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Determination of Benzethonium Chloride in Grapefruit Seed Extracts - a GC/MS alternative

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Dedicated to Acad. Dimiter Ivanov on the occasion of his 120th birth anniversary

Commercial grapefruit seed extracts, distributed on the market as food supplements, were analysed by gas chromatography-mass spectrometry (GC/MS). Presence of benzethonium chloride, a synthetic antimicrobial agent, in the range of 0.14-22.2% was found in 4 of 5 analysed commercial samples (one of them - especially developed for children). The presence of benzethonium chloride was additionally confirmed by high-performance liquid chromatography with diode-array detection (HPLC/DAD) and direct infusion electrospray ionization mass spectrometry (ESI-MS). This work demonstrates that GC/MS represents a simple, fast, selective and sensitive alternative approach for identification and quantitation of benzethonium chloride in commercial grapefruit seed extracts.

Key words: benzethonium chloride, Grapefruit seed extract, GC/MS

INTRODUCTION

Nowadays, there is a growing interest toward alternative medicine and consumption of herbal drugs and additives. At the same time, many of the herbal products available on the market have not been assessed by regulatory authorities and their chemical composition and pharmacological properties are not always well described. One of the products with most controversial fame in the natural products market is the "grapefruit seed extract" (GSE), labelled as an extract of the seeds and pulp of the common grapefruit (*Citrus paradisi*, Rutaceae). GSE has been promoted over the last three decades as a gentle non-toxic natural product with healing power against a variety of diseases, with very high antimicrobial efficacy and has been used as an ingredient for cosmetic and dermatological formulations, in dietary supplements as well as food/cosmetic preservative. However, in most of the cases, the compositions of commercial GSEs are not defined, and the methods of production being proprietary are not specified.

In 1991 the first analysis of commercial GSE was published by Nishina *et al.* [1], where methyl 4-hydroxybenzoate (methyl paraben), a preservative, and 2,4,4-trichloro-2-hydroxydiphenyl ether (triclosan), a microbicide and disinfectant were found by means of preparative HPLC. Using HPLC and ESI-MS, Sakamoto *et al*. [2] repeated the analysis of GSE, and compared the commercial GSE with ethanolic extracts of grapefruit seeds prepared by themselves. The presence of methyl paraben and triclosan in the commercial GSE (1.66% and 1.97%, respectively) was confirmed and no trace of these compounds was found in the ethanolic extract of grapefruit seeds. The antimicrobial activity as well as the content of preservative agents (methyl paraben and triclosan) in 6 commercially available GSE were examined by von Woedtke and co-workers [3], who additionally found *N*-Benzyl-*N*,*N*-dimethyl-2-{2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethoxy}ethanaminium chloride (benzethonium chloride), a synthetic antimicrobial agent commonly used in cosmetics and other topical applications, in these products. The five extracts containing one to three of these preservative agents showed high antimicrobial activity. At the same time, the only GSE product without synthetic preservatives along with the fresh extracts prepared from grapefruit seeds with glycerol, water and ethanol, did not exhibit any antimicrobial activity. The authors concluded that the antimicrobial activity attributed to GSE is due to the synthetic preservative agents with benzethonium chloride being responsible for the majority of activity. Takeoka *et al.* subsequently published two analyses of GSE formulations [4,5]. They have found using HPLC, ESI-MS, nuclear magnetic resonance (NMR) spectroscopy, and elemental

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analysis that benzethonium chloride is the main constituent in the analysed commercial GSE in form of a liquid concentrate and a concentrated powder (as 8% of the mass of the liquid GSE, higher amounts of benzethonium chloride were found in powder). Sugimoto *et al.* [6] carried out comprehensive research of the commercial GSE products which are used in Japan as food additives (13 products from 6 manufactures), dietary supplements (5 products from 4 manufactures), cosmetic materials (16 products from 10 manufactures) and disinfectants (7 products from 7 manufactures). By means of LC/MS and NMR analysis, synthetic disinfectant agents such as benzethonium or benzalkonium salts were detected in most of the commercial GSE products. Simultaneous identification and quantification of benzethonium chloride, methyl paraben and triclosan in 9 commercial GSE products, one pomegranate (*Punica granatum*, Punicaceae) seed extract, and a freshly prepared methanolic extract of grapefruit seeds, were performed by Avula *et al.* using HPLC/UV/MS [7]. Benzethonium chloride was found in 8 of 9 commercial GSE preparations. Only the commercial pomegranate seed extract (used as a control sample) and the fresh grapefruit seed extract were free of synthetic additives. An HPLC/UV/MS method was developed and validated by Ganzera *et al.* for simultaneous determination of 18 possible preservatives, disinfectants, and microbicides in GSE and tested on 9 commercial products used for eco-farming [8]. A method for the quantitative analysis of benzethonium chloride in GSE based on ^{1}H-NMR is also described in the literature [9].

Evidently, there is a serious problem with the adulteration of the commercial GSE, which proves the necessity of development and validation of new methods for analysis of benzethonium chloride. As seen, currently most of the methods are based on high–performance liquid chromatography. In addition to the above described, benzethonium chloride was determined by gas-liquid chromatography as reduction product obtained by treatment of the sample with sodium borohydrate and nickel (II) chloride by Kawase *et al.* [10].

Benzethonium chloride being quaternary ammonium salt (QAS) is non-volatile and at first sight gas chromatography could not be the most suitable method for its direct determination. However, the QAS are thermally unstable and decompose at high temperatures by cleavage at the quarternary nitrogen, forming tertiary amines and other neutral molecules [11-13]. Gas chromatography seems to be an ideal analytical technique for the determination of QAS because the heated inlet system of the instrument allows *in situ* decomposition of the salts. Consequently, thermal decomposition to neutral molecules, vaporization, and analysis of the characteristic products can be performed in a single step. An injection port pyrolysis method for the analysis of quaternary ammonium compounds (QAC) is reported by Lukazewsky *et al.* [14]. Direct injection GC/MS was used for the analysis of benzyl diethyl (2,6-xylylcarbamoylmethyl) ammonium benzoate, a QAS, in various Canadian denatured alcohol formulations [15].

Therefore, the aim of the current communication is to describe a fast, simple and sensitive direct GC/MS method for simultaneous identification and quantification of benzethonium chloride, based on the analysis of N,N-dimethyl-2-{2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethoxy}ethanamine as main pyrolysis product.

EXPERIMENTAL

Materials

Samples of commersial GSE, four of them in form of a liquid concentrate and one as a kid syrup, were purchased from drug stores in Bulgaria, Poland and UK. Benzethonium chloride reference material (99%) was delivered from Sigma-Aldrich. Solvents (methanol, chloroform, acetonitrile) of HPLC gradient grade (Sigma-Aldrich) and de-ionized water ASTM Type I were used. Solid-phase extraction columns SOLA SCX, 10 mg/1ml were purchased from Thermo Scientific.

Sample Preparation

Liquid-liquid extraction. Approximately 2 g of each GSE sample was mixed with 10 ml of water and extracted three times with 30 mL of chloroform. The combined chloroform extracts were evaporated either in a rotary vacuum evaporator or under stream of nitrogen. The dry residues were then dissolved in HPLC mobile phase and filtered through a membrane filter (PTFE, 0.22 μm) before the chromatographic analysis.

Solid Phase Extraction. Approximately 0.1 g of each sample was dissolved in 10 ml of methanol and then an aliquot of 0.5 ml was used for solid phase extraction.

Methods

High-Performance Liquid Chromatography (HPLC). The analyses were performed on a HPLC system consisting of an HP1100 liquid chroma-

tograph equipped with a manual injector (Rheodyne, model 7725), fitted with a 20 μL sample loop and a diode–array detector (G1365B DAD), and controlled by ChemStation software (Rev. B.04.03, Agilent Technologies). Analytical column ChromSep SS, Inertsil 5 ODS-2 (250 x 4.6 mm i.d. 5 μm) equipped with a ChromSep guard column (Varian, Palo Alto, CA) was used. The mobile phase was acetonitrile/water (80:20, v/v) containing 0.1% formic acid (pH 3) at a flow rate of 1.0 ml/min. The detector signal was monitored at 215 and 275 nm.

Gas Chromatography-Mass Spectrometry (GC/MS). The GC/MS analysis was performed on a HP gas chromatograph 6890 Series Plus coupled with a 5973 mass - selective detector (Hewlett–Packard, Palo Alto, CA). The ultra-inert fused silica capillary column DB-5ms UI (J&W Scientific, Folsom, CA) with 30 m column length, 0.25 mm i.d., 0.25 μm film thickness was used. The oven temperature was programmed from 80 to 300°C at a rate of 5°C /min, and a 10 min hold at 300°C was applied. Helium (99.999%) was used as a carrier gas at a constant flow rate of 0.8 ml/min. The split ratio was 1:10, the inlet temperature was set to 280°C and the transfer line temperature was 300°C. Mass - selective detector operated in electron impact ionization (EI) mode at 70 eV electron energy, the ion source temperature was set to 200°C, and the quadrupole temperature was 150°C.

Electrospray Ionization-Mass Spectrometry (ESI-MS). ESI-MS spectra were recorded on a DFS High Resolution magnetic-sector mass spectrometer (Thermo Scientific, Bremen, Germany) under the following operating conditions: positive ion scan mode, capillary temperature of 260°C and capillary voltage of 3.5 kV was applied. Direct infusion mode was used for the introducing of samples into the mass spectrometer via Harvard 11 Plus syringe pump (Harvard Apparatus, Holliston, Massachusetts, USA). Acetonitrile/water (30:70%, v.v) with 0.05% formic acid was used as a mobile phase at a flow rate of 0.05 ml/min. The instrument was controlled and the data was processed by Xcalibur™ software (Rev. 2.0 SR 1, Thermo Scientific).

Calibration Curves

The absolute calibration method (external standard method) was used to establish the calibration curve and quantify the analytes. 0.025 g of benzethonium chloride standard was weight and diluted in a 25 ml volumetric flask with methanol. Five working standard solutions with concentrations in the range 0.001-0.200 mg/ml were prepared from the stock standard solution with concentration 1 mg/ml. Each working standard solution was then analysed in triplicate by GC/MS, operating in full scan mode and the results were presented graphically, plotting peak area (for the peak at t_R =20.086 min) versus concentration.

RESULTS AND DISCUSSION

Benzethonium chloride, being quaternary ammonium salt is non-volatile, but, as seen in Fig. 1, its GC/MS chromatogram exhibits two main peaks. The first one is benzyl chloride (**1**), at retention time t_R=5,54 min, and the second one has been identified as N,N-dimethyl-2-{2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethoxy}ethanamine (**2**), at t_R=20.08 min. These peaks are result of the thermal dissociation of benzethonium chloride (favored pathway is shown in Scheme 1) in the GC inlet system, following the fact that QAS are unstable at high temperatures. In general, the N-benzyl cleavage is favored over the N-alkyl one in this process. The main pyrolysis product **2** exhibits GC/MS behavior, which makes it suitable for identification and low level quantification of benzethonium chloride.

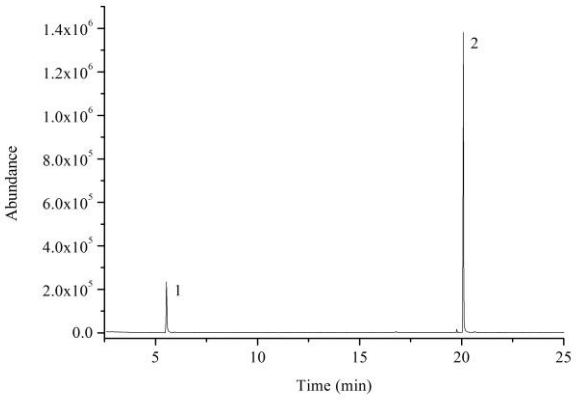


Fig. 1. GC/MS total ion current (TIC) chromatogram of the benzethonium chloride reference material.

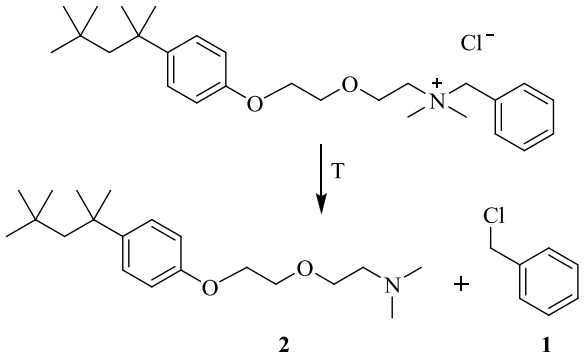
Linear calibration curve with regression coefficient of 0.9997 was established between the EI-MS response of **2** as a function of the benzethonium chloride standard solutions concentration. The corresponding equation is given below:

$$Y = 2.46 \times 10^7 . X + 4.74 \times 10^4 ,$$

where Y is the total ion current peak area of **2** and X is the concentration of benzethonium chloride in mg/ml.

The linearity was studied in concentration range from 0.001 to 1 mg/ml with limit of quantification

(LOQ) of 0.005 mg/ml (S/N=10) and limit of detection (LOD) of 0.001 mg/ml.



Scheme 1. Thermal dissociation of benzethonium chloride.

Glycerin, used as carrier in the commercial GSE formulations, is moderately volatile and need to be removed from the sample before the GC/MS analysis. Therefore two methods of extraction of benzethonium chloride from the commercial samples have been attempted, namely liquid-liquid extraction with chloroform and solid phase extraction (SPE). In the first case it was impossible to remove glycerin completely and always a white viscous product, containing sufficient amount of glycerin was obtained. The glycerin was fully removed by using only SPE and, therefore, this extraction method was finally selected for sample preparation. The recovery of the SPE, determined by standard addition method, was 0.98.

Using the above discussed procedure, five commercial GSE formulations, available in pharmacies as dietary supplements, with declared ingredients extract of seeds and pulp of grapefruit, plant glycerin and in some cases vitamin C, have been analysed. It is worth to underline that one of these products (sample 1) was introduced as stimulating immune activity syrup, especially designed for kids, with GSE content of 0.1%, containing in addition rose hips extract. The GC/MS analysis has shown availability of benzethonium chloride in four of the samples in range from 0.14 to 22.2%.The corresponding results are collected in Table 1.

These results are stunning, because according to the opinion of the Scientific Committee on Cosmetic Products and Non-Food Products, the scientific advisory body to the European Commission in matters of consumer protection SCCNFP/0762/03, the use of benzethonium chloride as a preservative in leave-on products is safe up to a maximum concentration of 0.1% and absolutely prohibited for internal use.

Table 1. Content of benzethonium chloride in the analysed commercial GSE.

Sample	Benzethonium chloride [%]
1	0.14 ± 0.01
2	8.96 ± 0.39
3	10.6 ± 0.28
4	22.1 ± 0.02
5	< LOD

Benzethonium chloride was identified in the commercial samples by using mass spectral libraries (NIST08, Wiley 275 and MS Search v.2.0), by the retention time of pyrolysis product **2** of the reference material and its mass spectrum with characteristic fragment ions (Fig. 2). As an additional piece of evidence the chromatograms of the analysed samples are shown in Fig. 3.

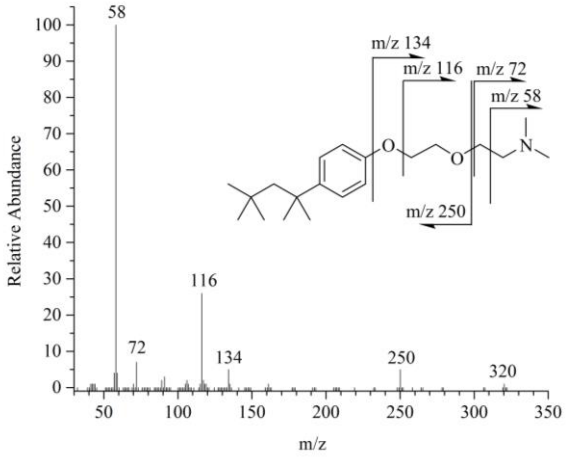


Fig. 2. EI-MS (70 eV) spectrum of **2**.

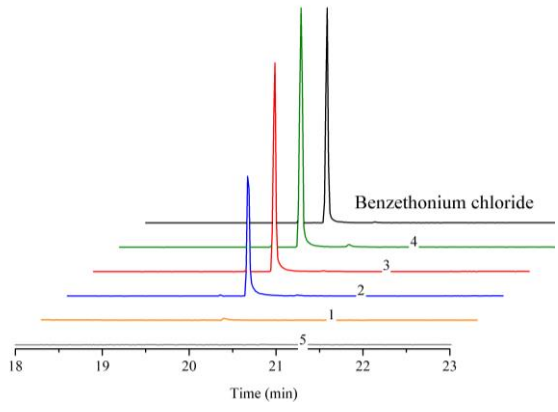


Fig. 3. GC/MS TIC chromatograms of commercial GSE samples.

The identification of benzethonium chloride was additionally confirmed by means of HPLC/DAD and ESI-MS. It is worth to underline that in both cases the samples were analysed at room temperature, which excludes thermal dissociation.

When HPLC/DAD (215 and 275 nm) was used a single peak at t_R = 8.35 min has been detected in the samples 1-4, which corresponds to the retention time of the benzethonium chloride standard. The commercial samples and the standard solution of benzethonium chloride in suitable concentrations have been analysed by direct infusion ESI-MS in full scan positive mode. The ESI-MS spectra of the reference benzethonium chloride and the samples 1-4, shown in Fig. 4, are identical, which once again confirms the presence of benzethonium chloride in the samples.

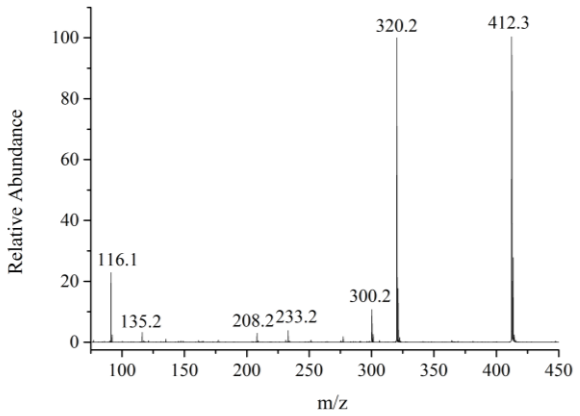


Fig. 4. Positive mode ESI-MS spectrum of benzethonium chloride as reference material and in the samples 1-4.

CONCLUSION

This work demonstrates that direct GC/MS represents a simple, fast, selective and sensitive alternative approach for identification and quantification of benzethonium chloride in commercial GSE. The proposed method seems to be suitable, with small modifications of the sample preparation procedure, for determination of benzethonium chloride and/or other synthetic microbicides based on quaternary ammonium compounds in various matrices (food additives, cosmetics, etc.).

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ОПРЕДЕЛЯНЕ НА БЕНЗЕТОНИЕВ ХЛОРИД В ЕКСТРАКТИ ОТ СЕМЕНА НА ГРЕЙПФРУТ – АЛТЕРНАТИВЕН ПОДХОД ЧРЕЗ ИЗПОЛЗВАНЕ НА ГАЗОВА ХРОМАТОГРАФИЯ С МАСПЕКТРАЛНА ДЕТЕКЦИЯ

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(Резюме)

Търговски екстракти от семена на грейпфрут, разпространени в търговската мрежа като хранителни добавки, бяха анализирани чрез газова хроматография – масспектрометрия (ГХ/МС). В четири от пет анализирани търговски продукта (един от които специално разработен за деца) беше намерено присъствие на бензетониев хлорид, синтетичен антимикробен агент, със съдържание от 0.14-22.2%. Наличието на бензетониев хлорид беше допълнително потвърдено чрез високо ефективна течна хроматография с фотометричен детектор с диодна матрица и масспектрометрия с електроспрей йонизация. Публикацията демонстрира възможностите на ГХ/МС като лесен, бърз, селективен и чувствителен алтернативен подход за идентификация и количествено определяне на бензетониев хлорид в търговски екстракти от семена на грейпфрут.