

Identification of Benzethonium Chloride in Commercial Grapefruit Seed Extracts

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Commercial grapefruit seed extracts (GSE) were extracted with chloroform. The solvent was evaporated, and the resulting solid was subsequently analyzed by high-performance liquid chromatography, electrospray ionization mass spectrometry, nuclear magnetic resonance (NMR) spectroscopy, and elemental analysis (by proton-induced X-ray emission [PIXE] analysis). The main constituent was identified as benzethonium chloride, a synthetic antimicrobial agent commonly used in cosmetics and other topical applications. This compound comprised 8.03% ($n = 2$) of the liquid GSE sample. Higher amounts of benzethonium chloride were found in powder GSE samples.

Keywords: *Benzethonium chloride; grapefruit seed extract; electrospray ionization mass spectrometry; PIXE analysis; antimicrobial activity*

INTRODUCTION

Grapefruit seed extract is promoted as a natural antimicrobial agent for both internal and external use. There are books devoted to this material which is said to be capable of treating various conditions such as eczema, acne, thrush, cold sores, sore throat, athlete's foot, colds, gastrointestinal infections, gastritis, gastric and duodenal ulcers, allergies, and parasitic diseases in a safe and efficacious manner (1, 2). The name implies that the product might be produced from a simple extraction of grapefruit seeds. However, the composition of commercial GSE is not defined, and its methods of production are proprietary and not specified. One commercial manufacturer states that GSE is made through a proprietary manufacturing process that involves the conversion of polyphenols in grapefruit seed and pulp to a quaternary ammonium compound that is stated to be the active antimicrobial agent.

There are various reports of the antimicrobial activity of GSE, although it is difficult to find data published in peer-reviewed scientific journals. The antimicrobial activity of GSE (ParaMycrocidin) was tested against 794 bacterial and 93 fungal strains (3). A 0.5% solution was found to be effective against gram-positive bacteria (*Streptococcus sp.*, *Staphylococcus aureus*, *Enterococcus sp.*), gram-negative bacteria (*Enterobacter sp.* and *E. coli sp.*), and various yeasts and molds (*Candida*, *Geotrichum*, *Aspergillus*, and *Penicillium sp.*). Oral administration of the same agent in patients with atopic eczema resulted in an inhibition of *Candida sp.*, *Geotrichum sp.*, and hemolytic *E. coli* growth, whereas *Staphylococcus aureus*, aerobic spore formers, and lactobacilli were only slightly inhibited. The antimicrobial effects of GSE (DF-100, Chemie Research & Manufacturing Co., Casselberry, FL) were evaluated by Cho et al. (4) who reported that DF-100 destroys microorganisms by disrupting the function of the microbial cell wall membrane and microbial spores. GSE has been evaluated in reducing

the levels of *Salmonella* on chicken skins. Dipping chicken skins in DF-100 solution for 1 and 3 min resulted in a 0.8 to 1.2 log reduction in the number of *Salmonella* for a 0.1% DF-100 solution and a 1.6 to 1.7 log reduction for a 0.5% DF-100 solution (5). Spraying chicken skins with 0.1% and 0.5% DF-100 reduced the *Salmonella* by 1.6 log and 1.8 log, respectively, whereas spraying with water reduced the number of *Salmonella* by 1.0–1.3 log (6). GSE has also been evaluated for potential use as an antimicrobial agent in the preservation of fruits, vegetables, and legumes. Cho et al. (7) investigated the use of GSE in preserving *Satsuma mandarin* fruit stored for eight weeks. They found that 80% of the control fruit was contaminated and decayed by *Penicillium sp.* while only 27% of 100-ppm-GSE-treated fruit and 13% of the 250-ppm-GSE-treated fruit was contaminated and decayed. Fresh fruits and vegetables treated with GSE and stored in polyethylene film (0.1 mm) at 10–15 °C had better color and texture than nontreated controls (8). Minimum inhibitory concentrations of GSE toward bacteria and fungi involved in the decay of fruits and vegetables were observed to be in the range of 250–500 ppm. Treatment of unshelled peanuts with grapefruit seed extract at 5000 and 10000 mg/kg was found to be ineffective in controlling the growth of total and aflatoxigenic fungi during storage (9). Calori-Domingues and Fonseca (10) found that treatment of unshelled peanuts with grapefruit seed extract was not efficient in controlling aflatoxin production during storage. Peanuts treated with GSE at 5000 and 10000 mg/kg had mean aflatoxin contamination ranging from 2757–56334 µg/kg and 688–5092 µg/kg, respectively, while the control had from 3362–108333 µg/kg. Of all of the chemicals tested only propionic acid was effective in controlling aflatoxin production. Treatments were considered efficient when the aflatoxin content ($B_1 + G_1$) remained under 30 µg/kg.

Grapefruit seeds are a rich source of limonoids, a group of chemically related triterpene derivatives (11). Grapefruit seeds contain the following limonoid aglycons: limonin, nomilin, obacunone, and deacetylномilin

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at concentrations of 19.06, 1.84, 1.86, and 1.10 mg/g dry seed, respectively (12). Limonin is intensely bitter and is primarily responsible for citrus juice bitterness. Ozaki et al. (12) also quantitated the levels of limonoid glucosides in grapefruit seeds and found deacetylnomilinic acid glucoside, nomilin glucoside, nomilinic acid glucoside, obacunone glucoside, limonin glucoside, and deacetylnomilin glucoside at concentrations of 0.75, 2.01, 0.89, 0.86, 1.48, and 0.68 mg/g dry seed, respectively.

Grapefruit peel contains relatively high concentrations of flavanone glycosides. Ortuña et al. (13) examined 10 grapefruit varieties and found the following peel (flavedo + albedo) constituents: narirutin (108–1042 mg/100 g FW), naringin (28–2509 mg/100 g FW), and neohesperidin (11–22 mg/100 g FW). On the basis of their occurrence as major grapefruit constituents it is expected that limonin, narirutin, and naringin would be present and detectable in GSE samples.

Recently, questions have arisen about the composition of GSE and whether some commercial samples have been adulterated with synthetic preservatives. Using HPLC and LC/MS, Sakamoto et al. (14) studied the composition of a commercial GSE sample and an ethanol extract of grapefruit seeds. They found that the HPLC chromatograms of these two samples were quite different. The preservative agents methyl 4-hydroxybenzoate (methyl paraben) and 2,4,4'-trichloro-2'-hydroxydiphenyl ether (triclosan) were identified in the commercial GSE but were not present in the ethanol extract of grapefruit seeds. Their identities were confirmed by comparing their retention times and absorption spectra with those of authentic standards, and triclosan was additionally confirmed by LC/MS using negative ion electrospray ionization. The presence of methyl 4-hydroxybenzoate and triclosan in several commercial GSE samples was later confirmed by von Woedtke et al. (15) who additionally found benzethonium chloride in these products. These workers utilized thin-layer chromatography (TLC) as their analytical method. Five of the six commercial grapefruit seed extracts tested contained between 1.25 and 10% benzethonium chloride and three samples had between 0.0125 and 0.025% triclosan. Methyl paraben was also detected in three GSE samples. The five extracts containing one to three of these preservative agents showed high antimicrobial activity against *Bacillus subtilis* SBUG 1, *Micrococcus flavus* SBUG 16, *Staphylococcus aureus* SBUG 11, *Serratia marcescens* SBUG 9, *Escherichia coli* SBUG 17, *Proteus mirabilis* SBUG 47, and *Candida maltosa* SBUG 700. However, only one of the six GSE samples tested did not contain any preservatives and this sample did not show any antimicrobial activity. Extracts prepared by extraction of grapefruit seeds and juiceless pulp using glycerol, water, and ethanol, as well as mixtures of these solvents, likewise did not have any antimicrobial activity. The authors concluded that the potent antimicrobial activity attributed to GSE is due to the synthetic preservative agents with benzethonium chloride being responsible for the majority of activity. One of the manufacturers has since claimed that their product does not contain benzethonium chloride and the error was due to the similarity in molecular weight of the quaternary ammonium compound (said to be formed in the proprietary manufacturing process) with that of benzethonium chloride. The goal of this research is to confirm the presence of benzethonium chloride in com-

mercial GSE using various analytical methods including HPLC, one- and two-dimensional NMR, PIXE analysis, and electrospray ionization MS.

EXPERIMENTAL PROCEDURES

Materials. Grapefruit seed extract (GSE liquid concentrate, unfiltered formula, NutriBiotic, Lakeport, CA) was purchased at a local store. The ingredients were stated to be 33% Citricidal and 67% vegetable glycerin (glycerol). Grapefruit seed extract powder concentrate was obtained from NutriTeam, Inc. (Ripton, VT). GSE powder is said to contain 50% quaternary ammonium compound (from grapefruit bioflavonoids), 30% silicon dioxide, and 20% USP vegetable glycerin.

Chemicals. Benzethonium chloride was purchased from TCI America (Portland, OR). Solvents were HPLC spectroscopic grade.

Extraction. Approximately 2 g of liquid GSE was mixed with 1 mL of water and extracted with 40 mL of chloroform in a separatory funnel. The chloroform layer was collected, and the oily residue was extracted with another 40 mL aliquot of chloroform. The combined chloroform extract was evaporated under a stream of nitrogen. The resulting white crystalline solid was dried in a desiccator containing CaSO₄. The solid was dissolved in HPLC mobile phase, and filtered through a 0.5- μ m disposable membrane filter before injection. The GSE powder was first extracted with diethyl ether, then after air-drying, the powder was extracted with methanol. The methanol extract was evaporated, redissolved in mobile phase and filtered before HPLC analysis.

High-Performance Liquid Chromatography (HPLC). The HPLC system consisted of an HP 1050 quaternary pump, a manual injector (Model 7125, Rheodyne, Rohnert Park, CA) equipped with a 20- μ L sample loop, and an HP 1040M diode array detector. The instrument was controlled and the data were processed by an HP ChemStation (G2180AA version A.03.02). The analytical column was a Phenomenex LUNA C18(2) (250 \times 4.6 mm i.d., 5 μ m, 100 Å, 17.8% carbon load; Phenomenex Inc., Torrance, CA) protected by a Supelguard LC-18-DB (Supelco, Inc., Bellefonte, PA) guard column. The mobile phase consisted of methanol–water (9:1, v/v) containing 0.1 M sodium perchlorate. The pH was adjusted to 3 by addition of phosphoric acid (16). The flow rate was 1.0 mL/min and the detector was set at 215 nm.

Electrospray Ionization–Mass Spectrometry. An HP 1100 liquid chromatograph equipped with a manual injector (Rheodyne, model 1725) fitted with a 20- μ L sample loop and an HP 1100 diode array detector (DAD) was coupled to an HP 1100 mass selective detector (MSD). Electrospray ionization (ESI) was utilized with the following mass spectrometer operating conditions: positive ion mode, gas temperature 350 °C with a nitrogen flow rate of 10 L/min, nebulizer pressure 25 psi, capillary voltage 4000 V, and fragmentor voltage was adjusted from 60 to 160 V. Extracts were introduced directly into the mass spectrometer. Methanol was used as the mobile phase at a flow rate of 0.2 mL/min. The instrument was controlled and the data was processed by an HP ChemStation (Rev. A.06.01 [403]).

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were obtained at 29 K from samples in CDCl₃ with TMS as an internal standard on a Bruker model ARX400 spectrometer at a frequency of 100.62 MHz for carbon and 400.13 MHz for proton. A 30° pulse at 2.3 s repetition rate was used for carbon, and a 90° pulse at a 7–8 s repetition rate was used for protons.

Elemental Analysis. Proton induced X-ray emission (PIXE) analysis was performed by Elemental Analysis Corporation (Lexington, KY). PIXE is an X-ray spectroscopic technique that provides simultaneous elemental analysis for the elements from sodium to uranium. The X-ray spectrum is initiated by energetic protons interacting with the electrons to create inner shell vacancies in the atoms of the sample material. The energies of the X-rays that are emitted when these vacancies

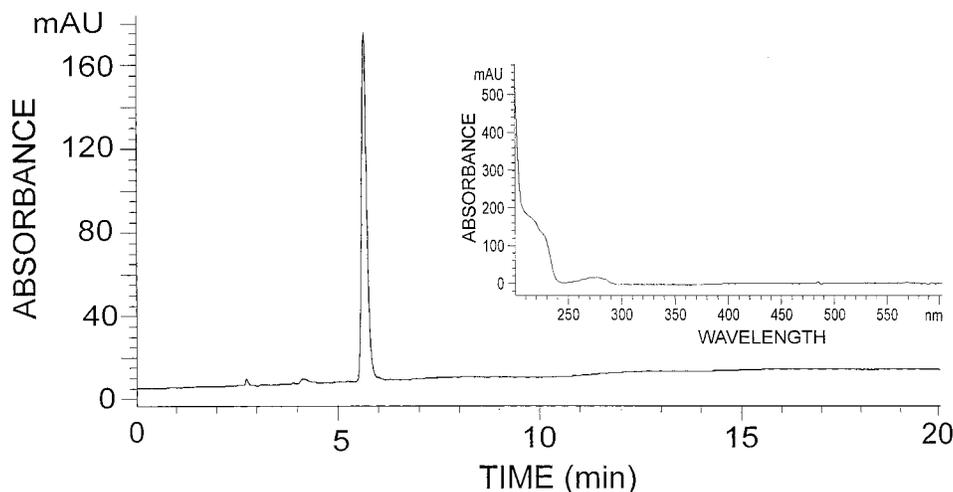


Figure 1. HPLC chromatogram (detection at 215 nm) of unknown solid extracted from commercial grapefruit seed extract (GSE). Conditions: column, Phenomenex LUNA C18(2), 25 × 4.6 mm i.d., 5 μm, 100 Å; mobile phase, methanol/water (9:1, v/v) containing 0.1 M sodium perchlorate (the pH of the mobile phase was adjusted to 3 by addition of phosphoric acid); flow rate, 1 mL/min. Inset: on-line UV spectrum of unknown solid from diode array detector.

are filled again are characteristic of the elements from which they originate while the number of X-rays of a certain energy is proportional to the mass of the corresponding element found in the sample.

RESULTS AND DISCUSSION

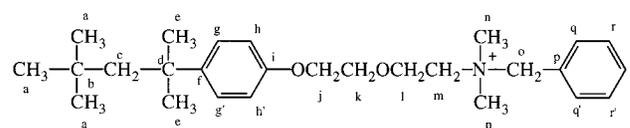
Chloroform extraction of liquid GSE followed by solvent evaporation produced a white crystalline solid that comprised 8.03% ($n = 2$) of the GSE sample. HPLC analysis of this solid revealed only one peak which eluted at 5.9 min (Figure 1). This retention time matched that of a standard of benzethonium chloride. The on-line UV spectrum of the unknown solid obtained on the diode array detector closely matched that of the benzethonium chloride standard. A mixture of the unknown solid and benzethonium chloride showed only one peak by HPLC.

Positive ion electrospray MS of the unknown solid showed a molecular ion at m/z 412 [M^+] that matched that of a standard of benzethonium chloride (Figure 2). Collision-induced dissociation (produced by increasing the fragmentor voltage) resulted in a very similar fragmentation pattern for both the unknown solid and the benzethonium chloride standard.

The presence of chloride in the unknown solid was confirmed by proton-induced X-ray emission (PIXE). The weight fraction of chlorine detected in the unknown solid was 6.99% whereas the weight fraction expected based on the molecular formula of benzethonium chloride ($C_{27}H_{42}ClNO_2$) was 7.91%.

The 1H and ^{13}C NMR spectra of the unknown solid (Table 1) closely matched those of the benzethonium chloride standard. The number of attached protons for ^{13}C signals was determined from DEPT90 and DEPT135 assays. One- and two-dimensional experiments were run for both nuclei. Nine aromatic protons were observed at δ 6.76 to 7.67. Some diagnostic resonances of the 1H NMR spectrum were those attributed to three tertiary methyl groups (9H; δ 0.70), two tertiary methyl groups (6H; δ 1.32), and two tertiary methyl groups (6H; δ 3.32). An AA'XX' spin system was observed for the para-disubstituted benzene. Inspection of the COSY spectrum revealed that the CH_2 group at δ 1.68 (c) was correlated to sets of CH_3 groups (3 tertiary methyl groups at δ 0.70

Table 1. 1H and ^{13}C NMR Spectral Data for Benzethonium Chloride (isolated from GSE) Dissolved in $CDCl_3$



		δ_H (ppm)	δ_C (ppm)
a	CH_3	0.70, 9H, s	31.75
b	C		32.25
c	CH_2	1.68, 2H, s	56.89
d	C		37.90
e	CH_3	1.32, 6H, s	31.62
f	C		142.72
g, g'	CH	7.24, 2H, m	127.12
h, h'	CH	6.76, 2H, m	113.65
i	C		156.00
j	CH_2	4.06, 2H, m	66.90
k	CH_2	3.85, 2H, m	69.81
l	CH_2	4.09, 2H, m	65.29
m	CH_2	3.91, 2H, m	62.67
n	CH_3	3.32, 6H, s	50.44
o	CH_2	5.03, 2H, s	69.80
p	C		127.73
q, q'	CH	7.67, 2H, m	133.51
r, r'	CH	7.35, 2H, m	129.00
s	CH	7.43, 1H, m	130.42

[a] and 2 tertiary methyl groups at δ 1.32 [e]), whereas the CH_2 group at δ 5.03 was correlated to both the two adjacent methyl group protons (δ 3.32, n) and the aromatic protons at δ 7.67 (q and q'). Cross-peaks at δ 6.76 (h and h') and δ 7.24 (g and g') were present for the aromatic protons on the para-disubstituted benzene. There was some ambiguity about the assignments of the CH_2 groups (δ 3.85 to 4.09) in the center of the molecule. COSY cross-peaks were observed at δ 3.85 and δ 4.06, and also at δ 3.91 and δ 4.09, revealing the pairing of the methylene groups but the absence of other cross-peaks for these protons made it difficult to assign their order. Assignments of the ^{13}C NMR signals were made by using a 2D heteronuclear shift correlation experiment.

In an effort to elucidate the shift assignments of four center CH_2 groups, NMR experiments were performed

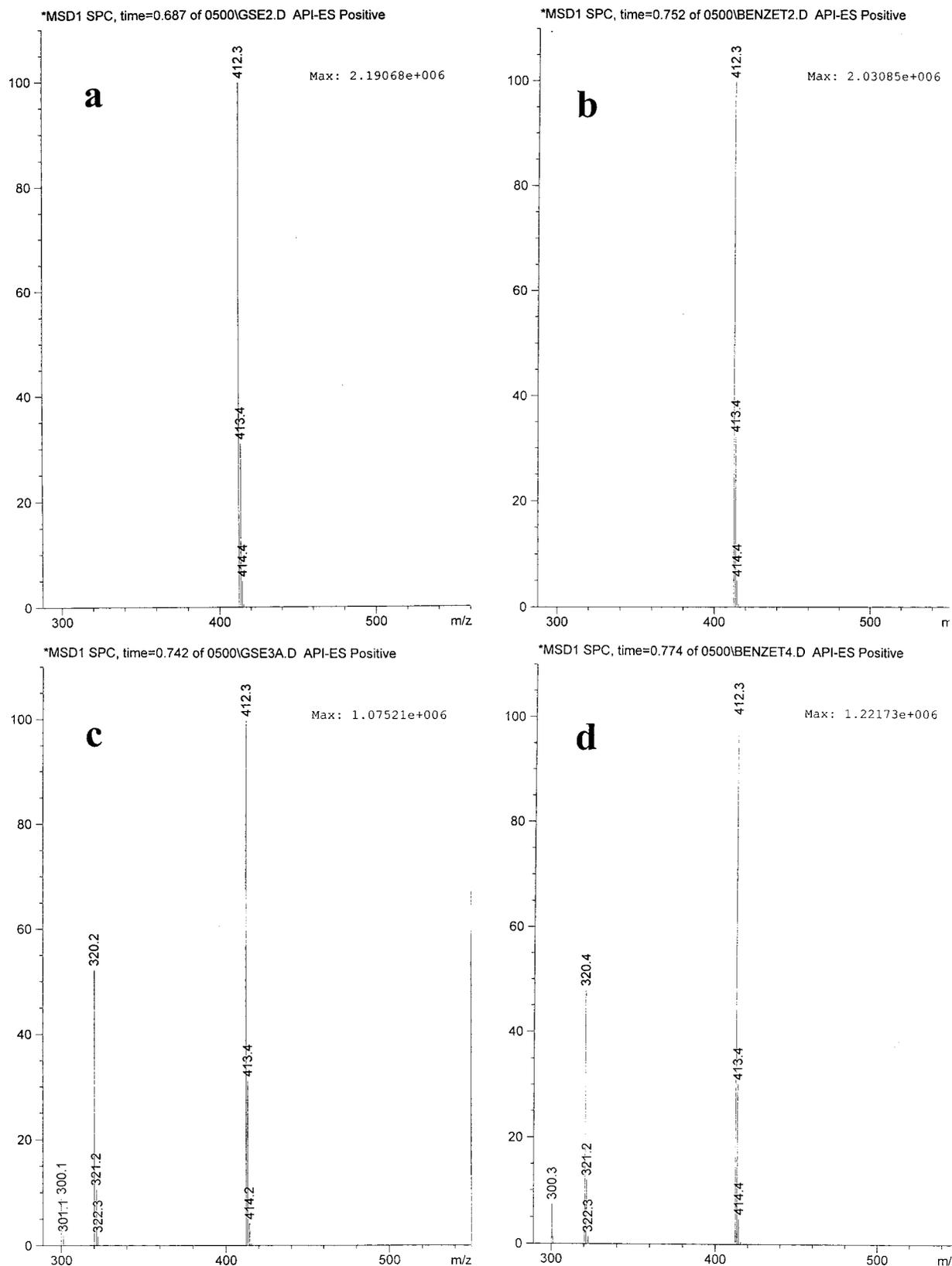


Figure 2. Electro-spray ionization mass spectra of (a) unknown solid, (b) benzethonium chloride standard (molecular ion at m/z 412), a and b were run with fragmentor voltage at 60 V; (c) unknown solid, (d) benzethonium chloride standard, c and d were run with fragmentor voltage at 160 V. See text for complete experimental conditions.

in pyridine. Better resolution of the four methylene groups (δ 3.95 to 4.36) was achieved in pyridine. The 2D homonuclear shift correlation for long-range couplings experiment showed that the two methyl group protons (e) were correlated to the aromatic protons (g

and g') and the other aromatic protons (h and h') on the para-substituted benzene were correlated to the oxy-methylene group at δ 4.17 which was assigned to position j. The CH_2 group furthest downfield at δ 4.36 was assigned to position m because it was correlated to

the two methyl group protons (n). This methylene group appeared to move from the third most downfield to the most downfield (of the four CH₂ groups) when the solvent was changed from CDCl₃ to pyridine-d⁵.

This work has conclusively demonstrated that benzethonium chloride is present in commercial GSE samples. Higher amounts of benzethonium chloride were present in powder GSE samples than in liquid GSE samples, although though we did not determine the exact concentration. This research confirms an earlier study (15) that found benzethonium chloride in commercial GSE samples. It seems unlikely that benzethonium chloride is formed during any extraction and/or processing of grapefruit seeds and pulp.

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