FOOD COMPOSITION AND ADDITIVES

Determination of 2-Hydroxy-4-Methoxybenzaldehyde in Roots of *Decalepis hamiltonii* (Wight & Arn.) and *Hemidesmus indicus* R.Br.

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The roots of *Decalepis hamiltonii* and *Hemidesmus indicus* are aromatic and possess the crystalline compound 2-hydroxy-4-methoxybenzaldehyde as the major compound (>90%) in their volatile oils. A gas chromatographic procedure was developed for the assay of 2-hydroxy-4-methoxybenzaldehyde in both fresh and dried roots of different origin. Benzyl butyrate was used as the internal standard. Among the methods tried, steam hydrodistillation was suitable for extraction of the volatile oils. The quantity of this aromatic compound varied from 0.03 to 0.54%.

The roots of *Decalepis hamiltonii* and *Hemidesmus indicus* belong to the Asclepiadaceae family and have food and pharmacological applications. The roots of *D. hamiltonii* are used extensively in pickles, whereas *H. indicus* is used in sherbet (soft drink) preparation and in many medicinal applications (1, 2). The extracts of *D. hamiltonii* have been reported to possess antibacterial, antimicrobial, and preservative qualities (3, 4). An isomer of vanillin, 2-hydroxy-4-methoxybenzaldehyde was reported as the major compound in the volatile oils of *D. hamiltonii* (96%) and *H. indicus* (91%). We reported (5, 6) the chemical composition of the volatile oils from these roots and confirmed that 2-hydroxy-4-methoxybenzaldehyde was the major compound. These plants and roots are seasonal and grow wild. The fresh roots of *D. hamiltonii* are available during monsoon in southern parts of India and are generally dried and preserved for various food and pharmaceutical applications (1, 2). The demand for these roots is increasing with the recent studies on their broad spectrum of biological activities and applications; however, because of deforestation, these species are becoming scant and vanishing (7). Studies on the propagation of these species through tissue culture are under progress at the Central Food Technological Research Institute (Mysore, India), hence the present study may find application in the analysis of wild as well as biotechnologically derived species. Until now, no standard analytical procedures have existed for the analysis of these volatile oils or estimation of 2-hydroxy-4-methoxybenzaldehyde; therefore, we report an assay procedure based on steam hydrodistillation and gas chromatographic (GC) analysis.

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Experimental

Samples of fresh roots of *D. hamiltonii* and *H. indicus* were obtained from Mysore District. The dried roots were procured from Kurnool (Andhrapradesh), Dindigul (Tamilnadu), and Mysore (Karnataka). Voucher specimens were deposited [Roots of *D. hamiltonii* (Wight & Arn.), MGM/LJM/4/2000; and *H. indicus* R.B.r, MGM/LJM/8/2000] at Manasa Gangotri Herbarium, University of Mysore (Mysore, India). The chemicals and solvents used were reagent (AR) grade. Glass-distilled water was used for distillation/extraction.

Preparation of Internal Standard Solution

A 5 μL volume of benzyl butyrate (Sisco-Chem Industries, Mumbai, India) was added to 1 mL ethanol. From this, 50 μL was added to each analytical sample as internal standard.

Cleanup and Processing of Plant Material for Extraction

The fresh root of *D. hamiltonii* (1.4 kg) was manually cleaned and the inner core (fibrous material) was removed. The fleshy portion was cut into small pieces of 0.5–1.5 cm length and immediately added to the water.

Isolation of Major Compound as Standard Reference

The cut pieces of fleshy root, 1.2 kg in 3 L water, were subjected to steam hydrodistillation, wherein the steam was generated in a separate flask and allowed to purge into the sample, which was kept under aqueous medium. The generated volatiles, along with the steam condensate, were collected and extracted with dichloromethane (500 mL × 5). The combined extract was dried over anhydrous sodium sulfate. The solvent was distilled off on a water bath <50°C and the residue was dried in a desiccator over P_2O_5 and paraffin wax to get the volatile oil (yield 9.5 g). This was crystallized from petroleum ether (60–80°C). White needlelike crystals separated and

Table 1. Determination of 2-hydroxy-4-methoxybenzaldehyde

Sample No.	Sample, amount	Estimated quantity, mg/mL[a]	Volatiles, dilution	Content, %
1	*D. hamiltonii* fresh root (100 g)	5.4 (±0.04)	100	0.54
2	*D. hamiltonii* dry root (100 g; Karnataka)	4.5 (±0.03)	100	0.45
3	*D. hamiltonii* dry root (100 g; AP)	5.2 (±0.05)	100	0.52
4	*D. hamiltonii* dry root (55 g; TN)	3.1 (±0.02)	10	0.06
5	*H. indicus* dry root (100 g)	2.9 (±0.01)	10	0.03

[a] All analyses were performed in triplicate, and mean values are given. Values in parentheses are standard deviations.

were filtered. The filtrate, which still contained the major constituent, was subjected to crystallization 3 times, and the minor constituents were separated out as mother liquor.

^{1}H NMR Analysis of the Major Constituent

^{1}H nuclear magnetic resonance (NMR) of the crystalline solid was recorded on a Varian EM 390 NMR spectrometer (90 MHz) using $CDC1_3$ as solvent and tetramethylsilane as the internal standard. Chemical shift (δ) values are expressed in ppm. ^{1}H NMR spectrum showed δ (ppm), 3.9 (s, 3H); 6.5 (d, J = 2 Hz; 1H); 6.6 (dd, J = 2.9 Hz; 1H); 7.5 (d, J = 9 Hz; 1H); 9.7 (s, 1H); and 11.6 (s, 1H).

Gas Chromatographic/Mass Spectrometric Analysis of the Major Constituent

The crystalline compound was also subjected to gas chromatographic/mass spectrometric (GC/MS) analysis on a Shimadzu GC-17A coupled with QP 5000 MS system under the following conditions: SPB-1 column (30 m × 0.32 mm, 0.25 μm film thickness); oven temperature program, 60°C (2 min)–2°C/min–250°C (5 min); injection port temperature, 225°C; detector temperature, 250°C; carrier gas, helium; flow rate, 1 mL/min. The mass spectral fragmentations observed were *m/z* (% abundance) 53(28), 63(25), 81(15), 95(30), 108(25), 134(3), l51(100), 152(20).

Preparation of Standard 2-Hydroxy-4-Methoxybenzaldehyde Solution

Stock solution was prepared by dissolving 100 mg crystalline 2-hydroxy-4-methoxybenzaldehyde in 5 mL ethanol in a volumetric flask. From this, working standard samples (1–12 mg/mL) were prepared by suitable dilution.

Preparation of Calibration Graph

From the working standard (1–12 mg/mL) solutions, 1 μL each was injected (in triplicate) into the capillary GC system under the following conditions. The sample solutions were subjected to GC analysis on Shimadzu gas chromatograph GC-14B (Shimadzu, Asia Pacific, Singapore) using SPB-1 (coated with polydimethylsiloxane) capillary column (30 m × 0.32 mm, 0.25 μm film thickness); oven temperature program, 60°C (2 min)–2°C/min–250°C (5 min); injection port temperature, 220°C; detector temperature, 250°C; carrier gas, nitrogen; flow rate, 30 mL/min. The peak area responses were obtained and a standard graph was prepared by plotting concentrations (μg) vs peak area responses.

Sample Preparation for Estimation of 2-Hydroxy-4-Methoxybenzaldehyde

The laboratory scale steam distillation method was used to extract the volatile oil. The steam was generated and introduced through the distillation head of the flask containing the fleshy portion of the roots (50–100 g *D. hamiltonii* or *H. indicus* root; Table 1). The purged steam along with volatiles were condensed, collected, and extracted with dichloromethane (25 mL × 3), and these extracts were dried with anhydrous sodium sulfate. The solvent was distilled off on a water bath (<50°C) and the volume was diluted to 1 mL with ethanol and preserved in a refrigerator (5).

GC/MS Analysis of Samples

The sample solutions (1 μL) each were subjected to GC/MS analysis on Shimadzu GC-17A coupled with QP 5000 MS system as described above.

GC Analysis of Samples

A 1 μL volume of each of the sample solutions was injected into the capillary GC system under the above-mentioned conditions. GC analysis of 1 mg in 1 mL solution showed no significant response, nor did analyses from 2 mg in 1 mL solution onward. Hence, this was considered the detection limit under these conditions.

Results and Discussion

The fresh and dried roots of *D. hamiltonii* and *H. indicus* procured from different regions (Table 1) were analyzed to determine the quantity of the 2-hydroxy-4-methoxybenzaldehyde present. Because this reference compound is not available from commercial sources, it was isolated from the fresh roots of *D. hamiltonii*. Freshly cut pieces were added to water immediately to avoid browning. They were then subjected to steam hydrodistillation and the reference compound was isolated from the condensate by fractional crystallization. In the

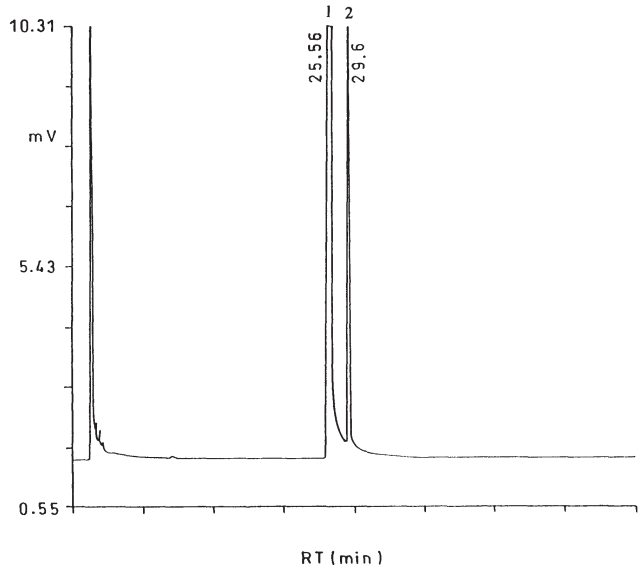

Figure 1. GC analysis of 2-hydroxy-4-methoxybenzaldehyde (reference compound) with benzyl butyrate (internal standard). Peak 1: 2-hydroxy-4-methoxybenzaldehyde; peak 2: benzyl butyrate.

mass spectrum of the 2-hydroxy-4-methoxybenzaldehyde, the strong molecular ion peak (*m/z* 152) and a stronger [M-1] ion peak *(m/z* 151) observed were characteristic of aromatic aldehydes. Generally, aryl aldehydes are characterized by large molecular ion peaks (8). The loss of H from the molecular ion is favored by aryl aldehydes because of the stability of $Ar\text{-}CO^+$. Subsequently, the M-1 ion (*m/z* 151) eliminates CO to give a peak at *m/z* 123 which, on further elimination of CO gives the peak *m/z* 95. The peak at *m/z* 134 (M-18) indicated either *meta* or *para* substitution of OCH_3 with respect to OH. Therefore, it was further confirmed that, in the mass spectrum, the peak at 134 corresponded to the *meta* effect, leading to the elimination of H_2O. The aromatic π electron system results in the formation of a relatively stable molecular ion, which frequently corresponds to the base peak in the spectrum (9).

The ^1H NMR spectrum of the crystalline compound showed signals at δ 3.9 (s, 3H) and 9.5 (s, 1H), indicating the presence of methoxyl and aldehyde groups, respectively. The sharp signal at δ 11.6 (s, 1H) was assigned to chelated hydroxyl, which could be placed on the *ortho*-position to the aldehydic group on the aromatic ring. Signals at δ 6.5 (d, 1H, J = 2 Hz); 6.6 (dd, 1H, J = 2 and 9 Hz); and 7.5 (d, 1H, 9 Hz) show the presence of the ABX pattern on the aromatic ring (10). As the signal at δ 7.5 assignable to the proton adjacent to the aldehydic group shows *ortho* coupling, the methoxyl group can be placed *para* to the aldehydic group. Hence, the structure of the major compound is established as 2-hydroxy-4-methoxybenzaldehyde.

The approximate retention times for 2-hydroxy-4-methoxybenzaldehyde and benzyl butyrate in GC analyses are 25.6 and 29.6 min, respectively (Figures 1 and 2). Because these 2 compounds are resolved closely, accuracy of quantification and reasonable quickness of analysis can be achieved. Hence, benzyl butyrate was found to be suitable as internal standard.

Initially, the calibration curve was constructed by plotting the concentration (1–12 mg 2-hydroxy-4-methoxybenzaldehyde) against peak area response and was found to be linear. The detector sensitivity under these conditions was observed from 2 ppm onward, i.e., the significant response of the peak was observed from the 2 mg solution onward. Therefore, this was considered as the detection limit. From the regression analysis, R^2 was found to be 0.9938. The isolation of the volatile constituents, which is a crucial step in sample preparation, was performed by steam hydrodistillation. This extraction by steam hydrodistillation was found to be better than the Clevenger distillation or simultaneous distillation extraction (SDE) methods (5), which gave smaller yields and presented handling problems such as blocking the side arm of the Clevenger unit by the crystalline product.

The GC/MS analysis of volatile oil samples has been used to identify and confirm the structure of the major constituent, and the 2-hydroxy-4-methoxybenzaldehyde was estimated in the volatiles based on the calibration graph. Table 1 shows that the fresh roots of *D. hamiltonii* contained 0.54% and dried roots from Karnataka, Andhrapradesh, and Tamilnadu contained 0.45, 0.52, and 0.06%, respectively. The dried root of *H. indicus* contained 0.03% 2-hydroxy-4-methoxybenzaldehyde. This variation in the quantity of the major constituent could be the basis for the evaluation of quality of the roots. The quantity may depend on the drying process, such as sun or shade drying and storage conditions.

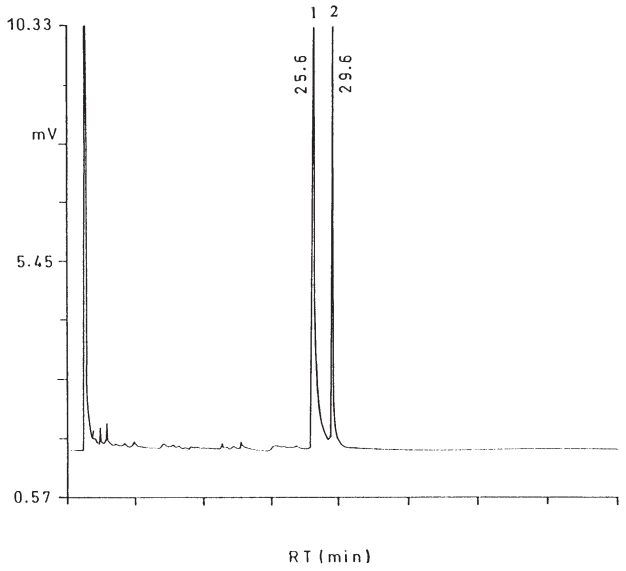

Figure 2. GC analysis of volatiles from the root of *D. hamiltonii* with benzyl butyrate (internal standard). Peak 1: 2-hydroxy-4-methoxybenzaldehyde; peak 2: benzyl butyrate.

Conclusions

The study indicates that the 2-hydroxy-4-methoxybenzaldehyde content of the roots of *D. hamiltonii* and *H. indicus* can be determined by using this analytical assay procedure to a near accuracy. This is a simple analytical procedure that, in turn, could be used to evaluate the quality of the roots of *D. hamiltonii* or *H. indicus*. Further, by adopting a suitable sample preparation procedure, this method may find application in determination of 2-hydroxy-4-methoxybenzaldehyde in plants propagated by biotechnological methods such as tissue culture and in some food products in which the roots are used as a food additive.

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