EXTRACTION OF THYMOL FROM DIFFERENT VARIETIES OF THYME PLANTS USING GREEN SOLVENTS

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Abstract. Thyme is a Labiatae plant which essential oil has demonstrated antiseptic and antispasmodic, properties [6, 7]. Thymol (2-isopropyl-5-methylphenol) is the main monoterpene phenol, isomeric with carvacrol, found in thyme essential oil. These compounds have shown antiinflamatory, immunomodulatory, antioxidant, antibacterial and antifungal properties. [8, 9]

In this work, the potential use of different green solvents, namely ethanol, limonene and ethyl lactate to extract thymol from thyme plants is studied. Ethyl lactate and limonene are agrochemical solvents, easily biodegradable, with polarities in the range of acetonitrile and hexane, respectively. Both solvents are recognized as GRAS (generally recognized as safe) and approved by the U.S. Food and Drug Administration as pharmaceutical and food additive. Further, high solubility of thymol in ethyl lactate has been recently determined and reported by the authors [30].

Pressurized liquid extraction (PLE) in an ASE 350 system using the three green liquid solvents at different extraction temperatures (60 ºC, 130 ºC, 200 ºC) was carried out employing *Thymus vulgaris* as model thyme variety. Then, the extraction of thymol from other thyme varieties (*Thymus zygis* and *Thymus citriodorus*) was studied. Extraction yield and thymol recovery obtained in the different extracts was quantified and compared. The three green solvents have shown good capacity to extract thymol from thyme plants.

Keywords: Pressurized Liquid Extraction (PLE), Thyme, Ethyl Lactate, Limonene, Thymol.

1. Introduction

In the European market there are a lot of products derived from natural plants, which are recognized to posses different biological properties, such as antioxidant, antiseptic, diuretic, stimulating the central nervous system, sedative, expectorant, digestive, etc. Some of these plants have been used in traditional medicine since ancient times and are available on market as infusions, tablets and/or extracts.

The genus *Thymus* (Lamiaceae family) is an aromatic plant that includes numerous species with quite different botanical characteristics and a broad chemical heterogeneity [1] and one of the most valued constituents of the herb (especially of the leaves) is the essential oil. Essential oil is formed by volatile aroma compounds and the main components are terpenes, sesquiterpenes and several oxygenated derivative compounds (alcohols, aldehydes, ketones, acids, phenols, ethers, esters, etc.) all of them responsible for the characteristic plant odor and flavor [2]. Thyme essential oil is appreciated for food flavoring [3] in cosmetic, perfumery [4] and in the pharmaceutical industries [5]. Among other properties, antimicrobial activity, antitussive, antispasmodic actions and antioxidative effects have been recognized as pharmacological actions [6, 7]. Among the constituents of thymus essential oil, which contribute to its biological activities, are thymol

and its isomer carvacrol.Thymol (2-isopropyl-5-methylphenol) is the main monoterpene phenol found in thyme essential oil. Thymol and carvacrol have shown antiinflamatory, immunomodulators, antioxidant, antibacterial and antifungal properties [8, 9].The variety most studied is, indeed, *Thymus vulgaris* [10-12]. Yet, particularly attention is focused on *Thymus zygis* [13], a thyme variety widespread over Portugal and Spain and *Thymus citriodorus*, a plant cultivated in the Mediterranean region [14].

Essential oil of thyme herb has usually been obtained by either steam distillation or traditional liquid extraction methods such as Soxhlet extraction, maceration or extraction under reflux. Although these methods are relatively simple, they suffer from several shortcomings as a long extraction time and a relatively high solvent consumption. Pressurized liquid extraction (PLE), a relatively recent solvent extraction technique, could, in principle, eliminate some of the drawbacks of the classical solvent extraction methods. PLE is based on the use of solvents at temperatures above their normal boiling points and pressures enough to keep the extracting fluid in the liquid state during the whole extraction process. By applying these conditions, faster extraction processes result, in which, typically, higher extraction yields are obtained with lower volumes of organic solvents [15, 16].

Several works have been reported about PLE extraction of essential oils from some raw herbal materials [17-20], however, a few works have been reported about PLE extraction of essential oil from thymus. In this sense, hexane, dichloromethane, ethyl acetate and water have been used as extraction solvents of essential oil from thyme herb [21-23].

Ethyl lactate (ethyl 2-hydroxypropanoate) is an agrochemical and economically viable alternative to traditional liquid solvents: it is fully biodegradable, non-corrosive, non-carcinogenic and non-ozone depleting. It was self-affirmed as GRAS (generally recognized as safe) and due to its low toxicity, it was approved by the U.S. Food and Drug Administration (FDA) as pharmaceutical and food additive. These characteristics have increased the attention to the use of ethyl lactate as a green solvent for the food industry. Several reported potential applications are related to the extraction of carotenoids from different plant matrix [24, 25], with the fractionation of edible oil compounds (squalene and tocopherol) [26, 27], with the extraction of gamma-linnolenic acid from the microalgae *Spirulina platensis* [28], and with the extraction of caffeine from green coffee beans and green tea leaves [29]. In addition, high solubility of thymol in ethyl lactate at different temperatures has been reported by the authors in a recent contribution [30].

As ethyl lactate, D-limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) is a non-toxic, non-corrosive, non-carcinogenic and fully biodegradable solvent and was approved by FDA as GRAS. This monoterpenic molecule is the major component of essential oils extracted from citrus peels and is a major by-product of the citrus fruits industry [31, 32]. Therefore, this solvent has been found to be a valuable alternative to the petroleum solvents and, for this reason, D-limonene has been used to remove oil from rice brand [33] and olive seeds [31], and lycopene from tomato [34].

The aim of this work was to test the potential use of different green solvents, namely ethanol, limonene and ethyl lactate to extract thymol from thyme plants. Pressurized liquid extraction was accomplished in an Accelerated Solvent Extraction system ASE 350 using the three green liquid solvents at different extraction temperatures (60 ºC, 130 ºC, 200 ºC) and it was carried out employing *Thymus vulgaris* as model thyme variety. Then, the extraction of thymol from other thyme varieties (*Thymus zygis* and *Thymus citriodorus*) was explored. Extraction yield and thymol recovery obtained in the different extracts was quantified and compared.

2. Material and methods

2.1 Chemicals

Ethanol was HPLC grade from Panreac. Thymol standard (≥ 99%), limonene (90%) and ethyl lactate (≥ 98%) was supplied from Sigma-Aldrich.

2.2 Thyme leaves preparation

The thyme samples (*T. vulgaris*, *zygis* and *citriodorus*) consisted of dried leaves obtained from a herbalist´s producer (Murcia, Spain). The samples were ground in a cooled mill and particle of sizes smaller than 500 μm were separated by using sieves and employed for the experiments. The whole sample was stored at -20 ºC until use.

2.3 Pressurized Liquid Extraction (PLE)

Thymol extractions were carried out in an Accelerated Solvent Extraction system ASE 350 from Dionex Corporation (Sunnyvale, CA, USA) equipped with a solvent controller unit. A scheme of the equipment is shown in Figure 1.

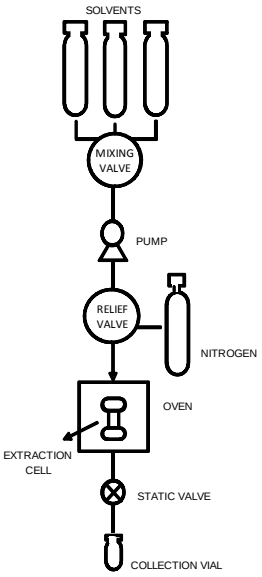

Figure 1. Scheme of ASE device employed in the present work.

The cells employed (10 ml capacity) were placed into an oven; each cell was filled with around 1 g of solid sample. After loading the sample into the extraction cell, the cell was filled with the corresponding solvent up to a pressure of 10 MPa (which ensures the liquid state of the three solvents employed at the three studied temperatures) and was heated-up to the desired temperature. In order to prevent over-pressurization of the cell, a static valve pulses open and close automatically when the cell pressure exceeds the set point. The solvent that escapes during this venting is collected in the collection vial. Then, a static extraction continues, in which all system valves are closed. After extraction the cell was washed with the solvent and subsequently the solvent was purged from cell using N_2 gas until complete depressurization was accomplished.

2.4 Determination of thymol by GC/MS analysis

The analyses of thyme extracts were carried out in Agilent 7890A System (Agilent Technologies. Santa Clara, California, USA) comprising a split/splitless injector, electronic pressure control, G4513A auto injector, a 5975C triple-axis mass spectrometer detector, and GC/MS Solution software. The column used was an Agilent HP-5MS capillary column (30 m × 0.25 mm i.d. and 0.25 µm phase thickness). The chromatographic method was as follows: oven temperature programming was 60 ºC isothermal for 4 min then increased to 106 ºC at 2.5 ◦C/min and from 106 ºC to 130 ºC at 1 ºC/min and finally from 130 ºC to 250 ºC at 20 ºC/min and this temperature was kept constant for 10 min. Sample injections (1 µL) were performed in split mode (1:10). Chromatography separation was carried out at constant pressure of 143.6 kPa and Helium (99.996 mass %) was used as a carrier gas. Injector temperature was 250 ºC and mass spectrometer ion source and interface temperatures were 230 ºC and 280 ºC respectively. Mass spectrometer was used in total ion current (TIC) mode and samples were scanned from 40 to 500 amu. Thymol was identified by comparison with standard mass spectra and compared with the mass spectra from library Wiley 229.

3. Results and discussion

In Table 1, the influence of the extraction time (10 and 20 min) on the extraction yield, the thymol concentration and the quantity of recovered thymol during the ASE extraction of *Thymus vulgaris* using ethyl lactate as solvent at 200 ºC is shown. The values given in the table correspond to the average values resulted

from duplicate experiments. Standard deviations are also given in the table. As it can be seen, the extraction yield obtained in 10 min represents 83 % of the yield obtained in 20 min. Further the recovery and concentration of thymol obtained in both experiments are very similar. Considering these results, 10 minutes were selected to study the effect of temperature on thymol recovery.

Table 1. Extraction yield (g of extract / g of sample × 100), thymol concentration (g thymol / g extract × 100) and thymol recovery (mg of thymol / g of sample) obtained in the ASE of *Thymus vulgaris* L. using ethyl lactate at 200 °C and two different extraction times: 20 and 10 minutes.

		extraction time	
		10 min	20 min
Solvent: ethyl lactate			
	extraction yield(%)	22.66 ± 0.26	27.29 ± 0.90
	thymol concentration (%)	3.75 ± 0.17	3.25 ± 0.03
	thymol recovery	8.50 ± 0.28	8.88 ± 0.38

Results obtained from the extraction of thymol from *Thymus vulgaris L*. using the three different solvents, at 60 °C, 130 °C and 200 °C and in 10 min of extraction time, are shown in Table 2. As it was expected, despite the used solvent, extraction yield increased with the temperature. On the other hand, ethyl lactate and limonene show very similar behavior: the thymol concentration decreased with temperature (see Figure 2) and only slight higher thymol recovery was obtained as increasing extraction temperature. It appears that higher extraction temperature results in a greater solubilization of compounds other than thymol. In the case of ethanol, the highest thymol concentration and recovery were observed at 130 °C.

Also, it should be noted that the highest concentrations of thymol in the extracts were obtained with limonene, due to its physicochemical character (it is part of plant essential oils), followed by ethyl lactate.

Comparing the results obtained in this work with data reported in the literature [22, 23], it was possible to attain thymol recoveries higher than those obtained with traditional methods, such as Soxhlet (≈ 6.8 mg/g of thymol recovery) and steam distillation (≈ 8.2 mg/g of thymol recovery). The recovery was very close to the supercritical fluid extraction (SFE) recoveries (≈10.1 mg/g) [22]. In addition, by using these "green solvents" the thymol recovered was slightly lower than PLE extraction with "non-green solvents" like hexane (≈ 10.7 mg/g), ethyl acetate (≈ 12.8 mg/g) or dichloromethane (≈ 12.2 mg/g). On the other hand, thymol recoveries were higher than those obtained with subcritical water extraction (SWE) (≈ 7 mg/g) [22, 23].

Table 2. Extraction yield (g of extract / g of sample × 100), thymol concentration (g thymol / g extract × 100) and thymol recovery (mg of thymol / g of sample) obtained in the ASE of *Thymus vulgaris* L. using ethyl lactate, ethanol and limonene.

		extraction temperature		
		60 °C	130 °C	200 °C
Solvent: ethyl lactate				
	extraction yield (%)	6.21 ± 0.03	8.02 ± 0.30	22.66 ± 0.26
	thymol concentration (%)	13.88 ± 0.10	11.78 ± 0.43	4.65 ± 0.02
	thymol recovery (mg/g)	8.62 ± 0.03	9.44 ± 0.01	10.53 ± 0.16
Solvent: ethanol				
	extraction yield (%)	10.55 ± 1.97	15.91 ± 0.36	21.84 ± 2.41
	thymol concentration (%)	6.59 ± 0.89	6.89 ± 0.78	4.88 ± 0.67
	thymol recovery (mg/g)	7.03 ± 2.23	10.98 ± 1.49	10.58 ± 0.28
Solvent: limonene				
	extraction yield (%)	4.37 ± 0.15	7.16 ± 0.54	9.52 ± 1.47
	thymol concentration (%)	18.15 ± 0.25	12.59 ± 0.49	10.06 ± 0.82
	thymol recovery (mg/g)	7.93 ± 0.39	9.00 ± 0.32	9.52 ± 0.70

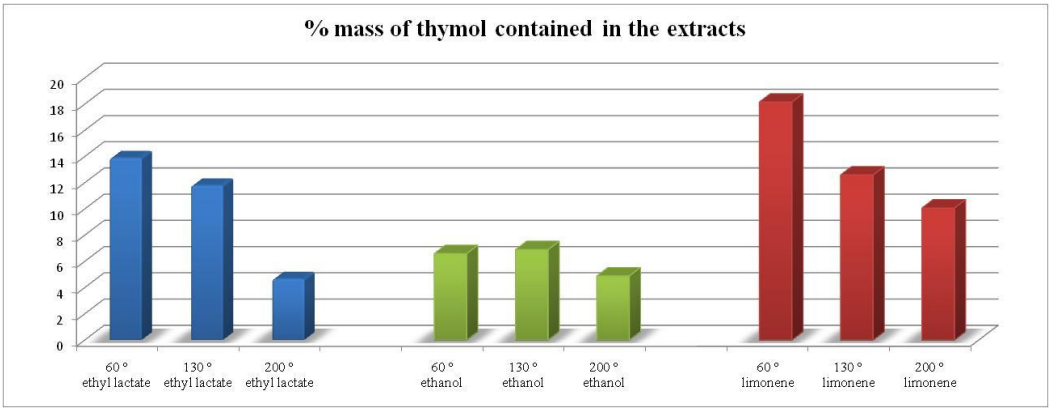

Figure 2. Concentration of thymol (%) contained in the extracts

Results obtained from the extraction of thymol from the other varieties of thyme (namely *Thymus zygis* and *Thymus citriodorus*) using the three different solvents at 60 °C and 10 minutes of extraction time are shown in Table 3. In the case of *Thymus zygis*, the extraction yields obtained were higher than those obtained from *Thymus vulgaris*, especially in the case of ethyl lactate and limonene. Additionally, the thymol concentration obtained from *Thymus zygis* (especially in the case of ethyl lactate and limonene) was lower than in the case of *Thymus vulgaris*. Limonene was the solvent which removed the highest quantity of thymol from *Thymus zygis*, followed by ethyl lactate. In the case of *Thymus citriodorus*, no presence of thymol was detected despite the solvent employed. These results agree with some reports in the literature [35] in which significant lower amounts of thymol were detected in the essential oil of *Thymus citriodorus* in comparison with the *Thymus zygis* and *Thymus vulgaris* varieties.

Table 3. Extraction yield (g of extract / g of sample × 100), thymol concentration (g thymol / g extract × 100) and thymol recovery (mg of thymol / g of sample) obtained in the ASE of *Thymus zygis* L. and *Thymus citriodorus* L. using ethyl lactate, ethanol and limonene at 60°C.

		Thymus zygis	*Thymus citriodorus*
Solvent: ethyl lactate			
	extraction yield (%)	9.25 ± 0.48	8.32 ± 0.21
	thymol concentration (%)	6.77 ± 0.10	-
	thymol recovery (mg/g)	6.26 ± 0.23	-
Solvent: ethanol			
	extraction yield (%)	12.09 ± 0.01	11.10 ± 1.56
	thymol concentration (%)	5.40 ± 0.02	-
	thymol recovery (mg/g)	6.53 ± 0.02	-
Solvent: limonene			
	extraction yield (%)	9.36 ± 0.17	11.35 ± 0.17
	thymol concentration (%)	8.96 ± 0.13	-
	thymol recovery (mg/g)	8.39 ± 0.03	-

4. Conclusion

The potential use of three green solvent namely ethyl lactate, ethanol and limonene in the extraction of thymol from three varieties of *Thymus* (*vulgaris*, *zygis* and *citriodorum*) was presented in this work. PLE is a suitable technology and the three green solvents show good capacity to extract thymol from thyme plants, obtaining higher yields of thymol in shorter extraction times with lower consumption of extraction solvents than the traditional extraction method like steam distillation.

Acknowledgements

The authors gratefully acknowledge the financial support from the Ministerio de Ciencia e Innovación (project 25506 FUN-C-FOOD) and Comunidad Autónoma de Madrid (ALIBIRD, project S-505/AGR-0153). D. Villanueva thanks for the JAE-pre fellowship given by Consejo Superior de Investigaciones Científicas (CSIC) of Spain.

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