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ARTICLE

Quantitative Determination of Pulegone in Pennyroyal Oil by FT-IR Spectroscopy

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Pulegone constitutes a monoterpene occurring in *Mentha* species and primarily in *Mentha pulegium* L. (pennyroyal). A major source of human exposure to pulegone is the use of pennyroyal essential oil in flavorings, confectionery and cosmetics. The rapid quantification of pulegone in hydrodistilled pennyroyal oils (which were also "spiked" to increase the validation range) by Fourier transform infrared spectroscopy (FT-IR) combined with partial least-squares (PLS) regression was evaluated, using the spectral region 1650−1260 cm^{-1}. Gas chromatography was applied as the reference method for pennyroyal oil samples, which ranged in pulegone content from 157 to 860 mg/mL. The two methods were subjected to statistical tests and proved equivalent in terms of accuracy and reproducibility (99% confidence level). The use of FT-IR spectroscopy could offer a viable alternative to the standard analysis procedures presently applied for quantification of valuable plant substances and could also provide the processing industry with a simple and high-throughput technique for the fast quality check of incoming raw materials such as pennyroyal oils.

KEYWORDS: Fourier transform infrared spectroscopy; PLS; quantitative analysis; *Mentha pulegium*; essential oil; hydrodistillation

INTRODUCTION

Pennyroyal oil is a volatile oil derived by steam distillation or hydrodistillation from European (*Mentha pulegium* L.) and American (*Hedeoma pulegioides* (L.) Pers.) species of pennyroyal, principally from the leaves and flowering tops (*1*). Historically, pennyroyal oil has been used as a folk remedy for dizziness or as an anticonvulsive and sedative (*2*), while it is still sometimes used in traditional medicine for abortions and the induction of menses (*3*, *4*). Nevertheless, there are no approved medicinal uses for pennyroyal oil (*1*). Leaves containing pennyroyal oil have been used as flavoring, as spice and for brewing teas (*3*). Because of the strong aromatic, mint-like pungent smell, pennyroyal oil is a constituent of alcoholic beverages and a frequently used raw material in flavorings, confectionery and cosmetics (*1*, *3*). It has been reported that the pungent smell of *Mentha pulegium* is associated with the presence of pulegone (*2*).

(*R*)-(+)-Pulegone, also referred to as pulegone, is a hepatotoxic monoterpene occurring in plant tissues from a number of species, including pennyroyal (*3*). The (*R*)-(+)-enantiomer comprises the major constituent of pennyroyal essential oils (*3*, *5*), and the toxicological database largely relates to this isomer (*6*, *7*), whereas the other enantiomer, (*S*)-(−)-pulegone, is rarely found in essential oils (*8*). Pulegone generally ranges from traces (< 0.1%) up to 90.7% in pennyroyal oil (*3*, *9*). However, it has been reported that the usual pulegone content in pennyroyal oil is 60−90%, especially when pulegone-type oils are mentioned (*10*). Hepatotoxicity of pulegone is due, at least in part, to its major metabolite, menthofuran (*8*) and results from the ingestion of pennyroyal oil as an abortifacient and as an infusion for the treatment of colic in children (*1*). Toxicological observations also indicate that pulegone is an insect neurotoxin (*11*). The European Commission set maximum levels for pulegone in the following foods and beverages: foodstuffs, 25 mg/kg; beverages, 100 mg/kg; peppermint or mint-flavored beverages, 250 mg/kg; and mint confectionery, 350 mg/kg (*7*). Pulegone may not be added as such to foodstuffs (*6*). Recommended limits of pulegone in cosmetic formulations are ≤1% (*1*). Conversely, pulegone is listed among the authorized synthetic flavoring substances in the USA (*12*).

To date the pulegone content occurring in essential oils, such as pennyroyal oil, along with related products is predominantly determined by chromatographic techniques (*5*, *12*, *13*). Gas chromatography (GC) has been established as the standard method for the analysis of essential oil volatile compounds, and since it is time-consuming, numerous attempts have been recently made to find alternative analytical options. In this context, molecular spectroscopy techniques such as mid-infrared (MIR) or near-infrared (NIR) in combination with suitable chemometric algorithms have been shown to be advantageous for rapid evaluation of essential oils and other plant-derived products (*14*−*17*). However, the peaks in the NIR region are broad and weak, as they are combinations and overtones of the sample functional groups (*18*).

The objective of this study was to develop a direct and rapid method to quantify pulegone, the compound which characterizes a pennyroyal essential oil, using Fourier transform mid-infrared

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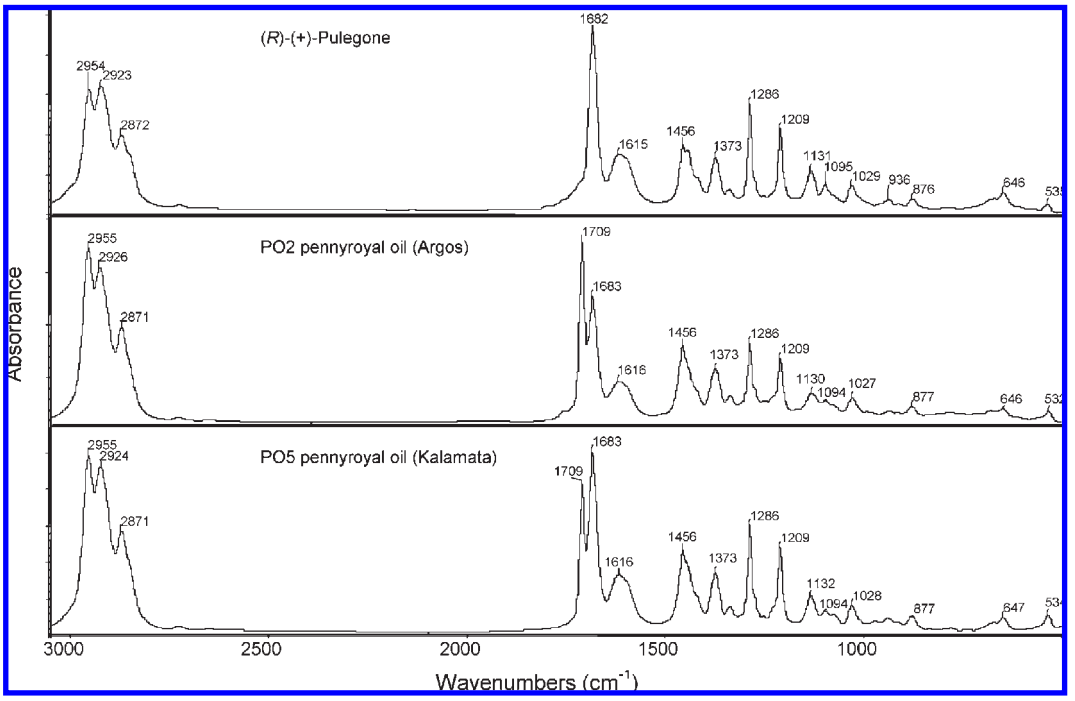

Figure 1. FT-IR spectra of pulegone and pulegone-rich pennyroyal oils over the spectral region 3050−500 cm^{-1}.

spectroscopy (FT-IR) associated with chemometric manipulation. Results were compared with those obtained using gas chromatography as a reference method.

MATERIALS AND METHODS

Plant Materials and Reagents. Aerial plant parts of six different fully flowered pennyroyal populations were provided from various collect areas around Greece: (1) Island of Samos (P1); (2) Argos (P2); (3) Evia (P3); (4) Island of Samothraki (P4); (5) Kalamata (P5) and (6) Island of Crete (Heraklion) (P6). All pennyroyal plants were collected in the years 2007−2008 during midsummer and were acquired from local retailers (i.e., health food stores and spice stores).

(*R*)-(+)-Pulegone, α-pinene, *β*-pinene, and (−)-menthol were purchased from Aldrich (Steinheim, Germany). (+)-Menthone was purchased from Fluka (Steinheim, Germany), while eucalyptol and limonene were purchased from Sigma (St. Louis, MO). Piperitone and *iso*-menthone were acquired from Extrasynthese (Genay, France). The purity grade of all the pure standard substances used was more than 98%.

Isolation of Essential Oils. A sample (200 g) of each pennyroyal population was ground in an electric blender and subsequently subjected to hydrodistillation for 4 h, using a Clevenger-type apparatus (*19*). The received pennyroyal essential oils from plant materials P1−P6 were named as PO1−PO6, respectively. Moreover, they were dried over anhydrous magnesium sulfate and, after filtration, stored in labeled sterile screw capped bottles at −22 °C until further use.

Analysis of Essential Oils. All isolated pennyroyal oils (PO1−PO6) were analyzed using a Hewlett-Packard 5890 series II gas chromatograph (GC) fitted with an HP-5 ms capillary column (30 m × 0.25 mm i.d., 0.25 *μ*m film thickness) and coupled with Hewlett-Packard 5972 mass selective detector, operating in EI mode at 70 eV. The injector and detector temperatures were set at 220 and 290 °C, respectively. The column temperature was gradually raised from 60 to 240 °C at a rate of 3 °C/min and then held isothermally for 10 min. The carrier gas was helium, with a constant flow rate of 1 mL/min. Diluted samples of 1.0 *μ*L (1/100 in diethyl ether, v/v) were injected manually in splitless mode. The identification of the major volatile compounds of essential oils was based on their relative retention time and their mass spectra in comparison with those observed by the aforementioned pure standard substances. For the other components, tentative identification was performed by matching their mass spectra and elution order with those obtained from NIST 98 and Wiley 275 libraries as well as literature data (*20*).

Sample Preparation. In order to develop the FT-IR method for determining pulegone, two different sets of samples were prepared: 31 samples with various concentrations of pulegone in ethanol (46.8−889.2 mg/mL) for calibration and 10 oil samples for validation. Concerning the latter set of samples, to expand the range of concentration of initial pulegone content in the five pennyroyal oils obtained, pennyroyal oil samples PO1−PO5 were "spiked" with authentic pulegone or PO6 oil and thus five more samples (SPO1−SPO5) were finally prepared so as to test the predictive ability of the FT-IR method.

Reference GC−FID Analysis. The specific amounts (reported as peak area) of pulegone contained in the pennyroyal oil samples (i.e., PO1−PO5 and SPO1−SPO5) were determined by gas chromatography−flame ionization detection (GC−FID) with the use of a Hewlett-Packard 5890 series II GC, equipped with an FID detector and an HP-5 ms capillary column. Detector and injector temperatures were set at 290 and 220 °C, respectively. A total of 8 pulegone standards ranging from 55 to 890 mg/mL (in ethanol) were used to build a calibration curve for quantification based on peak area. Triplicate analyses were performed, and the mean value was determined. The oven temperature was programmed individually for each set of samples. Standard samples: from 60 to 140 at 3 °C/min, then 25 °C/min up to 240 °C, followed by a 2 min hold at this temperature. Oil samples: from 60 to 160 at 3 °C/min and then 25 °C/min up to 240 °C, followed by a 2 min hold at this temperature. The carrier gas was helium at a constant flow rate of 1 mL/min. One microliter of each diluted sample (1/100 in ethanol, v/v) was injected manually in splitless mode.

FT-IR Spectroscopy. All FT-IR measurements were performed using a Nicolet 6700 FT-IR (Thermo Scientific) spectrometer operating in the 4000−400 cm^{-1} wavenumber range (mid-infrared), equipped with a deuterated triglycine sulfate (DTGS) detector, a Nichrome source and a KBr beamsplitter. The prepared samples were placed between ZnSe round crystal windows (25 mm x 2 mm) and the transmission path length was fixed at 15 *μ*m with a polytetrafluoroethylene (PTFE) spacer. The cell was then placed in the presslock demountable cell holder (Thermo Scientific), and the sample was scanned against a background air spectrum at constant temperature (25 °C). For each spectrum, 100 scans were accumulated at a resolution of 4 cm^{-1}. Spectra were collected in triplicate for each sample and averaged using the software supplied from the manufacturer of the spectrometer, OMNIC 7.3 (Thermo Electron Corp.). All spectra were smoothed using the "automatic smooth" function of the OMNIC 7.3 software, which uses the Savitsky-Golay algorithm (95-point moving second-degree polynomial). Subsequently the baseline was corrected using the "automatic baseline correct" function (polynomial).

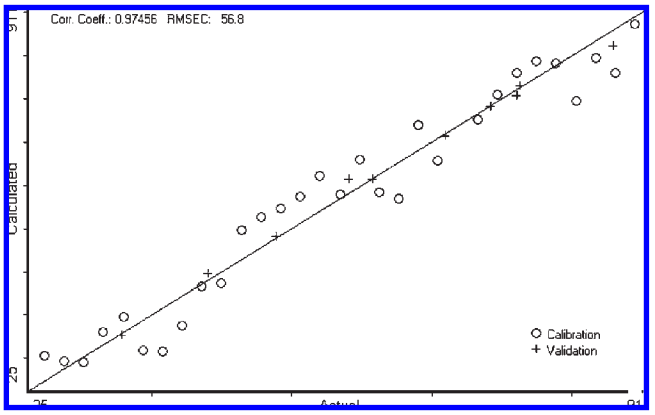

Figure 2. Partial least-squares (PLS) calibration model for the quantitative analysis of pulegone by FT-IR.

Chemometrics. Calibration model for the prediction of pulegone content in the different oil samples from FT-IR spectral data was obtained by partial least-squares (PLS) algorithm, using the TQ Analyst software (7.2.0.161 Release, Thermo Electron Corp.). The spectral data were mean centered and selected wavenumber ranges, as well as the full recorded spectral range, were evaluated for development of the PLS calibration model. The spectral region used for the PLS calibration was from 1650 to 1260 cm^{-1}. All data in the calibration set were examined carefully to detect and eliminate outlier samples and the resulting calibration model was established with 30 FT-IR spectra. Statistical accuracy of the calibration model was described by the correlation coefficient *r* and the overall errors between modeled and reference values, i.e. the root-mean-square error of calibration (RMSEC), the root-mean-square error of cross validation (RMSECV) using the leave-one-out approach and the root-mean-square error of prediction (RMSEP). The optimum number of PLS factors for pulegone quantification was determined by using the PRESS (predicted residual error sum of squares) calculation. PRESS values are indications of how closely a model fits the calibration data (*21*). The FT-IR method was further evaluated by computing the differences for reproducibility and accuracy between the predicted FT-IR data and the actual pulegone content values in the oil samples.

RESULTS AND DISCUSSION

Chemical Composition of Pennyroyal Oils. Analysis of the extracted pennyroyal essential oils (PO1−PO6) by GC−MS provided their relative percentage composition, which was taken into account in order to determine the spiking levels for the pulegone-type pennyroyal oils. Concerning PO1−PO5 oils, pulegone was found to be the main constituent (61.3−77.9%), followed by *iso*-menthone (10.6−18.5%), menthone (0.6−8.3%), piperitenone (0.3−3.2%) and *cis*-isopulegone (0−1.7%). In the case of PO6 oil, piperitone was the major ingredient (69.3%), followed by *iso*-menthone (24.8%). Limonene (1.8%) and menthone (1.6%) were detected in minor percentages. The results obtained for these pennyroyal populations by GC−MS analysis of their corresponding oils are in agreement with those reported for populations occurring in the same climatic zones of Greece (*9*).

Reference Quantitative Analysis. Gas chromatography in combination with flame ionization detection was used as a reference method due to its sensitivity and selectivity, properties that provide reliable analysis of volatiles such as pulegone (*5*, *12*). A calibration curve was created for the set of pulegone standard samples as the function of pulegone's peak area ($A_{pulegone}$ = pulegone's peak area/10^6 in GC−FID chromatograms) and pulegone's content. The empirical equation of calibration curve for pulegone was described as follows: $A_{pulegone}$ = (0.0539 ± 0.0004) × (mg of Pulegone), while the correlation coefficient was r = 0.9998. For the PO1−PO5 pennyroyal oil samples, the range of pulegone contents was 482−728 mg/mL. The addition of the spiked pennyroyal oil samples (SPO1−SPO5) increased the upper limit to 860 mg/mL and decreased the lower limit to 157 mg/mL (**Table 1**).

Table 1. Pulegone Content (± SD) in Pennyroyal Oils as Determined by Reference GC−FID Analysis and Predicted by FT-IR Spectroscopy[a]

	pulegone (mg/mL)			
sample[c]	GC−FID	FT-IR	*F*-test[b]	*t*-test[b]
SPO1	157 ± 4	153 ± 32	64.0	0.2
SPO2	281 ± 8	297 ± 16	4.0	1.3
SPO3	379 ± 31	381 ± 42	1.8	0.1
PO1	482 ± 33	515 ± 53	2.6	0.7
PO2	516 ± 28	514 ± 81	8.4	0.0
PO3	621 ± 18	613 ± 9	4.0	0.6
SPO4	686 ± 22	682 ± 25	1.3	0.2
PO4	723 ± 51	708 ± 37	1.9	0.3
PO5	728 ± 57	729 ± 14	16.6	0.0
SPO5	860 ± 24	823 ± 22	1.2	1.6

[a] The respective statistical *F*-test and *t*-test values are provided. [b] Confidence level: 99%. [c] PO1−PO5: isolated pennyroyal oil samples. SPO1−SPO5: spiked pennyroyal oil samples.

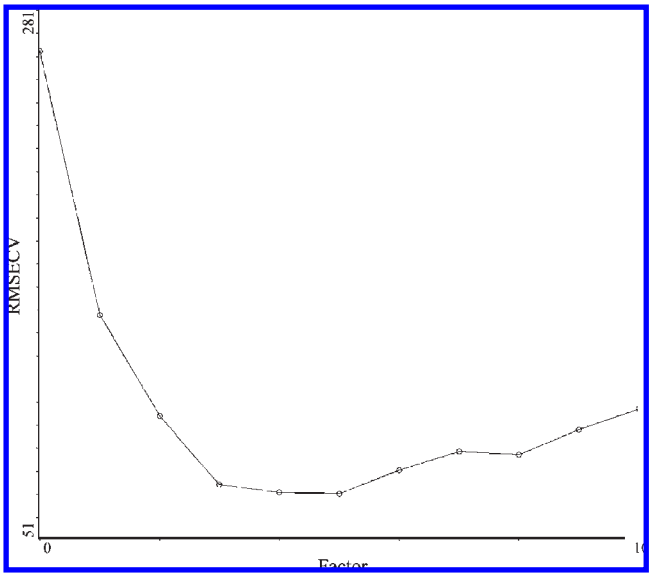

Figure 3. Plot of root-mean-square error of cross validation (RMSECV) values vs the different number of factors used in the cross validation and calibration of the PLS model for pulegone determination in pennyroyal oils. The optimum number of factors that resulted in the minimum RMSECV value and accordingly, minimum predicted residual error sum of squares (PRESS) value was 5.

Spectral Analysis. The typical FT-IR spectrum of pulegone (**Figure 1**) showed characteristic bands associated with methyl and methylene groups at ∼2954 cm^{-1} (C−H asymmetric stretching, $-CH_3$), 2923 cm^{-1} ($-CH_2-$ asym stretching, $-CH_3$ sym stretching due to $=C(CH_3)_2$) and 2872 cm^{-1} ($-CH_2-$ sym stretching, $-CH_3$ sym stretching due to $>CH-CH_3$) (*22*). The strong peak demonstrated at ∼1682 cm^{-1} is attributed to C=O (ketones) stretching vibration, while the broad band of medium intensity at ∼1615 cm^{-1} is assigned to C=C stretching vibration, which is conjugated with C=O (*22*). The "fingerprint region" between 1500 and 500 cm^{-1} provides complex but unique and reproducible spectral information with a significant contribution for substance identification (*22*). The band at 1456 cm^{-1} is due to $-CH_2-$ scissoring and $-CH_3$, $-CO-CH_2-$ asym deformation vibrations. The characteristic bands at 1373 and 1286 cm^{-1} are

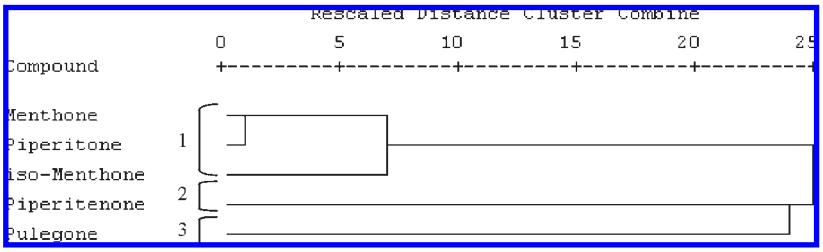

Figure 4. Hierarchical cluster analysis (Ward's algorithm) based on the FT-IR spectra (spectral range: 1650−1260 cm^{-1}) of the major substances contained in pennyroyal oils.

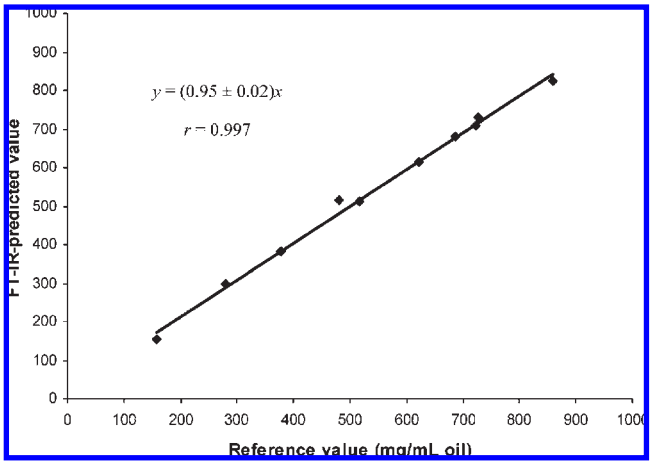

Figure 5. Reference values (GC−FID) vs partial least-squares (PLS) predicted values (FT-IR) of the pulegone content in pennyroyal oils.

assigned to C−H sym deformation of $>CH-CH_3$ and $=C-(CH_3)_2$, respectively (*22*). The band at 1209 cm^{-1} is due to C−H deformation vibration, whereas the weak peaks in the region 1131−936 cm^{-1} are attributed mainly to CH_3 rocking vibrations of the pulegone molecule (*22*). C−H wagging vibration and ring vibration are suggested for the band at 876 cm^{-1}, while the C−CO−C group in-plane deformation is related to the band at 646 cm^{-1} (*14*, *22*). The pulegone band occurring at 535 cm^{-1} is assigned to $-CH_2-$ rocking, C−C=O deformation and C=C skeletal vibration (*22*). Due to the significantly high pulegone content in the pennyroyal oils PO1−PO5, the corresponding FT-IR spectra present mostly the characteristic IR signals of pure pulegone (**Figure 1**). Additionally, pennyroyal oils display another typical peak at 1709 cm^{-1}, which is representative of *iso*-menthone and menthone C=O stretching (**Figure 1**) (*22*).

Quantitative Analysis of Pulegone by FT-IR. Only the data from the regions with features of interest were abstracted by the PLS calibration model, so that calibration standards especially representative of the samples to be analyzed are included (*23*). The best prediction results were produced by using spectral data in the 1650−1260 cm^{-1} wavenumber range. The data from this region produced the highest correlation coefficient r (0.97) and the lowest RMSEC (56.8) and RMSEP (17.1) (**Figure 2**). The results obtained for all oil samples used in prediction set are presented in **Table 1**. Regarding cross-validation, the values for r and RMSECV were 0.96 and 70.4, respectively. In addition, the maximum number of factors used to calculate the optimum PRESS value was 10, while the optimum number of factors that resulted in the minimum PRESS value was 5 (**Figure 3**).

Besides, this region differentiates pulegone from other pennyroyal oil substances due to the heterogeneity of the measured FT-IR spectra (**Figure 4**). Hierarchical cluster analysis was performed using SPSS 14.0 (SPSS Inc.) software for the 1650−1260 cm^{-1} spectral range, and the application of Ward's algorithm led to the clear discrimination between pulegone and the other oil major components.

A regression was derived by plotting the pulegone contents in the pennyroyal oil samples (PO1−PO5 and SPO1−SPO5) as determined by the GC−FID reference method against the FT-IR predicted values. The plot (**Figure 5**) shows the validation of the predictive model with the actual pulegone contents compared with the data obtained using FT-IR spectroscopy. The slope was 0.95, while r was 0.997. The F-test and t-test that were applied indicated that accuracy and reproducibility were high (99% confidence level), demonstrating that the PLS predicted pulegone contents were very close to the reference values (**Table 1**).

In conclusion, the FT-IR technique used was found to be a suitable analytical tool for the fast and accurate quantitative determination of pulegone in pennyroyal oil. Instead of the current standard chromatographic methods, which are resource- and time-consuming, the analysis by FT-IR is promising, with minimal personnel training, simple data acquisition and immediate predictions.

ABBREVIATIONS USED

Pennyroyal, *Mentha pulegium* L.; FT-IR, Fourier transform infrared; GC, gas chromatography; MS, mass spectrometry; FID, flame ionization detection; PLS, partial least-squares; RMSEC, root-mean-square error of calibration; RMSECV, root-mean-square error of cross validation; RMSEP, root-mean-square error of prediction; PRESS, predictive residual error sum of squares.

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