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# Direct Determination of Rosmarinic Acid in *Lamiaceae* Herbs Using Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) and Chemometrics

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Supporting Information

**ABSTRACT:** For the determination of rosmarinic acid (RA) directly in pulverized plant material, a method is developed using diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) without any physicochemical pretreatment of samples. The RA content of 11 samples of eight different *Lamiaceae* herbs, as determined by high performance liquid chromatography (HPLC), varied between 86 ± 1 mg/g (in lemon balm) and 12.0 ± 0.8 mg/g (in hyssop) of dried plant material. The 11 samples and 50 other additional samples, which were prepared by mixing initial samples with KBr, were measured using DRIFTS. The second derivative of the spectral region 1344−806 $cm^{-1}$ was used and the corresponding data were analyzed using partial least squares (PLS) regression. The correlation between infrared spectral analysis and HPLC measurements shows that the DRIFTS method is sufficiently accurate, simple, and rapid. The RA content of the 11 *Lamiaceae* samples determined by DRIFTS ranged from 81 ± 4 mg/g (in lemon balm) to 12 ± 3 mg/g (in hyssop) of dried plant material.

**KEYWORDS:** *diffuse reflectance infrared fourier transform spectroscopy, DRIFTS, rosmarinic acid, Lamiaceae, partial least-squares regression*

## INTRODUCTION

Rosmarinic acid (RA) (Figure 1), an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid, is an important naturally occurring phenolic bioactive compound. Its main activities are astringent, anti-inflammatory, antimutagen, antibacterial, antiviral, and anticancer.[1,2] RA occurs as a secondary metabolite in plants of the *Lamiaceae* family.[3,4]

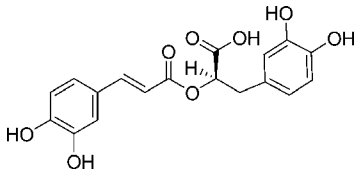


**Figure 1.** The molecular structure of RA.

In recent years, several chromatographic and spectroscopic methods of analysis have been developed for the determination of RA. All these methods require the extraction of RA from the plant material.

Ethanol[5] and methanol[6] are commonly used as extraction solvents of RA. It has been reported that the suitable solvent for plants of the *Lamiaceae* family is acidified methanol.[6] Modern extraction techniques commonly used to extract the RA in the case of the *Lamiaceae* family are ultrasound-assisted extraction,[6] microwave-assisted extraction,[7] and supercritical fluid extraction.[8] High performance liquid chromatography (HPLC) is the most common technique for determining RA.[6,9−11]

$^1$H NMR method has been proposed for the determination of RA in plant extracts.[12] Also RA was studied using FT-Raman spectroscopy.[13]

Finally, for the determination of RA in suspension cultures of *Lavandula officinalis*, a method has been developed on the basis of mid-FT-IR spectroscopy.[4]

In this work, we propose a method for the direct determination of RA in crude plant material of different *Lamiaceae* herbs without any physicochemical pretreatment, except drying and pulverizing, using diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) and partial least squares (PLS) regression analysis. The proposed method is accurate, simple, and rapid. Moreover, as a spectroscopic technique, it is low in cost.

## MATERIALS AND METHODS

**Plant Material, Chemicals, and Reagents.** Eleven herb samples (harvest 2010) of the *Lamiaceae* family were offered by Aetoloakarnania's Rural Cooperative of Aromatic, Pharmaceutical, and Energy Plant Cultivators (Agrotikos Syneterismos Kalliergiton Aromatikon, Farmakeftikon, Energiakon Fyton Aetoloakarnanias, ASKAFEFA), Greece. Namely, two samples of lemon balm (*Melissa officinalis*), two of sage (*Salvia officinalis*), two of oregano (*Origanum vulgare ssp. hirtum*), one of rosemary (*Rosmarinus officinalis*), one of basil (*Ocimum basilicum*), one of thyme (*Thymus vulgaris*), one of hyssop (*Hyssopus officinalis*), and one of savory (*Satureja hortensis*). Only the leaves of the above samples were used, with the exception of one oregano sample and the sample of hyssop, for which the leaves and flowers were used together. The samples were dried at room temperature and pulverized in a ball-mill (Retstch MM 2000) for 15 min.

The standard RA and methanol for HPLC were purchased from Sigma-Aldrich Corporation. Sufficient volume of extraction solvent

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**Table 1. The Actual Content of RA in 50 Mixtures of the Herb Samples and the Corresponding Calculated Content by DRIFTS−PLS Technique**

| serial no. | herb sample | content of RA by HPLC (mg/g dried sample) | mixtures of herb samples with KBr (mg of RA/g dried sample) actual values | mixtures of herb samples with KBr (mg of RA/g dried sample) calculated values by DRIFTS−PLS |
|---|---|---|---|---|
| 1 | lemon balm (1st sample) | 86 | 81 | 81 |
| 2 | | | 73 | 76 |
| 3 | | | 72 | 69 |
| 4 | | | 60 | 64 |
| 5 | | | 50 | 48 |
| 6 | | | 32 | 32 |
| 7 | | | 14 | 15 |
| 8 | lemon balm (2nd sample) | 72 | 67 | 71 |
| 9 | | | 58 | 57 |
| 10 | | | 46 | 48 |
| 11 | | | 37 | 36 |
| 12 | | | 22 | 21 |
| 13 | | | 20 | 24 |
| 14 | | | 16 | 16 |
| 15 | | | 15 | 16 |
| 16 | sage (1st sample) | 39.5 | 22 | 25 |
| 17 | | | 21 | 19 |
| 18 | | | 18 | 19 |
| 19 | | | 17 | 17 |
| 20 | | | 16 | 18 |
| 21 | | | 13 | 12 |
| 22 | sage (2nd sample) | 33 | 21 | 19 |
| 23 | | | 17 | 16 |
| 24 | | | 16 | 18 |
| 25 | | | 13 | 12 |
| 26 | savory | 56 | 37 | 39 |
| 27 | | | 28 | 26 |
| 28 | | | 26 | 29 |
| 29 | | | 19 | 15 |
| 30 | | | 17 | 14 |
| 31 | rosemary | 33 | 28 | 26 |
| 32 | | | 27 | 26 |
| 33 | | | 26 | 29 |
| 34 | | | 21 | 19 |
| 35 | | | 19 | 24 |
| 36 | | | 16 | 15 |
| 37 | | | 15 | 20 |
| 38 | basil | 20.2 | 16 | 13 |
| 39 | | | 14 | 15 |
| 40 | thyme | 33 | 24 | 23 |
| 41 | | | 19 | 19 |
| 42 | | | 17 | 21 |
| 43 | | | 16 | 20 |
| 44 | | | 15 | 25 |
| 45 | oregano (leaves and flowers) | 60 | 56 | 56 |
| 46 | | | 53 | 48 |
| 47 | | | 40 | 38 |
| 48 | | | 38 | 38 |


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**Table 1. continued**

| serial no. | herb sample | content of RA by HPLC (mg/g dried sample) | mixtures of herb samples with KBr (mg of RA/g dried sample) actual values | mixtures of herb samples with KBr (mg of RA/g dried sample) calculated values by DRIFTS−PLS |
|---|---|---|---|---|
| 49 | | | 24 | 22 |
| 50 | oregano (leaves) | 14.9 | 13 | 11 |

**Table 2. The Content of RA of 11 Samples of *Lamiaceae* Species Using HPLC and DRIFTS−PLS Technique**

| | mean content of RA ± SD (mg/g of dried material) ($n$ = 3) | |
|---|---|---|
| sample | HPLC | DRIFTS−PLS |
| lemon balm (leaves), 1st sample | 86 ± 1 | 81 ± 4 |
| lemon balm (leaves), 2nd sample | 72 ± 1 | 71 ± 6 |
| sage (leaves), 1st sample | 39.5 ± 0.6 | 38 ± 6 |
| sage (leaves), 2nd sample | 33 ± 1 | 31 ± 3 |
| oregano (leaves and flowers) | 60 ± 2 | 62 ± 3 |
| oregano (leaves) | 14.9 ± 0.5 | 11 ± 2 |
| rosemary (leaves) | 50 ± 1 | 48 ± 6 |
| basil (leaves) | 20.2 ± 0.6 | 20 ± 3 |
| thyme (leaves) | 33 ± 2 | 30 ± 4 |
| hyssop (leaves and flowers) | 12.0 ± 0.8 | 12 ± 3 |
| savory (leaves) | 56 ± 2 | 56 ± 6 |

was prepared using methanol and acidified distilled water (MeOH:-$H_2O$ (pH = 2.5), 60:40 v/v). The pH value of water was achieved by adding formic acid in water before mixing with methanol.

Then 100 mg of each sample and 10 mL of extraction solvent was added into an Erlenmeyer flask. The flask was then placed in an ultrasonic bath (Sonorex RK 255H, 35 kHz) for 10 min at 25 °C. The extract was filtrated under vacuum. The above procedure was repeated two more times using the same sample of plant material. Next, the filtered extracts were mixed, refiltered through a TeknoKroma (PTFE 0.45 $\mu$m) filter, their volume was adjusted to 30 mL by the addition of acidified methanol, and then stored at −20 °C.

Seven standard solutions (stock solutions) of RA were prepared with extraction solvent concentrations of 30, 60, 100, 150, 210, 270, and 330 mg·L$^{-1}$. The stock solutions were stored at −20 °C.

**Reference Analysis.** HPLC analysis was conducted using an Agilent model 1100 (Agilent Corporation, MA, USA) system equipped with a diode array detector. A reversed phase column Supelco (Discovery HS C18), length 250 mm, internal diameter 4.6 mm with material porosity of 5 $\mu$m was used. The column was thermostatted at 25 °C in a model 7971 column heater of Jones Chromatography Ltd. The HPLC system was controlled by Agilent Chemstation software. From the absorption spectra of samples solutions, absorptions at 260, 280, and 330 nm were obtained.

The analytes were eluted at a flow rate of 0.4 mL·min$^{-1}$ using the binary gradient of acidified water (pH = 2.5) (A) and methanol (B). The mobile phase consisted of 25% B during the initial 2 min, followed by a gradient increase of the percent of solvent B up to 90% for the next 38 min.[6] The sample injection volume was 20 $\mu$L. The samples were analyzed in triplicate. The retention time of RA was about 29.107 min for the stock solutions and 28.251−28.879 for the samples.

**FT-IR Spectra.** First, 50 additional samples were prepared by mixing initial herb samples of *Lamiaceae* family with appropriate KBr in the ball-mill for 15 min and calculating RA content based on the results of HPLC analysis. Specifically, there were prepared 15 lemon balm, 10 sage, five savory, seven rosemary, two basil, five thyme, and six oregano samples. The samples covered the concentration range from 86 to 12 mg RA/g of dried plant material (Table 1). Then infrared spectra of 11 triplicate initial herb samples and 50 triplicate mixture samples were recorded. Triplicate spectra were collected with a Thermo Nicolet 6700 FT-IR spectrophotometer (Thermo Electron Corporation, MA, USA) equipped with a deuterated triglycine sulfate (DTGS) detector at 4 cm$^{-1}$ resolution and 100 scans per sample using a Spectra Tech microcup (diameter 3 mm, height 2 mm) DRIFTS accessory. The background spectra were collected using pure dried KBr in powder form. The FT-IR spectra were smoothed, and their baselines were automatically corrected. Spectral data collection and processing was carried out using the software of the spectrophotometer (OMNIC version 7.3; Thermo Fisher Scientific Inc.). The average spectra of each sample were then calculated using the spectrophotometer software. Next, the second derivative spectra of the 1345−800 cm$^{-1}$ spectral region of each averaged spectrum were analyzed by PLS regression (TQ Analyst version 8.0.0.245; Thermo Fisher Scientific Inc.).

**Chemometrics.** The PLS model was constructed using a calibration set of 52 samples and a validation set of nine samples. Leave-one-out cross validation was used for the evaluation of the established calibration equation and for preventing overfitting of the calibration model.

Outliers in data calibration, if any, may affect adversely the model. Therefore, in the present work, outliers were detected on the basis of spectral or concentration difference with two widely used methods,

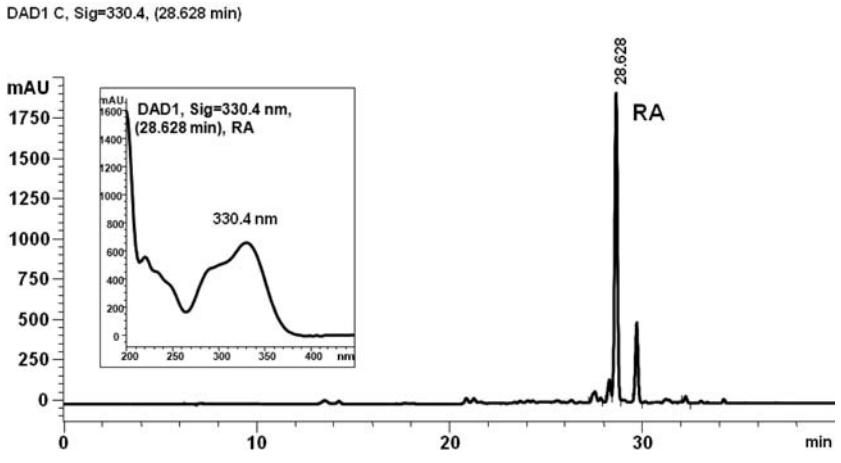


**Figure 2.** HPLC chromatogram and the corresponding UV spectrum of one lemon balm sample extract.

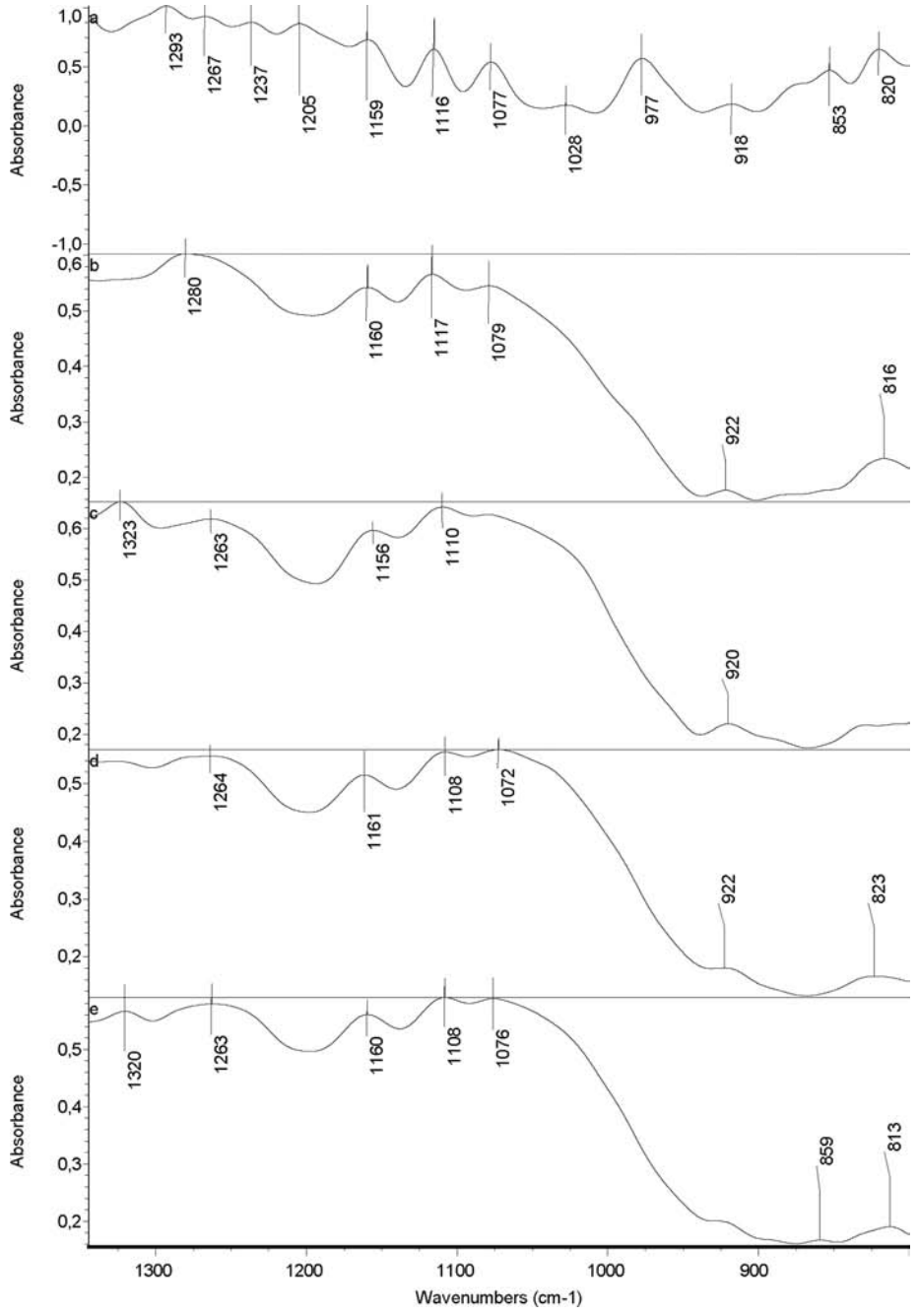


**Figure 3.** The spectral region 1345−800 $cm^{-1}$ of the FT-IR spectra: (a) rosmarinic acid, (b) lemon balm, (c) basil, (d) sage, (e) thyme.

Chauvenet test and Leverage diagnostic, which are ready to use in TQ Analyst.[14] Outliers were excluded from the final spectral analysis. Calibration model evaluation was based on correlation coefficient for calibration, root-mean-square error of calibration (RMSEC), root-mean-square error of cross validation (RMSECV), and root-mean-square error of prediction (RMSEP).[15]

Robust models should have lower RMSEC, RMSECV, RMSEP, and higher correlation coefficient for calibration but also small differences between RMSECV and RMSEP.[16]

Using the model derived from the PLS regression, the concentrations of RA in the 11 triplicate samples of the *Lamiaceae* family were calculated.

Specifically, the 33 FT-IR spectra were used by the DRIFTS-PLS model for the determination of the RA content in the 11 triplicate herb samples. For each herb sample, the raw data triplet was used for the estimation of mean value and standard deviation (Table 2).

## RESULTS AND DISCUSSION

**Reference Analysis.** Figure 2 shows a typical HPLC-chromatogram of one lemon balm sample methanol−acidified water extract and the corresponding UV spectrum of RA. The wavelengths of 260, 280, and 330 nm were chosen because they correspond to phenolic acids absorbance maxima. The retention time (28.628 min) of RA was determined using pure RA as an external standard. It was observed that the RA exhibits maximum absorbance at about 330 nm. Therefore, the peak area of RA at about 330 nm of triplicate samples was correlated with the RA concentrations of the stock solutions. The average peak areas were then calculated, hence the linear relationship, Average area of RA = (74 ± 1) × (RA concentration, $mg{\cdot}L^{-1}$) + (278 ± 224), ($r^2$ = 0.9994, $n$ = 3).


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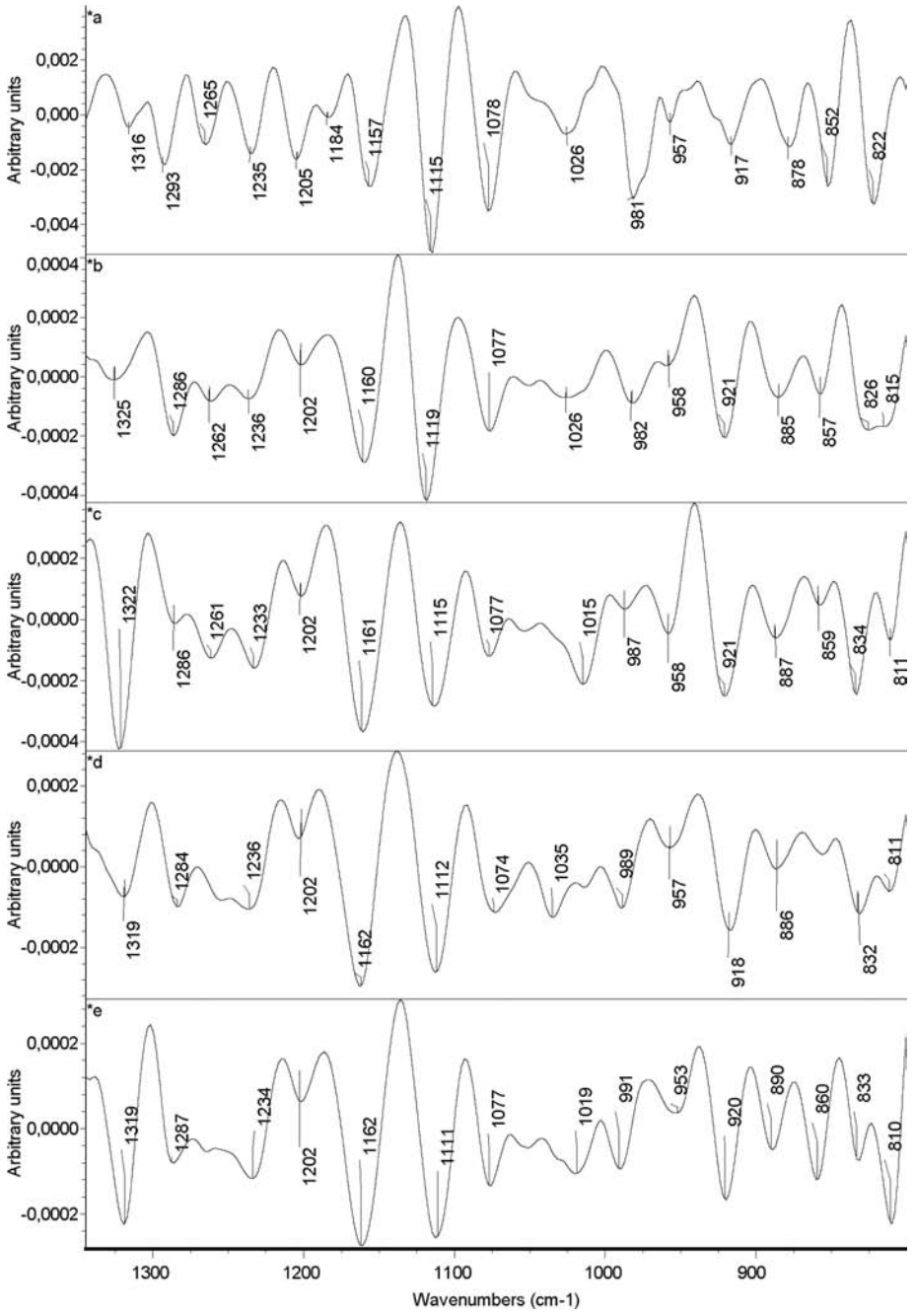


**Figure 4.** The 2nd derivative of spectral region 1345−800 $cm^{-1}$ of the FT-IR spectra: (*a) rosmarinic acid, (*b) lemon balm, (*c) basil, (*d) sage, (*e) thyme.

The *t* test was applied to estimate whether the constant term of the previous equation is statistically significant. Given that $t_{experimental} = 1.241 < t_{theoritical} = 2.571$ ($\alpha = 0.05$, df = 5), the constant term is not statistically significant, namely the calibration curve passes, practically, through the origin. Using the equation, average area of RA = (74 ± 1) × (RA concentration, $mg \cdot L^{-1}$), the concentrations of RA in the 11 samples of the *Lamiaceae* family were calculated and expressed as mg RA/g of dried sample. The RA content was found to range from 86 ± 1 mg/g (lemon balm) to 12.0 ± 0.8 mg/g (hyssop) of dried plant material (Table 2). Next, the concentrations of the remaining 50 samples, which came from the 11 plant samples after mixing appropriate KBr, were measured.

**Spectral Analysis.** The spectral region 1345−800 $cm^{-1}$ was primarily chosen because it is a part of the "fingerprint area" of RA. Additionally, in this region, there was no shift of the peaks observed in the spectra of the initial plant material when mixed with KBr. If such a shift had appeared, the preparation of additional samples would have been required. These observations allow a comparison between the spectra and their correlation with the content of samples in RA. Figure 3 shows the above spectral region of the FT-IR spectra of RA and four indicative *Lamiaceae* species (lemon balm, basil, sage, and thyme). The assignments of the RA peaks at 1293, 1267, 1237, 1205, 1159, 1116, 1077, 1028, 977, 918, 853, and 820 $cm^{-1}$ have been previously described.[12] The aforementioned peaks overlap with those of other components (cellulose, pectins,


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lignin, polysaccharides, etc.) of plant material at 1323−1320, 1280−1263, 1161−1156, 1117−1106, 1079−1072, 922−920, and 823−813 $cm^{-1}$. When comparing the FT-IR spectra of RA and the samples, there was not observed any peak that can be attributed only to RA. To reveal the peaks of RA, the second derivative of the above spectral region were used (Figure 4).

As seen in Figure 4, the minima of the spectra of *Lamiaceae* samples match the minima of the RA spectrum. The differences between the minima in the spectra of *Lamiaceae* samples are small.

These results suggest that there are interactions between the RA and other components of plant tissues and the peaks of RA overlap with those of other constituents. Thus, the use the PLS regression is needed.[17−19]

**Chemometrics.** The PLS regression shows a linear relationship between the calculated and the actual values of the RA content of the 61 samples. The software used 52 samples for calibration and nine for validation of the method. The spanned value found by the software was 100%. This value indicates that the calibration standards cover the concentration range (8.00−89.70 mg RA/g of dried plant material) very well and the number of standards is sufficient. A preliminary calibration curve was then obtained using the software TQ Analyst.

The existence of outliers affects the quality of the model.[16] Chauvennet test and Leverage diagnostic are more accessible for commercial application as they are ready to use in TQ Analyst and they are, also, two of the most widely used methods for outlier detection.[16] According to Cauvennet test, the spectral and concentration information for each sample is used to determine the Mahalonobis distance ($H$ statistic). If $H > 3.0$ for a sample, this sample is considered as an outlier.[16] In other words, if the deviation of a standard from the mean is so large that the probability of occurrence is less than 1/20, the standard is an outlier. According to the above test, two samples were characterized as outliers. The concentrations of the outliers were 14 and 17 mg RA/g of dried material, and they refer to the samples made by mixing the savory and basil plant samples with KBr, respectively.

The remaining 59 samples were then recalibrated and further outlier testing was applied. Outliers were not detected. The number of PLS factors was 10. The 10 factors describe the 99.9% of spectral and 98.5% of concentration variability, namely the model included practically all the differences among the spectra and between concentrations. The correlation coefficient of linear relationship was 0.992, RMSEC 2.43, RMSECV 4.53, and RMSEP 4.53. The low differences between RMSEC and RMSEP reveal the robustness of the model.

The RA content of 50 mixtures was calculated by the PLS model (Table 1).

**RA Determination in 11 *Lamiaceae* samples.** The RA content of 11 *Lamiaceae* samples was determined using the DRIFTS−PLS method. The results are presented in Table 2. Significant variability was observed among the examined herbs samples in regard to the RA content, with it ranging from 81 ± 4 mg/g (in lemon balm) to 12 ± 3 mg/g (in hyssop) of dried plant material.

In conclusion, the proposed method for the determination of RA using DRIFTS technique and PLS regression is simple, rapid, sufficiently accurate, cheap, and also uses the plant material without any physicochemical pretreatment except drying and pulverizing.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Calibration curve using HPCL and correlation curve between actual and calculated values using PLS for RA content in samples, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

DRIFTS, diffuse reflectance infrared Fourier transform spectroscopy; DTGS, deuterated triglycine sulfate; FT-IR, Fourier transform infrared; FT-Raman, Fourier transform Raman; HPLC, high performance liquid chromatography; MeOH, methanol; PLS, partial least squares; RA, rosmarinic acid; RMSEC, root-mean-square error of calibration; RMSECV, root-mean-square error of cross validation; RMSEP, root-mean-square error of prediction

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