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# Simultaneous identification and quantification by liquid chromatography of benzethonium chloride, methyl paraben and triclosan in commercial products labeled as grapefruit seed extract

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A HPLC method has been developed which permits the quantification of methyl paraben, benzethonium chloride and triclosan in various samples of grapefruit seed extract (GSE). The best results were obtained with a Phenomenex Gemini C18 column using gradient mobile phase of water (0.1% acetic acid) and acetonitrile (0.1% acetic acid) with a flow rate of 1.0 mL per minute. The detection wavelength was 254 nm for methyl paraben, and 275 nm for benzethonium chloride and triclosan. The main synthetic antimicrobial agent identified in commercial GSE samples was benzethonium chloride in concentrations from 0.29–21.84%. Positive ion electrospray MS of a commercial GSE sample showed a molecular ion at m/z 412 [$M^+$], which matched that of a standard of benzethonium chloride. Triclosan was detected in two samples at 0.009 and 1.13% concentrations; while methyl paraben was not detected in the samples analyzed.

## 1. Introduction

Commercial products labeled as grapefruit seed extract (GSE) are promoted as natural antimicrobial agents for both internal and external use (Cho et al. 1995; Ionescu et al. 1990; Takeoka et al. 2001; Xiong et al. 1998). The composition of commercial GSE is not defined, and its methods of production are said to be proprietary and are not specified. GSE has been evaluated for potential use as an antimicrobial agent in the preservation of fruits, vegetables and legumes (Cho et al. 1991). Questions have arisen about the composition of GSE and whether some commercial samples have been adulterated with synthetic preservatives. Preservative agents have been previously identified in commercial GSE preparations (Sakamoto et al. 1996). The homologous mixture commonly known as benzalkonium chloride has also been identified in commercial GSE (Takeoka et al. 2005). Thin layer chromatography (TLC) was used for the estimation of preservative agents (von Woedtke et al. 1999).

This work describes the quantitative HPLC analysis of commercial GSE samples and a methanolic extract of grapefruit seeds. A simple, precise and more sensitive HPLC analytical method is presented for the simultaneous detection and quantitation of three preservatives namely methyl paraben (**1**), benzethonium chloride (**2**), and triclosan (**3**) in various commercial GSE samples. Compound identity was further confirmed by electrospray ionization mass spectrometry (HRESI-MS).

## 2. Investigations, results and discussion

The separation of the three standard compounds in GSE under optimized conditions is shown in Fig. 1. These synthetic antimicrobial agents were baseline separated in less than 20 min after all separation parameters were carefully assessed. A Gemini C18 column (150 × 4.6 mm, 5 μm particle size) from Phenomenex produced the best peak symmetry and separation selectivity using the specified mobile phase, which was vital for a satisfactory result. Heating the column to 30 °C reduced the separation time without decreasing peak resolution. Peak identity of compound **2** was confirmed in an ESI-MS experiment in positive ESI mode, with a spectral signal at an *m/z* of 412.3 $[M]^+$.

The analytical method was validated in accordance with USP by determining several analytical and statistical parameters. Linearity of the detector response for the three standard compounds was confirmed between 100 and 0.5 μg/mL, with the detection limits of 0.05, 1.5 and 0.15 μg/mL

**Table 1: Calibration data [regression equation and correlation coefficient ($r^2$)] and limit of detection (LOD) for compounds 1–3**

| Analyte | Regression equation | $r^2$ | LOD (μg/ml) | LOQ (μg/ml) |
|---|---|---|---|---|
| **1** | $y = 7.79 \times 10^4$ x | 0.9999 | 0.05 | 0.11 |
| **2** | $y = 1.78 \times 10^3$ x | 0.9999 | 1.5 | 3.5 |
| **3** | $y = 1.01 \times 10^4$ x | 0.9999 | 0.15 | 0.40 |

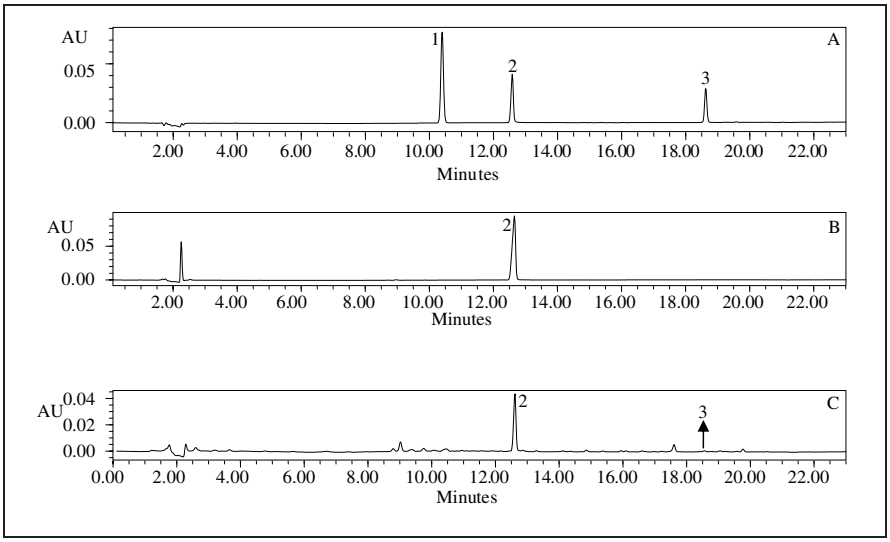


Fig. 1
Typical HPLC chromatograms of pure standards (**1**–**3**) at 275 nm (A) and commercial GSE (B & C)

for compounds **1**–**3** respectively (Table 1). Accuracy of the method was confirmed by performing a recovery experiment. Samples (GSE4 and GSE8) were spiked with a known amount of the standard compounds, extracted and analyzed. Compared to the theoretical amounts, recoveries of 98.7%–104.3% for compounds **1**–**3** were obtained. All standards and samples were injected in triplicate. Intra- and inter-day variation of the assay was determined and shown to be lower than 3.0%, with a maximum RSD of 2.92.

Nine commercial GSE samples, a pomegranate seed extract, and a grapefruit seed extract prepared from dried seeds were analyzed for methyl paraben, benzethonium chloride and triclosan. Benzethonium chloride in concentrations from 0.29–21.84% (Table 2) was found in various commercial GSE products. Triclosan was detected in two samples at concentrations between 0.009 and 1.13% while methyl paraben was not detected in any sample.

The mass spectrometric analysis was performed on an Agilent Series 1100 SL equipped with an ESI source. All acquisitions were performed under positive ionization mode with a capillary voltage of 4000 V. Nitrogen was used as nebulizer gas (30 psig) as well as drying gas at 10 L/min and drying gas temperature at 325 °C. Data acquisition and processing was done with the software Analyst QS. The identity of benzethonium chloride was confirmed by the appearance of a molecular ion at *m/z* 412 [$M^+$] matching the benzethonium chloride (**2**) standard. Peak purity and identity were verified by studying PDA and MS data, as well by spiking samples with reference compound. No impurities were found. Dried grapefruit seeds, commercial pomegranate seed extract (GSE6) and GSE9 did not show the presence of compounds **1**–**3**.

By the LC-UV method, the identification of compounds **1**–**3** in various samples was based on the retention times and the comparison of UV spectra with reference compounds. The UV spectrum and mass for compounds **1**–**3** (Fig. 2) and their presence in other samples identified were the same.

## 3. Experimental

### *3.1. Materials*

The standard compounds **1**, **2** and **3** were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and glacial acetic acid were of HPLC grade purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water for the HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Nine commercial grapefruit seed extracts (GSE1-GSE5 & GSE7-GSE10) and one commercial pomegranate seed extract (GSE6) were purchased online. Voucher specimens of all samples are deposited at the NCNPR.

### *3.2. Standard solution*

One milligram of each standard compound was placed in one 10 mL volumetric flask and dissolved in methanol (stock solution). Five additional calibration levels were prepared by diluting the stock solution with metha-

**Table 2: Percentage (%) or mg/serving size of antimicrobial agents methyl paraben (1), benzethonium chloride (2) and triclosan (3) found in commercial GSE**

| Product code | 1 | 2 | 3 | Serving size | GSE (Amount/serving) |
|---|---|---|---|---|---|
| GSE1 | ND | 5.97 mg/10 drops | ND | 10 Drops = 0.25 mL | 100 mg |
| GSE2 (powder) | ND | 20.37% | 0.009% | – | – |
| GSE3 | ND | 21.11% | ND | 2 tablets | 250 mg |
| GSE4 | ND | 0.29% | ND | 1 capsule | 50 mg |
| GSE5 | ND | 20.45% | 0.059% | 1 capsule | 125 mg |
| GSE6 (Pomegranate seed extract. No GSE) | ND | ND | ND | 1 capsule | – |
| GSE7 | ND | 20.95 mg/0.8 mL | ND | 0.8 ml | – |
| GSE8 | ND | 21.84% | 1.13% | 1 tablet | 250 mg |
| GSE9 | ND | ND | ND | 1 capsule | 250 mg |
| GSE10 | ND | DUL | ND | – | – |
| GS (*Citrus* x *paradisi* seed methanolic extract) | ND | ND | ND | – | – |

All % RSD were below 1.0
ND = Not Detected
DUL = Detected under limit of quantification

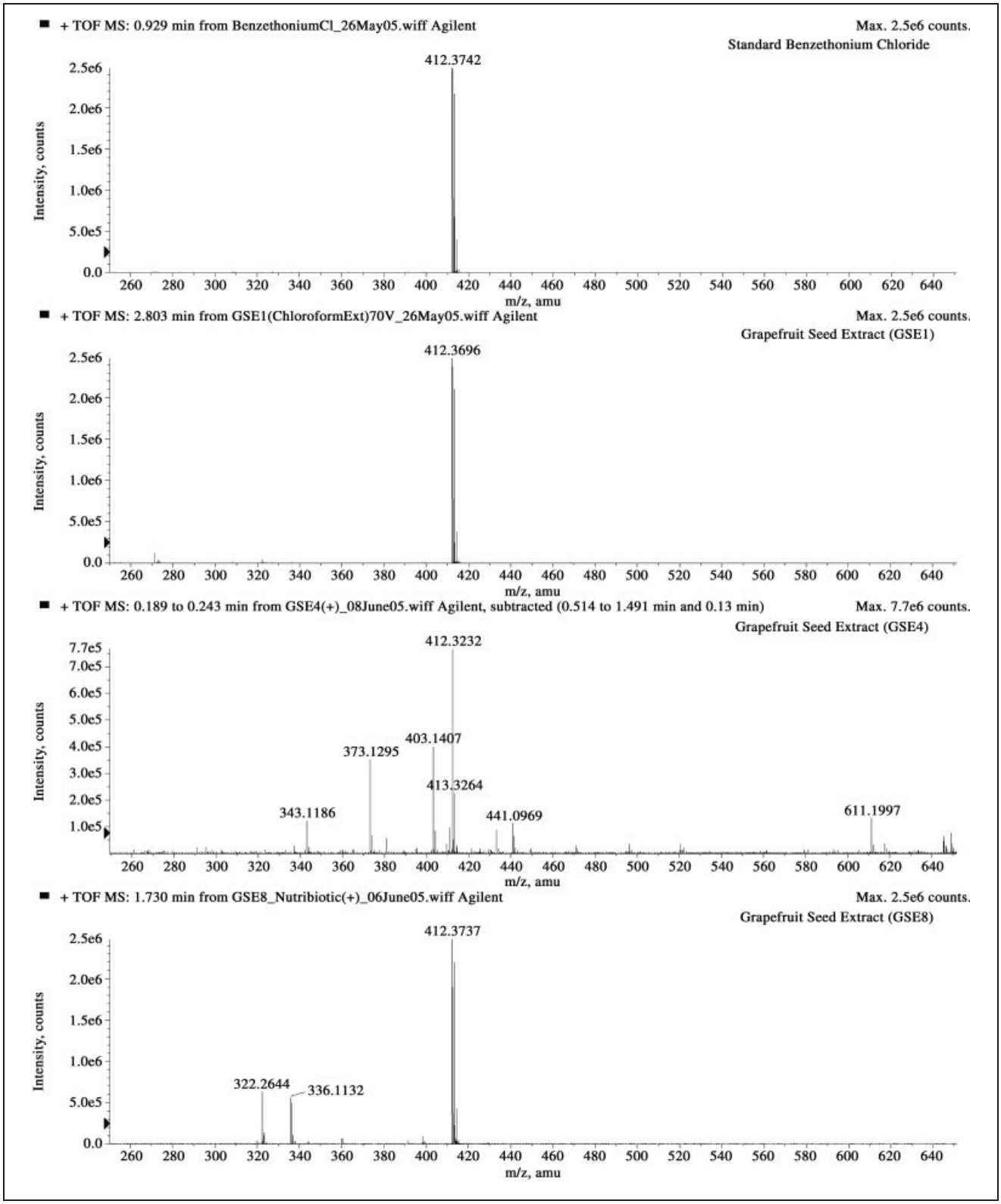


Fig. 2: ESI-MS of benzethonium chloride and its identification in various commercial grapefruit seed extracts

nol. The detector response was linear within the range of injected concentrations (100.0–0.5 µg/mL). Table 1 shows the calibration data and the calculated limits of detection, which were determined by serial dilution based on a signal to noise ratio of 3 : 1. Calibration curves generated by linear regression were based on peak area.

### *3.3. Sample preparation*

Finely powdered dried plant material (0.5 g), or an adequate amount of powdered solid GSE extract, or GSE powdered material taken from capsules, was sonicated in 2.5 mL methanol for 15 min followed by centrifugation for 15 min at 3300 rpm. The supernatant was transferred to a 10.0 mL volumetric flask. The procedure was repeated thrice and respective supernatants were combined. The final volume was adjusted to 10 mL with methanol. The aliquot of the liquid formulations (10 drops) were diluted with 5.0 mL of methanol and mixed thoroughly. 50 µL of all solutions were further diluted to 1.0 mL methanol. Prior to use all samples were filtered through a 0.45 µm nylon membrane filter.

### 3.4. Chromatographic conditions

A Waters 2695 Alliance Separations Module equipped with a 996 Photo-Diode Array detector (Waters, Milford, MA), using a Gemini column (150 × 4.6 mm; 5 μm particle size) from Phenomenex (Torrance, CA), and maintained at 30 °C was employed. The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% acetic acid, which were applied in the following gradient elution: 0 min, 80% A: 20% B held for 5 min; then 100% B over 15 min for the determination of methyl paraben, benzethonium chloride and triclosan. Solvent B was used as a wash for 5 min followed by re-equilibration with 80% A: 20% B for 15 min. Total run time was 20 min. The detection wavelength was 254 for methyl paraben, and 275 nm for benzethonium chloride and triclosan. Injection volume was 10 μL. Peak assignment was done by retention time and UV-spectral comparison. Data was collected and analyzed by Waters Millennium[32] software (Milford, MA) and tabulated by Microsoft Excel.

For peak confirmation an ESI-MS experiment, using an LC-MSD-TOF mass spectrometer from Agilent Technologies, was performed. Best results were obtained in positive ESI mode with fragmentation voltage set to 70 V, capillary voltage to 4000 V and source temperature to 325 °C.

### 3.5. Accuracy

A recovery experiment was performed to confirm the accuracy of the method. Samples (GSE4 and GSE8) were spiked with 1.0 mL of the standard stock solution and then extracted and analyzed under optimized conditions. The recovery rates were in the range from 98.7%–104.3% for compounds **1**–**3**.

### 3.6. Ruggedness

Intra- and inter-day assay: Precision of the method was determined by analyzing five individual samples of one specimen (GSE8) on three consecutive days. The samples were extracted and assayed under optimized conditions (Table 3).

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**Table 3: Intra- and inter-day precision of one sample (GSE8) assayed under optimized conditions**

| Compound | Intra-Day (n = 5) | | | Inter-Day (n = 3) |
|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | |
| **1** | – | – | – | – |
| **2** | 3.87 (1.48) | 3.83 (1.62) | 3.90 (1.40) | 3.86 (1.33) |
| **3** | 0.203 (2.55) | 0.201 (2.91) | 0.205 (2.92) | 0.20 (2.59) |

Values in mg/100 mg of tablet weight; relative standard deviation are given in parentheses

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