Separation and Identification of Eugenol in Ethanol Extract of Cloves by Reversed-Phase High-Performance Liquid Chromatography

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ABSTRACT: A method has been developed for the separation and identification of eugenol in the alcohol extract of cloves directly without having to previously separate other components. Extraction of eugenol with alcohol is followed by analysis with high-performance liquid chromatography. Separation by reversed-phase chromatography on low-polarity μBondapak C_{18} was achieved with isocratic elution. Tentative identification is based on chromatographic appearance of a peak with retention time 5.54 min for pure standard reference eugenol. The identification of eugenol was confirmed by gas chromatography/mass spectrometry analysis.
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KEY WORDS: Clove alcohol extract, eugenol, optimization, reversed-phase HPLC.

It is well known that cloves possess a phenolic compound, 4-allyl-2-methoxyphenol, commonly called eugenol, with a chemical formula of ($C_{10}H_{12}O_2$). Eugenol acts as an antioxidant on oleogenous foods, as an anti-carminative, antispasmodic, antiseptic in pharmacy, and also as an antimicrobial agent (1–3).

Kramer (4) identified eugenol and gallic acid as the two major antioxidants in clove through the use of thin-layer chromatography, ultraviolet (UV), infrared (IR), mass spectrometry (MS), and high-performance liquid chromatography (HPLC). Deyama and Horiguchi (5) investigated the steam-volatile constituents of air-dried clove buds by gas chromatography (GC) and IR, nuclear magnetic resonance (NMR), and MS. High-performance liquid chromatographic analysis of eugenol in pimento spice by means of UV and electrochemical detection was developed by Smith and Beck (6).

The object of the present work was to develop a direct HPLC procedure for the separation and identification of the major phenolic compound, eugenol, in the alcohol extract of clove without previous separation of other components in the extract.

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MATERIALS AND METHODS

Materials. Fresh green cloves were obtained from Kampung Batu Laut (Banting, Selangor, Malaysia). Analytical reagent-grade Univar (AJAX Chemicals, Auburn, Australia), 95% ethanol, was used as the extraction solvent. Eugenol with purity of 99.9% was purchased from Merck (Darmstadt, Germany). HPLC-grade, 99.9% methanol from Lab-Scan Limited (Dublin, Ireland) was used as the mobile phase in the liquid chromatographic separation.

Methods. Prior to the experiment, 500 g of dried cloves were ground in a blender (Moulinex Co., Bagnolex, France). Optimum particle size (250 μm) of the sample was selected by sieving with Test Sieves (Endecotts Ltd., London, England) based on a previous study (7). Ground cloves (50 g) of 250 μm particle size were agitated with 300 mL of 95% ethanol in a 500-mL Pyrex vessel with a hemispherical base with 11-cm diameter and a depth of 13 cm. The sample mixture was agitated for 2 h at a temperature of 50°C as described in a previous study (7).

The temperature during extraction was controlled by immersing the stirred flask in a thermostatically-controlled water bath, which was capable of maintaining the temperature to within ±0.1°C. The extract was filtered through Whatman No. 1 filter paper, followed by filtration of a 25-mL aliquot (twice) through Whatman No. 540 filter paper prior to HPLC analysis.

HPLC. Analytical separation of the sample was performed on a Hitachi (Tokyo, Japan) high-performance liquid chromatograph equipped with a 30 cm × 3.9 mm i.d. μBondapak C_{18} reversed-phase column obtained from Waters Associates (Milford, MA).

A Hitachi HPLC intelligent pump Model L-6200, in combination with a pressureless injection system, was used for solvent delivery and sample loading. Detection was performed with a Hitachi UV visible detector Model L-4000 set at 280 nm. A Hitachi chromato-integrator Model D-2500 was used in all computations. The mobile phases used in the HPLC analysis were prepared by mixing methanol (HPLC-grade 99.9% methanol) and double-distilled deionized water in required proportions. Particle removal and dissolving the

gas in the eluent were performed simultaneously by filtration through a membrane filter with pore size of 0.45 μm (Millipore Corporation, Bedford, MA). The eluent was again cleaned and degassed in an ultrasonic cleaner (Model Branson 220; SmithKline Company, Shelton, CT).

Sample preparation for analysis. A reference solution of eugenol was prepared by dissolving 50 μL eugenol in 10 mL methanol. The sample of clove alcoholic extract solution was prepared by dissolving 0.5 mL alcohol extract in 10 mL methanol.

Analytical separation. Analytical isocratic liquid chromatographic separation was performed with 20 μL of reference sample. Elution was with a mobile phase of methanol/water at a flow rate of 0.7 mL/min. The method suggested by Snyder and Kirkland (8) for determining the optimum mobile phase composition was adopted. Various elutions with methanol/water ratios of 60:40, 70:30, 80:20, 90:10, and 100% methanol were used. Mobile phase optimization, in which composition and elution profile were compared on an analytical scale, was attained with a methanol/water ratio of 80:20. The eugenol peak has a retention time of 5.54 min (Fig. 1).

Analysis of the 20-μL alcohol extract sample, eluted with the same mobile phase methanol/water ratio of 80:20 at a flow rate of 0.7 mL/min, indicated a significant peak with a retention time 5.54 min (Fig. 2).

GC/MS. GC/MS was performed on a Hewlett-Packard GC-quadrupole MS system Model 5989 A (Hewlett-Packard, Palo Alto, CA). The analyses were performed by using a capillary column of 25 m × 0.22 mm i.d., coated with SE 54 (methyl 5% phenyl silicone, film thickness 0.33 μm). The oven temperature of the GC was programmed from 80 to 250°C at a rate of 7°C/min, and was finally held at 250°C for 15 min. Helium at a flow rate of 1 mL/min was used as a carrier gas, and the spectrum was obtained by scanning from 50 to 400 a.m.u. The mass spectrometer was operated at an ionization voltage of 70 eV. Comparison of the mass spectra of the reference eugenol and eugenol peak of the alcohol clove extract sample confirmed the identity of the GC peaks.

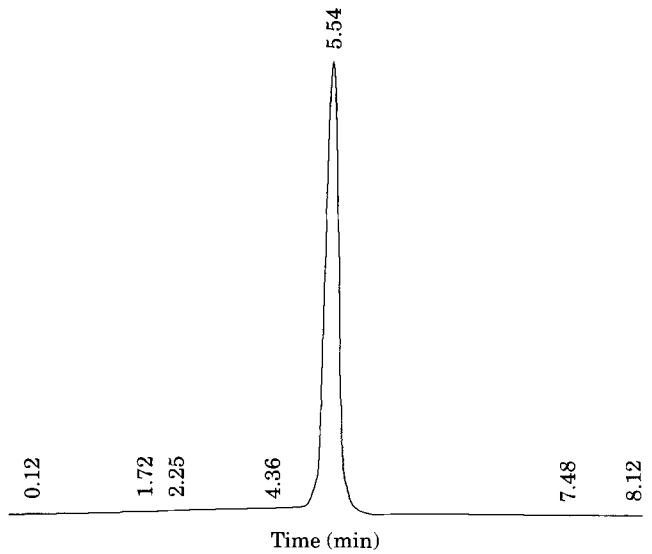

FIG. 1. High-performance liquid chromatogram of standard eugenol detected at 280 nm. Column, μBondapak C_{18} (30 cm × 3.9 mm i.d.; Water Associates, Milford, MA); mobile phase, methanol/water (80:20); flow rate, 0.7 mL/min; injection amount, 20 μL.

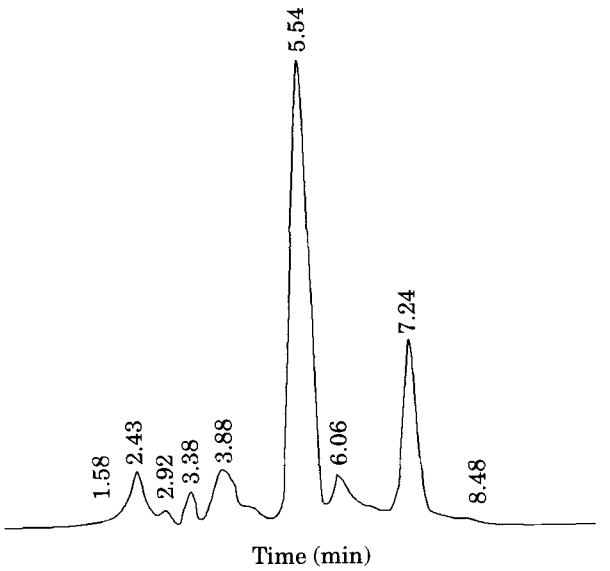

FIG. 2. High-performance liquid chromatogram of alcohol clove extract detected at 280 nm. Column, μBondapak C_{18} (30 cm × 3.9 mm i.d.) mobile phase, methanol/water (80:20); flow rate, 0.7 mL/min; injection amount, 20 μL. See Figure 1 for company source.

RESULTS AND DISCUSSION

Identification of eugenol in the clove alcoholic extract was made by comparing the chromatographic appearance of the peak and retention time of 5.54 min for the standard reference as presented in Figure 1. The peak at 5.54 min in Figure 2 is a parent peak due to elution of eugenol from the ethanolic extract and is comparable with the authentic sample of eugenol shown in Figure 1. The resolution of eugenol is adequate, and the separation time is less than 10 min with the mobile phase of methanol/water at a ratio of 80:20. The retention times and the chromatogram peak areas were reproducible for several different aliquots at 20-μL injection of the sample.

GC/MS of the alcoholic clove extract showed five peaks at retention times of 13.95, 15.26, 15.85, 16.84, and 19.67 min, respectively (Fig. 3). The mass spectrum of the first peak in Figure 3 is comparable with the mass spectrum of the authentic sample of eugenol (molecular weight 164): parent peaks of *m/e* are 164, 149, 137, 131, 121, 103, 91, 77, and 65. The second, third, and fourth peaks, at retention times of 15.27, 15.85, and 16.84 min, respectively, appear to be caryophyllene, α-humulene, and eugenol acetate, based on their mass spectrum index. The last peak, with retention time of 19.67 min, was not identified. However, no further work has been done on the confirmation of these peaks as they are outside the scope of this study.

It was found that 80% methanol/water is a suitable mobile phase for isolation of eugenol from fresh green cloves by re-

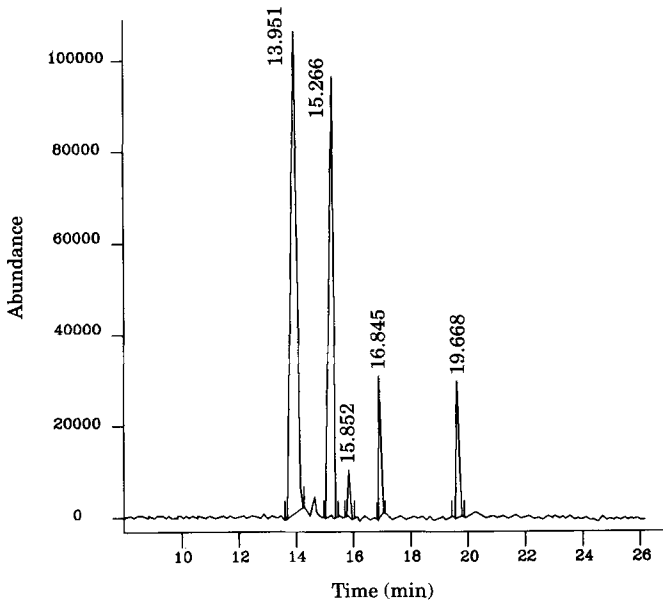

FIG. 3. Gas chromatogram of ethanol clove extract. Eugenol peak at retention time of 13.95 min.

verse-phase HPLC. Because the precise procedure is both simple and rapid, it can be used in routine analysis and quality control. In addition, the advantage of the method is that liquid chromatographic separation of eugenol is carried out at room temperature, thus avoiding the risk of thermal rearrangement and decomposition of eugenol that can occur in gas chromatography.

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