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Rapid Method for Simultaneous Quantitative Determination of Four Major Essential Oil Components from Oregano (*Oreganum* sp.) and Thyme (*Thymus* sp.) Using FT-Raman Spectroscopy

NIKOLAOS G. SIATIS, ATHANASIOS C. KIMBARIS, CHRISTOS S. PAPPAS, PETROS A. TARANTILIS, DIMITRA J. DAFERERA, AND MOSCHOS G. POLISSIOU*

Department of Science, Laboratory of Chemistry, Agricultural University of Athens, 75 Iera Odos, 118 55 Athens, Greece

A simple and rapid method for the quantitative determination of four major components found in oregano and thyme essential oils is presented. The method correlates the Raman peak intensity in the spectral region from 1800 to 600 cm^{-1} and the concentration percentage of each particular constituent in the sample. To achieve accurate quantification results and avoid the risk of overlapping peaks of unknown Raman-active substances in natural essential oils, the peaks must be analyzed. For this purpose, PEAKSOLVE software (Ver. 1.0.5) was used. Unknown samples were measured with the FT-Raman method, and the results were compared to those of the gas chromatographic (GC) analysis. The comparison was made at a confidence level of 99%, and the two methods scored equally in terms of repeatability and accuracy even at the edge of the method specifications. The new method can provide accurate results in very short times once the setup is complete and could be utilized in areas where vast amounts of samples must be analyzed.

KEYWORDS: FT-Raman; oregano; thyme; essential oil; quantitative; deconvolution

INTRODUCTION

Thyme and oregano are plants native to the Mediterranean region that have been used in traditional medicine and as spices in food for centuries. Their spice value and properties are due to the aromatic and other volatile compounds generally referred to as essential oils of these aromatic plants. Essential oils derived from these plants have valuable pharmacological properties that have been investigated by many scientists around the world (*1*−*7*).

The potential of application of these compounds as additives in foods is large, and their most common use is as preservatives for stored food (*8*). The industry has also taken serious interest in these applications and has launched a series of products based on essential oils as organic feed supplements for animals. The supporting arguments regarding the use of essential oils for animal stock dietary purposes are focused on their natural derivation and their medicinal properties.

Many different varieties of oregano and thyme appear to vary in their essential oil composition (*9*). Variability is observed among plants growing in different countries around the world (*10*−*13*) and also during the plant life cycle (*14*). All widely used varieties of thyme and oregano show a high content of four major constituents, *p*-cymene, γ-terpinene, thymol, and carvacrol. Those four elements usually add up to more than 90% of the essential oil (*10*, *11*, *13*).

Gas chromatography−mass spectrometry (GC−MS) is the most popular method for the determination of essential oil composition. Components existing in the essential oil can be identified by comparison of their relative retention times and their mass spectra. Fourier transform Raman spectroscopy (FT-Raman) and Fourier transform infrared (FT-IR) spectroscopy are techniques mainly used for qualitative determination of compounds based on their unique spectrum. Raman spectroscopy has been used for quantitative determination in mixture samples by our team for simultaneous determination of two compounds in mastic gum oil (*15*), and the challenge is toward more with one single measurement. FT-IR spectroscopy has also been used for quantification of lignin in wood (*16*). Vibrational spectroscopy methods provide accurate results in a short time (*15*−*17*), and when combined with the appropriate software can prove to be valuable tools for routine checks.

In this Article, we present a method in which quantitative determination of the four basic essential oil components from the most important Lamiaceae plants can be achieved in a rapid, simple, nondestructive way by applying FT-Raman spectroscopy. The method focuses on simultaneous quantification of the constituents rather than on single compound measurements. The analysis results provide adequate information for commercial classification of the product. A GC method was also used as a reference to the results. The GC and spectral quantification results are shown further in this Article. The aim of this work is to demonstrate the capacity of FT-Raman

* Author to whom correspondence should be addressed [telephone +30 210 529 4241; fax +30 210 529 4265; e-mail mopol@aua.gr].

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Table 1. Standard Solutions Composition

standard	thymol (%)	carvacrol (%)	*p*-cymene (%)	γ-terpinene (%)
1	85	3	7	5
2	70	10	6	14
3	55	20	5	20
4	45	30	15	10
5	35	40	14	11
6	30	50	4	16
7	25	60	9	6
8	15	70	12	3
9	10	80	3	7
10	3	85	8	4

spectroscopy in applications on the quantitative analysis field, with the use of the proper software tools where necessary.

MATERIALS AND METHODS

Plant Material. Cultivated oregano was provided by a commercial grower. Natural grown oregano (*Oreganum* sp.) and thyme (*Thymus* sp.) were collected from specific regions in Greece, air-dried, and kept in the dark until distillation.

Isolation of the Essential Oils. Five essential oils from thyme and oregano were isolated using the Clevenger apparatus for hydrodistillation for 4 h. Without any further treatment, essential oils were stored at −18 °C until analysis. The five samples were randomly characterized as essential oil 1, essential oil 2, essential oil 3, essential oil 4, and essential oil 5.

Chemicals and Standards. Pure thymol, carvacrol, *p*-cymene, and γ-terpinene were purchased from Sigma-Aldrich Co. (St. Louis, MO). Thymol is provided in solid phase by Sigma-Aldrich, and, to produce reliable calibration standard mixtures, it was necessary to further treat it to receive liquid-state thymol. The crystal state in which thymol was obtained does not allow good-quality spectra to be recorded. Liquid-state thymol is first obtained by fair heating in sealed vessel. When cooled, thymol will return to its solid-crystal form unless water molecules existing in traces are removed to inhibit crystal reformation. This is easily achieved with the addition of a few milligrams of anhydrous magnesium sulfate while in the liquid state. Liquid thymol can stay in this form for days at room temperature; however, for calibration standards and FT-RAMAN spectra, thymol was prepared minutes before measurement.

Ten standard mixtures were prepared containing the four basic essential oil components *p*-cymene, γ-terpinene, thymol, and carvacrol in different concentrations ranging between the lower and higher quantities most commonly found in oregano and thyme essential oils (**Table 1**). All standards were prepared minutes before measurement to ensure reliability.

GC Instrumentation and Conditions. The analysis of the essential oils was performed using a HP-5890 II GC equipped with a 30 m × 0.25 mm i.d., 0.25 μm HP-5 ms capillary column and flame ionization detector (FID). Helium was the carrier gas. Column temperature programming was as follows: starting temperature was 55 °C raised to 100 °C at 3 °C min, held for 5 min, and then raised to 220 °C at 5 °C/min. The GC−MS analyses were performed under the same conditions with GC, using an HP 5972 mass selective detector. Injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. For GC−MS detection, an electron ionization system was used with an ionization energy of 70 eV. Each sample was then analyzed three times with GC−FID to obtain the percentage concentration of each constituent and standard deviations.

All samples were injected manually and splitless after dilution (1/100 in methanol). The percentage composition of each component was calculated with the 100% method using chromatogram peak areas.

FT-Raman Instrumentation and Conditions. All samples were measured in triplets using a Nicolet 750 FT-Raman spectrometer, equipped with a Nd:YAG laser source that emits at 1064 nm. In addition, a CaF_2 beam splitter, an indium−gallium−arsenide (InGaAs) detector, and 180° backscattering geometry were used in the spectrometer. Routine procedures such as bench alignment and fine-tuning of the spectrometer were held before each batch of measurements. Sample cells were Wimad WG-SM NMR tubes of 4.97 mm outer diameter and 0.38 mm wall thickness. Optimum time/spectra quality was determined at 100 scans (3 min) with a resolution of 4 cm^{-1}. Each Raman spectrum was automatically smoothed and baseline corrected using the proper functions from the built-in spectrometer software Omnic 3.1.

RESULTS AND DISCUSSION

As mentioned before, the standard method for qualitative analysis and quantification of essential oil components is the GC−FID, GC−MS method. Characterization of these or similar compounds in essential oils has been previously published (*18*, *19*) on a qualitative analysis basis by FT-IR spectrometry. FT-Raman spectra of carvacrol, *p*-cymene, γ-terpinene, and treated thymol (as above) are shown (**Figure 1**) and discussed below.

In every spectrum, four regions can be analyzed from 1800 to 600 cm^{-1} (**Table 2**), the conjugated and nonconjugated double bond C═C vibrations from 1800 to 1500 cm^{-1}, the methyl and isopropyl bending region from 1500 to 1300 cm^{-1}, the stretching of the single C−C bond and C−O bonds between 1300 and 1000 cm^{-1}, and the breathing mode and out-of-plane C−H vibrations from 1000 to 600 cm^{-1}.

The spectrum of γ-terpinene shows no significant peak in the region 1300−1000 cm^{-1} as expected, and the strongest peak is at 1700 cm^{-1} assigned to nonconjugated double bonds of the 1,4-cyclohexadiene ring. The methyl and isopropyl C−H bending appears as a double broad band at 1428 cm^{-1}, and the ring-breathing mode shows at 756 cm^{-1} (*19*).

The C═C stretching vibration of the aromatic ring of *p*-cymene is shown at 1610 cm^{-1}, and the isopropyl methyl group symmetric bending vibration is shown at 1440 cm^{-1}. The asymmetric vibration of the same group appears at 1380 cm^{-1}. The single C−C bonds stretching are assigned to the peaks at 1210 and 1180 cm^{-1}, while the strong double peak at 804 cm^{-1} is assigned to the phenyl nucleus breathing mode. This peak's shape is probably affected by the different substituents of the para-substituted phenyl (*19*−*21*).

Table 2. FT-Raman Peak Positions, Assignments, and Observed Intensities in the Spectral Region of 1800–600 cm^{-1}

peak position	assignment	intensity[a]	γ-terpinene	*p*-cymene	thymol	carvacrol
1700	nonconjugated C═C stretching	s	+	−	−	−
1620–1590	conjugated C═C stretching vibration	s	−	+	+	+
1460–1440	isopropyl methyl (sym)	ms	+	+	+	+
1380	isopropyl methyl (as)	ms	+	+	+	+
1260–1180	stretching phenyl nucleus	w	−	+	+	+
1080–1110	C−O stretching	w	−	−	+	+
1060	$\gamma_{(C-H)}$ aromatic	ms	−	+	+	+
880	$\gamma_{(C-H)}$	w, ms	+	+	+	+
740–804	breathing mode (ring deformation)	vs	+	+	+	+

[a] w, weak; ms, moderate strong; s, strong; v, variable; vs, very strong.

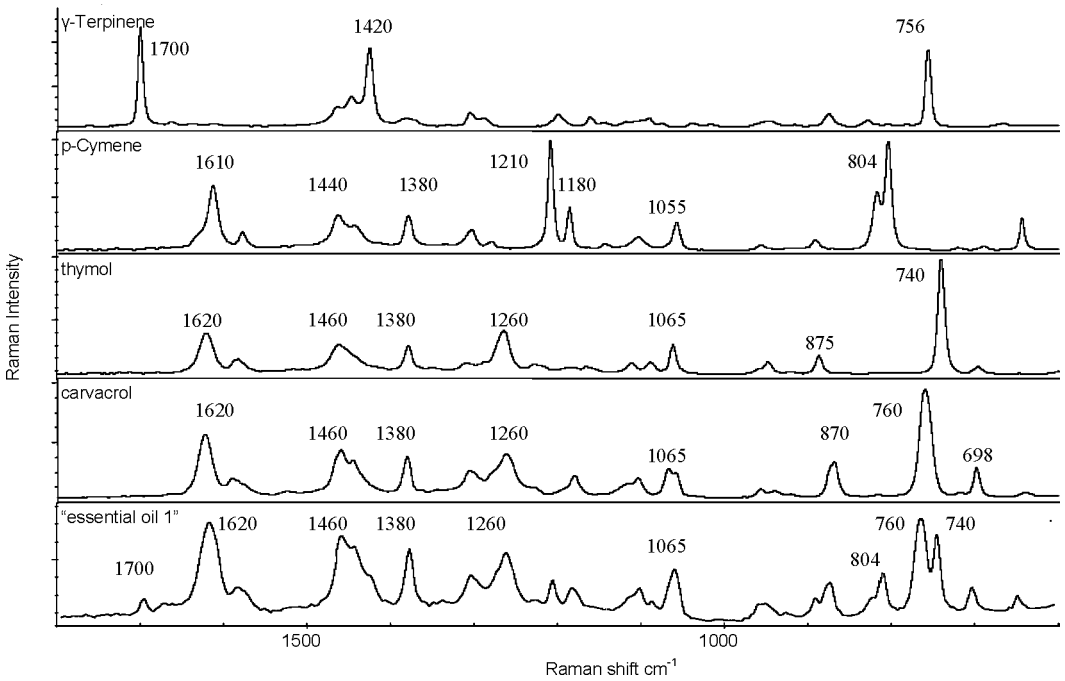

Figure 1. FT-Raman spectra of four pure individual compounds [carvacrol, thymol, γ-terpinene, *p*-cymene] and essential oil 1 [thymol 24.7%, carvacrol 46.6%, *p*-cymene 14.8%, γ-terpinene 4.46%].

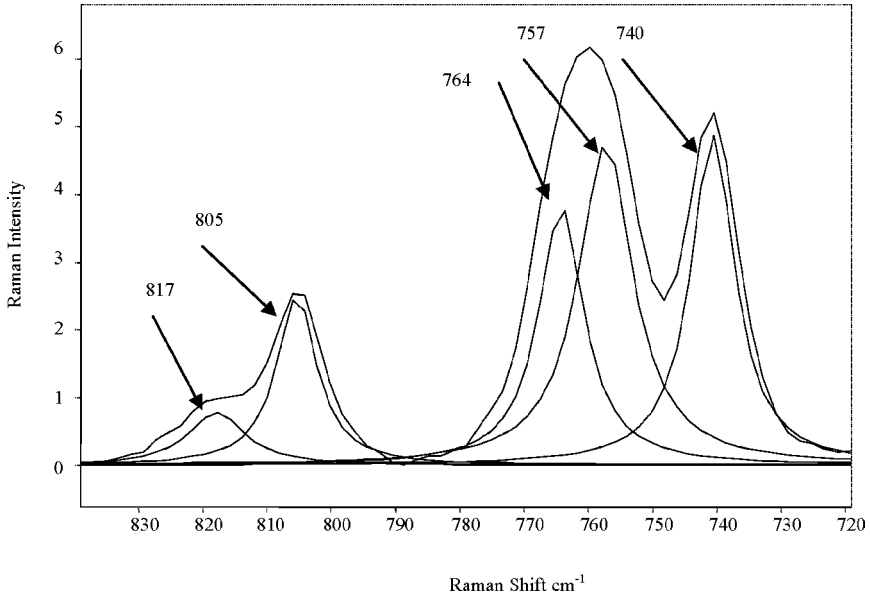

Figure 2. Deconvoluted FT-Raman spectrum region from 720 to 840 cm^{-1} of essential oil 1.

The FT-Raman spectra of carvacrol and thymol show the same peaks at 1620, 1460, and 1380 cm^{-1} assigned as above. The common peak at 1260 cm^{-1} not present in the spectra of *p*-cymene and γ-terpinene is assigned to the trisubstituted aromatic ring. The breathing mode vibrations for thymol and carvacrol appear at 740 and 760 cm^{-1}, respectively.

Quantitative Analysis. As mentioned above, quantification of essential oil compounds has been attempted on the basis of their infrared spectrum. By nature, the infrared spectra are more complex than Raman spectra (*18*) and involve a higher risk of overlapping peaks when natural essential oils are in question. The advantage of FT-Raman spectra in comparison to the infrared spectra is very clear as strong and sharp peaks result in lower mistakes in linearity. In such applications, FT-Raman spectroscopy is likely not be more reliable than FT-IR spectroscopy because overtones and combination bands are rarely observed in Raman spectroscopy except in the unusual situation where a Fermi resonance occurs.

FT-Raman spectra peaks used in the quantification procedure were chosen to be representative of the compound and of sufficient intensity. Hence, the peak at 1701 cm^{-1} will be used to quantify γ-terpinene as it uniquely identifies this component in standard mixtures. The peaks at 804 and 740 cm^{-1} will be used to quantify *p*-cymene and thymol, respectively. In the case of carvacrol, the strongest peak is at 760 cm^{-1}, but the γ-terpinene peak at 756 cm^{-1} presents a resolution issue. That makes it necessary to analyze the peak to obtain accurate peak height measurements for quantification. A characteristic deconvoluted spectrum is shown in **Figure 2**. The reasolved peaks' heights and percentage concentrations were used to produce three calibration curves for carvacrol, thymol, and *p*-cymene. The calibration curve for γ-terpinene was produced by the

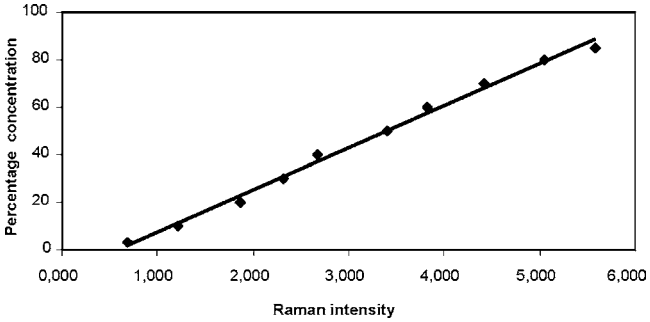

Figure 3. Calibration curve for carvacrol.

Table 3. Percentage Concentrations Obtained by GC–FID and Spectral Quantification

essential oil		GC–FID %[a]	Raman %[a]	F_{exptl}[b]	t_{exptl}[b]
1 (oregano)	*p*-cymene	14.8 ± 1.4	16.7 ± 0.2	49.00	1.900
	γ-terpinene	4.46 ± 0.45	4.77 ± 0.08	31.64	0.959
	thymol	24.7 ± 1.2	28.78 ± 0.46	6.81	4.900
	carvacrol	46.6 ± 1.8	56.5 ± 2.0	1.23	5.203
2 (oregano)	*p*-cymene	4.17 ± 0.76	5.078 ± 0.010	5776.00	1.689
	γ-terpinene	2.19 ± 0.39	2.27 ± 0.24	2.64	0.247
	thymol	0.29 ± 0.05			
	carvacrol	80.9 ± 2.1	74 ± 10	22.68	0.955
3 (oregano)	*p*-cymene	15.5 ± 1.4	16.90 ± 0.46	9.26	1.344
	γ-terpinene	2.2 ± 0.2	2.08 ± 0.17	1.38	0.646
	thymol	0.84 ± 0.04			
	carvacrol	73.20 ± 1.9	77.57 ± 0.30	40.11	3.213
4 (oregano)	*p*-cymene	5.82 ± 0.14	7.58 ± 0.12	1.36	13.499
	γ-terpinene	4.670 ± 0.088	5.54 ± 0.34	14.93	3.503
	thymol	2.515 ± 0.028	5.39 ± 0.37	174.62	10.958
	carvacrol	77.58 ± 0.28	57.9 ± 4.2	225.00	5.927
5 (thyme)	*p*-cymene	7.88 ± 0.58	9.68 ± 0.26	4.98	4.005
	γ-terpinene	6.41 ± 0.45	7.73 ± 0.16	7.91	3.909
	thymol	0.28 ± 0.03			
	carvacrol	72.45 ± 0.97	64 ± 12	153.04	0.993

[a] $n = 3$. [b] Confidence level of 0.99.

measured height of the peak at 1701 cm^{-1} because it is unique in every observed spectrum. The calibration equations are shown below:

$$p\text{-cymene (\%)} = (0.97 \pm 0.72) + (6.24 \pm 0.55)A; \quad r = 0.94, n = 3$$

$$\gamma\text{-terpinene (\%)} = -(0.18 \pm 0.33) + (6.31 \pm 0.19)A; \quad r = 0.99, n = 3$$

$$\text{thymol (\%)} = (3.34 \pm 0.85) + (5.31 \pm 0.11)A; \quad r = 0.99; n = 3$$

$$\text{carvacrol (\%)} = (-10.18 \pm 1.66) + (17.71 \pm 0.48)A; \quad r = 0.99; n = 3$$

where A is the intensity of specific peaks for each compound. The characteristic calibration curve for carvacrol is shown in **Figure 3**.

The quantification results comparison with the GC−FID analysis results for five natural essential oils is shown in **Table 3**, along with their standard deviations. The percentage concentrations fluctuate between 4.2 and 15.5 for *p*-cymene with the GC method and 5.1 and 16.9 with the Raman method. For γ-terpinene, the percentages were between 2.2 and 6.4, and 2.1 and 7.7 for the GC and Raman, respectively. For thymol, it is expected that in the cases that the concentration is lower than 3% the Raman method cannot detect it. Finally, for carvacrol, the percentage concentrations range between 46.6 and 80.9 for GC and 56.5 and 77.6 for Raman. The variation of the two methods is acceptable as shown by the *F*-test and *t*-test as follows.

The statistical tools of the *F*-test and *t*-test were used to determine the repeatability and sensitivity of the proposed methodology, respectively. The theoretical values for a confidence level of 0.99 are 99 for the *F*-test and 4.604 for the *t*-test. For the majority of the measurements, the percentage concentrations determined by the GC−FID method do not differ significantly from those calculated with the Raman method. It was shown that, even at the edge of the method limits, the spectral quantification is as accurate and reproducible as the standard method for the majority of the measurements.

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