, for example, dysmenorrhea, depression and chronic pain [1–5]. Therapeutic use still has to be proven by modern science, particularly in terms of effectiveness and safety. However, the popularity of natural remedies and a growing number of research showing positive effects on health make an average consumer even more prone to self-healing by consuming hydrosols [4–7].

Hydrosols on the European market are mostly sold as cosmetic ingredients or cosmetic products, and as such they are not intended for consumption. They can contain added ingredients, such as preservatives and cosolvents, which have not been shown to be safe for oral consumption. The additives are sometimes not reported in the ingredient list. Adulteration of hydrosols is also common [8,9]. Therefore, quality control of these products is of high importance. Considering this, it has to be emphasized that solvent extraction, as most commonly used sample preparation method for hydrosol analysis, fails to detect highly hydrophilic compounds.

Many studies have shown that hydrosols have antimicrobial, antioxidative and anti-inflammatory activities, making them highly interesting for the food industry [4,5,10–12]. There have been many studies showing their use as preservatives for fresh cut fruits and vegetables, as well as functional drinks. Hydrosols may inhibit tyrosinase, the main enzyme responsible for browning of fruits and vegetables [5,12–14]. They have also been shown to be appropriate for sanitizing tools, machinery and working surfaces used in food preparation process as they can be effective against biofilms [4,5,15–17].

Hydrosols are a coproduct in the production of essential oils by distillation. Their composition of volatile compounds is to some extent similar to essential oils, yet they contain more hydrophilic compounds. However, their analysis is complex, as the concentrations of volatiles are low [18]. The most common method of hydrosol analysis is gas chromatography coupled with mass spectrometry (GC–MS), which also allows researchers to identify the detected compounds.

Analytical methods can be carried out in different ways. Extraction of volatiles with an organic volatile solvent is most commonly performed [1,19–22]. After extraction, the concentration of volatile compounds in the analyzed sample is increased. The main disadvantage of organic solvent extraction is that the ratios between the detected compounds typically change in comparison to the original composition of a hydrosol due to the different partition coefficients between water and an organic solvent for each compound present in the hydrosol. A solid-phase microextraction (SPME) method can also be used to analyze the volatile compounds present in the gas phase above the hydrosol [23,24]. The disadvantages of the method are a low concentration of compounds in the volatile phase and different compositions of volatile and aqueous phases due to the different volatilities of the compounds present in a hydrosol. Direct hydrosol analysis is most rarely used. The reasons for this are poor compatibility of aqueous samples with GC columns and a low concentration of compounds in the hydrosol.

Helichrysum italicum is an up to 70 cm tall medicinal plant that belongs to the Asteraceae family. It is also called "everlasting" or "immortelle" because its inflorescences remain bright yellow after drying or the "curry plant" because of its characteristic, curry-like smell. *H. italicum* is traditionally widely used in European countries, particularly Italy, Spain, Portugal and Bosnia and Herzegovina. In these countries, the flowers and leaves of the plant are most commonly used in the treatment of health disorders such as allergies, colds, cough, skin, liver and gallbladder disorders, inflammation, infections and sleeplessness [25,26]. Increased interest in *H. italicum* has been noted recently, which is reflected in the growing body of evidence for the science-based medicinal use of the plant's preparations [27–35].

While the composition and effects of *H. italicum* essential oil are well studied, showing antimicrobial [25,36–39], anti-inflammatory [25,40], antioxidant [41] and anti-collagenase and anti-elastase activities [42], significantly less research has been done with *H. italicum* hydrosols. The most common compounds were shown to be alpha-terpineol, 3,5-dimethyloctane-4,6-dione, 2,4-dimethyl-heptane-3,5-dione, italidione I and II, linalool and nerol [43]. On the other hand, to the best of our knowledge, the biological effects of the hydrosol have not yet been investigated.

The purpose of our work is to identify the advantages and disadvantages of different methods of *H. italicum* hydrosol analysis with GC–MS. Emphasis is placed on qualitative analysis, repeatability, reliability and the presence of impurities due to sample handling. Conclusions can generally be applied to hydrosols of other plant sources since they have similar physicochemical properties.

2. Materials and Methods

2.1. Materials

The materials used in this study were as follows: hydrosol of *H. italicum* (Bonistra, Slovenia), n-hexane for GC–MS (SupraSolv, Merck, Darmstadt, Germany), NaCl (Carlo Erba Reagents, Val-de-Reuil, France), polypropylene centrifuge tubes (TPP, Trasadingen, Switzerland) and Water HPLC Gradient Grade (J.T. Baker, Phillipsburg, NJ, USA).

2.2. Extraction

Extractions of hydrosols were carried out in plastic and glass containers. The ratios of hydrosol:hexane used were 1:1 (vol:vol) and 10:1 (vol:vol). In some extractions, NaCl was added to the hydrosol phase at a concentration of 50 mg/mL, as this is the most commonly used concentration reported in the literature [18–20]. The mixtures of hydrosol and hexane

were shaken overnight at room temperature. All extractions were carried out three times, relative standard deviations were calculated and the results were averaged.

2.3. GC–MS Analyses

GC–MS analyses were performed using a GCMS-QP2010 Ultra system (Shimadzu Corporation, Kyoto, Japan) and data libraries NIST14 and FFNSC3 (Shimadzu Corporation, Kyoto, Japan). The GC system was equipped with a Rxi-5SilMS capillary column (length 30 m, internal diameter 0.25 mm, film thickness 0.25 µm, Restek, Bellefonte, PA, USA). The carrier gas was helium with a 1 mL/min constant column flow rate. The mass spectrometer ionization energy was 70 eV, the ion source temperature was 200 °C and the detector voltage was 1 kV. The injection volume of the samples was 1 µL, and the split ratio was 1:10 or 1:100. The injection port was set to 250 °C, and the interface temperature was 300 °C. In hexane samples, a 3.5 min solvent delay was used. A full scan was recorded in the mass range of 40–400 m/z with a scanning frequency of 5 Hz. The temperature program began at 40 °C, increased to 220 °C at 3 °C/min and was held at 220 °C for 15 min (total analysis time was 75 min).

2.4. GC–MS Data Processing

The list of detected compounds and their peak areas and area % were used for further analysis of the results and comparisons between methods. Peaks that appeared in blank samples (HPLC water, hexane extract of HPLC water, hexane) and peaks that did not appear in all three repetitions were removed from the list of compounds. The remaining compounds were then renormalized to the sum area of 100%. The percentage of area under the peak is not equal to the percentage of content, as the compounds may give different responses at the same concentration. However, it is a useful estimate of compound content, and it is also very suitable for our purpose to compare different methods of analysis.

3. Results and Discussion

GC–MS is a very sensitive technique that allows for the detection of compounds in the ppm range. This level of sensitivity allows for the detection of active pharmaceutical ingredients in wastewater [44,45] and other active compounds in environmental matrices [46]. It is therefore often difficult to determine where the detected compounds come from, as they may be compounds from the containers, the environment or impurities in the reagents used. To minimize the influence of solvent impurities, the solvents used for sample preparation must be of sufficiently high purity and marked to be used for GC–MS analysis, and samples must be handled in glass containers.

The results are shown in Tables 1–3 and Figure 1, representative raw GC-MS chromatograms are added as Figures S1–S9. In Table 1, all detected compounds are listed with area % detected by different methods. The number of compounds detected and their area % varies greatly in different methods; however, the compounds that are detected in area % larger than 5% were generally detected by all methods (except when using plastic containers). The area % of compounds varied among methods, mainly due to different total concentrations of detected compounds.

Table 1. Hydrosol compounds detected by different methods of analysis, their retention times and area % obtained. At the bottom are peak area ratios among eucalyptol, terpinen-4-ol, and alpha-terpineol.

		Method Used: Hydrosol or Extraction and Split Ratio Applied *								
t_{ret} [min]	Name	Hydr Direct S100	Hydr Direct S10	E 1:1 Plastic S100	E 1:1 Glass S100	E 1:1 Glass S10	E 1:1 Glass S S100	E 1:1 Glass S S10	E 10:1 Glass S100	E 10:1 Glass S S100
2.2	Heptane-2-one		1.52							
3.7	Pentan-3-one	3.44	2.00	2.84	2.79	1.62	3.39	1.95	0.63	0.75
3.7	Pentanol, <3->					0.15		0.21		

Table 1. *Cont.*

		Method Used: Hydrosol or Extraction and Split Ratio Applied *								
t_{ret} [min]	Name	Hydr Direct S100	Hydr Direct S10	E 1:1 Plastic S100	E 1:1 Glass S100	E 1:1 Glass S10	E 1:1 Glass S S100	E 1:1 Glass S S10	E 10:1 Glass S100	E 10:1 Glass S S100
4.3	Isopentyl alcohol					0.06		0.09		
4.4	Carbinol <sec-butyl->		2.07			0.58		0.83	0.09	0.15
4.6	Isopropyl ethyl ketone		1.08	3.17	1.62	1.18	1.86	1.30	0.85	0.92
5.1	3-Hexanol					0.29		0.34	0.10	0.14
6.9	4,6-Dimethyloctane-3,5-dione		0.61			0.73		0.74	0.77	0.73
7.5	Hex-(3Z)-enol					0.35		0.45		
8.0	Hexanol <n->					0.19		0.21		
8.3	3-Heptanol					0.21		0.23	0.14	0.17
11.2	Pentanoic acid, 2-hydroxy-, methyl ester					0.21		0.25		
11.6	Heptan-2-ol<6-methyl-					0.25		0.27		
11.7	3-Octanone					0.43		0.45		
12.8	Hept-5-en-2-one <6-methyl->					0.15			0.13	
13.6	3-Octanone, 2-methyl-					0.08				
15.0	Eucalyptol	55.49	31.52	62.32	53.05	34.70	54.64	34.63	42.35	39.51
16.9	Linalool oxide <cis->					0.46		0.46	0.16	0.24
17.6	Linalool oxide <trans->					0.29		0.29		0.12
17.9	Undecane <n->		2.45		2.04	2.20		2.28	1.16	1.41
18.3	Linalool	10.73	8.38		10.67	10.46	9.82	10.19	11.16	11.03
19.2	Fenchyl alcohol					0.27		0.26	0.27	0.30
19.6	Unknown 19.6					0.25		0.24	0.12	0.14
19.8	2,4-Diacetoxypentane or trans-4-(Hydroxymethyl)cyclohexanecarboxylic acid, O-acetyl					0.15		0.20		
20.2	Pinocarveol <trans->					0.25		0.25	0.27	0.26
20.5	Camphor					0.68		0.67	0.73	0.72
20.6	3,5-Heptanedione, 2,2,6-trimethyl-					0.16		0.16	0.24	0.23
20.8	Nerol oxide					0.20		0.18	0.22	0.21
21.7	Terpineol <delta->					0.37		0.42	0.26	0.32
21.7	Borneol		0.72			1.20		1.19	1.03	1.15
22.2	Terpinen-4-ol	14.93	13.57	15.09	14.47	14.41	14.53	14.67	14.14	14.64
22.5	Cymen-8-ol <para->					0.21		0.22		
22.6	Hex-(3Z)-enyl butyrate		0.46							
22.9	Terpineol <alpha->	15.42	14.23	16.58	15.36	14.07	15.76	14.51	12.92	14.38
23.2	Unknown 23.2					0.22		0.21		0.16
23.4	Unknown 23.4					0.30		0.27	0.27	0.28
23.7	Unknown 23.7					0.37		0.36	0.31	0.36
24.0	Carveol <trans->					0.08				
24.2	Nerol		1.66			2.12		1.95	1.94	1.96
24.3	4,6-Dimethyloctane-3,5-dione					0.90		0.85	0.96	1.06
24.5	Cyclopropane, trimethyl(2-methyl-1-propenylidene)- or Phenol <3-isopropyl-> or (1S,2R,5R)-2-(2-Hydroxypropan-2-yl)-5-methylcyclohexanol		3.65			0.22				
25.4	Geraniol					0.23		0.22	0.18	0.19
27.9	Guaiacol <4-vinyl->					0.30		0.29		
28.0	Unknown 28.0		1.18							
28.2	cis-Terpin hydrate		2.23							
29.8	Terpinyl acetate <alpha->					0.12		0.09		0.11
30.0	Eugenol		4.67			3.06		2.92	2.61	2.84
33.5	8-Decene-3,5-dione, 4,6,9-trimethyl-		4.42			3.05		2.74	3.42	3.12

Table 1. *Cont.*

		Method Used: Hydrosol or Extraction and Split Ratio Applied *								
t_{ret} [min]	Name	Hydr Direct S100	Hydr Direct S10	E 1:1 Plastic S100	E 1:1 Glass S100	E 1:1 Glass S10	E 1:1 Glass S S100	E 1:1 Glass S S10	E 10:1 Glass S100	E 10:1 Glass S S100
35.2	8-Decene-3,5-dione, 2,4,6,9-tetramethyl-		0.87			0.48		0.43	0.53	0.48
35.4	8-Decene-3,5-dione, 2,4,6,9-tetramethyl-		1.03			0.61		0.55	0.76	0.68
38.2	Elemicin		1.71			0.99		0.97	1.13	1.13
40.8	Rosifoliol or 2-Naphthalenemethanol, 2,3,4,4a,5,6,7,8-octahydro-.alpha.alpha.,4a,8-tetramethyl-, [2R-(2.alpha.,4a.beta.,8.beta.)]-					0.13			0.15	0.11
Peak area ratios:eucalyptol/terpinen-4-ol/alpha-terpineol		3.7/1/1	2.3/1/1	4.1/1/1.1	3.7/1/1.1	2.5/1/1	3.8/1/1.1	2.4/1/1	3.0/1/0.9	2.7/1/1

* Method used: Hydr direct—direct hydrosol analysis, E 1:1 plastic—extraction vol:vol ratio was 1:1 carried out in plastic container, E 1:1 glass—extraction vol:vol ratio was 1:1 carried out in glass container without NaCl added, E 1:1 glass S—extraction vol:vol ratio was 1:1 carried out in glass container with NaCl added, E 10:1 glass—extraction vol:vol ratio was 10:1 carried out in glass container without NaCl added, E 10:1 glass S—extraction vol:vol ratio was 10:1 carried out in glass container with NaCl added; S10—split 10 was applied, S100—split 100 was applied.

Table 2. Main characteristics of the methods used, that is, the number of detected compounds, maximal standard deviation of peak areas and area % for three repetitions of the same method, time consumption of sample preparation, and amount of sample used for analysis.

	Method Used: Hydrosol or Extraction and Split Ratio Applied *								
	Hydr Direct S100	Hydr Direct S10	E 1:1 Plastic S100	E 1:1 Glass S100	E 1:1 Glass S10	E 1:1 Glass NaCl S100	E 1:1 Glass NaCl S10	E 10:1 Glass S100	E 10:1 Glass NaCl S100
Number of compounds	5	21	5	7	48	6	43	32	34
Maximal area RSD	10%	46%	11%	8%	20%	10%	14%	41%	33%
Maximal area % RSD	6%	45%	10%	12%	20%	6%	15%	29%	20%
Sample preparation	simple	simple	long	long	long	long	long	long	long
Amount of sample	small	small	medium	medium	medium	medium	medium	large	large

* Method used: Hydr direct—direct hydrosol analysis, E 1:1 plastic—extraction vol:vol ratio was 1:1 carried out in plastic container, E 1:1 glass—extraction vol:vol ratio was 1:1 carried out in glass container; NaCl—NaCl was added to hydrosol before extraction; S10—split 10 was applied, S100—split 100 was applied.

Table 3. Comparison of ratios of peak areas after the second and the first extraction and some physical properties (GC–MS retention time, partition coefficient, boiling point, and topological polar surface) of three compounds found in *H. italicum* hydrosol. Physical properties were obtained from pubchem.ncbi.nlm.nih.gov.

t_{ret}. [min]	Compound Name	* E1:1 Glass S10	* E1:1 Glass S S10	* E10:1 Glass S100	* E10:1 Glass S S100	logP	T_{boil} [°C]	c_{water} [g/L]	Polar S [Å^2]
3.7	Pentan-3-one	0.17	0.12	0.55	0.53	0.99	102	47	17.1
15.0	Eucalyptol	0.01	0.01	0.08	0.08	2.74	176	3.5	9.2
22.9	α-Terpineol	0.03	0.02	0.23	0.16	2.98	219	7.1	20.2

* Ratios between peak areas in the second extraction to peak areas in the first extraction using the following method: E 1:1 glass—extraction vol:vol ratio was 1:1 carried out in glass container without NaCl added, E 1:1 glass S—extraction vol:vol ratio was 1:1 carried out in glass container with NaCl added, E 10:1 glass—extraction vol:vol ratio was 10:1 carried out in glass container without NaCl added, E 10:1 glass S—extraction vol:vol ratio was 10:1 carried out in glass container with NaCl added; S10—split 10 was applied, S100—split 100 was applied.

In Table 2, the main characteristics of the methods used are presented, that is, the number of detected compounds, maximal standard deviation of peak areas and area % for three repetitions of the same method, duration of sample preparation, and amount of a sample used. Peak area is an important feature for determining the compound's absolute concentration, while area % is commonly used to estimate the ratios among compounds present. Choosing the best method is not a straightforward procedure, as

there are many aspects that have to be considered. In our comparison of the evaluated methods, we will discuss the simplicity of the method, number of identified compounds, relative standard deviation of peak area and peak area %, and the smallest concentration of identified compounds.

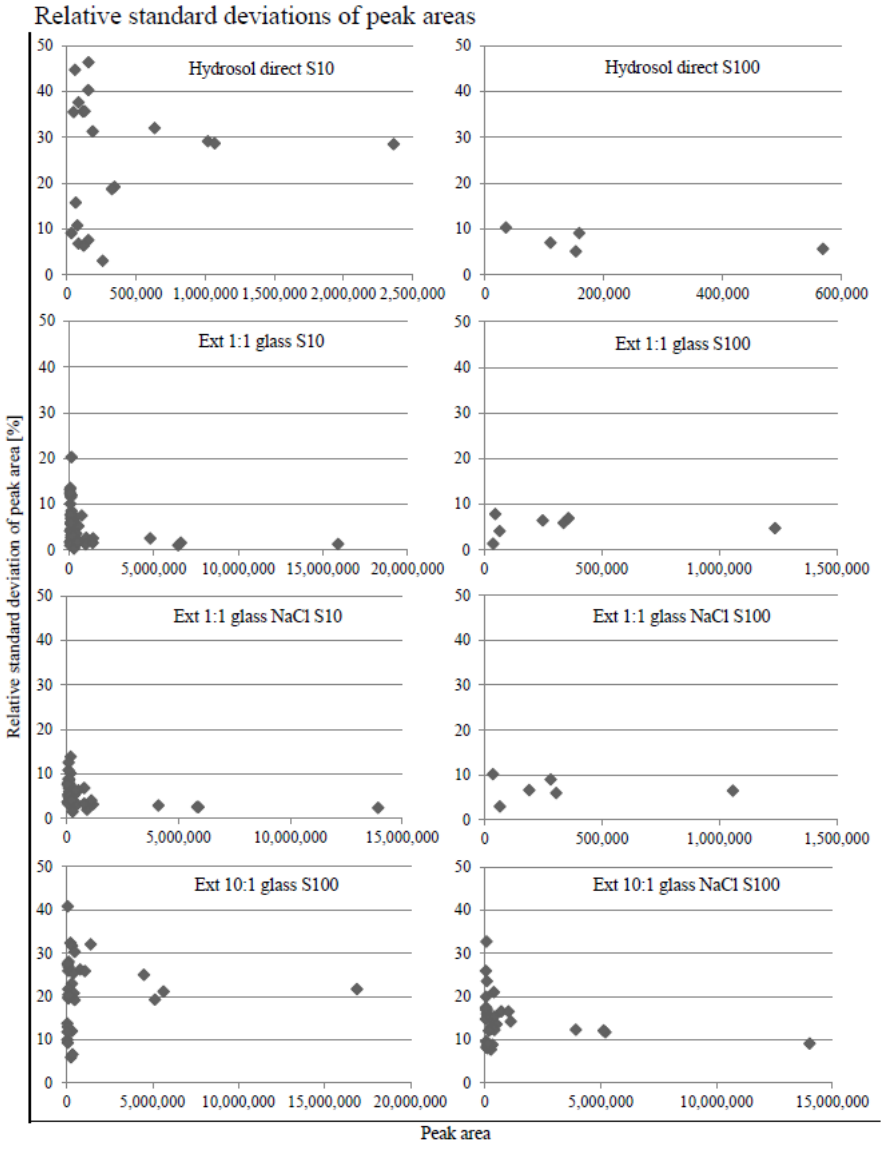

Figure 1. Relative standard deviation of peak areas (method used: Ext 1:1 glass—extraction vol:vol ratio was 1:1 carried out in glass container without NaCl added, Ext 1:1 glass NaCl—extraction vol:vol ratio was 1:1 carried out in glass container with NaCl added, Ext 10:1 glass—extraction vol:vol ratio was 10:1 carried out in glass container without NaCl added, Ext 10:1 glass NaCl—extraction vol:vol ratio was 10:1 carried out in glass container with NaCl added; S10—split 10 was applied, S100—split 100 was applied.).

Ratios among the main three compounds detected in each hydrosol sample in each analysis (eucalyptol, terpinen-4-ol, and alpha terpineol) were calculated (end of Table 1). The ratios are comparable between a direct hydrosol and 1:1 extraction analysis. However, there is a difference between using split 10 or split 100. This is probably due to the smaller detection limit in the split 100 mode. The consequence is a smaller relative decrease in the larger peak areas. A change in ratios is also observed in the 10:1 extraction, the reason for which is probably the higher concentrations of compounds in the hexane phase. Ratios observed in extraction in plastic containers are not reliable since there were many peaks

present from plastic that could change hydrosol peak areas. Extract from plastic containers produced over 300 peaks that were confirmed by blank samples of only hexane shaking overnight in plastic containers.

3.1. Method Simplicity

The simplest of the methods used is by far direct hydrosol analysis. There was no sample pre-preparation, and no organic solvents were used. Sample preparation using extraction is longer, since it takes time to prepare the mixture of sample and solvent, and the mixture has to be shaken for some time. We used overnight shaking to assure an equilibrium. However, in additional experiments, we concluded that the shaking time can be shorter, approximately 3 h (results not shown). Additionally, very little sample was used by direct hydrosol analysis; the volume of injected hydrosol was 1 µL. In hydrosol extraction, more sample is used, since extraction is difficult to carry out with very small volumes. About 1 mL of hydrosol is used for the 1:1 extraction (a larger volume can be used to make the solvent separation easier), and 10 times more sample is used for the 10:1 extraction. In the published literature, adding sodium chloride is often reported [18–20]. In our experiment, sodium chloride did not prove to be of much benefit, as it did not affect the area % significantly, while it decreased the number of detected compounds in the 1:1 extraction. We also compared extraction ratios of 1:1 and 10:1 (vol:vol, hydrosol:solvent). By using a 1:1 ratio, less sample was used. In conclusion, a direct hydrosol analysis is simplest; however, if extraction is used, the most convenient method is using a volume ratio of hydrosol to solvent of 1:1 with no sodium chloride, it uses less sample and gives good results.

3.2. Number of Detected Compounds (Method Sensitivity)

The method that has the highest sensitivity detects the largest number of compounds. The number of compounds that were detected with each method is presented in Table 2, and the compounds are listed in Table 1. The highest number of compounds identified was 48. These were obtained by the extraction method using a volume ratio of 1:1 and split 10 in the GC–MS program. When using the same method and adding sodium chloride, the number of identified compounds was slightly lower, at 43 compounds. Another method that identified many compounds involved using a volume extraction ratio of 10:1 and split 100; 32 compounds were identified, and when adding sodium chloride, the number of compounds identified was 34. The number of compounds identified in this extract would be higher if split 10 was used; however, the intensity of the largest peaks could harm the detector. When analyzing hydrosol directly, 21 compounds were identified by using split 10, and 5 compounds were identified using split 100. When analyzing the 1:1 extract with split 100, only a small number of compounds were identified; that is, 7 and 6 compounds, without and with sodium chloride, respectively.

3.3. Relative Standard Deviation

In all analysis cases, we noticed that the relative standard deviation had an inverse correlation to the peak area (Figure 1) and therefore also to the peak area %. The relative standard deviation is larger in compounds that are present in lower amounts.

The maximal relative standard deviations for the different methods are presented in Table 2. It was observed in our study that adding sodium chloride lowers the relative standard deviation of the peak area in extracted samples. The relative standard deviation of the peak area was lowest in the 1:1 extracted samples using split 100, where the maximal RSD was 8%. When split 10 was used, the maximal RSD was 20% with no sodium chloride used and 14% with sodium chloride. In the 10:1 extracted samples, the maximal RSD was 41% with no sodium chloride used and 33% with sodium chloride.

The standard deviation was the worst in direct hydrosol analysis using split 10, where the maximal RSD was 46%. When split 100 was used, the maximal RSD was 10%. In general, water samples have higher RSDs, and using split 10 gives higher RSDs compared

to split 100; however, in extracted samples, this is only true for compounds that are present in low concentrations (Figure 1). One of the possible reasons of higher RSDs for water samples compared to the hexane extracted samples is the difference in expansion of the sample when gasified. Water has higher density and lower molar mass compared to hexane; therefore, it expands more when evaporated. Consequently, it behaves differently in the GC system.

In conclusion, 1:1 extraction and using split 10 in GC–MS program gives the best results in terms of relative standard deviation. Relative standard deviation is comparable with a split 100 method in compounds that are detected with both methods, which is under 8% (Figure 1).

3.4. Smallest Area % of Detected Compounds

In terms of compound area %, all methods are good, since in all methods, compounds with area % larger than 3% were detected (Table 1). Methods that detected compounds with area % lower than 0.1 are the 1:1 extraction analyzed with split 10 and 10:1 extraction analyzed with split 100. A direct hydrosol analysis detected compounds at 0.4% when split 10 was used and at 3% when split 100 was used.

3.5. Extraction Efficiency

The efficiency of extraction from a hydrosol into the organic phase is an important parameter in assessing the applicability of the method. If the extraction does not have a high yield, it is difficult to estimate the content of volatile compounds in a hydrosol based on extract analysis. We also wanted to test the hypothesis of whether the ratios among compounds change with extraction. After the first extraction, the hydrosol was re-extracted, and the areas under the peaks in the first and second extractions were compared. This was done for three compounds that occur in all samples; the results are presented in Table 3. We found that the extraction efficiency was poorer for pentan-3-one than for eucalyptol and alpha-terpineol. At an extraction ratio of 1:1, the peak area of the second extract for pentan-3-one was 17% of the peak area of the first extract, with the addition of NaCl improving the extraction yield to 12%. The extraction yield was better for eucalyptol and alpha terpineol, where the peak area of the second extract was less than 3% of the peak area of the first extract. At an extraction ratio of 10:1, the extraction efficiency was much poorer.

Table 3 also shows some physical properties of the three compounds. Pentan-3-one has a shorter retention time, smaller log P, lower boiling point, higher water solubility, and higher topological polar surface. This explains its higher affinity for the aqueous phase and thus poorer extraction into hexane. Eucalyptol and alpha terpineol have similar retention times and logP, and the extraction efficiency differs slightly in the opposite direction than expected. This result can be explained by other physical properties. The boiling point would indicate better extraction efficiency for alpha-terpineol, and the water solubility and the topological polar surface explain why the extraction efficiency is lower for alpha terpineol compared to eucalyptol.

These results are important to understand the limitations of analyzing the extracts of a hydrosol. Extraction has different yields for different compounds, and the yields can differ significantly in some experimental setups, so the ratios among compounds can change substantially. However, if the aim of analysis is quantification of a compound for which the extraction yield is high, the extraction method is a good choice. An important drawback is also that highly water-soluble compounds cannot be detected. In this case, adulteration of a hydrosol with such compounds cannot be detected.

4. Conclusions

The main compounds of *H. italicum* hydrosol showed to be eucalyptol, alpha terpineol, terpinen-4-ol, linalool, eugenol, and 4,6,9-trimethyldec-8-ene-3,5-dione8-decene-3,5-dione,4,6,9-trimethyl. Some of these compounds were also found in *H. italicum* essential oil but not all [13,22].

In our experimental work, the best methods for hydrosol analysis were direct hydrosol analysis using split 10 and 1:1 extraction using split 10. The first of the two methods is simple, does not use organic solvents, and detects compounds with area % higher than 0.4. It allows for the identification of hydrophilic compounds that are not extracted into the organic solvent phase, and true ratios among compounds are obtained. It also enables the detection of added cosolvents (glycerol, surfactants). The main drawback of this method is its poor repeatability. Additionally, a problem can arise if the hydrosol is too diluted. However, in our experience, most hydrosols are suitable for the described method of analysis. If we are interested in compounds that are present in lower concentrations, direct hydrosol analysis should be supplemented by a 10:1 extraction. By using 1:1 extraction, the repeatability improves greatly; however, a slight change in the composition of volatile compounds occurs due to different extraction efficiencies for different compounds. The main drawbacks of 1:1 extraction are the length of sample preparation, that more sample is used, and the use of an organic solvent. It is also very important that extractions are carried out in glass containers. Additionally, an important drawback of extraction with organic solvents is that it does not detect adulteration of hydrosols with highly polar compounds. These are in some cases not stated in the ingredients list and can present harm if consumed.

Overall direct hydrosol analysis should be used for detecting adulteration with hydrophilic compounds and when an exact ratio among compounds is needed. The extraction method is better for detecting compounds in smaller concentrations and for quantitative analysis of compounds that have good extraction yield to organic solvent.

Conclusions can generally be applied to hydrosols of other plant sources, as there are many compounds that are common (terpenes and terpenoids) and are therefore expected to behave similar under these experimental conditions.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app121910040/s1. Raw GC-MS chromatograms of nine analysis presented methods.

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