

Article

Obtaining Aromatic Extracts from Portuguese *Thymus mastichina* L. by Hydrodistillation and Supercritical Fluid Extraction with CO₂ as Potential Flavouring Additives for Food Applications

Júlia C. Kessler ^{1,2,3}, Vanessa A. Vieira ^{1,2,4}, Isabel M. Martins ^{1,2,*}, Yaidelin A. Manrique ^{1,2}, Andreia Afonso ⁴, Patrícia Ferreira ⁴, Filipa Mandim ³, Isabel C. F. R. Ferreira ³, Lillian Barros ³, Alírio E. Rodrigues ^{1,2} and Madalena M. Dias ^{1,2}

- ¹ Laboratory of Separation and Reaction Engineering-Laboratory of Catalysis and Materials (LSRE-LCM), Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal; juliakessler@fe.up.pt (J.C.K.); vanessa.vieira@deifil.pt (V.A.V.); yjam@fe.up.pt (Y.A.M.); arodrig@fe.up.pt (A.E.R.); dias@fe.up.pt (M.M.D.)
- ² Associate Laboratory in Chemical Engineering (ALiCE), Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal
- ³ Centro de Investigação de Montanha (Mountain Research Center) (CIMO), Polytechnic Institute of Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; filipamandim@ipb.pt (F.M.); iferreira@ipb.pt (I.C.F.R.F.); lillian@ipb.pt (L.B.)
- ⁴ DEIFIL-Deifil Technology, Serzedelo, 4839-704 Póvoa de Lanhoso, Portugal; andreia.afonso@deifil.pt (A.A.); patricia.ferreira@deifil.pt (P.F.)
- * Correspondence: isa@fe.up.pt; Tel.: +351-22-508-1686



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Abstract: Humans often respond to sensory impulses provided by aromas, and current trends have generated interest in natural sources of fragrances rather than the commonly used synthetic additives. For the first time, the resulting aroma of a selected culture of *Thymus mastichina* L. was studied as a potential food ingredient. In this context, dried (DR) and fresh (FR) samples were submitted to carbon dioxide (CO₂) supercritical extraction (SFE) and hydrodistillation (HD) methods. The extracts were characterised according to their volatile composition by GC-MS, cytotoxicity against a non-tumour cell culture, and sensory attributes (odour threshold and olfactive descriptors). The most abundant aromas were quantified, and the analysis performed by GC-MS revealed an abundance of terpenoids such as thymol chemotype, followed by the precursors α -terpinene and *p*-cymene. DR and FR extracts (EX) obtained from SFE-CO₂ show the highest content of thymol, achieving 52.7% and 72.5% of the isolated volatile fraction. The DR essential oil (EO) contained the highest amount of terpenoids, but it was also the most cytotoxic extract. In contrast, SFE-CO₂ products showed the lowest cytotoxic potential. Regarding FR-OE, it had the lowest extraction yield and composition in aroma volatiles. Additionally, all samples were described as having green, fresh and floral sensory notes, with no significant statistical differences regarding the odour detection threshold (ODT) values. Finally, FR-EX of *T. mastichina* obtained by SFE-CO₂ presented the most promising results regarding food application.

Keywords: SFE-CO₂; natural odours; food ingredients; thymol rich extracts; terpenoids

1. Introduction

Lipophilic volatile compounds play a notable influence on the olfactory marketing strategy in the food, cosmetic and fragrance industries. Selective oils from natural origins are recognised as one of the main approaches to product improvement, whether to extend shelf life, in alternative medicine treatments or for sensory purposes [1,2]. The compounds extracted from plants, namely essential oils, have distinct chemical compositions related not only to the species itself but also to agriculture practices, soil properties, climate conditions

and stage of development [3]. Over the last century, advances in the knowledge of chemical routes have proven the contribution of the environmental state in the secondary metabolism of plants [4].

Chemically unstable and easily isomerized, volatile fractions obtained by conventional extraction procedures (e.g., HD, steam distillation (SD), maceration (MAC), and Soxhlet (SOX)) show significant chemical alterations. The presence of light, heat and the existence of an organic solvent are some of the conditions that promote the decomposition of unsaturated or esterified molecules [5]. The thermal hydrolysis effect was observed in HD products by the formation of the *p*-cymene molecule, an aromatic monoterpene and precursor of the thymol and carvacrol (both isomers) compounds [6,7]. This reaction can occur rapidly through dehydrogenated and oxygenated γ -terpinene, respectively [1,8]. However, in the last three decades, SFE has increased the naturalness of the odour of the extracted products [9]. SFE is a green extraction technology and typically uses CO₂ as a solvent at a critical pressure of 73.8 bar and a minimum temperature of 32.1 °C [5]. In addition to its safety, chemical stability, low cost and availability characteristics [10], CO₂ can change its solvent capability by changing pressure and temperature, thus affecting the density of the fluid and the extraction efficiency [7,11]. Generally recognised as safe by the Food and Drug Administration [9], CO₂ as a supercritical fluid is highly selective and favours the extraction of thermolabile bioactive compounds [7].

As shown by Costa et al. [12], by using a SFE-CO₂ process with gradual decompression, different compounds can be collected in order of their molecular weight in different separators. In this work, the first separator was designed to capture the heavier substances (mainly sesquiterpenoids such as β -caryophyllene), and the second separator was designed for lighter components such as monoterpenoids (e.g., γ -terpinene, linalool and camphor). Depending on the system pressure, no EX was recovered in both separators simultaneously, as shown by García-Risco et al. [13] using pressures from 150 to 400 bar and a temperature of 40 °C. No EX was obtained in the first separator at low pressure. Nevertheless, the product of the second separator has a similar chemical composition to those obtained in the severest extraction conditions, differing just in their relative concentration and yield.

Comparable results were shown for several raw materials [7,12–17]. Thereby, to guarantee the selectivity and high solubility of the volatile fraction, normally the indicated SFE-CO₂ conditions are around 80 to 100 bar and 40 to 50 °C [11,16]. Moreover, studies have reported a reduction of selectivity of the supercritical products when higher pressure and temperature values were applied, resulting in the co-extraction of long-chain lipids compounds [17].

Aiming to present these well-known concepts for traditional plant material from Northern Portugal, the main genotypes and sensory attributes of *T. mastichina* were studied. *T. mastichina* is an endemic and undershrub Iberian Peninsula herb, reaching more than 50 cm in height and blossoming from April to June [18]. Between the 215 species of genus *Thymus* [3], Portuguese *T. mastichina* shows a few explorative works, especially in the early 2000's [18–21]. The most embracing study so far was done by Salgueiro et al. [22] in 1997, giving significant advances in data information of the selected plant. Delgado et al. [23] studied *T. mastichina* collected from 20 different populations in Spain.

Regarding the numerous studies found in the literature about *Thymus* spp. aromatic plants, the chemical composition of its main molecules is listed in Table 1, where the species and origin of production, extraction procedures, respective conditions, yields and prevailing genotypes are also listed.

Thyme's dual activities highlight the herb for edible applications. First, the plant is widely used as a natural additive due to its expressive antimicrobial property against common food pathogens (e.g., *Staphylococcus aureus*) [23]. On the other hand, the terpenoid compounds (e.g., thymol, carvacrol, *p*-cymene) show desirable improvements in foods as flavouring agents [1]. Its pleasant aromatic properties play an interesting role in replacing synthetic ingredients generally introduced in industrial processes [9]. In fact, through the organoleptic characteristics of food products, the deepest human sensations and feelings

can be revealed. Thus, exploring these perceptions constitute a potential strategy for marketing purposes.

Table 1. Dataset of the main extraction results of *Thymus* species performed with HD and SFE-CO₂ methods over the last ten years.

Species	Origin	Yields	Main Volatile Compounds		References
			HD	SFE-CO ₂	
<i>T. fontqueri</i> , <i>T. x. citriodorus</i> and <i>T. zygis</i> subsp. <i>gracilis</i>	Portugal	HD (0.67 to 1.26%) and SFE-CO ₂ (<0.05 to 0.77%)	<i>T. fontqueri</i> : carvacrol (60.3%), γ -terpinene (25.8%) and <i>p</i> -cymene (6.3%); <i>T. x. citriodorus</i> : geraniol (76.1%), geranial (5.3%) and neral (3.7%) and <i>T. zygis</i> : thymol (41.0%), <i>p</i> -cymene (16.0%), and γ -terpinene (10.0%)	<i>T. fontqueri</i> : <i>p</i> -cymene (41.0%), γ -terpinene (25.8%) and carvacrol (2.6%); <i>T. x. citriodorus</i> : geraniol (66.5%), geranial (8.6%) and thymol (7.6%) and <i>T. zygis</i> : thymol (33.7%), <i>p</i> -cymene (10.5%) and carvacrol (8.7%)	[9]
<i>T. munbyanus</i> subsp. <i>coloratus</i> and <i>T. munbyanus</i> subsp. <i>munyanus</i>	Algeria	HD (0.09 to 0.11%) and SFE-CO ₂ (0.4%)	Camphor (11.7%), geranyl acetate (6.3%) and β -terpinyl acetate (5.1%)	<i>E</i> -nerolidol (2.0 to 13.7%), 4-terpineol (0.2 to 10.6%) and camphor (1.1. to 7.6%)	[10]
<i>T. lotocephalus</i>	Portugal	HD (0.30%) and SFE-CO ₂ (2.24 to 7.76%)	Linalool (10.4%), camphor (8.0%) and caryophyllene oxide (8.1%)	Camphor (1.2 to 7.9%), borneol (6.1 to 7.5%) and <i>cis</i> -linalool oxide (0.2 to 7.2%)	[12]
	Egypt	HD (1.00%) and SFE-CO ₂ (0.32 to 1.28%)	<i>p</i> -cymene (35.7%), thymol (33.2 %), and γ -terpinene (9.5%)	Thymol (45.2 to 82.6%), fenipentol (n.d. to 8.48%) and phytol isomer (n.d. to 7.2%)	[7]
	Portugal	~0.023 kg/kg	Thymol (41.6%), <i>p</i> -cymene (28.9%) and γ -terpinene (5.1%)	Thymol (36.3%), <i>p</i> -cymene (24.4%) and thymoquinone (6.2%)	[11]
<i>T. vulgaris</i> L.	Spain	0.8 to 1.1%	Thymol (35.4 to 41.6%), <i>p</i> -cymene (28.9 to 34.8%) and γ -terpinene (5.1 to 7.0%)	Thymol (19.5 to 40.8%), <i>p</i> -cymene (10.0 to 42.6%) and γ -terpinene (0.8 to 6.9%)	[15]
	Spain	-	Thymol (35.4%), <i>p</i> -cymene (34.7%) and γ -terpinene (7.0%)	Thymol (36.8%), <i>p</i> -cymene (28.6%) and γ -terpinene (4.1%)	[24]

HD: hydrodistillation, SFE-CO₂: dioxide carbon supercritical extraction, n.d.: not detected.

This work compares the performance of DR and FR *T. mastichina* extracts obtained from different extraction methods, namely SFE-CO₂ and HD. Descriptive and quantitative sensory assays (QDA) determine the fragrances attributes and odour detection threshold of samples and, finally, the cytotoxicity potential is defined within a safe concentration range for the addition of extracted oils in food products.

To the authors' best knowledge, this is the first time that the *T. mastichina* extracts from SFE-CO₂ and the HY fraction obtained in the HD extraction have been studied. Finally,

this work aims to present a proposal for the use of *T. mastichina* EX as a compound rich in odours and which can be used as a flavouring additive for food products.

2. Materials and Methods

2.1. Chemicals

Carbon dioxide (CAS 124-38-9, food industry-grade, 99.9%) was supplied from Linde (Lisbon, Portugal). Thymol (CAS 89-83-8, 98%), eucalyptol (CAS 470-82-6, 99%) and camphor (CAS 76-22-2, 96%) were acquired from Alfa Aesar (Madrid, Spain). α -pinene (CAS 80-56-8, 98%), thyme oil (CAS 8007-46-3, FCC, FG) and alkane C8–C40 (ref. 40147-U) standard solution were purchased from Sigma Aldrich (Madrid, Spain). Verbenone (CAS 1196-01-6, 99%) and *n*-hexane (CAS 110-54-3, 99%) were obtained from Supelco (Madrid, Spain).

2.2. Plant Material

T. mastichina herb was cultivated under controlled conditions at Deifil Technology (Póvoa de Lanhoso, Braga, Portugal). After the development of micropropagated seedlings from the same mother plant, the aerial material of the plant was collected in May 2020 and subjected to an air-drying process at 40 °C (Venticell, MMM Medcenter, Planegg, Germany) until constant weight, achieving $82.06 \pm 1.14\%$ (*w/w*) of water content.

2.3. Essential Oil and Hydrolate Isolation

The HD procedure was conducted with 75 g of FR and DR raw plant in duplicate and independent assays. The extraction was carried out using a conventional extraction apparatus. In this methodology, the essential oils of *Thymus* are evaporated by heating a mixture of water and plant materials followed by the liquefaction of the vapours in a condenser [25]. The extraction was carried out for 3 h with deionized water, as suggested by the European Pharmacopeia [26]. The experimental apparatus also comprises a condenser and a decanter to collect the condensate and to separate essential oils from water. Both products, essential oil and hydrolate, obtained in each extraction experiment were properly separated by their density differences. The HY fraction was kept under ultra-fast freezing as a preparation stage for the lyophilization process (55C, CoolSafe, Beverwijk, The Netherlands) under vacuum at -50 °C for 72 h. All solid fractions obtained were kept under -20 °C, while the oil fraction was stored at 4 °C until further analysis.

2.4. SFE-CO₂ *T. mastichina* L. Extraction

The supercritical extractor developed by Gomes et al. [27] was used for the SFE-CO₂ process, and following this work, the volatile compounds were obtained under fixed conditions of pressure and temperature, namely 80 bar and 50 °C. A stainless-steel extraction cell capable of containing 1 L of CO₂ and designed to perform experiments in static mode was inserted into the system with 30 g of *T. mastichina* plant using FR and DR samples. To guarantee full recovery of the extracted content after 2 h, a CO₂ re-alimentation cycle was performed. The products were collected through the regulation valves (VR1 operated at 40 bar and VR2 at 6 bar) inside 2 separators (S1 and S2). The recovered EX was stored at 4 °C for further characterization.

The extraction yields for both extraction methods was obtained by

$$\text{Yield (\%)} = \left[\frac{m_{\text{essential oil (g)}}}{m_{\text{sample (DW) (g)}}} \right] \times 100 \quad (1)$$

2.5. Volatile Composition Determined by GC-MS Assay

The volatile compounds of *T. mastichina* products were analysed by gas chromatography (GC) with an ion-trap mass spectrometer (MS) (TQ8040 NX Triple Quadrupole, Shimadzu, Japan) using a cross-bonded fused column (30 m \times 0.25 mm, 0.25 μ m film thickness) for low-polarity phases (Rxi-5Sil MS, Restek, Bellefonte, USA). The GC-MS

instrument operated with a splitless injector and automatic sampler (AOC-20s+i). All samples were diluted in *n*-hexane (GC grade), and a volume of 1 μL was injected at 290 °C, carried at 1 $\text{mL}\cdot\text{min}^{-1}$ by ultrapure He.

The isothermal oven program was initially set at 40 °C for 1 min, then raised to 200 °C for 2 min at a rate of 7 °C $\cdot\text{min}^{-1}$, followed by an increase to 250 °C for 2 min at 15 °C $\cdot\text{min}^{-1}$ and finally to 280 °C for 1 min at 20 °C $\cdot\text{min}^{-1}$. The mass scanning range was kept at *m/z* 40–500, while the ion and interface temperature were at 250 °C and 260 °C, respectively.

The resulting aroma fractions are presented in relative concentration (%), obtained by the GC peak areas for each identified compound. Molecules were discriminated based on the National Institute of Standards & Technology (NIST 21, 27, 107, 147, Gaithersburg, MD, USA) mass spectra. To confirm the identification, the retention time of the homologous series of alkanes (C8–C40), analysed under the same chromatographic conditions, was used to calculate the linear retention indices (LRI) by the Kovats retention index equation [28]. The comparison between the obtained LRI and those reported in the literature guarantees the precision of the development chromatographic method and allows for the quantification of the selected molecules according to the calibration curves of the respective analytical standards.

2.6. Odour Perception and Description of *T. mastichina* L. Volatile Compounds

Olfactory evaluations are affected by vapour pressure, relative composition in the gas and liquid phases, atmospheric pressure and temperature conditions, in addition to the psychophysics background [9].

In accordance with the recommendations of the International Standard ISO 8586:2012 [29], a panel of 12 judges was trained to discriminate the most promising samples of *T. mastichina* on the perception of minimum odour concentration, ODT, and index their respective sensory notes by Quantitative Descriptive Analysis (QDA). Subsequently, the commercial standard was incorporated from this study phase to evaluate the achieved products.

To carry out the experiments, a clean, ventilated and well-lighted room was used with a controlled temperature of 20 °C. This room was also free from annoying noises. Furthermore, in the face of the SARS-CoV-2 crises, social and hygienic precautions against the virus were also adopted.

2.6.1. Odour Detection Threshold (ODT)

Odour analysis was performed for the individual products following the instructions of ISO 13301:2018 [30]. Thus, samples in a concentration range of 1.0×10^{-5} to 1.0×10^{-2} $\mu\text{g}\cdot\text{mL}^{-1}$ covered with cotton and conditioned in polypropylene flasks were presented to each panellist. The odour provided by the solutions was properly inhaled, and the minimal odour perception was identified.

2.6.2. Quantitative Descriptive Analysis (QDA)

Aqueous samples with a concentration range of 1.0×10^3 $\mu\text{g}\cdot\text{mL}^{-1}$ were all evaluated individually by the panel members. The descriptors used in the sensory analysis were carefully selected in the literature according to the main compounds of *T. mastichina*. An in-depth discussion indicated woody, green, fresh, citrus, floral, sweet, spicy, fruity and oily as the appropriate odour notes. The methodology was based on ISO 11035:1994 [31], and panellists scored the odour notes on an unstructured scale between 0 “not very intense” and 9 “very intense” to record their perceptions.

2.7. Cytotoxicity Analysis

All aqueous and lipophilic-based products were subjected to a cytotoxic assay using a monkey non-tumour cell line, Vero (kidney cells, ATCC CCL81.4, Gaithersburg, MD, USA). Kidneys play an important role in vertebrate organisms due to their osmoregulatory and excretion functions. Therefore, as the envisioned application is for food purposes, the potential cytotoxic effect of the extracts was evaluated through these cells. The samples

were dissolved in aqueous DMSO (50%, *v/v*) at a final concentration of $8 \text{ mg}\cdot\text{mL}^{-1}$ and further diluted in the range of 400 to $6.25 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$. Following the protocol previously described by Barros et al. [32], the sulforhodamine B assay was performed, where ellipticine was used as a positive control, while the negative control was represented by a suspension of cells. The tests were performed independently (three independent assays) and in triplicate. The obtained results were expressed in GI50 values (the concentration that inhibited 50% of cell proliferation).

2.8. Statistical Analysis

The extraction yields, sensory ODT values and cytotoxicity analysis were carried out using ANOVA and Tukey tests ($\alpha = 0.05$) performed in Statistica StatSoft (version 64, Tulsa, OK, USA).

3. Results and Discussion

3.1. Extraction Yields of *T. mastichina* L. Product

Significant differences were observed in the values of extraction yields between the two methods (SFE-CO₂ and HD) and between types of samples (FR or DR) (Figure 1). The SFE-CO₂ assays resulted in a mix of extractable compounds, collected only in the S1 separator. FR-EX obtained from SFE-CO₂ showed the highest extraction yield ($16.25 \pm 0.23\%$), followed by DR-EX with $3.02 \pm 0.39\%$, determined in DW (Dry Weight). These results are an effect of the high water content in the raw material [33], which reached $82.06 \pm 1.14\%$ (*w/w*); this value was considered in the extraction yield calculations. Additionally, the differences can be explained by three possible phenomena: (i) the dissolution of water in the supercritical solvent increased the solubility of the *Thymus* compounds; (ii) the hydration of the samples decreased the mass transfer resistance by the diffusion mechanism of intracellular compounds to the particle surface of the *Thymus*; and (iii) there were significant losses of matter during the drying process [34]. Statistically, all HD products presented similar extraction yield values, ranging from $0.04 \pm 0.0004\%$ (EO-FR) to $0.62 \pm 0.02\%$ (EO-DR). Previous works report EO of *T. mastichina* of 1.3% [19], 2.3% [20] and 1.4 to 3.5% [18], far from the percentages achieved in the present study. Regarding the HY obtained for FR and DR plant samples, traces of solid fraction were obtained after the lyophilization process: 196.45 mg and 222.30 mg in FR- and DR-HY, corresponding to $0.36 \pm 0.05\%$ and $0.06 \pm 0.001\%$, respectively.

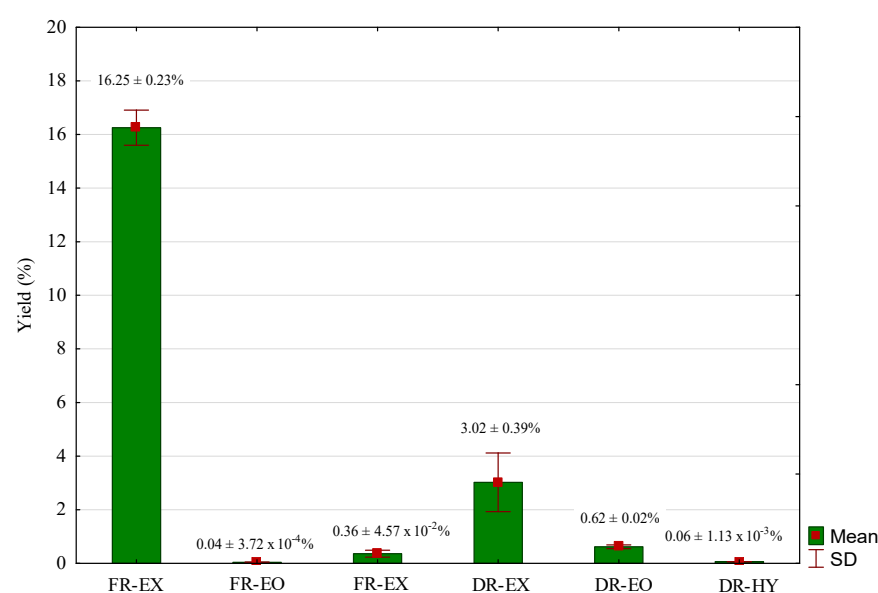


Figure 1. Extraction yields of DR and FR *T. mastichina* fractions obtained by SFE-CO₂ and HD method, presented in DW.

3.2. Chemical Composition of *T. mastichina* L. Product

The chemical variation of the *Thymus* family goes beyond the extraction method and offers a remarkable diversification according to the species and origin of growth [3]. However, as expected, the SFE-CO₂ method showed the highest selectivity in the extracted compounds in relation to the EO-HD results. The EO had a large source of different molecules, with a total of 39; this is as compared to 25 of the FR-EX from SFE-CO₂. Similar results were obtained in the DR raw material, with 30 molecules for the EO in comparison to 22 compounds from the DR-EX obtained by SFE-CO₂. Usually, HD extractions show a lower selectivity than SFE-CO₂. For example, Costa et al. [12] identified 44 and 20 compounds in *T. lotocephalus* extracts obtained by HD and SFE-CO₂ methods, respectively.

Table 2 and Figure 2 present the chemical composition of *T. mastichina* samples obtained in this work, and Table 3 presents the mass quantification of the main molecules. Thymol is the main molecule found in SFE-CO₂-EX, representing 72.4% and 52.7% or $1511.3 \pm 602.3 \mu\text{g}\cdot\text{g}^{-1}$ and $282.1 \pm 75.7 \mu\text{g}\cdot\text{g}^{-1}$ of FR and DR samples, respectively. In relation to the lyophilized HY, a relative composition of 98.6% to FR-HY and 11.5% to DR-HY was obtained. The high relative concentration of thymol was previously reported for *T. vulgaris* and *T. zygis* HY, 77.1% and 61.9%, respectively [35].

The precursors γ -terpinene and *p*-cymene also showed significant values, mainly in the EO samples. The FR sample was composed of 30.6% of γ -terpinene and 10.3% of *p*-cymene, while the DR-EO presented 35.5% and 17.6%, respectively. In contrast, the representativeness of both molecules was lower in SFE-CO₂-EX, reaching 13.6% (FR-EX) and 28.8% (DR-EX). Following the chemical route, the relative influence of thymol increases as the contribution of γ -terpinene and *p*-cymene decreases. Tohidi et al. [8] observed a positive correlation between the formation molecules, mentioning that *p*-cymene does not present itself as a final product.

As previously mentioned, the most abundant molecules present in the obtained extracts were quantified, and the results are shown in Table 3. The richest extract was the DR-EO, revealing $2565.5 \pm 208.7 \mu\text{g}\cdot\text{g}^{-1}$ of thymol, $609.4 \pm 28.3 \mu\text{g}\cdot\text{g}^{-1}$ of γ -terpinene and $337.0 \pm 20.3 \mu\text{g}\cdot\text{g}^{-1}$ of *p*-cymene molecules. In total, the sample presented an amount of $3700.7 \mu\text{g}\cdot\text{g}^{-1}$. As observed through the yield results, FR-HY had a higher total mass concentration ($2429.1 \mu\text{g}\cdot\text{g}^{-1}$) than the FR-EO fraction ($524.8 \mu\text{g}\cdot\text{g}^{-1}$), which suggests a considerable amount of the oil fraction solubilized in hydrophilic fraction. On the other hand, the HY of the DR plant was composed only of $4.3 \mu\text{g}\cdot\text{g}^{-1}$. Furthermore, the methodology to concentrate the organic products in HY is limited in terms of energy and time costs, although it is easy and cheap to produce them [35].

Different volatile compounds were previously referenced in Portuguese *T. mastichina* herbs. Eucalyptol, linalool and camphor stand out in EO obtained by the HD method [18,19,21,22]. β -pinene and limonene were also described as main molecules in samples cultivated in the North and South regions [19,20]. Although the present sample contains these compounds in smaller proportion, the chemotype corresponds to *T. vulgaris* plants collected in Algeria [44], Italy [45], Portugal [11], Romania [46] and Spain [15,24], or even to *T. zygis* species from Portugal [9].

Table 2. Chemical profile of the extracted products obtained from *T. mastichina* herb, analysed in GC-MS and obtained by SFE-CO₂ and HD methods.

No	Compound	LRI ^a	FR				DR				Sensory Descriptors					
			RT (min)	EX	EO	HY	EX	EO	HY							
1	α -Thujene	926	5988	tr	-	1.8	0.2	-	-	2.0	0.1	2.0	0.1	-	-	pine earthy, turpentine, fresh, sweet and woody [36,37]
2	α -Pinene	933	6140	0.6	0.1	0.6	0.0	-	-	1.5	0.0	1.1	0.0	-	-	herbaceous and green [9]
3	Camphene	949	6480	tr	-	tr	-	-	-	0.3	0.0	0.4	-	-	-	camphor, woody and herbal [36,37]
4	Sabinene	972	6963	tr	-	tr	-	-	-	-	-	tr	-	-	-	pine, turpentine, woody, terpenic, spicy and citrus [36,37]
5	β -Pinene	977	7067	-	-	tr	-	-	-	-	-	tr	-	-	-	green, piney, woody and green [37,38]
6	Amyl vinyl carbinol	980	7128	0.9	0.1	tr	-	-	-	0.5	0.0	0.3	0.0	-	-	mushroom, earthy, green and herbs [39–42]
7	β -Myrcene	988	7313	1.0	0.1	1.8	0.0	-	-	2.0	0.0	2.7	0.2	-	-	herbaceous, woody and floral [9]
8	3-octanol	997	7490	-	-	tr	-	-	-	-	-	tr	-	-	-	earthy, mushroom and herbal [37]
9	α -Phellandrene	1006	7681	-	-	tr	-	-	-	-	-	-	-	-	-	citrus, herbaceous and terpenic [37]
10	3-Carene	1016	7908	1.3	0.5	2.8	0.0	-	-	2.5	0.1	4.14	0.3	8.7	0.1	citrus and sweet [9,37]
11	<i>p</i> -Cymene	1023	8067	4.6	0.5	10.3	0.3	0.2	0.0	6.5	0.2	17.6	1.1	34.2	0.9	green, fresh, rubber, terpenic, woody and spicy [37,38]
12	D-Limonene	1029	8180	0.2	0.0	0.4	0.1	-	-	0.2	0.0	0.4	0.0	-	-	citrus, fresh and sweet [36,37]
13	Eucalyptol	1031	8239	1.1	0.2	0.9	0.1	-	-	1.2	0.0	0.5	0.0	-	-	woody, citrus, green, herbaceous and spicy [9,38,43]
14	β - <i>cis</i> -Ocimene	1047	8580	-	-	tr	-	-	-	-	-	tr	-	-	-	green and woody [9]
15	γ -Terpinene	1058	8809	9.1	0.4	30.6	0.7	0.3	0.1	22.4	0.8	35.5	1.7	45.6	0.7	herbaceous, woody, piney and fruity [37,38]
16	β -Terpineol	1070	9062	1.8	0.4	tr	-	-	-	1.5	0.8	0.2	0.0	-	-	woody, pungent and earthy [37]
17	Sabinene hydrate	1071	9090	-	-	tr	-	-	-	-	-	-	-	-	-	herbaceous, minty and oriental [9,37]

Table 2. Cont.

No	Compound	LRI ^a	FR								DR				Sensory Descriptors	
			RT (min)	EX		EO	HY		EX	EO	HY					
18	Terpinolene	1086	9414	-	-	tr	-	-	-	-	-	tr	-	-	-	herbaceous, woody and green [9]
19	β -Linalool	1099	9695	1.0	0.2	1.3	0.0	-	-	1.0	0.1	1.9	0.2	-	-	floral, lavender, citrus, woody and green [36–38]
20	Camphor	1147	10.719	0.4	0.2	-	-	-	-	0.4	0.1	-	-	-	-	camphoraceous and herbal [37]
21	Borneol	1172	11.263	0.4	0.1	tr	-	-	-	0.7	0.0	tr	-	-	-	herbaceous, oriental and woody [9]
22	4-Terpineol	1180	11.444	0.4	0.1	1.4	0.1	-	-	0.2	0.0	0.7	0.1	-	-	spicy, woody and oriental [9,37]
23	α -Terpineol	1194	11.743	0.1	0.0	tr	-	-	-	-	-	tr	-	-	-	terpenic, pine and woody [37]
24	<i>trans</i> -Dihydrocarvone	1201	11.900	-	-	tr	-	-	-	-	-	-	-	-	-	-
25	S-Verbenone	1206	11.993	0.4	0.1	-	-	-	-	-	-	-	-	-	-	camphoreous [37]
26	β -Citral	1240	12.691	-	-	tr	-	-	-	-	-	-	-	-	-	citrus [9,37]
27	Unknown	1247	12.827	0.8	0.3	-	-	-	-	0.3	0.1	-	-	-	-	-
28	Nerol	1249	12.873	-	-	tr	-	-	-	-	-	-	-	-	-	floral, citrus and green [9]
29	Unknown	1264	13.168	-	-	-	-	0.3	0.0	-	-	-	-	-	-	-
30	α -Citral	1277	13.440	-	-	tr	-	-	-	-	-	tr	-	-	-	citrus [9,37]
31	Bornyl acetate	1284	13.574	0.5	0.1	-	-	-	-	0.5	0.0	-	-	-	-	camphor, woody, pine, balsamic, herbal and spicy [37]
32	Thymol	1290	13.700	72.4	28.9	48.2	3.1	98.6	1.6	52.7	1.4	31.8	2.6	-	-	herbaceous [9,37]
33	Carvacrol	1298	13.857	1.0	0.6	tr	-	0.5	0.1	0.9	0.1	tr	-	11.5	1.3	herbaceous and woody [9]
34	Elixene	1334	14.546	-	-	tr	-	-	-	-	-	-	-	-	-	-
35	Thymol acetate	1344	14.750	0.4	0.0	tr	-	-	-	0.5	0.1	tr	-	-	-	thymol, sweet and balsamic [37]
36	β -Caryophyllene	1422	16.220	0.8	0.2	-	-	-	-	2.1	0.1	0.7	0.1	-	-	spicy, woody and sweet [9,37]
37	Copaene	1433	16.410	-	-	tr	-	-	-	-	-	-	-	-	-	woody, spicy and honey [37]

Table 2. Cont.

No	Compound	LRI ^a	FR								DR		Sensory Descriptors		
			RT (min)	EX		EO	HY		EX	EO	HY				
38	Citronellyl propionate	1438	16.505	-	-	tr	-	-	-	-	-	-	-	-	floral and green [37]
39	β -Farnesene	1454	16.785	-	-	tr	-	-	-	-	-	tr	-	-	woody, citrus and herbal [37]
40	α -Humulene	1458	16.872	-	-	tr	-	-	-	-	-	tr	-	-	woody [9,37]
41	Germacrene D	1483	17.317	0.8	0.1	tr	-	-	-	0.2	0.0	-	-	-	-
42	Germacrene B	1498	17.595	-	-	tr	-	-	-	-	-	-	-	-	woody, earthy and spicy [37]
43	Caryophyllene oxide	1585	19.088	-	-	tr	-	-	-	-	-	-	-	-	woody and herbaceous [9]
44	γ -Eudesmol	1612	19.725	-	-	tr	-	-	-	-	-	tr	-	-	-
45	α -Cadinol	1630	20.292	-	-	tr	-	-	-	-	-	-	-	-	herbaceous and woody [9,37]
46	Isopropyl myristate	1723	22.850	-	-	-	-	-	-	-	-	tr	-	-	-
47	Isopropyl palmitate	1927	26.102	-	-	-	-	-	-	-	-	tr	-	-	-
Identified total			90.2	100.0		99.7	99.8		100.0		100.0				

^a LRI: linear retention indices calculated through Kovat's retention index equation for series of alkanes C8–C40 (ref. 40147-U) using a cross-bonded fused column (Rxi-5Sil MS, Restek, Bellefonte, PA, USA) in GC-MS; EX: extract obtained by carbon dioxide supercritical fluid extraction; EO: essential oil obtained by hydrodistillation; HY: hydrolate obtained by hydrodistillation, tr: traces. Sensory description of volatile compounds mentioned in the literature.

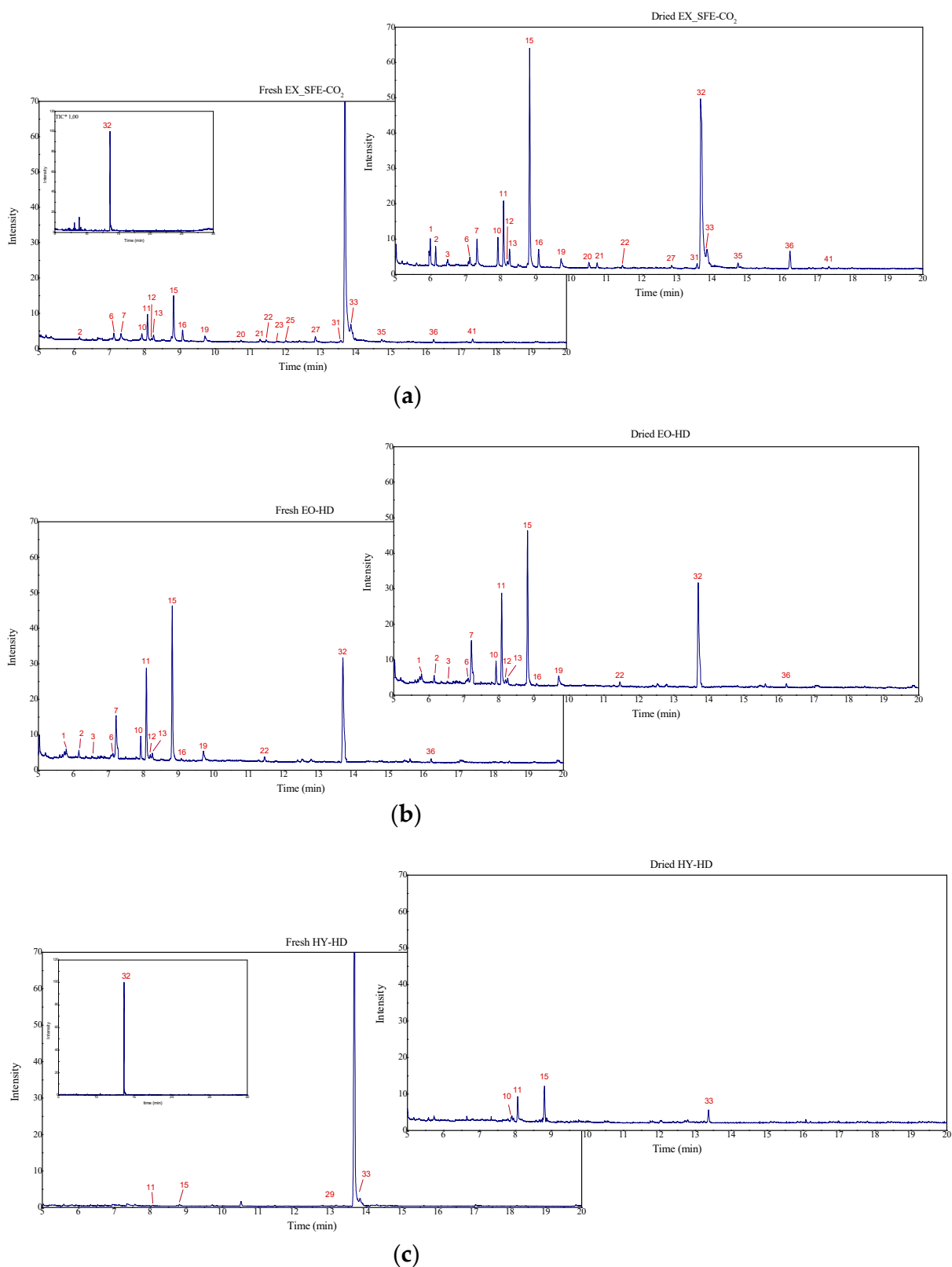


Figure 2. Chromatographic profile of FR and DR *T. mastichina* products analysed in GC-MS and identified as (a) EX obtained by SFE-CO₂, (b) EO extracted by HD and (c) HY provided from the HD method. The samples were analysed at different injection concentrations: (a) 2.5 g/L, (b) 0.01 g/L and (c) HY 0.1 g/L, respectively. The numbers at each peak represent the compounds identified in Table 2.

Table 3. Mass composition of the selected molecules found in *T. mastichina* products, expressed in μg of compound per g of plant, in DW.

Compound	Mass (μg Compound·g Plant ⁻¹ (DW))					
	FR			DR		
	EX	EO	HY	EX	EO	HY
α -Pinene	15.9 \pm 2.7 ^b	8.6 \pm 0.0 ^c	-	6.5 \pm 0.0 ^c	65.0 \pm 0.0 ^a	-
Camphor	23.8 \pm 11.6 ^a	-	-	4.8 \pm 0.7 ^b	-	-
Eucalyptol	34.3 \pm 7.4 ^b	18.6 \pm 1.1 ^c	-	7.0 \pm 0.1 ^d	123.8 \pm 9.7 ^a	-
γ -Terpinene	87.2 \pm 3.8 ^b	64.1 \pm 1.4 ^c	25.1 \pm 9.9 ^d	111.5 \pm 3.9 ^b	609.4 \pm 28.3 ^a	2.6 \pm 0.0 ^e
<i>p</i> -Cymene	49.5 \pm 5.1 ^b	29.0 \pm 0.7 ^c	14.8 \pm 2.6 ^d	55.6 \pm 1.5 ^b	337.0 \pm 20.3 ^a	1.7 \pm 0.0 ^e
<i>S</i> -Verbenone	61.8 \pm 21.1	-	-	-	-	-
Thymol	1511.3 \pm 62.3 ^b	404.4 \pm 25.9 ^c	2389.2 \pm 39.2 ^a	282.1 \pm 7.5 ^c	2565.5 \pm 28.7 ^a	-
Total	1783.8	524.8	2429.1	467.4	3700.7	4.3

Calibration curves: α -pinene ($y = 1.02 \times 10^{10}x - 2.16 \times 10^6$; $r^2 = 0.9987$; LOD = 8.73×10^{-4} g·L⁻¹; LOQ = 2.64×10^{-3} g·L⁻¹), camphor ($y = 9.03 \times 10^9x - 3.05 \times 10^6$; $r^2 = 0.9974$; LOD = 1.11×10^{-3} g·L⁻¹; LOQ = 3.37×10^{-3} g·L⁻¹), eucalyptol ($y = 1.05 \times 10^{10}x - 4.86 \times 10^6$; $r^2 = 0.9972$; LOD = 1.29×10^{-3} g·L⁻¹; LOQ = 3.91×10^{-3} g·L⁻¹), γ -terpinene ($y = 7.75 \times 10^9x - 4.89 \times 10^6$; $r^2 = 0.9966$; LOD = 1.44×10^{-3} g·L⁻¹; LOQ = 4.35×10^{-3} g·L⁻¹), *p*-cymene ($y = 7.13 \times 10^9x - 2.66 \times 10^6$; $r^2 = 0.9997$; LOD = 4.05×10^{-4} g·L⁻¹; LOQ = 1.23×10^{-3} g·L⁻¹), *S*-verbenone ($y = 7.60 \times 10^9x - 6.96 \times 10^6$; $r^2 = 0.9946$; LOD = 1.80×10^{-3} g·L⁻¹; LOQ = 5.47×10^{-3} g·L⁻¹), thymol ($y = 2.45 \times 10^9x - 1.31 \times 10^7$; $r^2 = 0.9977$; LOD = 2.53×10^{-3} g·L⁻¹; LOQ = 7.66×10^{-3} g·L⁻¹). Averages with different letters in the same line indicate significant difference with $\alpha = 0.05$. EX: extract obtained by carbon dioxide supercritical fluid extraction, EO: essential oil obtained by hydrodistillation, HY: hydrolate obtained by hydrodistillation, FR: fresh, DR: dry.

The thymol chemotype is a natural phenolic monoterpene group synthesized by the transformation of γ -terpinene and *p*-cymene precursors. Both thymol and carvacrol isomers are indicated as the end of the biosynthetic chain catalysed by the two-step oxidation of γ -terpinene under the action of the cytochrome P450 enzyme (γ -terpinene synthase), which is able to modify the polarity of the compounds and make them more water-soluble [47]. Geranyl diphosphate (GPP) plays an important role in the initial chemical route, followed by the α -terpineol intermediate [48]. Through a series of steps, the molecules are converted into γ -terpinene, which, by cyclization and carbonisation reactions, form the *p*-cymene molecule [3]. *p*-cymene is a by-product of the premature release of substrate from the active site [8,47], and, by terpene synthase (TPS) activity, it produces thymol or carvacrol molecules when it reaches the last step of biosynthetic conversion. The TPS offers a wide range of ideal characteristics to modify the metabolic profile of terpenes [3]. At the same time, the final products are formed directly via γ -terpinene hydroxylation [47,48].

Moreover, α -thujene, β -myrcene, 3-carene and β -caryophyllene had some influence on the chemical profile of *T. mastichina* plant, mainly in DR samples. *S*-verbenone was only found in FR-EX (0.4% or $61.8 \pm 21.1 \mu\text{g}\cdot\text{g}^{-1}$), while notable amounts of eucalyptol were measured in DR-OE (0.5% or $123.8 \pm 9.7 \mu\text{g}\cdot\text{g}^{-1}$) compared to the other lipophilic fractions. The resulting mass composition demonstrates that even a low relative concentration can indicate the presence of a compound rich in the molecules of interest.

3.3. Cytotoxicity Assay of *T. mastichina* L. Products

The most promising products were selected to perform the successive odour and cytotoxicity characteristics. Table 4 presents the cytotoxicity and ODT results. Concerning the cytotoxicity potential, significant differences were reached between EX, EO and HY. However, the values did not show statistical differences between the hydration state of FR samples. For example, in SFE-CO₂-EX, the GI50 values ranged from $235 \pm 7 \mu\text{g}\cdot\text{mL}^{-1}$ to $271 \pm 2 \mu\text{g}\cdot\text{mL}^{-1}$ in the FR and DR samples, respectively. Instead, the commercial EO was statistically equal in both EO obtained in this study, which reveals the average toxicological profile of $83.5 \pm 0.1 \mu\text{g}\cdot\text{mL}^{-1}$, while FR-EO and DR-EO reached $122 \pm 12 \mu\text{g}\cdot\text{mL}^{-1}$ and $65 \pm 1 \mu\text{g}\cdot\text{mL}^{-1}$, respectively. Nikolic et al. [49] showed higher cytotoxic activity in OE obtained from *T. serpyllum* ($7.02 \mu\text{g}\cdot\text{mL}^{-1} < \text{GI50} < 52.69 \mu\text{g}\cdot\text{mL}^{-1}$) and *T. algenensis*

EO ($62.12 \mu\text{g}\cdot\text{mL}^{-1} < \text{GI}_{50} < 64.79 \mu\text{g}\cdot\text{mL}^{-1}$). Furthermore, the results for *T. vulgaris* EO ($76.02 \mu\text{g}\cdot\text{mL}^{-1} < \text{GI}_{50} < 180.40 \mu\text{g}\cdot\text{mL}^{-1}$) ranged around the FR-OE cytotoxic potential [49], and the HY fraction did not show toxicity potential ($\text{GI}_{50} > 400 \mu\text{g}\cdot\text{mL}^{-1}$).

Table 4. Safe concentration ranges of the most promising *T. mastichina* products and commercial EO as defined by cytotoxic GI_{50} measurements and ODT values.

Test	FR ($\mu\text{g}\cdot\text{mL}^{-1}$)			DR ($\mu\text{g}\cdot\text{mL}^{-1}$)			Commercial EO Standard ($\mu\text{g}\cdot\text{mL}^{-1}$)
	EX	EO	HY	EX	EO	HY	
Vero cells (GI_{50})	235 ± 7^b	122 ± 12^c	$>400^a$	271 ± 24^b	65 ± 1^c	$>400^a$	83.5 ± 0.1^c
ODT value	$6.3 \times 10^{-4}a$	-	-	$5.0 \times 10^{-3}a$	$3.0 \times 10^{-4}a$	-	$1.0 \times 10^{-4}a$

Averages with different letters in the same line indicate significant differences with $\alpha = 0.05$. EX: extract obtained by carbon dioxide supercritical fluid extraction, EO: essential oil obtained by hydrodistillation, HY: hydrolate obtained by hydrodistillation.

3.4. Sensory Odour Evaluation of *T. mastichina* L. Products

The ODT results are expressed in Table 4, and according to the panellist's sensitivity, all the evaluated values were similar with 95% of confidence. The standard EO was the most easily detected product at a concentration of $1.0 \times 10^{-4} \mu\text{g}\cdot\text{mL}^{-1}$, while the DR-EO had the highest concentration value ($5.0 \times 10^{-3} \mu\text{g}\cdot\text{mL}^{-1}$). FR- and DR-EX showed a concentration of $6.3 \times 10^{-4} \mu\text{g}\cdot\text{mL}^{-1}$ and $3.0 \times 10^{-4} \mu\text{g}\cdot\text{mL}^{-1}$ as minimum values to reflect some sensory perception, respectively.

The human nose is a powerful tool to predict the dominant odours in a mixture [50], and, as already indicated above, the *T. mastichina* herb is comprised of volatile terpene compounds. Its influence on odour description was evaluated under controlled conditions to ensure the accuracy of human measurement, and the results were compared with the literature. The most pronounced attributes mentioned by the mean scores of 12 panellists were green, fresh and floral (Figure 3). The DR-EO was the only one with an oily note (0.7 points), with an emphasis on the green fragrance (4.4). The commercial EO has a strong green (3.3), fresh (2.6) and woody (1.0) description, like DR-EX. The FR-EX obtained by SFE- CO_2 exhibited the smoothest analysis among all samples, presenting green (2.2), spicy (1.1) and fresh (0.8) aromas. The intensity results agreed with the respective ODT values.

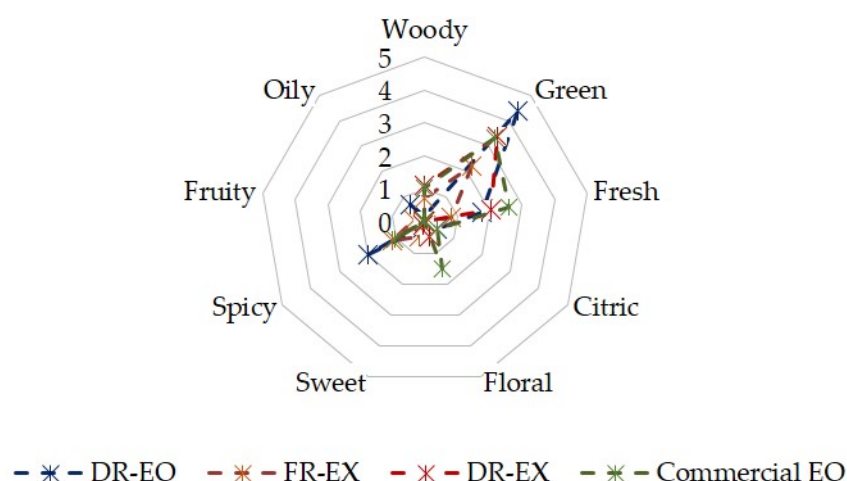


Figure 3. Sensory description and intensity of selected *T. mastichina* samples.

EX of *T. vulgaris* plant were related with spicy, pungent, herbaceous and phenol-like odour characteristics. The product was obtained with supercritical CO_2 under 167 bar and 40°C . Changing the pressure to 104 bar, the descriptors' intensity decreased, while the EO extracted by HD presented lower note values plus the burn attribute [7]. These

findings suggest the thymol molecule as the key to the thyme aroma [7,51]. Furthermore, this distinct odour profile has been pointed out for three different subspecies of Algeria *Thymus*, ranging from a sweet herbaceous fragrance, phenolic herbaceous odour and fresh camphoraceous notes [52].

Moreover, using validated models with ODT results, relative odour intensities and resulting odour values (OV), Teixeira et al. [9] classified three *Thymus* families. According to the data, herbaceous nuances prevailed in the samples, in addition to citrus, woody, floral and phenolic sensory attributes.

In fact, the herbaceous term codifies the aromatic characteristics provided by most Lamiaceae species [7,51] and offers a subjective interpretation of the odour description. Therefore, although it does not represent a fingerprint of the *T. mastichina*, the description of the odour presented is akin to the profile of the compounds previously mentioned. Furthermore, the obtained products revealed a promising opportunity to enhance the flavouring trajectory of processed foods in a safe range of cytotoxicity, especially to the FR-EX.

Scanning all samples and features analysed in this work, FR-EX obtained by SFE-CO₂ appears most promising. The sample reached a balance between the extraction yields, mass composition of the main compounds and toxicity value, and it has been rarely studied previously [21]. To the best of the authors' knowledge, this is the first time that the fresh *T. mastichina* species has been sensorially described.

4. Conclusions

Terpinene-rich extracts were isolated from *T. mastichina* herb by means of green technologies, namely HD and SFE-CO₂. The predominant thymol chemotype and its precursors, γ -terpinene and *p*-cymene, were the main molecules responsible for the sensory profile described as green, fresh and floral fragrance.

Additionally, no ODT results showed significant differences, and both SFE-CO₂ products achieved the lowest toxicity among the lipophilic products. Moreover, the FR-HY fraction showed a higher selectivity of the compound, although with a low yield on a DW. The analysed characteristics of the extracted products suggested the FR-EX obtained through SFE-CO₂ as the most promising sample to use as a flavouring additive for food applications.

According to the authors' best knowledge, the species *T. mastichina* was studied for the first time under supercritical conditions as well as regarding its sensory profile. Hydrodistillation and supercritical fluid extraction were applied to the respective final extraction yields for fresh and dry samples of thymus plant material. The extracts' chemical composition, sensorial attributes and cytotoxic potential were evaluated. Safe concentration ranges of the most promising *T. mastichina* products were defined by cytotoxicity measurements and ODT values, and the significant differences between the extraction yields, sensory ODT values and cytotoxicity analysis were achieved using statistical analysis, namely ANOVA and Tukey tests.

The resulting research presented an unexplored point of view for one of the most studied aromatic plants in recent decades and gives a suggestion for the use of *T. mastichina* extract as compound rich in odours and which can be used as a flavouring additive for food products.

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References

1. Nabavi, S.M.; Marchese, A.; Izadi, M.; Curti, V.; Daglia, M.; Nabavi, S.F. Plants belonging to the genus *Thymus* as antibacterial agents: From farm to pharmacy. *Food Chem.* **2015**, *173*, 339–347. [\[CrossRef\]](#)
2. Moghimi, R.; Ghaderi, L.; Rafati, H.; Aliahmadi, A.; McClements, D.J. Superior antibacterial activity of nanoemulsion of *Thymus daenensis* essential oil against *E. coli*. *Food Chem.* **2016**, *194*, 410–415. [\[CrossRef\]](#)
3. Tohidi, B.; Rahimmalek, M.; Trindade, H. Review on essential oil, extracts composition, molecular and phytochemical properties of *Thymus* species in Iran. *Ind. Crops Prod.* **2019**, *134*, 89–99. [\[CrossRef\]](#)
4. Emami, Z.E.; Siadat, S.A.; Bakhshandeh, A.; Pirbalouti, A.G.; Hashemi, M.; Maggi, F.; Morshedloo, R.M. Application of combined fertilizers improves biomass, essential oil yield, aroma profile, and antioxidant properties of *Thymus daenensis* Celak. *Ind. Crops Prod.* **2018**, *121*, 434–440. [\[CrossRef\]](#)
5. Fornari, T.; Vicente, G.; Vázquez, E.; García-Risco, M.R.; Reglero, G. Isolation of essential oil from different plants and herbs by supercritical fluid extraction. *J. Chromatogr. A* **2012**, *1250*, 34–48. [\[CrossRef\]](#)
6. Mancini, E.; Senatore, F.; Del Monte, D.; De Martino, L.; Grulova, D.; Scognamiglio, M.; Snoussi, M.; De Feo, V. Studies on chemical composition, antimicrobial and antioxidant activities of five *Thymus vulgaris* L. essential oils. *Molecules* **2015**, *20*, 12016–12028. [\[CrossRef\]](#)
7. Morsy, N.F.S. Production of thymol rich extracts from ajwain (*Carum copticum* L.) and thyme (*Thymus vulgaris* L.) using supercritical CO₂. *Ind. Crops Prod.* **2020**, *145*, 112072. [\[CrossRef\]](#)
8. Tohidi, B.; Rahimmalek, M.; Arzani, A. Essential oil composition, total phenolic, flavonoid contents, and antioxidant activity of *Thymus* species collected from different regions of Iran. *Food Chem.* **2017**, *220*, 153–161. [\[CrossRef\]](#)
9. Teixeira, M.A.; Rodrigues, A. Coupled extraction and dynamic headspace techniques for the characterization of essential oil and aroma fingerprint of *Thymus* species. *Ind. Eng. Chem. Res.* **2014**, *53*, 9875–9882. [\[CrossRef\]](#)
10. Bendif, H.; Adouni, K.; Miara, M.D.; Baranauskienė, R.; Kraujalis, P.; Venskutonis, P.R.; Nabavi, S.M.; Maggi, F. Essential oils (EOs), pressurized liquid extracts (PLE) and carbon dioxide supercritical fluid extracts (SFE-CO₂) from Algerian *Thymus munbyanus* as valuable sources of antioxidants to be used on an industrial level. *Food Chem.* **2018**, *260*, 289–298. [\[CrossRef\]](#) [\[PubMed\]](#)
11. Coelho, J.P.; Cristino, A.F.; Matos, P.G.; Rauter, A.P.; Nobre, B.P.; Mendes, R.L.; Barroso, J.G.; Mainar, A.; Urieta, J.S.; Fareleira, J.M.N.A.; et al. Extraction of volatile oil from aromatic plants with supercritical carbon dioxide: Experiments and modeling. *Molecules* **2012**, *17*, 10550–10573. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Costa, P.; Gonçalves, S.; Grosso, C.; Andrade, P.B.; Valentão, P.; Bernardo-Gil, M.G.; Romano, A. Chemical profiling and biological screening of *Thymus lotocephalus* extracts obtained by supercritical fluid extraction and hydrodistillation. *Ind. Crops Prod.* **2012**, *36*, 246–256. [\[CrossRef\]](#)
13. García-Risco, M.R.; Vicente, G.; Reglero, G.; Fornari, T. Fractionation of thyme (*Thymus vulgaris* L.) by supercritical fluid extraction and chromatography. *J. Supercrit. Fluids* **2011**, *55*, 949–954. [\[CrossRef\]](#)
14. Da Rocha, B.C.A.; Kawase, K.Y.F.; Coelho, G.L.V. Comparison of SPME and SFE for the Determination of Volatile Constituents in *Thymus vulgaris* L. Lamiaceae. *IJENS* **2014**, *14*, 35–43.
15. Grosso, C.; Figueiredo, A.C.; Burillo, J.; Mainar, A.M.; Urieta, J.S.; Barroso, J.G.; Coelho, J.A.; Palavra, A.M. Composition and antioxidant activity of *Thymus vulgaris* volatiles: Comparison between supercritical fluid extraction and hydrodistillation. *J. Sep. Sci.* **2010**, *33*, 2211–2218. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Petrović, N.V.; Petrović, S.S.; Džamić, A.M.; Ćirić, A.D.; Ristić, M.S.; Milovanović, S.L.; Petrović, S.D. Chemical composition, antioxidant and antimicrobial activity of *Thymus praecox* supercritical extracts. *J. Supercrit. Fluids* **2016**, *110*, 117–125. [\[CrossRef\]](#)
17. Šojić, B.; Tomović, V.; Kocić-Tanackov, S.; Kovačević, D.B.; Putnik, P.; Mrkonjić, Ž.; Đurović, S.; Jokanović, M.; Ivić, M.; Škaljac, S.; et al. Supercritical extracts of wild thyme (*Thymus serpyllum* L.) by-product as natural antioxidants in ground pork patties. *LWT Food Sci. Technol.* **2020**, *130*, 109661. [\[CrossRef\]](#)
18. Miguel, M.G.; Duarte, F.L.; Venâncio, F.; Tavares, R. Comparison of the main components of the essential oils from flowers and leaves of *Thymus mastichina* (L.) L. ssp. *mastichina* collected at different regions of Portugal. *J. Essent. Oil Res.* **2004**, *16*, 323–327.

19. Miguel, M.G.; Simões, M.; Figueiredo, A.C.; Barroso, J.G.; Pedro, L.G.; Carvalho, L. Composition and antioxidant activities of the essential oils of *Thymus caespitosus*, *Thymus camphoratus* and *Thymus mastichina*. *Food Chem.* **2004**, *86*, 183–188. [CrossRef]
20. Miguel, M.G.; Guerrero, C.; Rodrigues, H.; Brito, J.C.; Duarte, F.; Venâncio, F.; Tavares, R. Main components of the essential oils from wild Portuguese *Thymus mastichina* (L.) L. ssp. *mastichina* in different developmental stages or under culture conditions. *J. Essent. Oil Res.* **2004**, *16*, 111–114.
21. Miguel, M.G.; Simões, M.; Figueiredo, A.C.; Barroso, J.M.G.; Pedro, L.G.; Carvalho, L.M. Evaluation of antioxidant activity of *Thymbra capitata*, *Thymus mastichina* and *Thymus camphoratus* essential oils. *J. Food Lipids* **2005**, *12*, 181–197. [CrossRef]
22. Salgueiro, L.R.; Vila, R.; Tomàs, X.; Cañigueral, S.; da Cunha, A.P.; Adzet, T. Composition and variability of the essential oils of *Thymus* species from section *Mastichina* from Portugal. *Biochem. Syst. Ecol.* **1997**, *25*, 659–672. [CrossRef]
23. Delgado, T.; Marinero, P.; Asensio-S.-Manzanera, M.C.; Asensio, C.; Herrero, B.; Pereira, J.A.; Ramalhosa, E. Antioxidant activity of twenty wild Spanish *Thymus mastichina* L. populations and its relation with their chemical composition. *LWT Food Sci. Technol.* **2014**, *57*, 412–418. [CrossRef]
24. Grosso, C.; Coelho, J.A.; Urieta, J.S.; Palavra, A.M.F.; Barroso, J.G. Herbicidal activity of volatiles from Coriander, Winter Savory, Cotton Lavender, and Thyme isolated by hydrodistillation and supercritical fluid extraction. *J. Agric. Food Chem.* **2010**, *58*, 11007–11013. [CrossRef]
25. Rassem, H.H.A.; Nour, A.H.; Yunus, R.M. Techniques for extraction of essential oils from plants: A review. *Aust. J. Basic Appl. Sci.* **2016**, *10*, 117–127.
26. COE. *European Directorate for the Quality of Medicines*, 6th ed.; European Pharmacopoeia: Strasbourg, France, 2007.
27. Gomes, P.B.; Mata, V.G.; Rodrigues, A.E. Production of rose geranium oil using supercritical fluid extraction. *J. Supercrit. Fluids* **2007**, *41*, 50–60. [CrossRef]
28. Zellner, B.d.A.; Bicchi, C.; Dugo, P.; Rubiolo, P.; Dugo, G.; Mondello, L. Linear retention indices in gas chromatographic analysis: A review. *Flavour Fragr. J.* **2008**, *23*, 297–314. [CrossRef]
29. International Organization for Standardization. 8586:2012. *Sensory Analysis—General Guidelines for the Selection, Training and Monitoring of Selected Assessors and Expert Sensory Assessors*, 12th ed.; ISO: Geneva, Switzerland, 2012; p. 28.
30. International Organization for Standardization. 13301:2018. *Sensory Analysis—Methodology—General Guidance for Measuring Odour, Flavour and Taste Detection Thresholds by a Three-Alternative Forced-Choice (3-AFC) Procedure*, 4th ed.; ISO: Geneva, Switzerland, 2018; p. 28.
31. International Organization for Standardization. 11035:1994. *Sensory Analysis—Identification and Selection of Descriptors for Establishing a Sensory Profile by a Multidimensional Approach*, 12th ed.; ISO: Geneva, Switzerland, 1994; p. 26.
32. Barros, L.; Pereira, E.; Calhella, R.C.; Dueñas, M.; Carvalho, A.M.; Santos-Buelga, C.; Ferreira, I.C.F.R. Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of *Chenopodium ambrosioides* L. *J. Funct. Foods* **2013**, *5*, 1732–1740. [CrossRef]
33. Rodríguez, J.; Ortuño, C.; Benedito, J.; Bon, J. Optimization of the antioxidant capacity of thyme (*Thymus vulgaris* L.) extracts: Management of the drying process. *Ind. Crops Prod.* **2013**, *46*, 258–263. [CrossRef]
34. Balachandran, S.; Kentish, S.E.; Mawson, R. The effects of both preparation method and season on the supercritical extraction of ginger. *Sep. Purif. Technol.* **2006**, *48*, 94–105. [CrossRef]
35. Andrés, M.F.; González-Coloma, A.; Muñoz, R.; De la Peña, F.; Julio, L.F.; Burillo, J. Nematicidal potential of hydrolates from the semi industrial vapor-pressure extraction of Spanish aromatic plants. *Environ. Sci. Pollut. Res.* **2018**, *25*, 29834–29840. [CrossRef]
36. Xiao, Z.; Ma, S.; Niu, Y.; Chen, F.; Yu, D. Characterization of odour-active compounds of sweet orange essential oils of different regions by gas chromatography-mass spectrometry, gas chromatography-olfactometry and their correlation with sensory attributes. *Flavour Fragr. J.* **2016**, *31*, 41–50. [CrossRef]
37. The Good Scents Company. Available online: <https://www.thegoodscentscompany.com/> (accessed on 15 September 2021).
38. Miyazaki, T.; Plotto, A.; Baldwin, E.A.; Reyes-De-Corcuera, J.I.; Gmitter, F.G., Jr. Aroma characterization of tangerine hybrids by gas-chromatography-olfactometry and sensory evaluation. *J. Sci. Food. Agric.* **2012**, *92*, 727–735. [CrossRef]
39. Jensen, S.; Oestdal, H.; Skibsted, L.H.; Larsen, E.; Thybo, A.K. Chemical changes in wheat pan bread during storage and how it affects the sensory perception of aroma, flavour, and taste. *J. Cereal Sci.* **2011**, *53*, 259–268. [CrossRef]
40. Birch, A.N.; Petersen, M.A.; Hansen, Å.S. The aroma profile of wheat bread crumb influenced by yeast concentration and fermentation temperature. *LWT Food Sci. Technol.* **2013**, *50*, 480–488. [CrossRef]
41. Wang, Y.; Zhao, J.; Xu, F.; Wu, X.; Hu, W.; Chang, Y.; Zhang, L.; Chen, J.; Liu, C. GC-MS, GC-O and OAV analyses of key aroma compounds in Jiaozi Steamed Bread. *GOST* **2020**, *3*, 9–17. [CrossRef]
42. Paravisini, L.; Sneddon, K.A.; Peterson, D.G. Comparison of the aroma profiles of intermediate wheatgrass and wheat bread crusts. *Molecules* **2019**, *24*, 2484. [CrossRef] [PubMed]
43. Caputi, L.; Aprea, E. Use of terpenoids as natural flavouring compounds in food industry. *Recent Pat. Food Nutr. Agric.* **2011**, *3*, 9–16. [CrossRef] [PubMed]
44. Abdelli, W.; Bahri, F.; Romane, A.; Höferl, M.; Wanner, J.; Schmidt, E.; Jirovetz, L. Chemical composition and anti-inflammatory activity of Algerian *Thymus vulgaris* essential oil. *Nat. Prod. Commun.* **2017**, *12*, 611–614. [CrossRef]
45. Shabnum, S.; Wagay, M.G. Essential oil composition of *Thymus vulgaris* L. and their uses. *J. Res. Dev.* **2011**, *11*, 83–94.
46. Borugã, O.; Jianu, C.; Mișcă, C.; Goleț, I.; Gruia, A.T.; Horhat, F.G. *Thymus vulgaris* essential oil: Chemical composition and antimicrobial activity. *J. Med. Life* **2014**, *7*, 56–60. [PubMed]

47. Crocoll, C.; Asbach, J.; Novak, J.; Gershenzon, J.; Degenhardt, J. Terpene synthases of oregano (*Origanum vulgare* L.) and their roles in the pathway and regulation of terpene biosynthesis. *Plant Mol. Biol.* **2010**, *73*, 587–603. [[CrossRef](#)] [[PubMed](#)]
48. Lima, A.S.; Schimmel, J.; Lukas, B.; Novak, J.; Barroso, J.G.; Figueiredo, A.C.; Pedro, L.G.; Degenhardt, J.; Trindade, H. Genomic characterization, molecular cloning and expression analysis of two terpene synthases from *Thymus caespititius* (Lamiaceae). *Planta* **2013**, *238*, 191–204. [[CrossRef](#)]
49. Nikolić, M.; Glamočlija, J.; Ferreira, I.C.F.R.; Calhelha, R.C.; Fernandes, Â.; Marković, T.; Marković, D.; Giweli, A.; Soković, M. Chemical composition, antimicrobial, antioxidant and antitumor activity of *Thymus serpyllum* L.; *Thymus algeriensis* Boiss. and Reut and *Thymus vulgaris* L. essential oils. *Ind. Crops Prod.* **2014**, *52*, 183–190. [[CrossRef](#)]
50. Teixeira, M.A.; Rodríguez, O.; Mota, F.L.; Macedo, E.A.; Rodrigues, A.E. Evaluation of Group-Contribution Methods to Predict VLE and Odor Intensity of Fragrances. *Ind. Eng. Chem. Res.* **2011**, *50*, 9390–9402. [[CrossRef](#)]
51. Sonmezdag, A.S.; Kelebek, H.; Selli, S. Characterization of aroma-active and phenolic profiles of wild thyme (*Thymus serpyllum*) by GC-MS-Olfactometry and LC-ESI-MS/MS. *J. Food Sci. Technol.* **2016**, *53*, 1957–1965. [[CrossRef](#)]
52. Benomari, F.Z.; Djabou, N.; Moumani, M.; Hassani, F.; Muselli, A.; Costa, J. Chemical variability of essential oils of three subspecies of *Thymus munbyanus* Boiss. & Reut. from Western Algeria. *J. Essent. Oil Res.* **2020**, *32*, 474–484.