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ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · SEPTEMBER 2006

Impact Factor: 3.8 · DOI: 10.1021/np060180b · Source: PubMed

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Qualitative and Quantitative Analysis of the Active Components of the Essential Oil from *Brickellia veronicaefolia* by Nuclear Magnetic Resonance Spectroscopy[#]

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Received April 24, 2006

The composition of the spasmolytic essential oil of the medicinal species *Brickellia veronicaefolia* was established by NMR spectroscopy in addition to GC-MS analysis and HPLC studies. Seven major compounds, representing ca. 86% of the oil, were identified as benzyl 2,6-dimethoxybenzoate (**1**), 2-hydroxybenzyl 2′-methoxybenzoate (**2**), chamazulene (**3**), β-caryophyllene (**4**), germacrene D (**5**), bicyclogermacrene (**6**), and β-eudesmol (**7**). A sensitive and accurate analytical ^{1}H NMR method has been developed for the quantification of the major compounds in the essential oil of *B. veronicaefolia*. The method was validated using benzyl 2,6-dimethoxybenzoate (**1**) and β-caryophyllene (**4**), two of the active principles in the oil, and successfully applied to the determination of these pharmacologically active compounds in three different batches of the oil collected in different geographical regions and/or seasons.

Brickellia veronicaefolia (Kunth) Gray (Asteraceae) is a bitter-tasting shrub that grows in the oak and pine woodlands of Mexico from Coahuila to Oaxaca. The commercial drug consists of the dried aerial parts of the plant, and it is extensively commercialized in Mexico for the treatment of gastrointestinal discomforts including stomachache, biliary colic, dyspepsia, arthritis, local inflammations, and infectious diseases.[1] In addition, some herbal preparations containing *B. veronicaefolia* are widely used to cure gastritis.

Early phytochemical studies of the aerial parts of this species resulted in the isolation and characterization of several 6-methoxyflavonols and labdane-type diterpenes.[2−5] In addition, the antioxidant and hypoglycemic activities of the extract and a flavonoid, centauridin, isolated from the plant have been described.[6,7] More recently we demonstrated the antispasmodic properties of a CH_2Cl_2−MeOH (1:1) extract prepared from the aerial parts of the plant. Bioassay-guided fractionation of the active extract led to the isolation of smooth muscle relaxant salicylic acid and benzyl benzoate derivatives, including benzyl 2,6-dimethoxybenzoate (**1**) and 2-hydroxybenzyl 2′-methoxybenzoate (**2**) (Figure 1), as well as substantial amounts of chamazulene (**3**).[8] The isolates were characterized from physical data and induced a concentration-dependent inhibition of the spontaneous contractions of the guinea-pig ileum with IC_{50}'s ranging from 1.49 to 4.96 μM.

Despite the continued popularity of *B. veronicaefolia*, there are no procedures for quality control and/or standardization of its crude drug and herbal preparations.[9] Therefore, the present study was undertaken in order to develop a suitable analytical method to quantify the most important active principles of the oil of this Mexican species. The ultimate goal would be the establishment of quality control procedures for the crude drug and herbal preparations of this widely commercialized Mexican plant. Two different strategies were initially envisaged: a classical GC procedure and a more contemporary approach based on ^{1}H NMR spectroscopy. The GC method was considered because it is well-known that many pharmacologically active components in herbal medicines are volatile. GC analysis of the volatile oils has a number of advantages, in particular, its high sensitivity of detection and the fact that the components can be readily identified using GC-MS analysis. In addition, the extraction of the volatile oil is relatively straightforward and can be standardized. Finally, this type of analysis renders a reasonable "fingerprint", which can be used to identify the plant.[10]

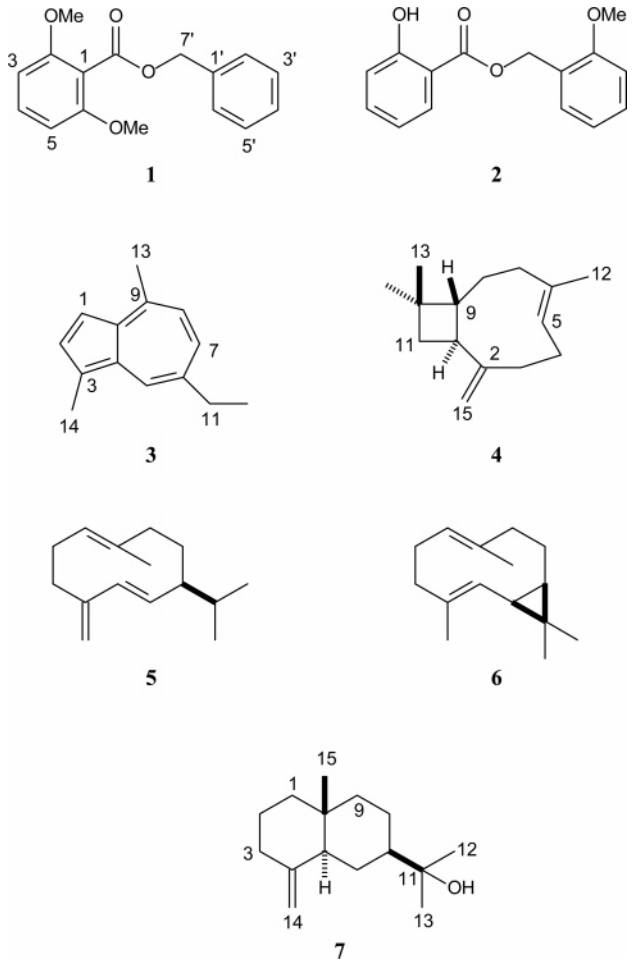

Figure 1. Structures of the main components (**1**−**7**) present in the essential oil of *B. veronicaefolia*.

On the other hand, NMR spectroscopy has proved to be useful for quantification of individual components in crude extracts,

[#] Taken in part from the Ph.D. thesis of B. Rivero submitted to Posgrado en Ciencias Químicas, UNAM, 2006.

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10.1021/np060180b CCC: $33.50
Published on Web 07/27/2006

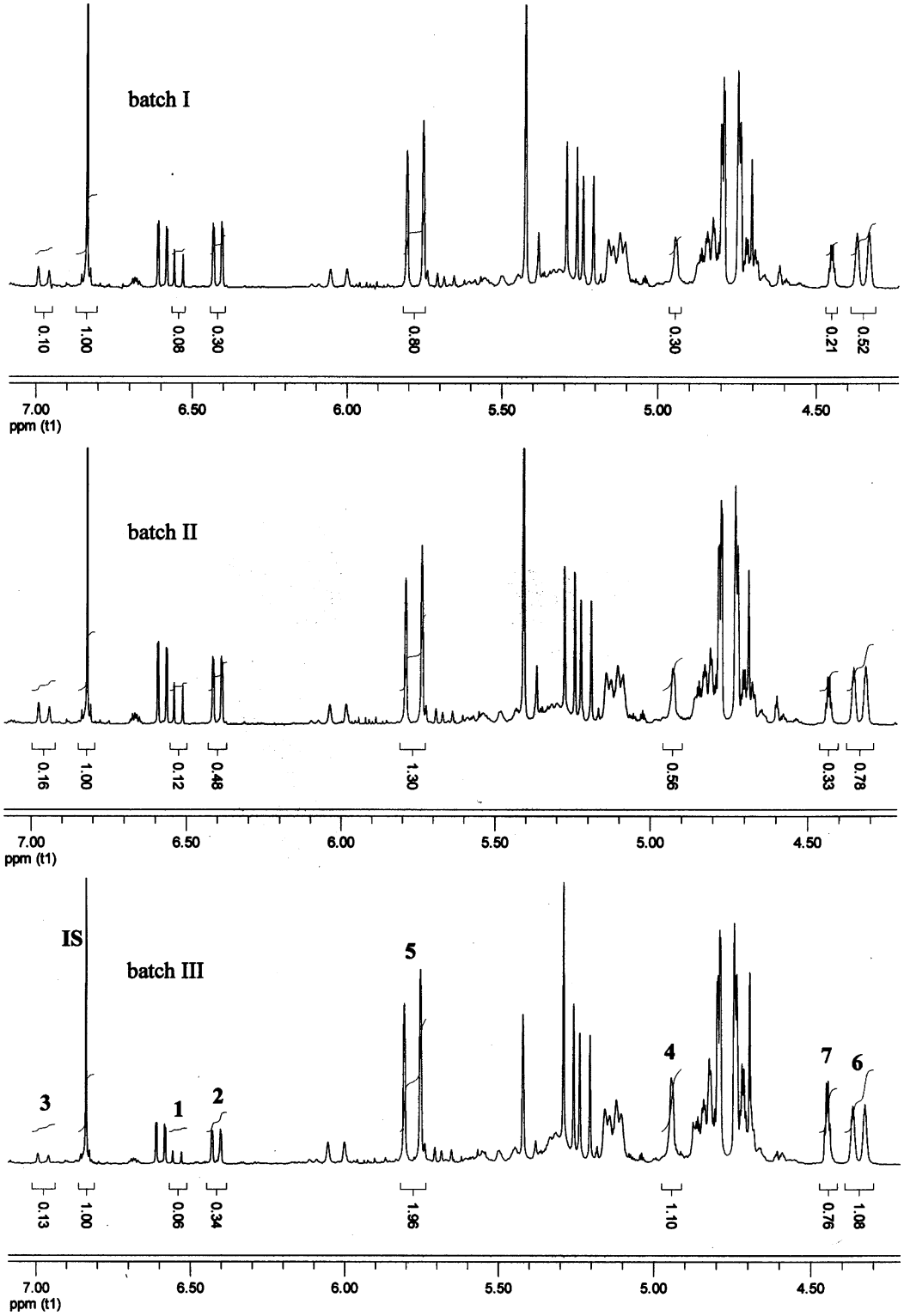

Figure 2. ^{1}H NMR spectra (300 MHz in $CDCl_3$) of *B. veronicaefolia* essential oil collected in three different locations. The δ 7.10−4.20 region shows key signals for quantitative analysis of **1**, benzyl 2,6-dimethoxybenzoate; **2**, 2-hydroxybenzyl 2′-methoxybenzoate; **3**, chamazulene; **4**, β-caryophyllene; **5**, germacrene D; **6**, bicyclogermacrene; and **7**, β-eudesmol. 1,4-Dimethoxybenzene was used as internal standard (IS).

essential oils, or dietary preparations without the need of fractionation or isolation procedures.[11−13] This methodology is particularly valuable when the studied compounds show at least one well-resolved signal in the region from 3.0 to 10.0 ppm. Quantitative NMR spectroscopy offers a comprehensive validation of reference compounds of natural products that can compete with or even surpass chromatographic validation based on molecular analysis.[12] In particular, ^{1}H NMR spectroscopy allows the precise determination of the sample content as well as the amount and nature of nonactive and/or marker compounds, providing useful fingerprints for the identification of herbal drugs, which can be applied in the certification and quality control of reference compounds. Further-

more, due to the sophisticated design of contemporary NMR instruments, calibrated and standardized analytical conditions are readily accessed.

Results and Discussion

The active volatile oil of *B. veronicaefolia* (batch II) was qualitatively analyzed by GC-MS and comprised benzyl benzoates and sesquiterpenoids. Seven major compounds, representing ca. 86% of the oil, were identified, i.e., benzyl 2,6-dimethoxybenzoate (**1**),[8] 2-hydroxybenzyl 2′-methoxybenzoate (**2**),[14] chamazulene (**3**),[15] β-caryophyllene (**4**),[16] germacrene D (**5**),[17] bicyclogermacrene (**6**),[18] and β-eudesmol (**7**).[19] HPLC separation of the oil yielded compound **1**, **2**, **3**, and **5**. The isolates were characterized by comparison of its physical data with literature data and those of authentic samples.[8,15,17] The presence of compounds **1**, **2**, and **7** with known spasmolytic properties seems to account for the antispasmodic activity of the oil.[8,20] The presence of **3** could be related to the attributed anti-inflammatory properties of the plant.[21]

The oil from three additional samples (batches I, IIa, and III), collected in different geographic regions and seasons, was analyzed using the same GC-MS methodology. In all batches the qualitative composition remains constant and the dominant components were **4**−**6**. However, the proportions of the analyzed constituents, including the active substances and reference standards in combination with the essential oil, show variation, which precludes the validation of the procedure with accuracy, linearity, and precision according to international guidelines and pharmacopoeia requirements. All attempts probably failed because of variations in the stability and volatility of the sesquiterpenes under the analysis conditions, as observed in recent studies particularly for germacrene D, bicyclogermacrene, and β-caryophyllene,[22] which account for 60% of the oil components of *B. veronicaefolia*. Therefore, in our case, the classical GC-MS procedure was not adequate for a rigorous quantitative analysis of the essential oil.

Considering that NMR spectroscopy has also proved to be useful for quantification of single compounds in crude extracts, essential oils, or dietary preparations without the need of fractionation or isolation procedures,[11,12] we proceeded to develop a procedure to estimate the amount of compounds **1**−**7** using ^{1}H NMR. Furthermore, the method was validated, according to standard procedures,[23] using compounds **1** and **4**. Compounds **1** and **4** were chosen for the validation process because of their accessibility as standards. Thus, **1** was synthesized and **4** commercially purchased. Repeated ^{1}H NMR analysis of the oils prepared from different batches revealed that the NMR profiles stayed unchanged. The results of the validation procedure indicated that the method was selective, linear, accurate, and precise in the range of concentrations tested.

For optimal analysis conditions, the characteristic NMR signals of the individual components need to be identified.[12] Therefore, the assignment of all NMR signals for the seven components was first carried out. As observed in Figure 2, the ^{1}H NMR spectra of the three analyzed oils clearly revealed characteristic and well-defined key resonances for each component. In the low-field region (Figure 2), between δ_H 7.10 and 6.20, the signals due to H-3/H-5 in **1** (δ_H 6.54, d, J = 8.8 Hz) and the one ascribable to H-3′ (δ_H 6.42, dd, J = 8.4, 1.0 Hz) in **2** were used to quantify these two components. For chamazulene (**3**), the key resonance appeared at δ_H 6.97, d, J = 10.8 Hz (H-5). In the δ_H 5.90−4.30 region the olefinic signals selected for the analysis of **4**−**7** resonated at δ_H 4.95 (br s, H-15a), 5.78 (d, J = 15.9 Hz, H-5), 4.33 (d, J = 11.5 Hz, H-5), and 4.48 (m, H-14), respectively. The signal of the internal standard (1,4-dimethoxybenzene) appeared at δ_H 6.84 as a singlet and was not overlapped by the signals of the essential constituents. This standard was selected because of its stability and solubility and because it displays only two sharp singlets in the ^{1}H NMR spectrum.[12]

The integration of the key resonances in the ^{1}H NMR spectrum allowed quantification of the different constituents of the oil by comparison with the area corresponding to the signal of the standard. The accuracy of the method was checked by adding to the oil (50.0 mg) known amounts of **1** (0.05 to 0.5 mg) and **4** (1.3 to 13.0 mg). The peak areas corresponding to each compound increased proportionally to the added concentration of the added standards (**1** and **4**). Although calibration curves were not needed for quantification of individual components because the integration of the peaks is always proportional to the amount of compounds, the calibration curves for **1** and **4** were determined in the range 0.05−0.5 and 1.30−13.0 mg/mL in order to evaluate the linearity of this method. Each calibration curve is shown in Figure 3. The linearity parameters (r^2) for **1** and **4** were higher than 0.997 and 0.998, respectively. The repeatability of the method was demonstrated by determining the levels of **1** and **4** in three batches whose NMR profile remained unchanged during at least three consecutive days.

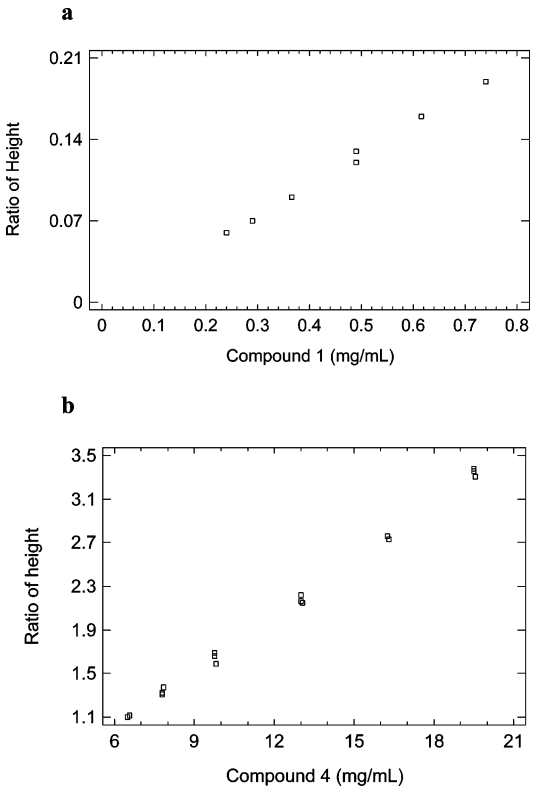

Figure 3. Plot of the area of H-3/H-5 of benzyl 2,6-dimethoxybenzoate (a) and H-15 of β-caryophyllene (b) versus the amounts of each compound.

Once the procedure was validated, we analyzed the most widely used OTC preparation of *B. veronicaefolia*. The NMR and TLC analyses of the essential oil prepared from this herbal mixture did not contain the compounds identified and/or quantified in the crude drug of *B. veronicaefolia* (Table 1). The results of such analyses are very important because the lack of appropriate regulations in Mexico have resulted in many commercialized phytomedicines commonly consumed by people being misidentified or containing adulterants or less effective substitutes. The statistical analysis by ANOVA followed by a Tukey's test indicated that there is a significant difference among the means of three different batches. Therefore, the NMR quantification developed in this study can also be applicable for seasonal and geographic variation studies of *B. veronicaefolia*.

In summary, quantitative NMR spectroscopy has proven to be an excellent analytical tool for both qualitative and quantitative analysis of the essential oil of *B. veronicaefolia*, offering several advantages over the classical chromatographic methods. In addition, the developed method complied with the international requirements for the quality control of herbal pharmaceutical products.

Table 1. Contents of Benzyl 2,6-Dimethoxybenzoate (**1**), 2-Hydroxybenzyl 2′-Methoxybenzoate (**2**), Chamazulene (**3**), β-Caryophyllene (**4**), Germacrene D (**5**), Bicyclegermacrene (**6**), and β-Eudesmol (**7**) Quantified by ^{1}H NMR Spectrum of the Essential Oils (mg/g ± SD)

sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**
batch I	0.47 ± 0.01	3.51 ± 0.01	0.85 ± 0.01	3.27 ± 0.12	7.63 ± 0.26	4.42 ± 0.17	2.06 ± 0.02
batch II	0.24 ± 0.01	2.40 ± 0.05	0.63 ± 0.04	6.52 ± 0.03	11.44 ± 0.21	6.30 ± 0.3	4.91 ± 0.20
batch IIa	0.28 ± 0.01	2.19 ± 0.04	0.52 ± 0.03	1.91 ± 0.12	3.31 ± 0.25	2.86 ± 0.19	1.29 ± 0.06
batch III	0.08 ± 0.01	0.67 ± 0.01	0.05 ± 0.02	10.78 ± 0.12	4.49 ± 0.16	1.30 ± 0.06	0.04 ± 0.01

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. IR spectra were obtained using KBr disks on a Perkin-Elmer FT 1605 spectrophotometer. Open column chromatography was carried out on silica gel 60 (70−230 mesh, Merck). Analytical TLC was performed on precoated silica gel 60 F254 plates (Merck). The 300 MHz ^{1}H NMR spectra were recorded at 295 K on a Varian Mercury 300 equipped with a direct detection 5 mm probe using tetramethylsilane (TMS) as the zero reference point. EI-MS were registered on a JEOL SX 102 mass spectrometer.

Plant Material. Two different batches of the aerial parts of *B. veronicaefolia* were collected in the States of Michoacán (batch I) and Mexico (batches II and IIa). A third sample, batch III, was purchased at the Mercado de Sonora in México City. Batch I was collected on May 20, 2004. Batches II and IIa were cropped on December 10, 2003, and June 25, 2005, respectively. Finally, batch III was acquired on April 16, 2004. Voucher specimens of *B. veronicaefolia* (BCR-1M, BCR-2M, BCR-2MA, and BCR-3M, respectively) were deposited in the National Herbarium (MEXU), Instituto de Biologia, UNAM.

Essential Oils from the Aerial Parts. The essential oils of the aerial parts of each batch (I−III) were prepared by hydrodistillation from 150 g of plant material, according to a procedure previously described.[9] The yields were 0.35, 0.27, 0.39, and 0.43 g, respectively.

Isolation of Compounds 1−3 and 5. The essential oil from batch II (100 mg) was subjected to chromatographic separation by HPLC. The HPLC system consisted of a Waters instrument equipped with Waters 996 UV photodiode array detector (900) set at 264 nm, using a μPorasil column (19 × 300 mm). Control of the equipment, data acquisition, processing, and management of chromatographic information were performed by the Millennium 2000 software program (Waters). The mobile phase was hexane and the flow rate 6 mL/min. This process allowed the isolation of **1** (t_R = 15.1, 4 mg), **2** (t_R = 14.0, 6 mg), **3** (t_R = 6.2, 15 mg), and **5** (t_R = 13.8, 10 mg) identical to authentic samples.

GC-MS Analysis of the Oils. The GC-MS measurements were conducted on a Hewlett-Packard 5989A spectrometer. The GC column was HP-5MS (30 m × 0.31 mm i.d.). The typical linear temperature programming was from 70 to 250 °C, at a rate of 20 °C/min, and the carrier gas was He (1 mL/min). Solutions of samples were prepared in $CHCl_3$ (1 mg/mL) and injected (1 μL) using the split injection method. Major constituents of the essential oils were identified by matching their 70 eV mass spectra with those of the reference library. In the case of benzyl 2,6-dimethoxybenzoate (**1**), 2-hydroxybenzyl 2′-methoxybenzoate (**2**), chamazulene (**3**), and β-caryophyllene (**4**) the identities were established by comparison with authentic samples. The t_R's (retention times) for **1**−**7** in the case of batch II were 6.58, 6.37, 5.18, 4.00, 4.24, 4.31, and 4.90 min, respectively.

Preparation of Compound 1. To a solution of benzyl alcohol (4.8 mmol, 527 mg) and 2,6-dimethoxybenzoyl chloride (1.2 mmol, 240 mg) in 10 mL of acetonitrile was added dropwise Et_3N (675 μL). The reaction mixture was stirred at room temperature during 3 h, poured into cold H_2O, and extracted with CH_2Cl_2 (15 mL × 3). The organic phase was dried over Na_2SO_4 and concentrated in vacuo. The crude reaction mixture was purified by open column chromatography on silica gel 60 (70−230 mesh, Merck, ∼100 g) eluting with hexane−CH_2Cl_2 (1:1) to yield 510 mg of pure compound identical to the natural product. Analytical TLC was performed on precoated silica gel 60 F_{254} plates (Merck).

NMR Measurements. Samples were prepared with 50.0 mg of the essential oil, 0.8 mL of $CDCl_3$, and 100 μL of the internal standard solution and transferred to 5 mm NMR tubes. The internal standard solution was prepared dissolving 10 mg of pure 1,4-dimethoxybenzene (99.9%), purchased from Sigma-Aldrich Co. (St. Louis, MO), in 1 mL of $CDCl_3$. The spectra were acquired using the standard *s2pul* sequence, a 45 deg pulse (9.0 μs), a relaxation delay of 1 s, a spectral width of 4505 Hz, and an acquisition time of 3.636 s in 64K data points. A total of 256 scans were recorded for each sample, yielding an analysis time of ca. 21 min per sample. The digital resolution was kept below 0.4 Hz. NMR files were Fourier transformed with the Mestre-C 4.0 program (Mestrelab Research, Santiago de Compostela, Spain) to determine the peak integrals. Phase adjustment and baseline correction were applied prior to integration of the δ 7.10−4.20 region, where diagnostic resonances were found for the seven analyzed substances.

^{1}H NMR Data of Compounds 1−7 in $CDCl_3$. **1**: δ 7.44 (2H, br d, J = 7.5 Hz, H-2′ and H-6′), 7.35 (2H, m, H-3′ and H-5′), 7.34 (1H, m, H-4′), 7.27 (1H, t, J = 8.3 Hz, H-4), 6.54 (2H, d, J = 8.3 Hz, H-3 and H-5), 5.38 (2H, s, H-7′), 3.80 (6H, s, OCH_3-2,6). **2**: δ 11.42 (1H, s, OH), 7.47 (1H, br d, J = 8.0 Hz, H-6), 7.38 (2H, br t, J = 7.2 Hz, H-4 and H-4′), 7.32 (2H, t, J = 7.3 Hz, H-5 and H-5′), 6.59 (1H, dd, J = 8.4, 1.0 Hz, H-3), 6.42 (1H, d, J = 8.4 Hz, H-3′), 5.41 (2H, s, H-7′), 3.84 (3H, s, OCH_3). **3**: δ 8.16 (1H, br d, J = 1.8 Hz, H-8), 7.61 (1H, br d, J = 3.6 Hz, H-2), 7.38 (1H, dd, J = 10.2, 2.1 Hz, H-6), 7.21 (1H, d, J = 3.9 Hz, H-3), 6.98 (1H, d, J = 10.8 Hz, H-5), 2.84 (2H, q, J = 7.8 Hz, H-11), 2.83 (3H, s, H-9), 2.65 (3H, s, H-10), 1.34 (3H, t, J = 7.6 Hz, H-12). **4**: δ 5.40−5.20 (1H, m, H-5), 4.95 (1H, br s, H-15a), 4.88−4.83 (1H, 2 br s due to conformational mixture, H-15b), 1.62 (3H, br s, H-12), 1.00 (3H, br s, H-14), 0.98 (3H, br s, H-13). **5:** δ: 5.78 (1H, d, J = 15.9 Hz, H-5), 5.25 (1H, dd, J = 16.0, 9.9 Hz, H-6), 5.13 (1H, m, H-1), 4.79 (1H, m, H-15), 4.74 (1H, m, H-15′), 1.66 (3H, d, J = 1.5 Hz), 0.86 (3H, d, J = 7.2 Hz, H-13), 0.81 (3H, d, J = 6.9 Hz, H-12). **6**: δ 4.80 (1H, m, H-1), 4.33 (1H, d, J = 11.5 Hz, H-5), 1.65 (3H, s, H-15), 1.46 (3H, s, H-14), 1.07 (3H, s, H-13), 1.0 (3H, s, H-12). **7**: δ 4.67 (1H, dd J = 1.8 Hz, H-14a), 4.48 (1H, m, J = 1.8 Hz, H-14b), 2.20 (1H, ddd, J = 14, 10, 5 Hz, H-3a), 1.90 (1H, ddd, J = 14, 4, 2 Hz, H-3b), 1.69 (1H, dd, J = 10, 4 Hz, H-5), 1.57 (2H, m, H-6), 1.55 (2H, m, H-8), 1.52 (2H, m, H-2), 1.45 (2H, m, H-9), 1.35 (2H, m, H-1), 1.31 (1H, m, H-7), 1.2 (6H, s, H-12 and 13), 0.62 (3H, s, H-15).

Specificity, Linearity, Accuracy, and Reproducibility of the NMR Analytical Method. The specificity of the method was established for each test substance by demonstrating the lack of interference among them, as well as from the internal standard and the solvent. The linearity of the method was evaluated by measuring the relationship between the NMR detector response and the sample concentration employing a regression analysis of the response data. Five samples containing 50.0 mg of the oil, 1.0 mg of 1,4-dimethoxybenzene, and increasing amounts (0.050, 0.125, 0.250, 0.375, and 0.500 mg) of benzyl 2,6-dimethoxybenzoate (**1**) and (1.30, 3.25, 6.50, 9.75, and 13.00 mg) of **4** (99.9%, Sigma St. Louis, MO) in 0.8 mL of $CDCl_3$ were prepared; after each addition of **1** and **4**, the ^{1}H NMR spectrum of the resulting sample was recorded in triplicate. Altogether 15 spectra were registered. All validation parameters were obtained by analyzing the three oil batches (I−III) in triplicate.

Acknowledgment. This work was supported by grants from CONACyT (C01-018) and DGAPA-UNAM (IN 212005). B.R. acknowledges the scholarship awarded by CONACyT to carry out graduate studies.

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NP060180B