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Presence of chemical additives and microbial inhibition capacity in grapefruit seed extracts used in apiculture

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Summary

American foulbrood, caused by *Paenibacillus larvae* subsp. *larvae* (White 1906) is one of the most serious diseases of honey bees, causing beekeepers and health workers to make difficult, complex decisions and leading to the development of 'organic' treatments, such as grapefruit seed extract, with minor residue problems in the end product. This study evaluates the chemical composition of grapefruit seed extracts using gas chromatography/mass spectrometry for the detection of benzethonium chloride, cetrimonium bromide and decyltrimethylammonium chloride. The results obtained suggest a close correlation between the microbial effect and the presence of chemical additives in the samples analysed.

Keywords

Additives, American foulbrood, Grapefruit seed extracts, Inhibition capacity, *Paenibacillus larvae*.

Introduction

American foulbrood, caused by *Paenibacillus larvae* subsp. *larvae* (White 1906), is a serious disease of honey bees, requiring complex decisions by beekeepers and health workers, i.e. the need to tackle the disease with appropriate treatment and the need to minimise residues in honey, to protect the consumer.

The sometimes indiscriminate use of antibiotics to combat the vegetative form of the bacteria can lead to the marketing of honey bee products containing traces of inhibitors (tetracycline, oxytetracycline and sulfathiazole). Recently published data from researchers in this sector has revealed the presence of antibiotics in honey and royal jelly samples (2).

In light of these issues, numerous 'organic' treatments have been developed in recent years. These offer reduced environmental impact and minimal residues in the end products, especially in honey (1, 3, 4, 6, 10). One of these treatments is grapefruit seed extract (GSE).

In a previous study (9), we tested the inhibition capacity of GSE in regard to *in vitro* growth of *P. larvae* subsp. *larvae* and of the bacteria normally used in micro-biological tests. The field efficacy of the extract was tested in an experimental study (5). Tests revealed that the product provided excellent inhibition against the disease: two years later, the beehives still show no signs of American foulbrood.

Some authors have analysed samples of commercial GSE, after it was suggested that some of them contained artificial preservatives. Results indicated the presence of a number of synthetic compounds (methylparaben, triclosan, benzethonium chloride and cetrimonium bromide), with concentrations varying from 1.25% to 19% (7, 8, 12, 14).

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Further studies postulated a relationship between the microbial activity and the presence of synthetic compounds; additive-free GSEs showed no microbial inhibition activity (11, 13). This study evaluates the chemical composition of GSEs using high resolution gas chromatography/low resolution mass spectrometry (HRGC/LRMS) for the detection of benzethonium chloride, cetrimonium bromide and decyltrimethyl-ammonium chloride, correlating their presence with *in vitro* antimicrobial activity.

Materials and methods

Sampling and experimental design

Seventeen GSE samples (4 powder and 13 liquid preparations) were analysed for benzethonium chloride, cetrimonium bromide and decyltrimethylammonium chloride using HRGC/LRMS.

The samples of Italian and European origin were provided by beekeepers and/or commercial companies.

The same samples were subjected to *in vitro* inhibition tests against the following bacteria: *Bacillus subtilis* BGA, *Bacillus cereus* K250, *Bacillus cereus* 11778, *Micrococcus luteus* 9341.a and then against *P. larvae.* The procedures used for these tests were those indicated in our previous study (9).

Sample preparation

The preparation was as follows: 25 mg (powder preparations) or 50 µl (liquid samples) were taken from each GSE sample and dissolved or diluted with 20 ml with acetone/methanol 50:50 v/v. Samples were filtered through 0.2 µm, 25 mm discs (Whatman, Clifton, New Jersey) The solutions were then injected directly in the HRGC/LRMS. The concentrations of the test analytes were calculated by calibration against the concentration-response curve obtained from standards diluted to various concentrations.

Reference standards and reagents

The reference standards used were benzethonium chloride (Sigma-Aldrich, Toufkirchen), cetrimonium bromide (Dr Ehrenstorfer-Schafer, Augsburg) and decyltrimethylammonium chloride (Anatrace, Inc. Maumee, Ohio). Pesticide analysis grade acetone and methanol were used (Carlo Erba, Milan).

The stock solutions of the three 1 000 mg/l standards were obtained by dissolving 50 mg of pure standard in acetone/methanol (80:20 v/v) in 50 ml volumetric flasks. Working solutions of 1, 10, 100 and 250 mg/l were obtained by diluting the stock solutions in isooctane.

Chromatographic conditions

A Trace high resolution gas chromatograph with a Trace DSQ 70 low resolution mass spectrometer detector (Thermo Finnigan, San Jose, California) was fitted with a split-splitless injector set to a temperature of 270°C with a split time of 1 min and a split flow of 40 ml/min. A DB-5, 60 m × 0.25 mm, 0.25 µm capillary column (J&W Scientific, Folsom, California) was used for the separation. The following column ramp programme was used: 98°C held for 2 min, 25°C/min up to 160°C, 2 min hold, 4°C/min up to 210°C, 8 min hold, 10°C/min up to end temperature of 320°C, 10 min hold (total 40 min). The line transfer and source temperatures were set at 260°C and 240°C, respectively. Electron ionisation was obtained by setting the source voltage at 70 eV. The mass spectrum was obtained by full scan from 50 to 450 m/z. The quantitative analysis was performed by monitoring the fragment at m/z = 58. The injection volume was 1 µl.

Results and discussion

Presence of chemical additives

HRGC-LRMS analysis revealed the presence of benzethonium chloride (Fig. 1), cetrimonium bromide (Fig. 2) and decyltrimethyl-ammonium chloride (Fig. 3). The percentage concentrations found are summarised in Table I.

Of the 17 samples analysed, 14 tested positive for benzethonium chloride, five for cetrimonium bromide and one for decyltrimethylammonium chloride. All samples containing cetrimonium bromide also contained a low concentration of

benzethonium chloride. Only two samples (nos 2 and 3) were free of the three preservatives.

Benzethonium chloride was present in both powder and liquid samples, while cetrimonium bromide was not found in any powder sample.

Benzethonium chloride showed the widest concentration range, from a minimum of 0.003% in sample no. 8 to a maximum of 21.500% in sample no. 4. Cetrimonium bromide ranged from a minimum of 3.202% (sample no. 5) to a maximum of 11.656% (sample no. 1).

Figure 4 shows the percentage of additives present in each grapefruit seed extract sample.

Microbial inhibition capacity

In a previous paper (9), the authors conducted microbial inhibition tests against various bacterial strains. The results can be summarised as follows:

- not all grapefruit seed extracts inhibited *P. larvae* and the test bacteria
- the inhibition capacity varied from sample to sample: some presented a highly accentuated inhibition halo, while others were almost at the detection limit
- there was no direct correlation between the extract dilution factor and the extent of the inhibition halo.

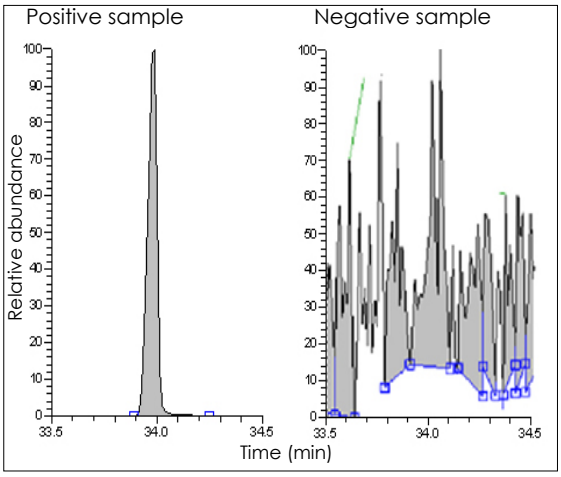

Figure 1
Benzethonium chloride
Mass chromatogram (m/z 58) for a positive and negative sample

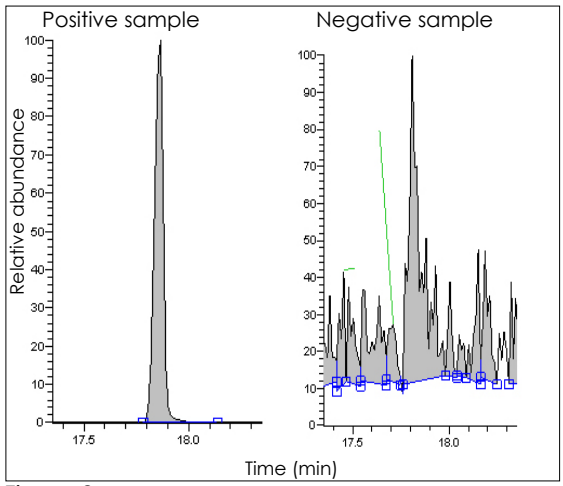

Figure 2
Cetrimonium bromide
Mass chromatogram (m/z 58) for a positive and negative sample

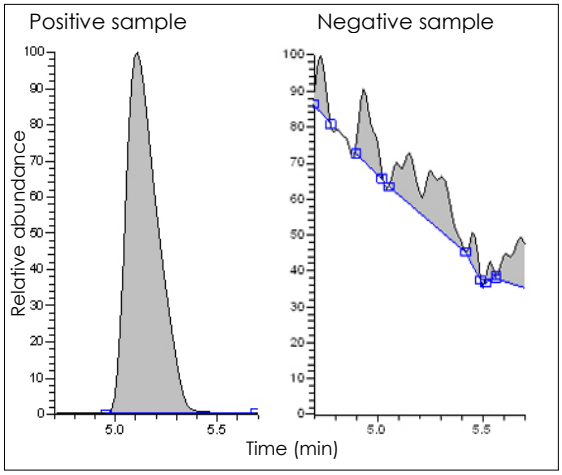

Figure 3
Decyltrimethylammonium chloride
Mass chromatogram (m/z 58) for a positive and a negative sample

Table I gives a comparison between the concentration of benzethonium chloride, cetrimonium bromide and decyltrimethyl-ammonium chloride present in the GSE samples analysed, together with their *in vitro* inhibition capacity.

It can be seen that the only preservative-free samples (nos 2 and 3) are also the only samples that did not produce an inhibition halo.

Table I
Percentage of preservatives and inhibitory capacity of grapefruit seed extract samples

Sample No.	Benzethonium chloride (%)	Cetrimonium bromide (%)	Decyltrimethyl-ammonium chloride (%)	Inhibitory capacity (*Paenibacillus larvae*)	Inhibitory capacity (test bacteria)[a]
1	0.564	11.656	ND	+	+
2	ND	ND	ND	–	–
3	ND	ND	ND	–	–
4[b]	21.500	ND	ND	+	+
5	0.013	3.202	ND	+	+
6[b]	13.774	ND	ND	+	+
7	0.066	ND	ND	+	+
8	0.003	ND	ND	+	+
9[b]	12.541	ND	ND	+	+
10	8.318	ND	ND	+	+
11	5.206	ND	ND	+	+
12	12.522	ND	ND	+	+
13[b]	7.273	ND	ND	+	+
14	0.066	8.050	ND	+	+
15	0.013	4.602	ND	+	+
16	0.014	9.977	ND	+	+
17	ND	ND	10.320	+	+

a) test bacteria: *Bacillus subtilis* BGA, *Bacillus cereus* K250, *Bacillus cereus* 11778, *Micrococcus luteus* 9341.a
b) commercial powder preparations, while the others were liquid formulations

ND not detected

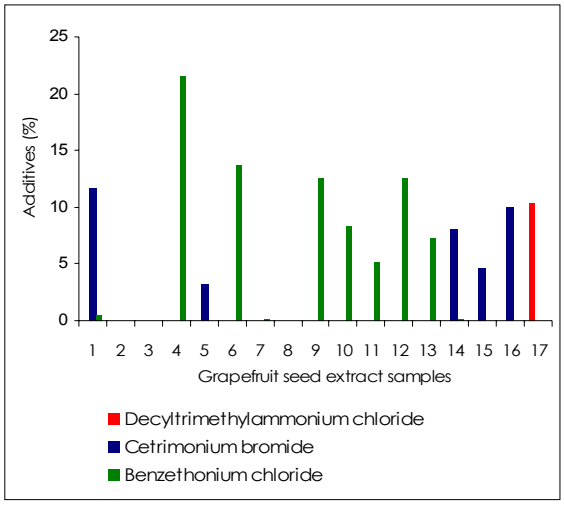

Figure 4
Percentage of additives in the samples analysed

Conclusions

The results obtained demonstrate that only samples which contained one or more preservatives showed any bacterial inhibition activity. The only GSE samples that did not inhibit growth of the test bacteria or of *P. larvae* were those in which none of the three compounds was present.

It can therefore be postulated that the inhibition capacity of GSE in the samples tested was due to the presence of the three preservatives analysed and not to any compound naturally present in the extracts.

It can thus be concluded that the only GSE preparations that possess any microbial inhibition capacity are those to which chemical additives have been added during the industrial processing of the product.

References

1. Cremasco S., Mutinelli F. & Irsara A. 1994. Attività degli oli essenziali nelle malattie delle api. *Ape nostra amica*, **16** (2), 4-10.

2. European Commission (EC) 2004. Rapid Alert System for Food and Feed (RASFF) annual report on the functioning of the RASFF 2004. EC, Health & Consumer Protection Directorate-General, Directorate D, Food Safety: production and distribution chain, Brussels, 38 pp (ec.europa.eu/food/food/rapidalert/report2004_en.pdf accessed on 16 December 2006).
3. Fiorentini L. & Castello M.C. 2002. Proprietà antibatteriche di alcuni olii essenziali. *Large Anim Rev*, **8** (6), 87-88.
4. Heggers J.P., Cottingham J., Gusman J., Reagor L., McCoy L., Carino E., Cox R., Zhao J.G. & Reagor L. 2002. The effectiveness of processed grapefruit-seed extract as an antibacterial agent. II. Mechanism of action and *in vitro* toxicity. *J Altern Compl Med*, **8** (3), 333-340.
5. Langella V., Semprini P., Di Fabio F., Calvarese S., Pasini B., Falda M.T. & Panella F. 2003. Indagine preliminare sull'efficacia dell'estratto di semi di pompelmo nella lotta contro la peste americana. *Vet Ital*, **XXXIX** (47), 49-54.
6. Lee Reagor B.S., Jean Gusman B.S., Lana McCoy M.T., Ekith Carino M.T. & Heggers J.P. 2002. The effectiveness of processed grapefruit-seed extract as an antibacterial agent. I. An *in vitro* agar assay. *J Altern Compl Med*, **8** (3), 325-332.
7. Okayama A., Sadogawa T., Nadajima C. & Hayama T. 1996. Biological properties and antibiotic susceptibility of *Bacillus larvae* originated from American foulbrood of honeybee in Japan. *J Vet Med Sci/Jap Soc Vet Sci*, **58** (5), 439-441
8. Sakamoto S., Sato K., Maitani T. & Yamada T. 1996. Analysis of components in natural food additive grapefruit seed extract by HPLC and LC/MS. *Bull Natl Inst Health Sci*, **114**, 38-42.
9. Semprini P., Langella V. & Calvarese S. 2004. Proprietà antibatteriche dell'estratto di semi di pompelmo nei confronti di *Paenibacillus larvae* subsp. *larvae*. *Vet Ital*, **40** (2), 39-45.
10. Sharamon S. & Baginski B.J. 1998. Le virtù terapeutiche dei semi di pompelmo. *Tecniche Nuove*, Milan.
11. Shimanuki H. & Knox D.A. 1997. Bee health and international trade. *Rev Sci Tech*, **16** (1), 172-176.
12. Takeoka G., Dao L., Wong R.Y., Lundin R. & Mahoney N. 2001. Identification of benzethonium chloride in commercial grapefruit seed extracts *J Agric Food Chem*, **49**, 3316-3320.
13. Tirillini B. 2000. Grapefruit: the last decade acquisitions. *Fitoterapia*, Suppl. 1, S 29-37.
14. von Woedtke T., Schlüter B., Pflegel P., Lindequist U. & Jülich W.D. 1999 Aspects of the antimicrobial efficacy of grapefruit seed extract and its relation to preservative substances contained. *Pharmazie*, **54**, 452-456.