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Development and Validation of an HPLC/UV/MS Method for Simultaneous Determination of 18 Preservatives in Grapefruit Seed Extract

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Grapefruit seed extracts are used in cosmetics, food supplements, and pesticides because of their antimicrobial properties, but suspicions about the true nature of the active compounds arose when synthetic disinfectants such as benzethonium or benzalkonium chloride were found in commercial products. The HPLC method presented herein allows the quality assessment (qualitative and quantitative) of these products for the first time. On the basis of a standard mixture of 18 preservatives most relevant for food and grapefruit products, a method was developed allowing the baseline separation of all compounds within 40 min. Optimum results were obtained with a C-8 stationary phase and a solvent system comprising aqueous trifluoroacetic acid, acetonitrile, and 2-propanol. The assay was fully validated and shown to be sensitive (LOD $\leq$ 12.1 ng on-column), accurate (recovery rates $\geq$ 96.1%), repeatable ($\sigma_{rel} \leq$ 3.5%), precise (intra-day variation $\leq$ 4.5%, interday variation $\leq$ 4.1%), and rugged. Without any modifications the method could be adopted for LC−MS experiments, where the compounds of interest were directly assignable in positive ESI mode. The quantitative results of several products for ecofarming confirmed previous studies, as seven out of nine specimens were adulterated with preservatives in varying composition. The samples either contained benzethonium chloride (2.5−176.9 mg/mL) or benzalkonium chloride (138.2−236.3 mg/mL), together with smaller amounts of 4-hydroxybenzoic acid esters, benzoic acid, and salicylic acid.

KEYWORDS: Grapefruit; *Citrus paradisi*; pesticide; preservative; LC; LC−MS

INTRODUCTION

Grapefruits (*Citrus paradisi* Macf., Rutaceace), especially extracts prepared from dried grapefruit seeds (GSE), are major ingredients in a number of cosmetics and dietary supplements, for which there are claims of antioxidant and antimicrobial effects (*1*−*3*). They are used to treat various conditions such as eczema, cold sores, colds, gastritis, allergies, ulcers, and parasitic diseases; even the successful therapy of HIV infections is reported (*4*−*6*). These products mainly contain seed extracts prepared by proprietary manufacturing processes (extraction of grapefruit seeds and smaller amounts of pulp with glycerine at approximately 150 °C, combined with UV-radiation or catalytic conversion using "natural" enzymes) that claim to transform polyphenols natively present in the plant into the actual antimicrobial agents (*6*, *7*).

Numerous reports describing the activity of GSE against various fungal and bacterial strains can be found in the literature. For example, Ionescu et al. reported on potent antibacterial and antifungal characteristics of the commercial product ParaMycrocidin against Gram-positive (*Staphyllococcus* and *Enterococcus* ssp.) and Gram-negative (e.g. *Pseudomonas* ssp.) bacteria, as well as against yeasts and molds (e.g. *Aspergillus* and *Candida* ssp.) (*8*). Spraying vegetables (garlic, onion, and soybean sprouts) and fruits (tangerine) with GSE significantly prolonged their shelf lives (*9*, *10*), whereas antibacterial effects against oral bacteria in saliva suggested the use of GSE as antiseptic mouth rinse (*11*).

Despite these interesting biological properties, doubts about the nature of the actual antibacterial constituents in GSE emerged over the last years. Von Woedtke and co-workers found preservatives (e.g. benzethonium chloride, methyl paraben) in several commercial grapefruit seed extracts by TLC. Only these adulterated extracts were active against a number of test germs in vitro, whereas self-made extracts as well as samples free of preservatives showed no antimicrobial activity at all (*12*). Three HPLC studies are reported as well, but none of them enabled the quantitative determination of all compounds of interest in one analytical run (*13*−*15*). Additionally, these methods were not validated, present only fragmentary quantitative results for a limited number of samples, and focus on the analysis of GSE used for medicinal or cosmetic applications.

More recently GSE is also advertised as a natural and environmentally friendly pesticide in ecofarming; several commercial products are available in Europe for that purpose already. On the basis of the label information, they claim to be active against bacteria, viruses, and phytopathogen fungi such

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Table 1. Calibration Data and Percent Recovery Rates (high, medium, and low spike; sample GSE-9) for Preservatives Found in GSE Products, Including Regression Equation, Correlation Coefficient (R^2), and Limit of Detection and Quantitation (LOD and LOQ; values in ng on-column)

compd	regression equation	R^2	LOD	LOQ	rec 1 (high)	rec 2 (medium)	rec 3 (low)
2	$y = 8.703x^{-6}$	0.9999	2.4	9.7	100.8	99.5	98.8
4	$y = 2.131x^{-5}$	0.9976	7.8	24.4	96.1	97.2	101.9
5	$y = 4.292x^{-6}$	0.9981	1.5	4.9	97.2	98.6	99.1
8	$y = 9.919x^{-6}$	0.9999	4.5	15.8	101.9	98.4	98.2
16	$y = 4.179x^{-5}$	0.9998	12.1	34.6	98.2	99.4	98.4
17	$y = 2.476x^{-5}$	0.9999	7.8	25.2	100.1	102.2	98.1
19	$y = 4.357x^{-5}$	0.9999	8.8	26.4	97.6	98.8	98.0
20	$y = 3.727x^{-5}$	0.9994	9.7	31.1	97.4	97.3	98.4

as *Fusarium*, *Phytium*, *Verticillium*, or *Botrytis* subspecies; diluted with water they can be utilized in gardens and orchards (e.g. citrus fruits, apricots, apples, and grapes) as well as used to protect ornamental plants. Information regarding the exact chemical composition of these products is not available. Owing to the aforementioned suspicions, several agricultural GSE products were analyzed for a possible contamination with preservatives in this study. For that purpose an HPLC method enabling the separation of 18 preservatives most relevant for food was developed and validated; LC−MS experiments were performed for an unambiguous peak assignment.

EXPERIMENTAL PROCEDURES

Materials. Commercial products for eco-farming (liquid, GSE-1−8; powder, GSE-9; all samples except GSE-8 claimed to contain GSE) were supplied by the Agriculture and Forestry Research Institute, Laimburg, Pfatten, Italy. Reference specimens of all samples are deposited at the Institute of Pharmacy, University of Innsbruck, in Innsbruck, Austria.

Chemicals. Reference compounds were purchased from Fluka (Buchs, Switzerland) or Aldrich (Sigma-Aldrich, Steinheim, Germany): 4-hydroxybenzoic acid (**1**), methyl 4-hydroxybenzoate (**2**), sorbic acid (**3**), benzoic acid (**4**), salicylic acid (**5**), methyl benzoate (**6**), chlorhexidine diacetate (**7**), propyl 4-hydroxybenzoate (**8**), 4-chloro-3-hydroxytoluol (**9**), ethyl benzoate (**10**), benzyl 4-hydroxybenzoate (**11**), butyl 4-hydroxybenzoate (**12**), propyl benzoate (**13**), benzyl benzoate (**14**), butyl benzoate (**15**), benzalkonium chloride (C12; **16**), benzethonium chloride (**17**), 2,4,4′-trichloro-2′-hydroxydiphenyl ether (**18**), benzalkonium chloride (C14; **19**), and benzalkonium chloride (C16; **20**). All reference compounds had a purity of ≥98%. Benzalkonium chloride is a mixture of several homologues (C12, 64.8%; C14, 31.7%; C16, 3.5%; assignment based on peak area and LC−MS results).

All solvents and acids used (methanol, acetonitrile, 2-propanol, trifluoroacetic acid) were of analytical grade and purchased from Merck (Darmstadt, Germany). Nanopure water (Barnstead, Dubuque, IO) was used for all HPLC analyses.

Sample Preparation. Liquid samples were prepared by dilution with methanol, either in the ratio 1:500 (GSE-1−4) or 1:100 (GSE-5−8). For sample GSE-9 (powder), 20 mg of the material was dissolved in 5.00 mL of methanol. If necessary, the solutions were filtered through a 0.45 μm nylon membrane filter (SRP 15, Machery Nagel, Düren, Germany) prior to HPLC-analysis.

Calibration. Calibration curves were established for six preservatives present in the GSE products investigated (**2**, **4**, **5**, **8**, **16**, **17**, **19**, and **20**). For that purpose, 5.0 mg of each compound was placed in one 10-mL volumetric flask and dissolved in methanol. Beginning with this solution, four additional calibration levels were prepared by a 1:2 serial dilution with methanol. Within the range of concentrations injected (6.2−500 μg/mL) the detector response was linear; see **Table 1** for calibration data (limits of quantitation and detection were determined by serial dilution and calibration curves were generated by linear regression based on peak area). Standard solutions were stable for at least 30 days if stored at 4 °C (confirmed by reassaying the solution).

Table 2. Intra- and Interday Precision of Assay, if Sample GSE-3 Is Analyzed Five Times per Day on Three Consecutive Days[a]

compd	intraday ($n = 5$) day 1	intraday ($n = 5$) day 2	intraday ($n = 5$) day 3	interday ($n = 3$)
2	98.69(2.7)	100.72(4.4)	98.92(4.1)	99.44(1.1)
8	49.65(3.3)	50.22(4.5)	51.01(4.2)	50.29(1.4)
16	992.01(1.6)	998.77(3.8)	987.77(1.0)	992.85(0.6)
19	333.14(1.8)	335.03(3.4)	321.51(4.1)	331.56(2.2)
20	49.54(2.4)	52.12(4.4)	48.12(2.2)	49.92(4.1)

[a] Values in μg/mL sample solution; relative standard deviations are in parentheses.

Analytical Method. Method development and quantification studies were performed on a LaChrom Elite HPLC system (Merck-Hitachi, Tokyo, Japan), equipped with L-2200 autosampler, L-2100 quarternary pump, L-2300 column oven, and L-2400 UV-detector. An optimum separation of 18 preservatives (**1**−**20**) was achieved on a Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 5 μm) from Agilent (Palo Alto, CA) and a mobile phase comprising 0.05% TFA in water (A) and a 1:1 mixture of acetonitrile and 2-propanol, containing 0.05% TFA (B). Separations were performed by gradient elution (85/15 A/B in 20 min to 65/35 A/B, in 25 min 25/75 A/B), followed by a 5 min column wash (10A/90B) and a reequilibration period of 10 min. Flow rate, temperature, and injected sample volume were adjusted to 1.0 mL/min, 40 °C, and 10 μL, respectively. Detection was performed at 205 nm.

For confirmation of peak purity, ruggedness of the assay, and LC−MS studies an HP 1100 HPLC system, equipped with photodiode array detector, autosampler, and column heater was used (Agilent, Waldbronn, Germany). The same analytical conditions as mentioned above were applied. For LC−MS experiments, an Esquire 3000 iontrap mass spectrometer (Bruker-Daltronics, Bremen, Germany) was connected to the LC apparatus. Best results were obtained in positive ESI mode, with nebulizer, dry gas, and probe temperature set to 30 L/min (nitrogen), 10 L/min (nitrogen), and 350 °C, respectively; the solvent split ratio was 1:3.

Method Validation. The HPLC method was validated for linearity (see calibration), limit of quantitation and detection, accuracy, peak purity, precision, repeatability, and ruggedness.

Limit of quantitation (S/N ratio of 10) and limit of detection (S/N ratio of 3) were determined by serial dilution of standard solutions containing the relevant compounds and determined to be 34.6 and 12.1 ng on-column (**16**) and below; accuracy was confirmed by spiking sample GSE-9 with three concentrations of the quantified standard compounds. The spiked samples were assayed under optimized conditions and recovery rates were between 96.1% (**4**, high spike) and 102.2% (**17**, medium spike; **Table 1**).

Peak purity and identity were confirmed by LC−MS experiments. Precision (intra- and interday) of the assay was verified by analyzing sample GSE-3 5-fold on three consecutive days; for detailed results, see **Table 2**. Repeatability was confirmed by evaluating the consistency of retention times and standard deviations; a maximum relative standard deviation of 3.5% (compound **20** in sample GSE-9) was observed for triplicate injections (**Table 3**). Finally, ruggedness of the developed assay can be concluded by the fact that the same results were obtained on two different HPLC instruments (LaChrom Elite and HP 1100).

RESULTS AND DISCUSSION

Earlier reports indicated the presence of preservatives in GSE products. Thus, we aimed to develop an analytical method suitable for the qualitative and quantitative determination of a maximum number of food-relevant preservatives in this matrix. Most of these compounds are derivatives of benzoic or 4-OH-benzoic acid and quarternary amines, like benzethonium chloride

Table 3. Quantitative Analysis of Preservatives in Different GSE Products for Ecofarming[a]

product	2	4	5	8	16	17	19	20
GSE-1	–	–	–	–	–	176.90(0.2)	–	–
GSE-2	17.89(1.4)	–	–	9.13(1.5)	167.15(1.6)	–	69.07(1.8)	–
GSE-3	9.88(0.1)	–	–	4.97(0.2)	99.38(0.2)	–	33.86(0.2)	4.96(0.3)
GSE-4	–	–	–	–	–	72.45(1.0)	–	–
GSE-5	–	–	–	–	–	–	–	–
GSE-6	–	–	–	–	–	2.50(2.0)	–	–
GSE-7	–	–	–	–	–	2.48(2.1)	–	–
GSE-8[b]	–	–	–	–	–	–	–	–
GSE-9	0.31(2.0)	0.13(0.9)	0.05(0.4)	0.12(0.8)	3.47(1.7)	–	1.15(1.6)	0.31(3.5)

[a] Values in mg/mL, except sample GSE-9 (values in %); relative standard deviations are given in parentheses ($n = 3$). [b] Contains no GSE according to product label.

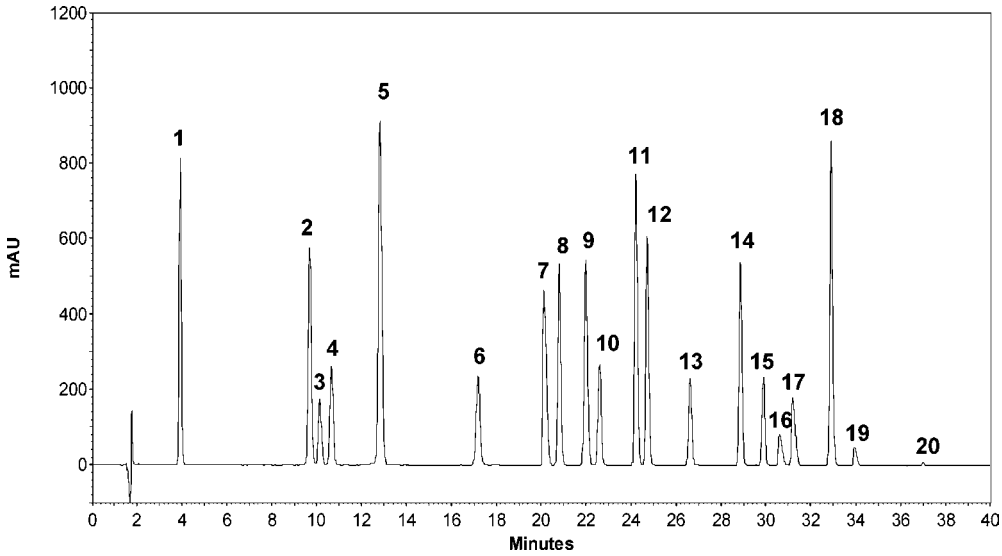

Figure 1. Separation of 18 preservatives under optimized HPLC conditions (column, Zorbax Eclipse XDB-C8, 150 × 4.6 mm, 5 μm; mobile phase, 0.05% TFA (A), acetonitrile/2-propanol = 1/1 containing 0.05% TFA (B); gradient, 85/15 A/B in 20 min to 65/35 A/B, in 25 min 25/75 A/B; flow rate, 1.0 mL/min; sample volume, 10 μL; temperature, 40 °C; detection, 205 nm; baseline subtracted). Peak assignments: 4-hydroxybenzoic acid (**1**), methyl 4-hydroxybenzoate (**2**), sorbic acid (**3**), benzoic acid (**4**), salicylic acid (**5**), methyl benzoate (**6**), chlorhexidine diacetate (**7**), propyl 4-hydroxybenzoate (**8**), 4-chloro-3-hydroxytoluol (**9**), ethyl benzoate (**10**), benzyl 4-hydroxybenzoate (**11**), butyl 4-hydroxybenzoate (**12**), propyl benzoate (**13**), benzyl benzoate (**14**), butyl benzoate (**15**), benzalkonium chloride (C12; **16**), benzethonium chloride (**17**), 2,4,4′-trichloro-2′-hydroxy-diphenyl ether (**18**), benzalkonium chloride (C14; **19**), benzalkonium chloride (C16; **20**).

and benzalkonium chloride. Aminergic compounds are generally difficult to separate on reversed-phase material (*14*−*17*), and to our knowledge no method for the simultaneous quantification of benzethonium chloride (**17**) and the different constituents of benzalkonium chloride (C12, **16**; C14, **19**; C16, **20**) in biological matrixes has been reported so far.

Owing to the number of analytes and their diverse chemical nature, the HPLC assay had to be carefully optimized in order to give satisfactory results. Concerning the stationary phase, out of a number of different columns tested (C-8, C-12, C-18, and CN material; 3, 4, and 5 μm particle size; 80−300 Å pore size), the best results were obtained with a Zorbax Eclipse XDB-C8 from Agilent. As several of the analytes are acids, another necessity for the separation of the 18 reference compounds (**Figure 1**) was the use of an acidic mobile phase. The method should be adaptive for LC−MS studies; thus, 0.05% TFA was added to the mobile phase. The use of weaker, volatile acids such as acetic or formic acid was less advantageous. Of equal importance on the results was the use of a 2-propanol−acetonitrile mixture as mobile phase. Only a 1:1 mixture of these solvents allowed the baseline separation of all compounds of interest within less than 38 min. Using pure acetonitrile, methanol, or 2-propanol instead resulted in the coelution of several compounds [e.g. benzyl 4-hydroxybenzoate (**11**) and butyl 4-hydroxybenzoate (**12**), or benzalkonium chloride (C12, **16**) and benzethonium chloride (**17**)]. A column temperature of 40 °C significantly reduced the required separation time and improved peak symmetry at the same time.

To confirm that an analytical method is suitable for its intended use, it has to be validated. In the present case these investigations were performed according to ICH guidelines (*18*). Data presented in **Table 1** indicate the linearity of the assay within the tested range (6.2−500 μg/mL), combined with sensitivity and accuracy. The latter was confirmed by spiking one sample at three concentration levels with standard compounds representing 50, 100, and 150% of the expected value. In **Table 2** results of precision studies are presented. Both intraday as well as interday precision were found to be well within accepted criteria with maximum deviations of 4.5% (**8**, day 2) and 4.1% (**2**), respectively. Repeatability of the assay was deduced by very stable retention times over the whole study (approximately 400 injections) and relative standard deviations below 5% for multiple injections (**Table 3**).

The instrument used for method development was equipped with a single-wavelength detector. Peak assignment based on retention times will not be sufficient in the present case, as several of the analytes show a very similar behavior in that respect. Therefore, additional LC/DAD/MS experiments were

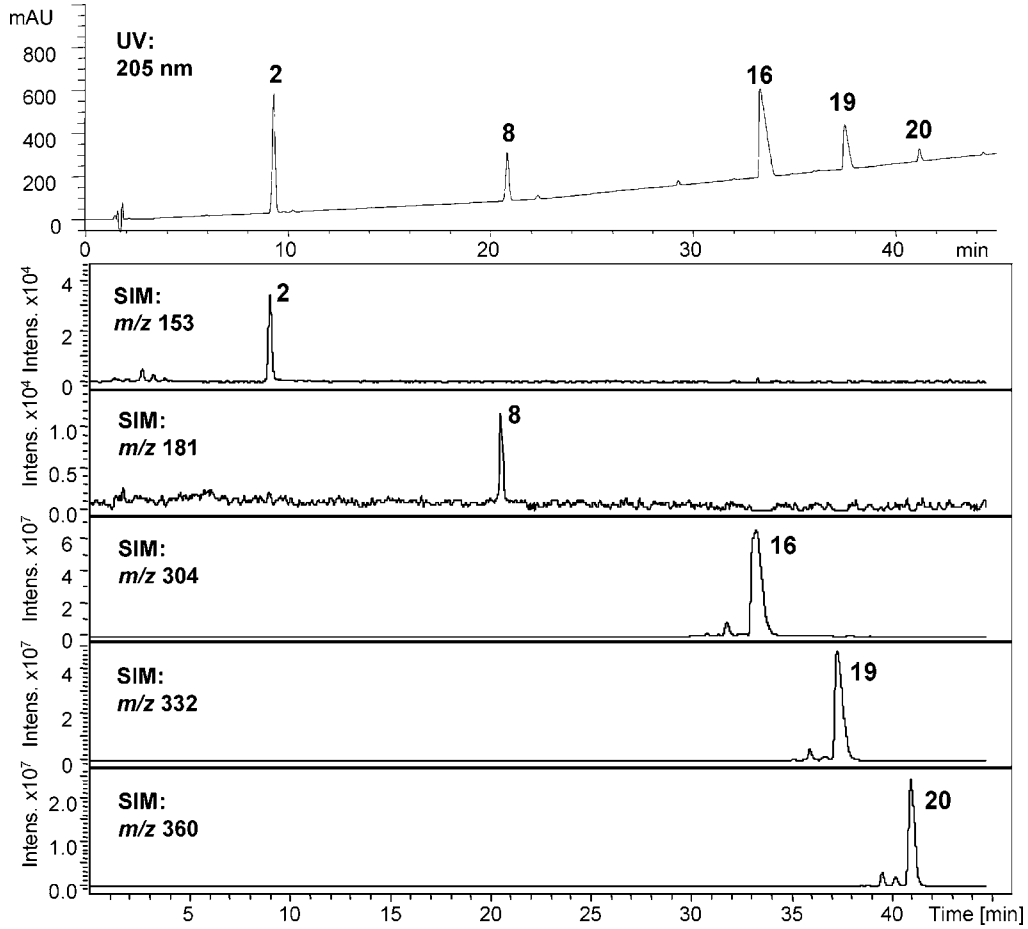

Figure 2. LC–MS analysis for the assignment of individual preservatives in sample GSE-3 [HPLC-conditions as for **Figure 1**; MS conditions, positive ESI mode; nebulizer, 30 L/min (nitrogen); dry-gas, 10 L/min (nitrogen); probe temperature, 350 °C; solvent split ratio, 1:3].

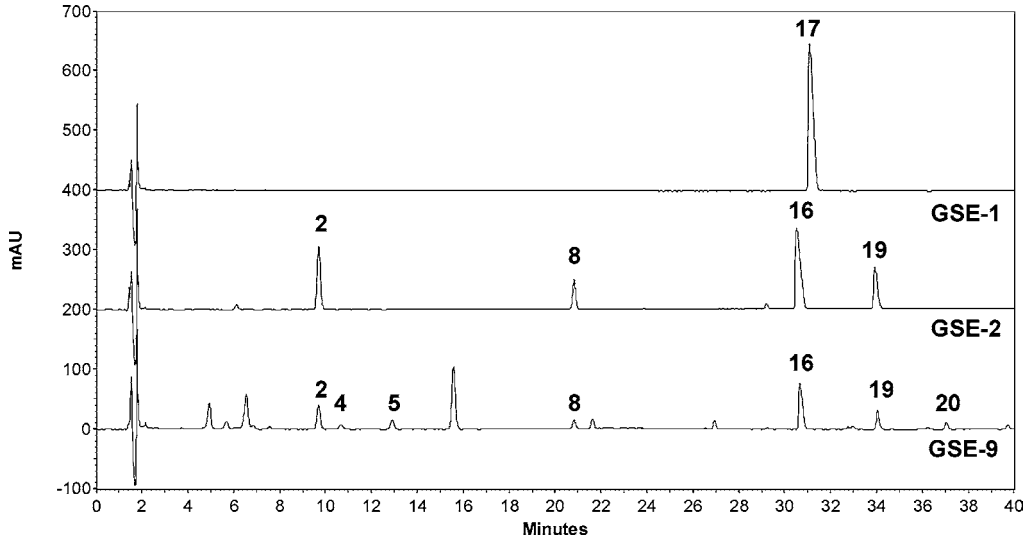

Figure 3. Analysis of three commercial grapefruit seed extracts (GSE-1, GSE-2, and GSE-9) under optimized conditions; separation conditions and peak assignment as for **Figure 1**.

performed. Spectral information from a diode-array detector, in combination with the peak purity option in the utilized software (chemstation Rev. A.09.01; threshold value set to 975), confirmed that all quantified peaks were free of impurities. MS spectra were recorded in positive ESI-mode and allowed the unambiguous assignment of all peaks of interest (**Figure 2**).

For the analysis of commercial GSE products (mostly concentrates that need to be diluted with water before being sprayed on the crops) no special sample pretreatment was required. Liquid samples were simply diluted with methanol; the only solid sample (GSE-9) was dissolved in methanol and then membrane filtered.

Typical sample chromatograms are presented in **Figure 3**, and **Table 3** combines the obtained quantitative results. They show that all GSE products except GSE-5 and -8 contained preservatives. The latter, intended for ecofarming, too, contained no grapefruit but other herbal extracts (e.g. *Equisetum arvense*, *Arnica montana*, *Thymus vulgaris*). The remaining products

could be divided into two groups. Some of them only contained benzethonium chloride (**17**), in ratios ranging from 2.5 (GSE-6, GSE-7) to 176.9 mg/mL (GSE-1); this is a 70-fold difference. The second group contained several preservatives, with benzalkonium chloride being most dominant (total of homologues C12−C16, 138.2−236.3 mg/mL), followed by hydroxybenzoic acid derivatives **2** (9.8−17.9 mg/mL) and **8** (5.0−9.1 mg/mL). Sample GSE-9 showed a rather similar composition, but additionally small amounts of **4** (benzoic acid) and **5** (salicylic acid) were found. Except for the latter, all determined preservatives are commonly used synthetic antimicrobial agents whose formation in the plant or during the extraction process is very unlikely. They show toxic pharmacological properties for humans or animals in part (*12*−*15*) and undoubtedly are not compatible with the principles of ecofarming.

CONCLUSIONS

Organic food is a rapidly growing market segment; thus, these products are attractive for fraud and adulteration. This affects not only the consumer but also the producer, who is confronted with an ever-growing number of "environment-friendly" pesticides. With respect to cosmetic and medicinal GSE products, this problem has been pinpointed previously, as the presence of preservatives, harmful to human health has been reported. Yet, the published data remained fragmentary, leaving room for questions and improvements. The here presented HPLC method will be very useful to close these gaps. For the first time it is possible to quantify all GSE-relevant preservatives in one analytical run by a fully validated assay. The method is simple yet selective, it requires no special sample preparation or equipment other than HPLC, it is fast (less than 40 min for 20 compounds), and it is applicable to commercial products. Because of the large number of analytes and their prevalence in diverse commercial products, this method will be valuable for a broad range of other applications as well.

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