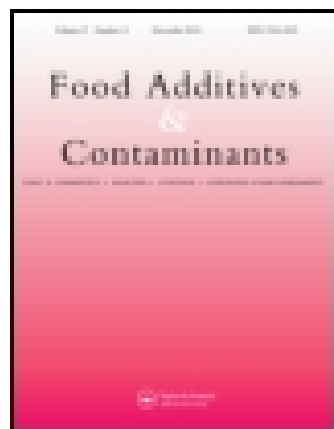




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Foreign matter contaminating ethanolic extract of propolis: a filth-test survey comparing products from small beekeeping farms and industrial producers

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Propolis is a resinous material collected by honeybees from the exudates and buds of plants. It has been widely used as a remedy by humans since ancient times, as well as for dietary supplements and cosmetics. European legislation recently focused on the quality and hygiene standards of foods, including beehive products, and extensive efforts have been made to avoid the presence of chemical contaminants, whilst in contrast few studies have investigated the magnitude of contamination by physical ones. We conducted a filth-test survey to evaluate the contamination of ethanolic extract of propolis by foreign materials. We also compared the abundance of contaminants in propolis extracts currently marketed by small beekeepers and industrial producers. We found different foreign materials in the ethanol extract of propolis. Contaminants differed in abundance, with a higher number of carbon particles (small beekeepers: 2.70 ± 0.63 ; industrial producers: 1.25 ± 0.49 ; mean ($n/30$ ml) $\pm$ SE) and other inorganic fragments (small beekeepers: 3.50 ± 0.31 ; industrial producers: 3.88 ± 1.11) than arthropod fragments (small beekeepers: 0.30 ± 0.21 ; industrial producers: 0.38 ± 0.26) and mammal hairs (small beekeepers: 0.10 ± 0.10 ; industrial producers: 0.38 ± 0.26). No differences in the abundance of foreign matter between propolis from small beekeepers and industrial producers were found, allowing us to point out an increased awareness by small producers of issues inherent in hygiene management. Contamination of propolis extracts by animal body parts, such as insect fragments, mites and rodent hairs, indicates poor management of hygiene in the production process and low effectiveness of the filtration phase. Animal-borne contaminants can act as pathogen vectors as well as introducing dangerous allergens when ingested or applied to human skin. The filth-test applied to ethanolic propolis extract quality control can be considered a promising tool, also for small beekeeper activities, since it is cheap and allows the quick interpretation of results.

Keywords: allergens; arthropods; beehive products; quality control; rodent contaminants

Introduction

Propolis, also known as ‘bee glue’, is a resinous, dark-coloured material that honeybees collect from the exudates and buds of plants, mixed with wax and bee enzymes. The term ‘propolis’ is derived from the Greek *pro* = in defence or for and *polis* = city, reflecting its importance to honeybees, since they use it to seal the walls of their hives, to strengthen the borders of combs and to embalm dead invaders, avoiding contamination by pathogen microorganisms (Bankova et al. 2000; Sforcin 2007). Humans have used propolis as a remedy since ancient times, as well as for dietary supplements and cosmetics. Currently, propolis is very popular in folk and alternative medicine (Król et al. 2013). The biological properties of propolis have been widely investigated in the last 40 years. It has been shown that it has a number of biological and pharmacological properties, including immunomodulatory, antitumor, anti-inflammatory, antioxidant, antibacterial, antiviral, antifungal and anti-parasite activities (Sforcin & Bankova 2011). This has led to a considerable interest of consumers in propolis-containing products marketed by health-food stores, and Bankova (2005) has pointed out

the potential of propolis for the development of new drugs. In particular, ethanolic extracts of propolis are commonly used worldwide for their therapeutic properties, with special reference to their stimulant action on the organism’s immune system (Sforcin & Bankova 2011; Król et al. 2013).

Recently, European legislation has focused on the quality and hygiene standards of food, including beehive products, mainly through the ‘Hygiene Package’ (i.e. European Commission Regulation Nos 852/2004, 853/2004, 854/2004 and 882/2004). This encouraged European industry to raise hygienic standards, at the same time respecting both nutritional and safety aspects (Neethirajan et al. 2007; Trematerra et al. 2011; Trematerra 2013). The ‘Hygiene Package’ strongly focuses on the direct involvement of the beekeeping industry to respond to the organoleptic quality and safety of beehive products (Canale et al. 2014). In this context, to ensure the adequate protection of consumers, reliable and rapid quality control methods are of key importance. Extensive efforts have been carried out to evaluate the presence of chemical contaminants in beehive products

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(e.g. honey: Panseri et al. 2014; pollen: Stoner et al. 2013; royal jelly: Martínez-Domínguez et al. 2014; propolis: Woisky & Salatino 1998; Pérez-Parada et al. 2011; see also Bogdanov 2006 for a review), while only little research has investigated the range of physical contaminants (e.g. insect fragments, mites, mammal hairs and feather barbules, animal excreta pellets, sand, plastic, metal and glass particles) in such products (e.g. honey: Loi & Canovai 2002; Liebezeit & Liebezeit 2013; Canale et al. 2014), despite the potential importance of such contaminants as allergens and vectors of pathogens. For beekeepers it is crucial to have effective, easy-to-use tools to monitor the presence of foreign particles in beehive products before the commercialisation phase. The filth-test method (FT) is a simple, cheap effective tool to evaluate contamination by foreign matter in a number of foods and beverages, such as cereals, flour, milk and derivatives, tomato sauce, beer, and beehive products (Donadini et al. 2004; Martini et al. 2007; Porporato et al. 2009; Trematerra et al. 2011; Canale et al. 2014). The identification of foreign particles using FT can help in the easy tracking of the source of contamination, providing useful information to implement corrective actions in production processes (see Canale et al. 2014 for a recent example on honey).

This study reports results of an FT survey investigating foreign matter contamination in Italian ethanolic propolis. We evaluated the abundance of physical contaminants such as carbon particles, other inorganic fragments (e.g. metal, glass and plastic) and animal body parts (e.g. insect body parts, mites, vertebrate hairs and feather barbules). Differences in foreign matter contamination were assessed in ethanolic extracts of propolis currently marketed by both small beekeeper farms (i.e. beekeepers with about 200 hives; Canale et al. 2014) and industrial producers (beekeepers with more than 1000 hives).

Materials and methods

Eighteen samples of ethanolic propolis extracts were purchased in local markets, shops, and small and big supermarkets in Central Italy (Florence, Pisa, Lucca and Reggio Emilia). We analysed 10 samples of propolis from small beekeeper farms and eight propolis samples from big industrial producers. Following the method described by Canale et al. (2014), the FT was carried out by the 1:1 dilution of propolis extracts in pure ethanol and double-vacuum filtration of the obtained solution using dishes of filter paper (5A, ashless, pore size: 10 µm; Advantec MFS, Inc., Dublin, CA, USA). Each sample of ethanolic extract of propolis (30 ml) was transferred into a 100 cm³ beaker and mixed with an equal amount of ethanol (20°C). It was filtered twice with vacuum Buchner funnel, by the use of filters of paper (diameter 100 mm). Since the solvent can contain a small number of fibres and granular particles

(Liebezeit & Liebezeit 2013), all ethanol used in this study was filtered over glass microfibre filters (GF/C, Whatman Ltd, Maidstone, UK) before use (see also Canale et al. 2014). Filter paper dishes were examined under a photomicroscope (Leica ES2, Germany) for the detection of foreign matter. Specimens were mounted on light microscopy slides in a tiny drop of Berlese's solution and examined through light microscopy (Zeiss Primo Star, Germany).

For each propolis sample, the total number of different impurities found on the filter paper dish was recorded. On the basis of the classification established by Domenichini (1997), Loi and Canovai (2002), and Canale et al. (2014), the propolis foreign matter was divided into three categories: fragments of animal origin, carbon particles and other inorganic fragments (i.e. glass, metal, plastic and textile particles). Fragments of animal origin were classified as arthropod body parts and hairs of mammals (e.g. rodents) (Trematerra et al. 2011; Canale et al. 2014).

Differences in the abundance of propolis foreign matter were analysed by JMP 7 (SAS, 1999) by using a weighted general linear model with two fixed factors:

$$y = X\beta + \varepsilon$$

where y is the vector of the observations (e.g. the number of foreign particles); X is the incidence matrix; β is the vector of fixed effects (i.e. fixed effect 1 = the kind of foreign matter; fixed effect 2 = the origin of tested propolis: from small beekeeper farms or from industrial producers); and ε is the vector of the random residual effects. A probability level of $p < 0.05$ was used for the significance of differences between values.

Results and discussion

We found different foreign materials in ethanolic propolis extracts. Results showed that different kinds of foreign materials significantly differed in their abundance ($F = 21.557$; d.f. = 3; $p < 0.001$), with a higher number of carbon particles (small beekeepers: 2.70 ± 0.63 ; industrial producers: 1.25 ± 0.49 ; mean ($n/30$ ml) $\pm$ SE) and other inorganic fragments (small beekeepers: 3.50 ± 0.31 ; industrial producers: 3.88 ± 1.11) than arthropod fragments (small beekeepers: 0.30 ± 0.21 ; industrial producers: 0.38 ± 0.26) and mammal hairs (small beekeepers: 0.10 ± 0.10 ; industrial producers: 0.38 ± 0.26) (Figure 1). No differences in the abundance of foreign matter between propolis from small beekeeper farms and industrial producers were found ($F = 0.2675$; d.f. = 1; $p = 0.6068$), allowing us to point out an increased awareness and knowledge by small producers on issues inherent in the hygiene management of this product. No significant effect of the interaction propolis origin*kind of foreign matter was found ($F = 1.488$; d.f. = 3; $p = 0.226$).

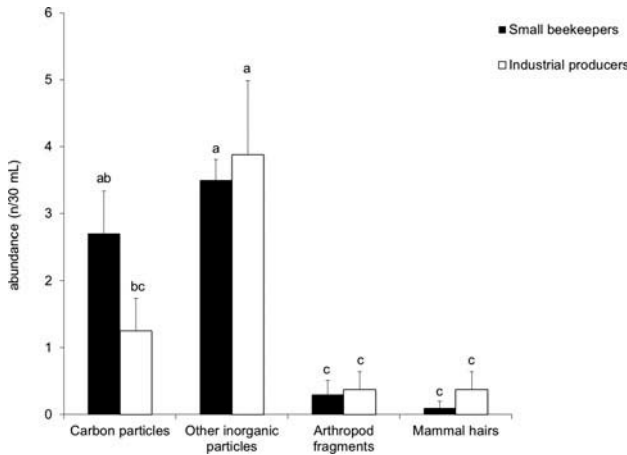


Figure 1. Mean abundance of foreign matter in Italian ethanolic extract of propolis from small beekeeper activities and industrial producers: carbon particles, other inorganic particles (glass, metal, plastic and paper), arthropod body parts (insects and mites), and mammal hairs. T-bars indicate standard errors. Different letters above each column indicate significant differences (general linear model, Tukey–Kramer HSD test, $p < 0.05$).

We found a high number of fragments of carbonaceous origin in propolis extracts. These contaminants are usually due to an overuse of smokers or unsuitable combustibles used during beekeeping practices (Porporato et al. 2009). Industrial carbonaceous dust sources or fires in the close proximity of apiaries can act as additional sources of carbon particles, which may deposit on plant secretions, leading to propolis contamination. Furthermore, we also found a noticeable contamination of propolis extracts by other inorganic foreign particles (e.g. metal, glass, fabric and plastic fragments). The means ($\pm$ SE) of such abiotic impurities were 3.50 ± 0.31 and 3.88 ± 1.11 fragments/sample for small beekeeper farms and big industrial producers, respectively. This may be due to a number pollution sources in the extraction and/or packaging phases (see also Liebezeit & Liebezeit 2013; Canale et al. 2014).

Among foreign materials of animal origin, we found arthropods fragments and mammal hairs. Arthropod fragments were mainly represented by insect cuticular body parts (four fragments: two from small local beekeepers, two from big industrial activities), while only a mite was found (from a propolis extract produced by a small beekeeper activity). Following the keys reported by Domenichini (1997), we also found two propolis samples contaminated by rodent hairs (one from a small local beekeeper, one from an industrial activity). Contamination of propolis extracts by animal body parts, such as insect fragments, mites and rodent hairs, indicates inaccurate management of hygiene in the production process and low effectiveness of the filtration process (see also Martini et al. 2007; Porporato et al. 2009; Canale et al. 2014). In particular, animal-borne contaminants can act as dangerous allergens

and/or pathogen vectors when ingested or applied on human skin. The arising effects can be also confounded with the ones generated by allergy to propolis *sensu stricto* (Basista & Filipek 2012; De Groot 2013). To avoid contamination by animal fragments, filtration process should be carried out with high hygienic standards and anti-insect nets should be adopted by beekeepers to prevent/reduce ethanolic propolis extract contamination by arthropods during the extraction and packaging phases.

Overall, FT applied to propolis extract quality control can be considered a promising tool for its production process, also for small beekeepers, since FT is cheap and allows the quick interpretation of results.

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