



Phytochemical, Antioxidant and Antimicrobial Parameters of Essential Oils and Hydrosols of Colombian Thyme and Rosemary Obtained Using Two Different Steam Distillation Methods

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Abstract

Two procedures of steam distillation of *Rosmarinus officinalis* and *Thymus vulgaris* were compared by phytochemical, antioxidant and antimicrobial analysis of their respective essential oils and hydrosols using respectively GC/FID and GC/MS analysis, free radical scavenging activity on ABTS and dilution and diffusion method against 3 bacteria and 2 fungi. Differences in bioactivity results are discussed taking into account changes in molecular content of essential oils and hydrosols obtained by the two different distillation methods. Although no significant difference was observed between the two procedures with *Rosmarinus officinalis*, that was not the case with *Thymus vulgaris*. Significant differences were noted, not only in their respective chemical composition especially with the thymol and p-cymene percentage with respectively a change from 39% with conventional steam distillation to 27% with adapted steam distillation for thymol and from 28% with the first distillation to 37% with the second one for p-cymene. These phytochemical composition changes have an impact on their biological activities results and consequently on the therapeutic activities expected which shows the importance of distillation method selection.

Keywords: *Rosmarinus officinalis*, *Thymus vulgaris*, Essential oils, Hydrosols, Antioxidant, Antibacterial.



Introduction

Essential oils (EOs) are aromatic and volatile liquids extracted from plant material using different methods. One of these methods is steam distillation in which an aqueous phase called hydrosol (HD) is obtained. Unlike EOs, there has been few research about HDs.

Seasons,^{1,2} climate,³ photoperiodism,³ geographical situation,⁴⁻⁶ phenological stages⁷ and other ecosystemical factors, and distillation methods, used to obtain EO, have all been shown to impact on chemical composition, as described by Peng et al.⁸ and Sefidkon et al.⁹ These chemical compositions changes have consequences on biological activities.

Besides, the comparison of extraction methods enables important technological decisions in the industry regard-

ing different aspects such as cost, efficacy and security. Steam distillation is one of the oldest extraction processes of EOs. The hot steam produced from water, breaks the structure of plant cells, releasing the volatile molecules from the cellulosic substrate. The steam charged with aromatic molecules is condensed through a serpentine condenser, which is located in an enclosure, which is cooled using a cold-water stream. Finally the EO is obtained generally in the upper phase and the HD in the lower phase. This last one corresponds to condensed steam.

Two common European aromatic plants, *Thymus vulgaris* (Thyme) and *R. officinalis* (Rosemary), were selected because of their antimicrobial and antioxidant activity, which has been reported by various researchers, among them, Pasqua and Feo,¹⁰ Snoussi et al.,¹¹ Kumar et al.,¹²



Imelouane et al,¹³ Hajlaoui et al,¹⁴ Zaouali et al,¹⁵ Miladi et al.¹⁶ The research of these authors was used to compare the results obtained in this study. HDs have not been researched as extensively as EOs and their use in industry is much less important in spite of their important indirect production. However, HDs could offer many benefits for agriculture as input or for human health as proposed by Bosson¹⁷; who specifies different indications for HD uses. The author states that Thyme HD chemotype thymol can be used to detoxify and purify the organism. Thyme EO chemotype thymol is indicated to treat infectious pathologies and weariness. With a high p-cymene concentration, this EO is indicated for treating rheumatism and arthrosis. Rosemary EO chemotype camphor is used to treat myalgia, muscular cramps, rheumatism, hypertension, and hepatomegaly as well as heart weakness.¹⁸ This work compares the EOs and HDs obtained through the conventional distillation method with those resulting from an adapted steam distillation method developed for 100% natural phytotherapeutic products elaboration.

Materials and Methods

Plant Material

Six batches of aerial parts of *Thymus vulgaris* (Thyme) and the leaves and flowers of *Rosmarinus officinalis* (Rosemary) were collected from the Farmaverde cooperative farm located in Usme, in the south of Bogota, Colombia. Plant material was collected during blooming, early in the morning, in sunny weather, and was naturally dried in the shade on shelves. Six batches of 71 kg of fresh Thyme were collected and 21 kg of dried material were obtained from each batch (TvDC1, TvDC2 and TvDC3 for the conventional distillation and TvDF1, TvDF2 and TvDF3 for the adapted distillation) used for distillation. Each batch is differenced after with “EO” or “HD” depending if it con-

cerns EO or HD from each batch. Eight kilograms were obtained from three batches of 27 kg of fresh Thyme, dried and macerated as subsequently explained. Six batches of 48.5 kg of fresh Rosemary material were collected; 28.5 kg of dried material were obtained from each batch for distillation (RoDC1, RoDC2 and RoDC3 for the conventional distillation and RoDF1, RoDF2 and RoDF3 for the adapted distillation). Three batches of 12 kg of the same fresh vegetal material was dried to obtain the three batches of 7 kg used for the maceration.

Distillations

Distillations were performed in the FARMAVERDE cooperative (Bogota, Colombia) which lent us his equipment. Three batches were distilled using the conventional steam distillation procedure and three by using the adapted steam distillation. In the latter method a macerate obtained from a cold and hot maceration (until boiling) of a part of vegetal material was used as the steam source for a posterior distillation with new vegetal material. The 2 procedures are described in Figure 1. In the adapted steam distillation, macerations of Thyme (8 kg) and Rosemary (7 kg) were prepared using 70 L of distilled water in anaerobic conditions during 24 hours at 20°C and then heated to boil; they were naturally cooled and filtered. The macerate was used as the steam source for the distillation. For each distillation, the first 13.5 L of HD was recovered, and EO were dried using anhydrous sodium sulfate (Na₂SO₄). Both EOs and HDs were kept refrigerated at 4°C until their evaluation.

Test Microorganisms and Medias

All the microbial strains were obtained from the strain collection at the Pontificia Universidad Javeriana (CM-PUJ), Bogotá, Colombia.

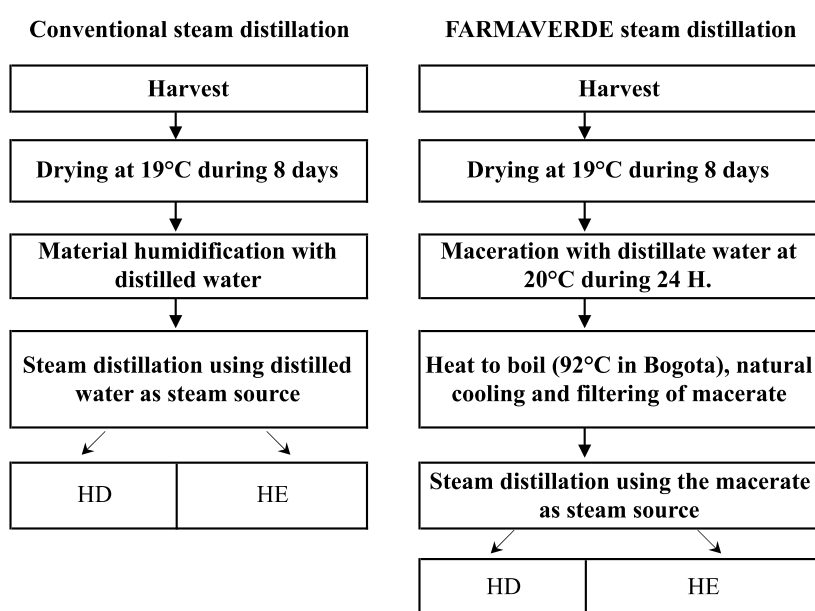


Figure 1. Conventional and Adapted Distillation Methods.

Bacteria

Escherichia coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 25923 were used. All the cultures were kept at -70°C in 20% glycerol in Tryptic soy broth (TSB), and grown at 37°C for 24 hours to obtain the sub-cultures, which were inoculated on Tryptic soy agar (TSA) at 37°C for 24 hours. Working cultures were prepared from sub-cultures in Mueller-Hinton broth at 37°C for 4 hours and adjusted to a concentration of $1.5 \cdot 10^8$ UFC/ml using 0.5 Mac Farland standard (Biomérieux Inc.).

Fungi

Candida albicans CMPUJH022 was maintained at -70°C in 20% glycerol in Oxoid malt extract broth (Basingstoke, Hampshire, England) and grown in Malt extract at 37°C for 24 hours to obtain the sub-cultures inoculated on Sabouraud agar (Merck, Germany) at 37°C for 24 hours. Working cultures were prepared from subcultures on Sabouraud agar at 37°C for 4 hours, and adjusted to a concentration of $1.5 \cdot 10^8$ UFC/ml using 0.5 Mac Farland standard (Biomérieux Inc., Craponne, France).

Aspergillus niger ATCC 16404 was grown on Sabouraud agar for 5 days at 25°C. Conidiospores were recovered from this culture using 2 ml of sterile saline solution, which was aliquoted by 100 µl for later use. To obtain monospore cultures, 100 µl of conidiospores solution was massively seeded on Sabouraud agar, then, filter paper discs (5 mm) were placed on the agar and the culture was incubated for 5 days at 25°C.

Gas Chromatography (GC-FID)

The GC-FID analyses of EOs were carried out on a 3900 gas chromatograph (Varian, Holland), equipped with a flame ionization detector. The column used was an Elite 5 MS column (30 m × 0.25 mm × 0.25 µm) (Perkin Elmer, Holland). Helium carrier gas was used with a flow rate of 1 mL/min. The oven temperature program was initially at 50°C for 2 minutes, rate of 4°C/min up to 160°C for 5 minutes, rate of 8°C/min up to 220°C for 2 minutes, rate of 15°C/min up to 280°C for 5 minutes. Injector and flame ionization detector temperatures were 250°C and 290°C respectively. 1 µL of each EO was injected with a split ratio of 1:200.

Mass Spectrometry Analysis (GC-MS)

GC-MS analyses were carried out with an Agilent Technologies-6850 Series II gas chromatograph (GC) with a DB-5MS (5% phenyl methylpolysiloxane) column (60 m × 0.25 mm × 0.25 µm) (Agilent Tech., USA) and an HP-INNOWAX column (60 m × 0.25 mm × 0.25 µm) (Agilent Tech., USA), equipped with an Agilent 5975B mass selective (MS) detector used in the full scan mode in order to monitor mass unit from 30 to 500 m/z with an electronic impact of 70 eV. Helium carrier gas was used with a flow rate of 1 mL/min. The injector and detector

temperature were respectively 250°C and 230°C. The quadrupole temperature was 150°C. The DB-5MS oven temperature program with DB-5MS column was 120°C during the first minute, then, 5°C/min until 250°C for 5 minutes, 30°C/min until 320 for 5 minutes and the last 5 minutes at 300°C. The oven temperature program with HP-INNOWAX column was 60°C during the first minute, then, 6°C/min until 220°C; the last 3 minutes at 220°C. One microliter of each EO was injected with a split ratio of 1:200 and HD preparation for injection was explained previously.

Simultaneous extraction and concentration of compounds from the vapor phase of the HDs was carried out using the Head Space solid-phase microextraction (HS-SPME) technique, and a fused silica fiber coated with 65 µm thick PDMS/DVB acquired from Supelco (Bellefonte, PA, USA). Chromatographic analysis was performed with the same chromatograph, columns and conditions used previously, except the injection, which was performed in splitless mode using the SPME device (Bellefonte, PA, USA) for injection.

Identification of EO and HD constituents was conducted by comparing the mass spectra with those reported in the NIST (2005) library and the retention index relative to n-alkanes with those reported by Adams¹⁹ as well as other literature data.

ABTS Free Radical-Scavenging Activity

The antioxidant activity of EO and HD was assessed by evaluating the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical scavenging activity, as reported by Re et al.²⁰ The ABTS radical obtained from Sigma-Aldrich (St. Louis, MO, USA) was prepared by the reaction of 20 mg/l ABTS mixed with 2.5 mg/l potassium persulfate ($K_2S_2O_8$) (Sigma-Aldrich, St. Louis, MO, USA) in deionized water kept in darkness for 16 hours at room temperature. Its absorbance was adjusted to 0.73 at 740 nm. First, 6 serial 2-fold dilutions were prepared for each sample to define their optimal concentration ranges and continue with 4 dilutions ranges. These dilutions were prepared using ethanol and 10 µl of each dilution, which were added to 240 µl ABTS. Then, 2 fold concentrations ranges were defined respectively for Thyme and Rosemary EOs; 0.75 to 6 and 250 to 2000 µL/L. Each experiment was performed 3 different times in triplicate. The initial absorbance at 740 nm was read and then every 5 minutes, during a total of 120 minutes. The inhibition percentage was calculated using the equation $(\%) = (A_0 - A_t) / A_0 \times 100$ where A_0 is the control absorbance; this is ABTS without sample and A_t the absorbance of ABTS with sample at t time. The sample concentration required to reduce ABTS to a 50% (IC50) was obtained by calculating the inhibition percentage against sample concentrations. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) purchased from Sigma-Aldrich (St. Louis, MO, USA) was used as a synthetic antioxidant reference.

Antimicrobial Activity Assay

Microwell Dilution Method

The microwell dilution method with the BIOSCREEN C Microbiological Growth Analyser (Labsystems, Helsinki, Finland), as reported by Medina et al,²¹ was applied to define antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*. The BIOSCREEN C reports optical density that represent the turbidity caused by cell growth. A 150 µl of bacterial solution with Mueller-Hinton broth (MHB) for bacteria or with malt extract (Oxoid Ltd, Basingstoke, Hampshire, England) for *C. albicans* was prepared with the working culture and prepared, as described previously, before adding 150 µl of each EO and HD dilution of the sample prepared with dimethyl sulfoxide (DMSO - Sigma-Aldrich, St. Luis, MO, USA).

Three wells with 300 µl of MHB alone were used as negative controls and three others ones used as positive controls with gentamicin (Oxoid Ltd) (100 µg/ml) for *E. coli* and *P. aeruginosa*, vancomycin (Oxoid Ltd) (100 µg/ml) for *S. aureus*, voriconazol (Sigma-Aldrich, St Louis, USA) (10 µg/ml) for *A. niger* and amphotericin B (Sigma-Aldrich, St Louis, USA) (1250 µg/ml) for *C. albicans*.

Lastly, the minimum bactericidal concentration (MBC) and minimum fungicidal concentrations (MFC) were confirmed by pouring 5 µl of each test solution into Petri dishes for *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans* during 24 hours at 37°C and applying thiazolyl blue tetrazolium bromide [3-(4,5-dimethyl thiazol-2-yl)2, 5-diphenyltetrazolium bromide] (MTT, Sigma Aldrich, St. Louis, MO, USA) assay for *E. coli*, *P. aeruginosa* and *S. aureus* to evaluate cellular viability as reported by Mosmann.²² Each experiment was performed in three different times in triplicate.

Agar Dilution Method

The agar dilution method was used to define the minimum fungicidal concentrations (MFC) of EOs and HDs against *Aspergillus niger* as reported by Hammer et al,²³ using modifications. Potato dextrose agar (PDA) media was prepared with serially diluted EOs and HDs at 30°C; before its solidification, it was poured into small Petri dishes. Then, a disc with monosporic cultures of *A. niger*, prepared as described previously, was placed in the Petri dish center and the preparation was incubated at 25°C for 5 days. MFC was defined as the lowest concentration without growth. Each assay was repeated three times in triplicate. Mean values were defined for MFC. Three negative controls were prepared with PDA alone, and three positive controls were prepared with voriconazole at 10 µg/ml. The concentrations ranges used for EOs and HDs were the same than those used for microwell dilution method.

Statistical Analysis

The variance analysis was conducted and the differences between variables were assessed for significance using

a one-way analysis of variance (ANOVA) with a SPSS 11 program. Differences at $P < .01$ were considered statistically significant.

Results and Discussion

Chemical Composition of Essential Oil and Hydrosol

The 6 batches of Rosemary and Thyme EOs obtained by the 2 different steam distillation methods yielded, respectively, 0.3% and 0.1% based on the weight of the dry plant material.

Relative compositions of EOs and HDs are presented in Table 1 and 2. EO results confirmed the chemotype camphor for the 6 Rosemary EOs, chemotype thymol for the Thyme EOs obtained by steam distillation and adapted steam distillation but with an higher p-cymene content in this last group. In the 3 Rosemary EOs obtained by the conventional steam distillation, 11 compounds were identified representing between 88.8% and 89.0%. The 3 Rosemary EOs obtained with adapted method contained 10 compounds, which represent between 86.0% and 87.6%. In all the Rosemary HDs, 8 compounds were identified and represent 78.12% to 81.8% in HDs obtained by steam distillation, and 79.6% to 92.0% in HDs obtained by adapted steam distillation.

In the 6 Thyme EOs, 9 compounds were identified representing between 86.5% to 87.2% in the EOs obtained by steam distillation, and 85.7% to 86.6% in EOs obtained by adapted steam distillation. In HDs obtained by steam distillation and adapted steam distillation, 5 compounds were identified representing from 80.3% to 87.5% and 88.4% to 93.1%, respectively.

Rosemary EO compositions were homogenous in 8 group of distillation method. No significant qualitative or quantitative difference was observed between EOs obtained by steam distillation or the adapted steam distillation except for the weak presence of bornyl acetate in the first group and its absence in the second one. The chemical composition comparison between Rosemary HDs obtained by steam distillation showed no significant differences.

Inversely, Thyme EOs obtained by the two different steam distillation methods showed quantitative differences especially in terms of percentages of their respective major constituents, which attribute their chemotypes. All EOs obtained by steam distillation showed a thymol chemotype with a percentage between 36.1% and 39.8% and EOs obtained by adapted steam distillation showed the same chemotype but with an higher p-cymene percentage between 36.8% and 37.7%. Thyme HDs obtained, showed mainly qualitative differences. Although the corresponding EOs showed a p-cymene chemotype, HDs obtained by adapted steam distillation did not contain this compound; oddly, HDs obtained by steam distillation did. All Thyme HDs obtained by adapted steam distillation contained 1,8-cineole and camphor with ranges 3.7%-5.1% and 7.1%-9.4% and Thyme HDs obtained by conventional steam distillation did not show these molecules which

Table 1. Composition of *Rosmarinus officinalis* and *Thymus vulgaris* Essential Oils Obtained by 2 Steam Distillation Methods

Compounds	RI ^b	RI ^c	<i>Rosmarinus officinalis</i> Essential Oil ^a						<i>Thymus vulgaris</i> Essential Oil ^a					
			RoCEO1	RoCEO2	RoCEO3	RoFEO1	RoFEO2	RoFEO3	TvCEO1	TvCEO2	TvCEO3	TvFEO1	TvFEO2	TvFEO3
Monoterpene Hydrocarbons														
α-Pinene	935	1038	10.1	10.2	10.2	8.5	9.8	9.4	1.2	1.0	1.0	1.2	1.2	1.1
Camphene	952	1087	8.7	8.8	8.7	7.7	8.6	8.3						
β-Pinene	980	1129	7.1	7.1	7.1	6.3	6.8	6.6						
β-Myrcene	991	1173	2.9	2.9	2.9	2.8	2.9	2.8	1.3	1.1	1.1	1.5	1.5	1.5
α-Phellandrene	1008	1183	1.2	1.2	1.2	0.8	1.0	1.0						
α-Terpinene	1019	1198							1.7	1.5	1.3	1.5	1.7	1.4
p-Cymene	1033	1297							28.4	28.1	29.3	37.7	36.8	36.8
γ-Terpinene	1060	1264	1.4	1.4	1.4	1.0	1.2	1.2	11.6	9.5	7.4	10.8	12.3	11.0
Oxygenated Monoterpenes														
1,8-Cineole	1037	1233	21.1	21.1	21.0	21.5	21.4	20.9						
Linalol	1107	1556							3.2	3.1	3.1	3.9	3.8	3.9
Camphor	1156	1557	29.2	29.0	29.0	31.2	29.4	30.3						
Terpinen-4-ol	1179	1638	4.0	4.0	4.0	3.9	4.2	4.5	1.3	1.3	1.3	1.2	1.2	1.2
Verbenone	1213	1816	2.1	2.1	2.1	2.5	2.2	2.4						
Bornyl acetate	1286	1611	1.1	1.1	1.1									
Sesquiterpene Hydrocarbons														
β-Caryophyllene	1423	1648							2.2	2.2	2.2	1.3	1.4	1.2
Aromatic Compounds														
Thymol	1315	2411							36.1	39.4	39.8	27.3	26.7	27.6
Total identified			89.0	88.8	88.8	86.0	87.6	87.3	87.0	87.2	86.5	86.3	86.6	85.7

^aRoC (1 to 3) and TvC (1 to 3) are the molecular relative percentage indicated by GC-FID for essential oils obtained by the conventional steam distillation with Rosemary and Thyme respectively and RoF (1 to 3) and TvF (1 to 3) correspond to the molecular relative percentage of essential oils obtained by adapted steam distillation method of the two species.

^bRI are Kovats retention index on an Elite-5 MS column.

^cRI are Kovats retention index on an HP-INNOWAX column.

Table 2. Composition (Area %) of *Rosmarinus officinalis* and *Thymus vulgaris* Hydrosols Obtained by 2 Steam Distillation Methods

Compounds	RI ^a	<i>Rosmarinus officinalis</i> Hydrosol ^a						<i>Thymus vulgaris</i> Hydrosol ^a					
		RoCHD1	RoCHD2	RoCHD3	RoFHD1	RoFHD2	RoFHD3	TvCHD1	TvCHD2	TvCHD3	TvFHD1	TvFHD2	TvFHD3
Monoterpene hydrocarbons													
p-Cymene	1290							2.3	1.5	2.1			
Oxygenated monoterpenes													
1,8-Cineole	1226	26.9	26.4	28.0	29.3	28.5	24.5				5.1	3.7	4.1
Linalool	1554							3.2	3.2	2.3			
Camphor	1555	37.7	35.9	30.4	41.0	36.3	36.2				9.4	7.1	7.4
Terpinen-4-ol	1637	2.4	2.4	3.1	3.4	2.5	2.6	1.5	1.5	1.1	1.8	1.7	1.6
Verbenone	1813	3.4	3.7	5.0	4.2	3.6	4.3						
Bornyl acetate	1608	1.2	0.7	1.1	1.1	1.3	1.6						
α-Terpineol	1824	1.9	2.1	3.0	2.4	2.0	2.2						
Borneol	1879	5.7	5.0	8.0	7.4	5.6	4.4						
Aliphatic ketones													
3-Octanone	1267	2.6	1.9	1.2	3.2	3.0	3.8						
Aromatic compounds													
Thymol	2408							74.7	63.8	62.1	71.7	71.3	72.6
Carvacrol	2440							5.8	13.1	12.8	5	5	4
Total identified		81.8	78.1	79.8	92.0	82.9	79.6	87.5	83.0	80.3	93.1	88.4	90.0

^aRoCHD (1 to 3) and TvCHD (1 to 3) are the molecular relative percentage indicated by GC-MS for hydrosols obtained by the conventional steam distillation with Rosemary and Thyme respectively and RoFHD (1 to 3) and TvFHD (1 to 3) correspond to the molecular relative percentage of hydrosols obtained by adapted steam distillation method of the two species. ^aRI are Kovats retention index on an HP-INNOWAX column.

Table 3. ABTS Free Radical Scavenging Activity of Thyme and Rosemary Essential Oils and hydrosols Obtained by the 2 Steam Distillation Methods

Essential oils	20 min	120 min	Hydrosols	20 min	120 min
TvDCEO1	3.1 ± 0.2	2.9 ± 0.3	TvDCHD1	3420.9 ± 73.9	3028.1 ± 118.8
TvDCEO2	3.5 ± 0.3	3.1 ± 0.2	TvDCHD2	3194.4 ± 215.6	2893.5 ± 181.2
TvDCEO3	2.9 ± 0.2	2.6 ± 0.2	TvDCHD3	3206.9 ± 177.7	2876.2 ± 173.1
TvDCEO123	3.1 ± 0.3	2.9 ± 0.3	TvDCHD123	3274.1 ± 127.3	2932.6 ± 83.1
TvDFEO1	4.2 ± 0.1	3.7 ± 0.1	TvDFHD1	3269.4 ± 198.5	2913.8 ± 191.2
TvDFEO2	4.8 ± 0.3	4.2 ± 0.5	TvDFHD2	3443.4 ± 183.0	3036.2 ± 200.7
TvDFEO3	4.3 ± 0.4	3.9 ± 0.3	TvDFHD3	3362.3 ± 224.4	3013.0 ± 192.0
TvDFEO123	4.4 ± 0.3	3.9 ± 0.2	TvDFHD123	3358.4 ± 87	2987.7 ± 65.0
RoDCEO1	3704.4 ± 251.3	1516.7 ± 54.5	RoDCHD1	>>40000	>>40000
RoDCEO2	4035.1 ± 237.3	1544.6 ± 189.2	RoDCHD2	>>40000	>>40000
RoDCEO3	4119.8 ± 153.1	1673.5 ± 82.0	RoDCHD3	>>40000	>>40000
RoDCEO123	3953 ± 219.5	1578.3 ± 83.6	RoDCHD123	>>40000	>>40000
RoDFEO1	5443.3 ± 153.0	2333.0 ± 41.0	RoDFHD1	>>40000	>>40000
RoDFEO2	5048.4 ± 216.2	2169.5 ± 143.7	RoDFHD2	>>40000	>>40000
RoDFEO3	5266.7 ± 600.7	2356.5 ± 108.0	RoDFHD3	>>40000	>>40000
RoDFEO123	5252.8 ± 197.8	2286.3 ± 101.9	RoDFHD123	>>40000	>>40000
Trolox	8,8 +/- 0,7	8,3 +/- 0,8			

^a Due to the limited samples number the distribution normality was tested by *t* test. Assuming the sample normality, statistically, the differences were significant at *P* < .01.

could be obtained by hydrolysis during maceration. HDs obtained by conventional distillation contained linalool with a percentage between 2.3% and 3.2% and the other HDs group obtained by adapted distillation does not have this molecule but has carvacrol from 7.1% to 9.4%, molecule which is missing in the first group.

The content differences between TvDCEOs and TvDFEOs showed that the 2 distillation methods used in our research can also have an impact on EO chemical composition as Jordan et al.¹ showed in their research on *Thymus vulgaris* and *T. hyemalis*, in which they underscore the relationship between the phenological stage and the dynamic of the sequence γ -terpinene \rightarrow p-cymene \rightarrow thymol.

ABTS Free Radical Scavenging Activity of Essential Oils and Hydrosols

The antioxidant activity of EOs and HDs from the 12 batches was measured by using the free radical ABTS assay to evaluate their capacity to scavenge this radical. Results are expressed by IC₅₀ in μ l/L and presented in Table 3. Rosemary HDs did not show any antioxidant activity with ABTS until 40 μ l/mL, the maximum concentration used for HDs to assess this activity.

IC₅₀ was evaluated intentionally at 20 minutes and at 120 minutes because these times correspond to the steady phase beginning of the antioxidant reaction of Rosemary

EOs and HDs and Thyme HDs especially, which contrary to Thyme EOs have a slower reaction. More variability of the standard deviations was observed too between the results obtained at 20 and 120 minutes for Rosemary EOs obtained by the 2 different methods.

Significant differences were observed between antioxidant activities of Thyme EOs obtained by steam distillation and Thyme EOs obtained by adapted steam distillation probably due to the first ones thymol relative concentration (between 36.1% and 39.8%), which is higher than the second ones (between 26.7% and 27.6%). However, no significant difference was observed between HDs obtained by steam distillation and HDs obtained by adapted steam distillation, which present a homogenous behavior into and between each batches issued from the 2 distillation methods. IC₅₀ obtained with the different Thyme HDs are similar to that obtained by Aazza et al²⁴: 3000 mg/mL at 6 minutes with a Thyme HD chemotype Carvacrol.

The lower IC₅₀ observed in the Rosemary EOs obtained by the steam distillation method in comparison with the Rosemary EOs obtained by the adapted steam distillation could not be explained by the comparison of the chemical composition of identified compounds, specified previously, with a relative percentage over 1%. Some weak variations between the identified molecules or between minor compounds (<1%) from the 2 EOs could, possibly, have an

Table 4. MBC and MFC ($\mu\text{L}/\text{mL}$) of Thyme and Rosemary EOs and HDs obtained by 2 steam distillation methods

	MBC			MFC	
	<i>E. Coli</i>	<i>P. Aeruginosa</i>	<i>S. Aureus</i>	<i>C. Albicans</i>	<i>A. Niger</i>
TvDCEO1	0.4	0.4	0.2	0.2	0.4
TvDCEO2	0.4	0.4	0.2	0.2	0.4
TvDCEO3	0.4	0.4	0.2	0.2	0.4
TvDFEO1	0.8	0.8	0.4	0.2	0.8
TvDFEO2	0.8	0.8	0.4	0.2	0.8
TvDFEO3	0.8	0.8	0.4	0.2	0.8
RoDCEO1	7.5	7.5	3.75	3.75	10
RoDCEO2	7.5	7.5	3.75	3.75	10
RoDCEO3	7.5	7.5	3.75	3.75	10
RoDFEO1	7.5	3.75	3.75	3.75	10
RoDFEO2	7.5	3.75	3.75	3.75	10
RoDFEO3	7.5	3.75	3.75	3.75	10
RoDCHD1	>500	>500	>500	>500	>250
RoDCHD2	>500	>500	>500	>500	>250
RoDCHD3	>500	>500	>500	>500	>250
RoDFHD1	>500	>500	>500	>500	>250
RoDFHD2	>500	>500	>500	>500	>250
RoDFHD3	>500	>500	>500	>500	>250
TvDCHD1	>500	250	250	250	250
TvDCHD2	>500	250	250	250	250
TvDCHD3	>500	250	250	250	250
TvDFHD1	>500	250	250	250	250
TvDFHD2	>500	250	250	250	250
TvDFHD3	>500	250	250	250	250

Abbreviations: MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; EOs, essential oils; HDs, hydrosols.

impact on the antioxidant activity variation.

Antimicrobial Activity

The minimum bactericidal and fungicidal concentrations of the EOs and HDs obtained by the 2 steam distillation methods are shown in Table 4. Rosemary HDs did not present any activity against the bacteria and fungi tested up to 500 $\mu\text{L}/\text{mL}$. All Rosemary EOs obtained by the 2 different distillation methods showed the same minimum microbicidal concentration except against *P. aeruginosa*. Contrary to the antioxidant activity, which showed better results with the EOs obtained by the conventional steam distillation, the Rosemary EOs obtained by the FARMAVERDE steam distillation showed the lowest MBC against this *Pseudomonas* specie. However, these minimum microbicidal concentrations of Rosemary EOs against the different strains were low in comparison with those obtained with all Thyme oils, which showed activity between 0.2 and 0.8 $\mu\text{L}/\text{mL}$.

With all the strains, except *C. albicans*, the EOs obtained

by steam distillation showed minimum microbicidal concentration, 2-times lower than the EOs obtained by adapted steam distillation method. This difference could also be explained by the difference of thymol content between the two EOs. Moreover, Thyme HDs showed a minimum microbicidal activity of 250 $\mu\text{L}/\text{mL}$ against *P. aeruginosa*, *S. aureus*, *C. albicans* and *A. niger*. Although this concentration is very high for a preservative, it could be used at this concentration or higher as an active ingredient to promote product conservation with an antioxidant activity superior to 3 $\mu\text{L}/\text{mL}$, but ensuring that thymol concentration does not present any risk.

As shown by our results, the antimicrobial activity of derived EOs can vary according to the quantitative relationship between thymol and p-cymene. The latter, as reported by Burt,²⁵ is not an effective antibacterial, but it can cause the swelling of the cytoplasmic membrane, helping the action of others antimicrobial molecules like carvacrol or thymol. Consequently, depending on the ratio thymol/p-cymene, Thyme EOs can have more or less

antimicrobial and antioxidant activity.

The comparison of the chemical composition, and antioxidant and antimicrobial activity of EOs and HDs obtained by steam distillation using the plant macerate as steam source with those obtained by conventional steam distillation did not show the differences that we were expecting on the improvement of the biological activity of EOs and HDs. However, these results allowed us to underscore two important facts in the use of EOs. Firstly, the importance and consequences of chemotype definition, not only at the research level in order to objectively compare different bioactivity results from different authors, but also at the industrial level to ensure the desired biological activity as a preservative or its potential therapeutic use. Secondly, the consequence of chemical composition changes on the biological activity and consequently its extrapolation from a therapeutic point-of-view.

Therefore, if consumers have concerns about the security of different synthetic preservatives, its substitution by EO or HD should be correctly controlled too. This is the main challenge for industry and it requires consistency and accuracy at the agricultural and process level.

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