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Botanical Adulterants Program
American Botanical Council • the American Herbal Pharmacopoeia • the University of Mississippi's National Center for Natural Products Research

Grapefruit Seed Extract Laboratory Guidance Document

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Grapefruit Seed *Citrus paradisi*. Photo ©2017 Steven Foster

Keywords: *Citrus paradisi,* Rutaceae, grapefruit seed extract, GFSE, adulterant, adulteration, benzalkonium chloride, benzethonium chloride, triclosan

1. Purpose

The case of synthetic microbicides marketed as grapefruit seed extract (GFSE) differs from the other botanicals addressed thus far by the Botanical Adulterants Program (BAP), in that the adulteration does not consist of substitution by or inclusion of other botanicals, but rather the inclusion of one or more synthetic microbicidal compounds (disinfectants) in the products. Therefore, this Guidance Document presents a review of the analytical technologies used to differentiate and identify the various microbicides that have been reported from commercial GFSE products, as well as methods to separate and identify natural grapefruit seed constituents.

2. Scope

We are unaware of any pharmacopoeial standards or monographs for grapefruit seed extract. In addition, some of the commercial GFSE products are reportedly prepared from dried, ground seeds that are boiled in water and distilled; that distillate is then treated with ascorbic acid, hydrochloric acid, and ammonium chloride under heat and pressure; this treatment purportedly produces microbicidal compounds resembling benzethonium chloride from the flavonoids in the grapefruit seeds.[1] However, there are no known natural or synthetic chemical pathways whereby the natural constituents of grapefruit seed could be transformed into such compounds, using the reagents listed above under the conditions described. It simply defies the logic and state of our knowledge of synthetic organic chemistry and biosynthesis. More interestingly, the majority of published analyses of GFSE products report only man-made microbicidal compounds and no compounds typical of grapefruit or any citrus fruit (e.g., flavonoids, limonoids, or essential oils).

Complicating the selection of an analytical method is the observation that the microbicidal compounds detected in GFSE products have changed over time. Therefore, the ideal analytical method should be flexible enough to detect and quantify not only any and all microbicides previously found in GFSE, but perhaps also any other similar, commercially available microbicidal compounds.

The recommendation of a specific method or methods in this Laboratory Guidance Document for testing GFSE materials does not take away the responsibility of laboratory personnel to demonstrate adequate method performance in their own laboratory using accepted protocols outlined in the 21 CFR Part 111 and by AOAC International, ISO, WHO, and ICH.

3. Common and Scientific Names

3.1 Common name: grapefruit seed extract (GFSE)

3.2 Other common names:

French: Extrait de pépins de pamplemousse
German: Grapefruitkern Extrakt
Italian: Estratto di semi di pompelmo
Spanish: Extracto de semilla de pomelo

3.3 Accepted Latin binomial: *Citrus paradisi* Macfad.[2]

3.4 Synonyms: *Citrus* x *paradisi*[3]

3.5 Botanical family: Rutaceae

4. Botanical Description

Grapefruit, *Citrus paradisi*, is believed to have originated in Barbados as a hybrid of sweet orange (*C. sinensis*) and shaddock (*C. grandis*); both of those species had been introduced to Barbados from southern Asia in the seventeenth century. Grapefruit was first described in 1750, but was not distinguished botanically from pomelo until the 1830s.[4] The fruit is large, compared to other citruses, and is characterized by a sour/acidic to semi-sweet taste.

5. Identification and Distinction using Macroanatomical Characteristics

The dried seeds of *Citrus paradisi* are not easily distinguished from other *Citrus* spp. seeds on a macroanatomical basis, although they are generally larger than most other citrus seeds, certainly lemon and lime. Like all citrus seeds, they are white, with a thin shell over a pith layer protecting a seed kernel.

6. Identification and Distinction using Microanatomical Characteristics

No report of microanatomical distinction of grapefruit seeds from seeds of other *Citrus* spp. was found.

7. Genetic Identification and Distinction

Only one paper describes the use of DNA analysis to differentiate 38 grapefruit and 3 pomelo (*Citrus maxima*) samples by RAPD and SSR markers.[5] In that study, only two grapefruit samples clustered closely with the pomelos, and the remainder were subdivided into three closely related groups. However, DNA analysis would not be useful in the case of GFSE, since the process of preparing much of the marketplace product uses heat, pressure, and acid treatments, very likely decomposing the DNA of the seed material. DNA analyses would have to be conducted on the untreated raw material. Here the presence of grapefruit seed, relative to some other seed raw material, could be confirmed, but this would have no bearing on the presence of synthetic microbicides in the final products.

8. Chemical Identification and Distinction

There is minimal information in the literature on differentiating grapefruit seeds from those of other citrus species. There is one report of the application of HPLC to distinguish the extracts of seeds of four citrus varieties – ruby red grapefruit, sour orange (*C. aurantium*), Nova tangerine, and Cleopatra mandarin.[6] However, the latter two varieties were shown by this analysis to be the same species, *C. reticulata*. Nonetheless, the method did allow differentiation of grapefruit seed extract from those of the other citrus seeds examined; not surprisingly, the two different *C. reticulata* samples were not easily distinguished from one another.

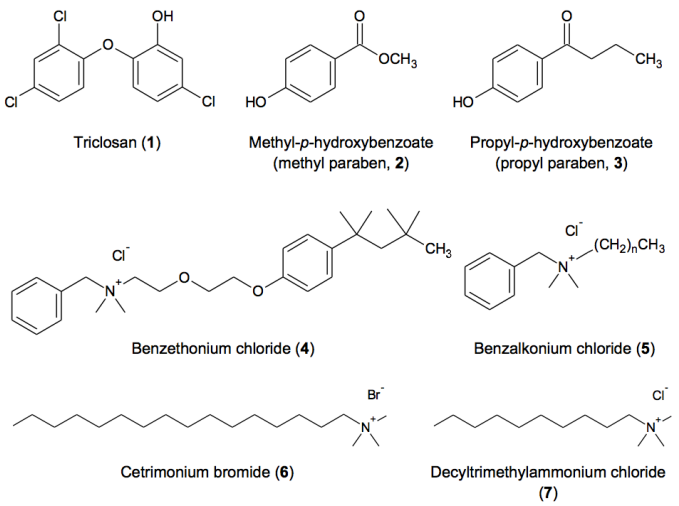
The very recent paper by Avula et al.[7] reports improvement and expansion of a previously reported UHPLC-UV-MS method,[8] whereby the reported modifications make it possible to resolve and identify not only the suspected adulterant microbicides, but also the limonoids and flavonoids expected in a true extract of grapefruit seed. Thus, one should now be able to look at a GFSE product with one analytical method to examine whether it is made from grapefruit seeds or other citrus (e.g., lemon and orange seeds are abundantly available as byproducts of the juice industry) and/or whether it contains any adulterating synthetic microbicides.

8.1 Chemistry of *Citrus paradisi* and potential adulterants

The secondary metabolites of grapefruit seeds are predominately limonoids and flavonoids, both being bitter. Naringin, a diglycoside of the common flavanones naringenin (**8**), is far and away the dominant flavonoid in grapefruit seeds. Limonoids are a unique subset of triterpenes in which the conventional triterpene skeleton is significantly oxidized and cleaved in one or more places. Seven limonoids, along with seven limonoid glycosides (a sugar attached to the triterpene core), have been reported from grapefruit seeds.[9-16] Limonin (**9**), the most abundant of grapefruit seed limonoids, comprises ~0.5% of the dry weight of the seeds,[15] and the total limonoid content could approach 1%.[16]

It is noteworthy that none of the published analyses of commercial grapefruit seed extracts have indicated the presence of either limonoids or flavonoids in those products, even though limonoid glycosides have been isolated from citrus seeds extracted with aqueous acid in the presence of pectinase.[17-19] Instead, a series of 13 analyses of commercial GFSE products over a span of more than two decades has revealed the presence of a number of synthetic microbicides, shown in Figure 1. Any commercially available quaternary ammonium salt with at least one lipophilic (hydrophobic) ring or chain could conceivably come into play as an adulterant in purported GFSE products. This class of compounds exerts its considerable microbicidal effect by lysing cell membranes. For comparison purposes, Figure 2 illustrates the structures of **8** and **9**, representative of the flavanones and limonoids, respectively, present in grapefruit seed.

Figure 1. Structures of the principal disinfectants/microbicides found in products labeled "Grapefruit Seed Extract"



8.2 Laboratory methods

Table 1 lists the different analytical methods used to analyze commercial GFSE products for adulteration and considers the key advantages and disadvantages of each technique.

Complicating the evaluation of the various published analytical methods for GFSE adulteration and potential selection of a method to use is the fact that the adulterant microbicides found in GFSE commercial products changed over time. Table 2 summarizes the chronological record of the appearance of the various GFSE adulterant microbicides.

The 2001 paper by Terreaux et al.[24] was not included in our original review of the adulteration of GFSE[31], because it was not uncovered in several literature searches. Terreaux et al. reported an HPLC-UV analysis of 17 commercial GFSE products, 9 of which contained 4. Six of those samples had high levels of this adulterant, 6.7-20.4%.

8.2.1 TLC

The method of von Woedtke et al.[22] was evaluated in this review.

Comments: Both UV and colorimetric approaches were used for determining the presence of the adulterant microbicides, but their concentration could only be estimated by comparison of spot intensity to different concentrations of reference standards. However, this relatively inexpensive and rapid analysis can qualitatively distinguish grapefruit seed components from synthetic microbicides quite readily, providing a quick yes/no answer regarding adulteration. Further analyses might be necessary to assess more thoroughly the quality of the material being analyzed.

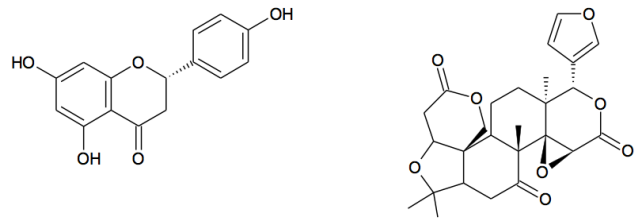
Note: An example of a HPTLC analysis of GFSE is shown in Figure 3.

8.2.2 HPLC-UV

Methods described in the following literature were evaluated in this review: Terreaux et al.,[24] Spitaler et al.,[25] and Avula et al.[8]

Comments: Since all but two of the reported adulterant microbicides (including the most commonly encountered ones) from GFSE have strong aromatic chromophores and reference standards for all those compounds are available, HPLC-UV can be employed for both qualitative and quantitative analyses of the most frequently observed adulterants. It should be noted that, while **6** and **7** have only been

Figure 2. Structures of naringenin and limonin, representative of the flavanones and limonoids, respectively, present in authentic grapefruit seed



Naringenin (**8**) Limonin (**9**)

Table 1. Summary comparison of different approaches to determine adulterants in GFSE

Method	Applicable to	Pro	Contra
TLC	liquid or powder products	quick, inexpensive basic systems affordable for smaller labs reference compounds available	high-end equipment expensive more qualitative than quantitative some products (e.g., glycerin extracts) may require time-consuming sample preparation
HPLC-UV	liquid or powder products	standard equipment in many laboratories most compounds of interest have chromophores reference compounds available	equipment is costly some of the synthetic microbicides do not have a chromophore
HPLC-MS HPLC-ESIMS	liquid or powder products	equipment increasingly common in laboratories reference compounds available	equipment is very costly
HPLC-UV-MS	liquid or powder products	equipment increasingly common in laboratories reference compounds available	equipment is very costly
GC-MS	liquid or powder product	standard equipment in many laboratories reference compounds available unknown microbicides may be identified using commercially available libraries	equipment is costly some products (e.g., glycerin extracts) may require time-consuming sample preparation
^{1}H-NMR	liquid or powder products	reference compounds available	equipment is very costly sensitivity is lower compared to other methods

reported once from GFSE products, they might well have evaded detection in any UV-based analytical method (and might not easily be discerned by NMR, either, depending on the complexity of the sample being analyzed).

8.2.3 HPLC-MS

Methods, including HPLC-MS, HPLC-ESIMS and HPLC-ESIMS/MS, described in the following literature were evaluated in this review: Sakamoto et al.,[21] Takeoka et al.,[23,26] Ganzera et al.,[27] and Sugimoto.[29]

Comments: All of these methods used reversed phase HPLC coupled to a mass spectrometer, most often positive ion ESI (electrospray ionization), to confirm the identity of and quantify the various adulterants, after using UV detection in separate HPLC analyses to determine which adulterants were present. This method offers the added advantage of being able to detect those microbicides that do not have a chromophore; in fact, the quaternary ammonium microbicides are already ionized and readily detected by MS in the positive ion mode.

8.2.4 UHPLC-UV-MS

The recently reported method of Avula et al.[7] was evaluated in this review.

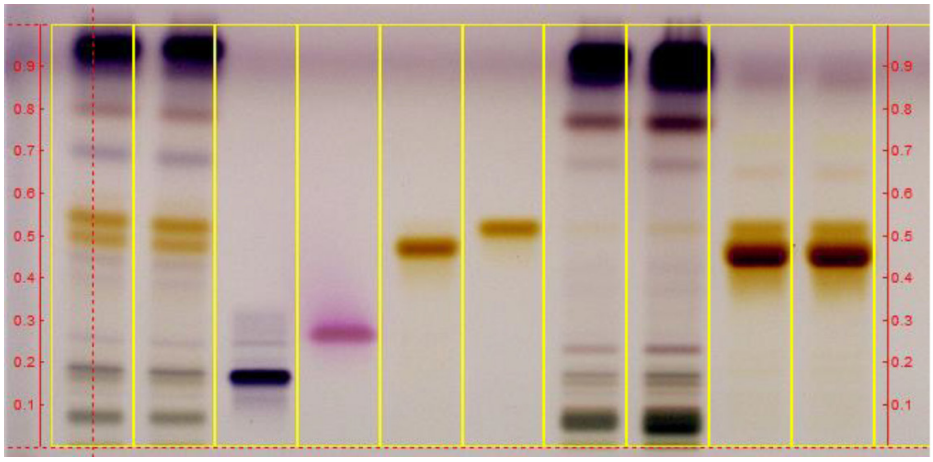
Comments: This study, the most recent publication in the series of 13 analyses, is the first to provide a method for simultaneous detection and quantification of both the expected limonoids and flavonoids known to occur naturally in grapefruit seeds and also the adulterant synthetic microbicides that have been all too frequently observed in commercial GFSE products.

8.2.5 GC-MS

The method described in the following literature was evaluated in this review: Spinosi et al.[26]

Comments: This study only looked at three adulterant microbicides (**5-7**) as part of an investigation of supposed organic treatments (GFSE) for diseases of honeybees. GC-MS should be a sensitive and effective method for detection of any of the quaternary ammonium compounds, the various parabens and even **1**; all are relatively volatile, and the quater-

Figure 3: HPTLC analysis of grapefruit seed extract. Image provided by Nature's Way Brands Inc. (Green Bay, WI)



Lanes 1,2: Grapefruit pectin; lane 3: escin; lane 4: benzethonium chloride; lane 5; hesperidin; lane 6: naringin; lanes 7,8: grapefruit seed, reference material; lanes 9,10: commercial grapefruit seed extract

Stationary phase: Silica gel 60, F_{254}, HPTLC plates.

Concentrations: Extracts: 100 mg/mL; Pure compounds: 1 mg/mL

Application volume: 5 µL

Mobile phase: n-Butanol: water: acetic acid (5:4:1) (v/v/v)

Detection: Anisaldehyde reagent, observation under white light

Table 2. Time sequence of the detection of adulterants in GFSE products

year, first author and ref	1[a]	2[a]	3[a]	4[a]	5[a]	6[a]	7[a]
1991 Nishimia et al.[20]	√	√					
1996 Sakamoto et al.[21]	√	√					
1999 von Woedtke et al.[22]	√	√		√			
2001 Takeoka et al.[23]				√			
2001 Terreaux et al.[24]				√			
2004 Spitaler et al.[25]		√	√	√	√		
2005 Takeoka et al.[26]					√		
2006 Ganzera et al.[27]		√	√	√	√		
2007 Avula et al.[8]	√			√			
2007 Spinosi et al.[28]				√		√	√
2008 Sugimoto et al.[29]				√	√		
2008 Bekiroglu et al.[30]				√			
2016 Avula et al.[7]				√			

nary compounds are already ionized. It should be noted that this is an excellent method for detecting synthetic microbicides like **6** and **7**, which lack a UV chromophore; MS is the most effective detector for this type of adulterant, and might be the reason the authors detected these compounds, which had not been reported in any of the other investigations prior to 2007. A further benefit of GC-MS is that the mass spectral analysis (ECI-MS) gives a richer fragmentation pattern than other ionization methods, allowing mass spectral library matching of the mass spectra produced.

8.2.6 ^{1}H-NMR

Methods described in the following literature were evaluated in this review: Takeoka et al.[23,26] and Bekiroglu et al.[30]

Comments: Takeoka et al. used NMR only to confirm the structure of **4**[23] and **5**,[26] while Bekiroglu et al. validated a quantitative ^{1}H-NMR method for the detection and quantitative analysis of **4** in GFSE products. In their work, Bekiroglu et al. found that the limit of quantification exceeded 20 mg/mL, based on the high signal to noise ratio of the NMR data. They also found that three different operators running 6 analyses on the same sample preparation obtained standard deviations of 0.8, 0.9 and 1.3 mg/mL, respectively, in line with previous reports that the handling of NMR data by operators is the highest impact factor of influence on the quality of a qNMR analysis.[32]

9. Conclusion

None of the published methods has been evaluated for the detection and quantification of all the known adulterants of GFSE, but the most recent contribution by Avula et al. is the most inclusive.[7] This is likely due to the continuing change in the composition and content of microbicidal compounds in commercial GFSE products over time (see Table 2). The HPLC-UV methods of Avula et al.[8] and Ganzera et al.[27] have been validated, making them attractive methods to develop further for all the potential adulterants. The only drawback to this idea is that **6** and **7** (detected by Spinosi et al.[28]) do not contain a UV chromophore. That leads to the suggestion that an HPLC-MS or GC-MS method might be the most appropriate approach for analyzing GFSE products for any of the known or suspected adulterants. Fortunately, reference standards are available for all the potential adulterants, facilitating development and validation of an analytical method.

In response to reviewer requests, we include here information provided by commercial analytical laboratories that offer analyses of GFSE products; two laboratories responded to our request for such information.

One laboratory performs both the Avula HPLC-UV method[8] and an HPTLC method for **1**, **2**, **4**, and **5**.

A second laboratory performs an unspecified HPLC-UV method for all the microbicides reported herein, and also offers FTIR (Fourier Transform Infrared) and mass spectrometry methods.

Grapefruit Seed *Citrus paradisi*. Photo ©2017 Steven Foster

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