Taylor & Francis
Taylor & Francis Group

Journal of Apicultural Research

ISSN: 0021-8839 (Print) 2078-6913 (Online) Journal homepage: https://www.tandfonline.com/loi/tjar20

Comparison of qualitative characteristics of propolis extracts using different purification methods

Dimitra Graikini, Alexandros Papachristoforou & Ioannis Mourtzinos

To cite this article: Dimitra Graikini, Alexandros Papachristoforou & Ioannis Mourtzinos (2019): Comparison of qualitative characteristics of propolis extracts using different purification methods, Journal of Apicultural Research, DOI: 10.1080/00218839.2019.1653813

To link to this article: https://doi.org/10.1080/00218839.2019.1653813

Published online: 06 Sep 2019.

Submit your article to this journal

Article views: 10

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=tjar20

Journal of Apicultural Research, 2019
https://doi.org/10.1080/00218839.2019.1653813

IBRA INTERNATIONAL BEE RESEARCH ASSOCIATION
Taylor & Francis
Taylor & Francis Group
Check for updates

ORIGINAL RESEARCH ARTICLE

Comparison of qualitative characteristics of propolis extracts using different purification methods

Dimitra Graikini[a†], Alexandros Papachristoforou[b,c*†] and Ioannis Mourtzinos[a]

[a]*Laboratory of Food Chemistry-Biochemistry, Department of Food Science and Technology, Faculty of Agriculture, Aristotle University, Thessaloniki, Greece;* [b]*Department of Food Science and, Nutrition University of the Aegean, Lemnos, Greece;* [c]*Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus*

(Received 28 December 2018; accepted 10 April 2019)

Purification of extracts is an essential procedure in order to remove wax and other undesirable ingredients while retaining the polyphenolic fraction which contains most of bioactive components. This study investigates how different purification methods affect the qualitative characteristics of propolis extracts. Five different purification methods were conducted both for ethanolic and methanolic extracts: (a) centrifugation for 30 min at 370 g; (b) centrifugation at −5 °C for 10 min at 1850 g twice with a 15 min interval; (c) centrifugation at 3340 g for 2.5 min twice with a 15 min interval; (d) filtration through a 0.22 μm polyethersulfone membrane; (e) filtration through a 0.45 μm pore size nylon membrane filter using Buchner vacuum system. Methods were evaluated with reference to the post-treatment antioxidant activity (radical scavenging), total phenolic content and total flavonoid content of each extract. Two different methods of centrifugation were found to be the most effective and stable: centrifugation at 3340 g for 2.5 min or for 10 min at 1850 g, at −5 °C.

Keywords: Propolis purification; centrifugation; filtration; antioxidant activity; total polyphenol content; total flavonoid content

Introduction

Propolis is a resinous substance produced by honey bees by blending mandibular enzymes, waxes, pollen and, most importantly, collected natural resins (Bankova, 2005; Marcucci, 1995). Bees use propolis as coating material to fill small holes and seal segments of their hive or nest, to repair combs and to narrow the entrance of the hive to foil invaders (Hausen & Wollenweber, 1988; Silici & Kultuca, 2005; Silva-Carvalho, Baltazar, & Almeida-Aguiar, 2015).

The chemical synthesis of propolis is affected by many factors, such as geographic and botanical origin, climate and harvesting season, and it determines its bioactivity (Calegari et al., 2017; Huang, Zhang, Wang, Li, & Hu, 2014; Isla et al., 2009). Typically, propolis is composed of resin and vegetable balsam (50%), wax (30%), pollen (5%), essential and aromatic oils (5%) and various other substances (5%) (Burdock, 1998), forming a "sticky gum". For human consumption, propolis is mainly used in extracts. Extracts are also prepared during research procedures. Such extracts can usually be obtained through the application of ethanol or methanol (Bankova et al., 2016). Other organic solvents such as chloroform or hexane have also been used for propolis extraction (dos Santos Pereira et al., 1998; Negri, Salatino, & Salatino, 2003; Righi et al., 2011) while aqueous solutions have also been tested in some cases (Nagai, Inoue, Inoue, & Suzuki, 2003; Gulcin, Bursal, Sehitoglu, Bilsel, & Goren, 2010; Moura et al., 2011).

Main bioactive ingredients of propolis are phenolic compounds, such as flavonoid aglycones, phenolic acids and their esters, and terpens. Its extracts have been found to present several beneficial properties to human health. Propolis has antioxidant, antimicrobial, anti-inflammatory, healing, anesthetic, anticariogenic, anti-viral, and anticarcinogenic (Bodini, Sobral, Favaro-Trindade, & Carvalho, 2013; Chaillou & Nazareno, 2009; Hashemi, 2016; Lotfy, 2006) properties. As a result, propolis has been used as an additive to food, beverages and cosmetic products and is marketed as "health food" and "nutricosmetic", respectively, (Bernardi et al., 2013; Melliou, Stratis, & Chinou, 2007). Since many consumers prefer natural and less-processed products (Aizpurua-Olaizola et al., 2016; Spinelli, Conte, Lecce, Incoronato, & Del Nobile, 2015), propolis seems to have potential for many applications in the food industry.

As stated above, propolis may contain waxes, fatty acids and non-polar substances, originating mainly from beeswax, but also materials from plants and the percentage of these constituents may reach 35–40% in some samples (Dobrowolski et al., 1991; Papay, Toth,

*Corresponding author. E-mail: alpapach@aegean.gr
†Both authors have contributed equally.

© 2019 International Bee Research Association

Soltesz, Nagy, & Litkei, 1985). These substances can be retained in propolis extracts, since the extraction solvents are mainly organic (Cottica et al., 2011), incommoding the manipulation of the material both in laboratory and industrial section. Processed propolis is a highly pigmented sticky gum with different physicochemical properties and composition from the raw material (Pérez-Parada et al., 2011). Thus, though propolis can be consumed raw from the colony, its value and properties increase through purification following extraction with solvents. Kalogeropoulos, Konteles, Troullidou, Mourtzinos, and Karathanos (2009) supported that purification should be applied in all propolis diluted in solvents. Purification should remove the inert material and preserve the polyphenolic fraction, which is considered to contribute most to the observed biological activity than other propolis constituents (Kumazawa, Ahn, Fujimoto, & Kato, 2010; Lahouel, Boulkour, Segueni, & Fillastre, 2004).

In order to receive clean and pure extracts, many procedures have been adopted. Kumazawa, Hamasaka, and Nakayama (2004) applied centrifugation for 24 h to obtain a clear suspension from their ethanolic extracts. The same method, with modifications in duration, temperature and speed, was performed by Ahn et al. (2007), Ahn, Kumazawa, Hamasaka, Bang, & Nakayama (2004) and Hatano et al. (2012). Many other wax-removing techniques are commonly applied, including specific pore-size filters (Al Naggar, Sun, Robertson, Giesy, & Wiseman, 2016; Chaillou & Nazareno, 2009; Kalogeropoulos et al., 2009) or vacuum filtration (Busch et al., 2017; Cottica et al., 2011; Nader, El-Agamy, & Suddek, 2010; Sawah & Kav, 2010). However, the above studies only use these procedures as a preliminary step for other experiments and do not investigate the effectiveness of the purification of the material. As yet, a standard for an optimal purification method does not exist.

The present study investigated how different methods of propolis purification affect its extracts. Five methods were tested to compare qualitative characteristics of extracts, such as antioxidant activity, total polyphenolic and total flavonoid content. Four of these methods concern protocols currently existing in literature and one concerns a new experimental method that emerged from data from preliminary tests of the centrifugation of propolis in various time and speed combinations. Both ethanolic and methanolic extracts were used for the comparison and the qualitative indicators were measured before and after each intervention.

Materials and methods

Sampling

Propolis samples were collected from hives of *Apis mellifera macedonica* on the island of Samothrace, Greece (40°27′53.09″N, 25°31′46.26″E). The colonies in the selected apiary were in good health and managed according to organic apiculture. To ensure optimum quality of the collected propolis, hives were fitted with propolis traps provided by ANEL (Athens, Greece), Apicultural Company. The traps are made from low-density polyethylene (LDPE) suitable for food, and during preliminary analyses no residues were detected in the propolis samples taken from the traps (data not shown). When each trap was filled with propolis, it was removed and placed in a freezer at −18 °C for 24 h. The propolis was then removed from the trap and stored at −18 °C until extraction.

All propolis samples were obtained between June and July 2018. To avoid variability amongst the propolis, only one sample was chosen from a single colony and all extracts were prepared from a single propolis load.

Extraction preparation

Crude propolis samples were frozen (−18 °C) and ground to fine powder of 50–70 μm in a chilled grinder (Kenwood, KMM060). Then extraction was obtained by the use of two different solvents: methanol and ethanol. The methanolic extracts (PME) were prepared according to Cuesta-Rubio et al. (2007) with slight modifications: 1 g of pulverised crude propolis was extracted by stirring into a 20 mL volume of absolute methanol for 5 min. The ethanolic extracts (PEE) were prepared as described by Bankova et al. (2016). A sample of the powdered propolis (0.667 g) was extracted in an ultrasonic bath for 20 min at 20 °C with 20 mL of absolute ethanol.

Then, all extracts were filtered in ambient temperature with a paper filter of 8 μm pore size and the solutions were kept in tightly sealed bottles stored at −18 °C. From the initial propolis collected, six separate PME ($n = 6$) and six PEE ($n = 6$) samples were prepared.

Purification methods

Method A: As described by Gregoris and Stevanato (2010), extracts were filtered through a strainer to remove insoluble residual beehive products, i.e., wood fragments, bee bodies, etc. The suspensions were left to sediment and the supernatant was centrifuged for 30 min at 370 g using an Eppendorf 5415 D Centrifuge.

Method B: Purification of this method was obtained according to Busch et al. (2017). In order to remove all remaining wax, extracts were kept in a freezer at −18 °C for 10 h and centrifuged at −5 °C at 1850 g for 10 min twice with a 15 min interval (when samples were kept at −18 °C). A Sigma 3K30 was used for the centrifugation.

Method C: Extracts were removed from freezer where they were stored at −18 °C for 10 h and were centrifuged at 3340 g for 2.5 min using an Eppendorf 5415 D Centrifuge. The supernatant was transferred to new tubes and was placed for 15 min at −18 °C. Then

Table 1. Antioxidant activity, total phenolic content and total flavonoid content of ethanolic and methanolic extracts before and after each purification method.

		AA (Trolox Eq. μmol/mL)	TPC (mg Gallic Acid Eq/g propolis)	TFC (mg Querquetin Eq/g propolis)
PEE	**Initial**	33.07 ± 2.39 (23.92–38.84)[c]	113.85 ± 8.14 (86.70–142.57)[c]	43.35 ± 1.39 (38.23–47.45)[c]
	Method A	49.88 ± 1.12 (47.12–54.55)[b]	298.11 ± 16.82 (242.92–362.21)[a]	82.67 ± 5.37 (64.52–103.21)[a]
	Method B	47.91 ± 1.04 (45.16–51.68)[b]	183.44 ± 8.58 (165.29–212.63)[b]	48.82 ± 1.205 (45.93–53.40)[bc]
	Method C	57.10 ± 1.49 (50.54–60.7)[a]	178.54 ± 4.97 (158.66–194.64)[b]	56.78 ± 1.94 (48.58–61.35)[ab]
	Method D	53.11 ± 1.80 (47.23–57.86)[ab]	171.76 ± 6.66 (151.09–193.69)[b]	52.5 ± 1.61 (46.84–58.25)[abc]
PME	**Initial**	44.64 ± 1.73 (37.49–49.51)[c]	177.12 ± 9.11 (143.51–206.95)[b]	54.04 ± 1.36 (48.81–58.25)[c]
	Method A	53.82 ± 1.44 (48.77–57.86)[b]	401.41 ± 31.76 (310.14–491.92)[a]	112.71 ± 9.60 (81.149–140.84)[a]
	Method B	63.65 ± 1.37 (59.25–67.43)[a]	254.6 ± 7.35 (229.67–281.74)[ab]	77.76 ± 1.98 (71.85–82.81)[ab]
	Method C	62.96 ± 2.13 (55.79–68.98)[a]	244.81 ± 12.59 (204.11–281.74)[ab]	76.07 ± 4.07 (61.50–88.40)[ac]
	Method D	52.44 ± 1.71 (46.92–57.44)[b]	182.8 ± 3.29 (171.92–194.64)[b]	63.53 ± 1.54 (57.19–68.38)[bc]

Mean standard error and minimum-maximum. Superscript letters (a,b,c) express statistical significance for $P < 0.05$. PEE. Propolis ethanolic extract; PME, Propolis methanolic extract, AA, antioxidant activity; TPC, total phenolic content; TFC, total flavonoid content.

centrifugation was repeated and the new supernatant was stored in new tubes until further analysis. The above method is novel and has been chosen from preliminary tests implementing various centrifugation speeds and durations (data not shown).

Method D: The specific method is based on filtration (Frozza et al., 2013). Extracts were filtered through a 0.22 μm polyethersulfone membrane (TPP, Techno Plastic Products, Switzerland).

Method E: This last method is based on vacuum filtration as described by Žilius, Ramanauskienė, Juškaitė, and Briedis (2016). Methanolic and ethanolic extracts were filtered through a 0.45um pore size nylon membrane filter, using Buchner vacuum filtration system. Vacuum pressure was set at 1015 mbar.

Chemicals and agents

The 2, 2-diphenyl-picrylhydrazyl (DPPH•) was obtained from Scientific Industries Inc. (N.Y., U.S.A.). Trolox and gallic acid were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Folin-Ciocalteau and monohydrated sodium phosphate were obtained by Merck (Darmstradt, Germany). AlCl3 were from Fisher Scientific (Fair Lawn, NJ).

Determination of the antioxidant activity (AA)

For AA determination, antioxidant activity, the DPPH• quench method was used, based on the experimental study by Arnous, Makris, and Kefalas (2002) with slight modifications. Aliquot of 0.1 mL of sample was added to 3.9 mL DPPH•; (100 μM in MeOH). Mixing was conducted by vortex (Scientific Industries Inc., N.Y. U.S.A.) and then the sample was stored for 30 min in a dark place. Absorbance of the samples was measured at 515 nm using quartz cuvettes, at a UV-Vis spectrophotometer (UV-1800, Shimadzu Co., Kyoto, Japan). The absorbance of the control (containing DPPH• reagent without the addition of a sample) was also measured.

Trolox™ equivalents (mM TRE) were determined from linear regression, after plotting %ΔA515 of known concentration of trolox™ against, where $\%\Delta A_{515} = \frac{A_{515}^{t=0} - A_{515}^{t=30}}{A_{515}^{t=0}} \times 100$ ($t = 0$: the absorbance of the control reaction at time 0, $t = 30$: the absorbance in the presence of the sample of the extracts after 30 min of reaction). Results were expressed as μmol TRE per g of dry propolis weight.

Determination of total polyphenol content (TPC)

The total polyphenol yield (TPC) from propolis extract was determined using the Folin-Ciocalteau method, as adapted in microscale by Arnous et al. (2002), with slight modifications. In a tube, 3.16 mL of distilled water, 0.04 mL of sample and 0.2 mL of Folin-Ciocalteau reagent were mixed. After shaking and resting for 1 min, 0.6 mL of sodium carbonate (20% w/v in distilled water) were added, the sample was mixed with vortex and stored in the dark for 120 min. Absorbance of the samples was measured at 750 nm using quartz cuvettes, at a UV-Vis spectrophotometer and the final results were expressed as mg gallic acid equivalents (GAE) per g of dry propolis weight.

Determination of the total flavonoids content (TFC)

A previously published protocol to determine TFC was used (Chang, Yang, Wen, & Chern, 2002) with slight modifications. To a 0.5 mL solution of appropriate dilution of extract or standard at a suitable concentration, 0.05 mL of $AlCl_3$ solution (2% $AlCl_3$ in a methanol/acetic acid, 95/5, v/v solution) and 0.7 mL of 5% acetic acid methanolic solution are added gradually. Absorbance of the complex formed is measured at 415 nm as a reference solution after a time period of 30 min. The absorbance was obtained at 415 nm (A415) using deionized water as blank and the total flavonoid concentration (CTFn) was calculated from a standard curve constructed with quercetin as the calibration standard. Yield in total flavonoids is expressed as mg quercetin equivalents (QE) per gram of dry propolis weight.

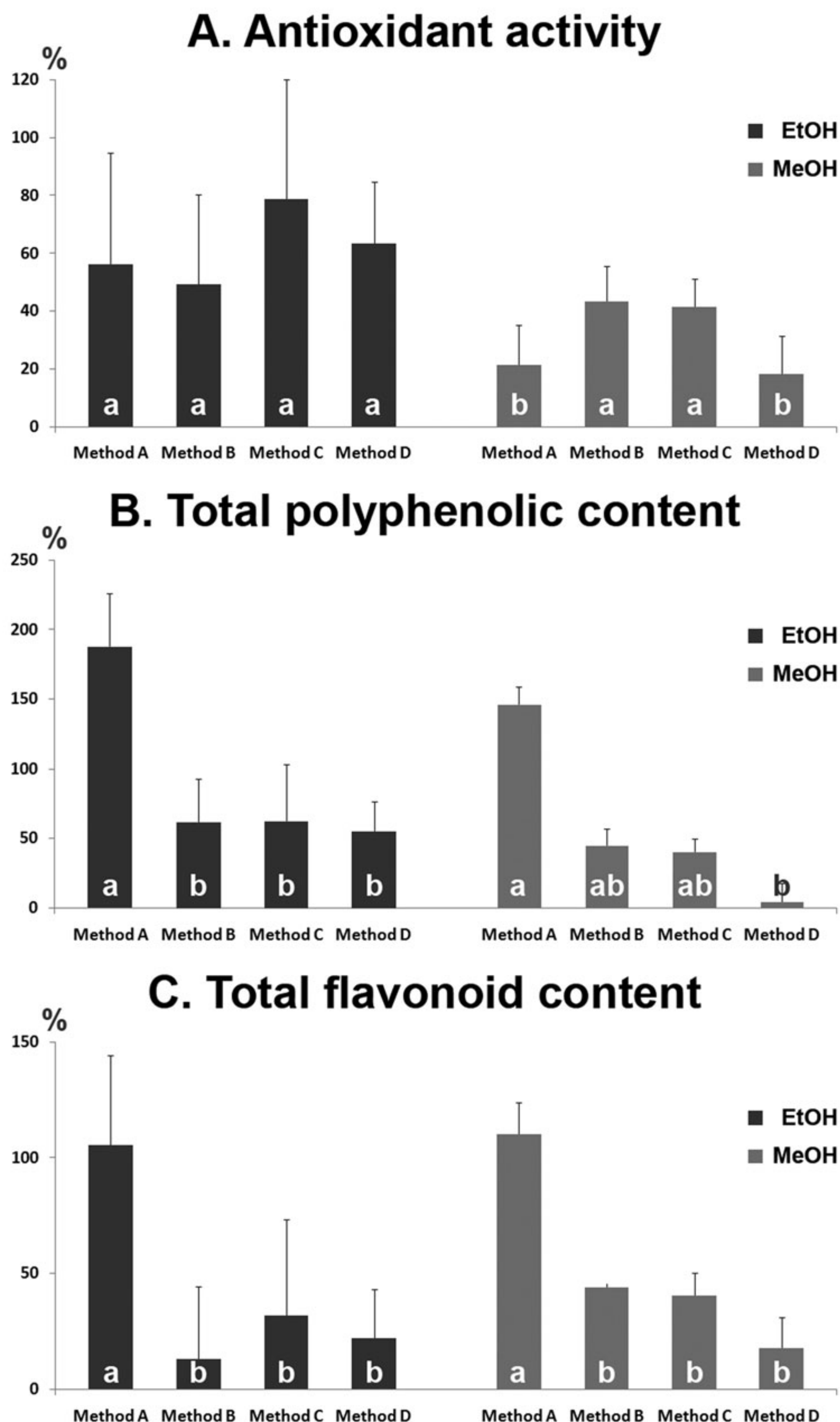

Figure 1. Percentage of increase (% and Standard Error of Means) of the antioxidant activity (A), total polyphenolic content (B), total flavonoid content (C); after each purification method for the ethanolic and the methanolic extracts in relation to the initial extracts.

Statistical analysis

Statistical analyses were conducted through the GraphPad Instat V3.10 software. An unpaired t-test was used for the comparison between ethanolic and methanolic extracts before and after purification. For the comparison of means between different purification

methods, parametric One-Way ANOVA and nonparametric Kruskal–Wallis tests were applied. Each purification method was repeated 6 times ($n = 6$) at the initial extract. A total of 36 replications (6×5 purification method plus 6 for control) was used for each One-Way ANOVA, both for ethanolic and methanolic extracts.

Results

Initial extraction

The comparison of propolis PEE and PME demonstrated differences between all three qualitative indicators. PME presented significantly higher values (Table 1) and were therefore examined and compared separately. All purification methods tested resulted in extracts that presented higher AA, TPC and TFC. Although exhibiting high values in all qualitative indicators, the extracts which were purified with Method E (Buchner vacuum filtration system) demonstrated huge deviation amongst replications, especially during the TPC evaluation (sd = 64.58, 98.26 for PEE and PME, respectively). During the implementation of this method, the volume of the extract was gradually decreasing, probably because of the evaporation of the solvent from the device. Therefore, Method E was excluded from the trials because it was considered unreliable.

Evaluation of methods implementing double centrifugation

The extracts which were purified with Methods B and C (double centrifugation) exhibited higher values in AA, TPC and TFC when compared with the initial extracts. However, extracts of the second centrifugation presented slightly decreased qualitative indicators (though not significant) when compared with extracts after the first application. This decrease involved all three qualitative indicators. Therefore, the values from PEE and PME derived from a second centrifugation were not taken into consideration.

Effect of purification methods on the antioxidant activity

The DPPH• free radical scavenging activity of samples after each purification method tested is shown in Table 1. The percentage of increase of each method, compared to the values of their initial extracts, is presented in Figure 1. Both PEE and PME presented increased AA after every purification treatment (Table 1, Figure 1A).

In tests of the antioxidant capacity of ethanolic extracts, samples treated with Method C expressed the highest values of AA (mean: 57.1 Trolox Eq. mmol/mL). Samples after purification with Method D also exhibited increased AA (53.11 Trolox Eq. mmol/mL) though differences with Method C were not significant. No differences were observed on the increase of the percentages between methods tested (Figure 1A). The methanolic extracts showed a slightly different pattern. PME samples treated with purification Method B expressed the highest antioxidant activity with an average of 63.65 Trolox Eq. mmol/mL (Table 1) though there was no difference with Method C (62.96 Trolox Eq. mmol/mL). The increase of the AA was also the highest in samples treated with Method B and C and significantly different when in comparison with the other two methods (A, D) (Figure 1A).

Effect of purification methods on the total polyphenol content

PEE purified with Method A showed significantly higher polyphenolic content (mean: 298.11 mg GA Eq/g propolis) than any other method tested (Table 1). Similarly, the increase of TPC in samples after treatment with Method A was also the highest among all samples (Figure 1B). Method A also presented the highest polyphenol content from all PME samples after purification (401.41 mg GA Eq/g propolis) though no significant differences were observed between Methods B and C. Methods B, C and D presented higher values from the unpurified samples though differences were not significant. Method A also demonstrated the highest increase of TPC (significantly different with Method D) though differences were not significant with Methods B and C (Figure 1B).

Effect of purification methods on the total flavonoid content

Total flavonoid content obtained from PEE purified with Method A produced the highest values (82.67 mg QE/g propolis) and increase (Figure 1C), though PEE treated with Method C (56.78 mg Q Eq/g propolis) and Method D (52.5 mg Q Eq/g propolis) presented no significant differences of values compared to Method A. Method A differed significantly from Method B regarding the values and the percentage of increase. PME samples treated with Method A expressed significantly the highest percentage of increase compared to other methods tested (Figure 1C). Furthermore, Method A presented also the highest values of TFC from all other methods (112.71 mg Q Eq/g propolis) though differences were not significant with Methods B and C (Table 1).

Discussion

Results from this study indicate that the process of purification through centrifugation, filtering and vacuum induces improvement of propolis quality by boosting its antioxidant activity and increasing its total phenolic and flavonoid content. The five methods tested demonstrated an increase in all three qualitative indicators, yet this increase was not equal after the application of each method.

When antioxidant activity was measured, purified ethanolic extracts showed a significantly higher increase than the methanolic ones. After the implementation of methods A, C and D, no significant differences were observed between the ethanolic and the methanolic samples. These findings are important because ethanol, unlike methanol, is a non-toxic solvent (food grade). Therefore most commercial products should be extracted using ethanol rather than methanol, which is mainly used for experimental purposes (Sforcin & Bankova, 2011; Bankova et al., 2016; Shi, Yang, Zhang, & Yu, 2012).

Increase of the antioxidant activity was higher for method C both for PEE and PME. However, method A increased significantly the TPC and TFC compared to the other methods tested. Although it has not been a subject of the present study, this interesting contradiction might be explained by the difference in temperature of centrifugation between methods A and C and by the nature of Greek propolis.

Though it is generally accepted that the antioxidant activity of propolis is due to its phenolic constituents (Banskota et al., 2000; Gardana, Scaglianti, Pietta, & Simonetti, 2007; Hashemi, 2016), many researchers have shown that Greek and Mediterranean propolis is in general, rich in terpenoid substances and its high antioxidant activity is mainly a result of diterpenes rather than polyphenols (Kalogeropoulos et al., 2009; Melliou & Chinou, 2004; Popova et al., 2012). However, the propolis sample used during the present study was also very rich in polyphenolic compounds. Polyphenols and flavonoids in propolis and plant extracts in general, have been found to be rather thermal-stable (González, Gómez, Tereschuk, & Molina, 2009; Volf, Ignat, Neamtu, & Popa, 2014). Longer centrifugation at a relatively high temperature as applied in method A, may lead to more effective wax and organic waste removal without any negative effect on the polyphenolic content, but it may also lead to chemical changes of the terpenoid compounds (including diterpenes) and consequently to lower antioxidant activity. Since most of the terpenoids determined in Greek and Mediterranean types of propolis are highly volatile (Popova, Graikou, Chinou, & Bankova, 2010), short-term centrifugation at low temperature, as obtained in method C, might protect these compounds from degradation and result in high antioxidant activity of the purified extracts. In any case, the above assumptions need to be investigated through future experimentation.

Some of the methods tested expressed much more variability than others. The deviation in the purification using the Buchner vacuum filtration system was extremely high and during its implementation the volume of the extract decreased gradually, probably because of evaporation of the solvent from the device. Applying vacuum to clean propolis samples may be rapid but it does not lead to reliable qualitative indicator results. Therefore, it should not be recommended as a treatment, at least for experimental purposes, since it could lead to inaccurate results.

Taking all factors into consideration, purification using centrifugation is the most efficient method of improving propolis quality. This may be because wax is easily removed as a uniform mass after agglomeration during the process. The pre-treatment resting of samples at low temperature ($-18\,^{\circ}C$) may be important since low temperature agglomerates wax in solvents. Nevertheless, based on the results of the present study, a double-step centrifugation is not recommended since it does not further contribute in antioxidant activity or the polyphonolic and flavonoid content of the final extract.

Based on the results of the present study, it is concluded that purification of propolis through centrifugation is an essential procedure that should be applied both for experimental and commercial purposes. It can increase antioxidant activity, total polyphenolic and flavonoid content and consequently the value of propolis as a medicinal food. The research demonstrated the variability, time and effort required for each method. Single centrifugation for a short time and at high speed as described in Method C (2.5 min at 3340 g) can induce the highest increase of both TPC and TFC, while method A (centrifugation for 30 min, at 370 g) can achieve the highest antioxidant activity both for PEE and PME.

Acknowledgments

The authors are grateful to the Apicultural Company ANEL (Athens, Greece) for supporting the study. Alexandros Papachristoforou's research is supported by Vita (Europe) Ltd. The authors also thank Stephen Fleming for editing the manuscript. Finally, authors thank Dukas Hailas for his valuable assistance at the apiaries at Samothraki.

Disclosure statement

No competing financial interests exist.

ORCID

Alexandros Papachristoforou http://orcid.org/0000-0001-8123-6848

References

Ahn, M. R., Kumazawa, S., Hamasaka, T., Bang, K. S., & Nakayama, T. (2004). Antioxidant activity and constituents of propolis collected in various areas of Korea. *Journal of Agricultural and Food Chemistry*, *52*(24), 7286–7292. doi:10.1021/jf048726s

Ahn, M. R., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F., & Nakayama, T. (2007). Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chemistry*, *101*(4), 1383–1392. doi:10.1016/j.foodchem.2006.03.045

Aizpurua-Olaizola, O., Navarro, P., Vallejo, A., Olivares, M., Etxebarria, N., & Usobiaga, A. (2016). Microencapsulation and storage stability of polyphenols from *Vitis vinifera* grape wastes. *Food Chemistry*, *190*, 614–621. doi:10.1016/j.foodchem.2015.05.117

Al Naggar, Y., Sun, J., Robertson, A., Giesy, J. P., & Wiseman, S. (2016). Chemical characterization and antioxidant properties of Canadian propolis. *Journal of Apicultural Research*, *55*(4), 305–314. doi:10.1080/00218839.2016.1233700

Arnous, A., Makris, D. P., & Kefalas, P. (2002). Correlation of pigment and flavanol content with antioxidant properties in selected aged regional wines from Greece. *Journal of Food Composition and Analysis*, *15*(6), 655–665. doi:10.1006/jfca.2002.1070

Bankova, V. (2005). Chemical diversity of propolis and the problem of standardization. *Journal of Ethnopharmacology*, *100*(1–2), 114–117. doi:10.1016/j.jep.2005.05.004

Bankova, V., Bertelli, D., Borba, R., Conti, B. J., da Silva Cunha, I. B., … Zampini, C. (2016). Standard methods for *Apis mellifera* propolis research. *Journal of Apicultural Research*, *58*, 1–49. doi:10.1080/00218839.2016.1222661

Banskota, A. H., Tezuka, Y., Adnyana, I. K., Midorikawa, K., Matsushige, K., Message, D., … Kadota, S. (2000). Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and China. *Journal of Ethnopharmacology*, *72*(1–2), 239–246. doi:10.1016/S0378-8741(00)00252-X

Bernardi, S., Favaro-Trindade, C. S., Trindade, M. A., Balieiro, J. C. C., Cavenaghi, A. D., & Contreras-Castillo, C. J. (2013). Italian-type salami with propolis as antioxidant. *Italian Journal of Food Science*, *25*(4), 433–440.

Bodini, R. B., Sobral, P. J. A., Favaro-Trindade, C. S., & Carvalho, R. A. (2013). Properties of gelatin-based films with added ethanol–propolis extract. *Lwt - Food Science and Technology*, *51*(1), 104–110. doi:10.1016/j.lwt.2012.10.013

Burdock, G. A. (1998). Review of the biological properties and toxicity of bee propolis (propolis). *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *36*(4), 347–363.

Busch, V. M., Pereyra-Gonzalez, A., Šegatin, N., Santagapita, P. R., Ulrih, N. P., & Buera, M. P. (2017). Propolis encapsulation by spray drying: Characterization and stability. *LWT*, *75*, 227–235. doi:10.1016/j.lwt.2016.08.055

Calegari, M. A., Prasniewski, A., Silva, C. D., Sado, R. Y., Maia, F., Tonial, L., & Oldoni, T. L. (2017). Propolis from Southwest of Parana produced by selected bees: Influence of seasonality and food supplementation on antioxidant activity and phenolic profile. *Anais da Academia Brasileira de Ciências*, *89*(1), 45–55. doi:10.1590/0001-3765201620160499

Chaillou, L. L., & Nazareno, M. A. (2009). Chemical variability in propolis from Santiago del Estero, Argentina, related to the arboreal environment as the sources of resins. *Journal of the Science of Food and Agriculture*, *89*(6), 978–983. doi:10.1002/jsfa.3542

Chang, C. C., Yang, M. H., Wen, H. M., & Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*, *10*(3), 178–182.

Cottica, S. M., Sawaya, A. C., Eberlin, M. N., Franco, S. L., Zeoula, L. M., & Visentainer, J. V. (2011). Antioxidant activity and composition of propolis obtained by different methods of extraction. *Journal of the Brazilian Chemical Society*, *22*(5), 929–935. doi:10.1590/S0103-50532011000500016

Cuesta-Rubio, O., Piccinelli, A. L., Fernandez, M. C., Hernández, I. M., Rosado, A., & Rastrelli, R. (2007). Chemical characterization of Cuban propolis by HPLC-PDA, HPLC-MS, and NMR: The brown, red, and yellow cuban varieties of propolis. *Journal of Agricultural and Food Chemistry*, *55*(18), 7502–7509. doi:10.1021/jf071296w

Dobrowolski, J. W., Vohora, S. B., Sharma, K., Shah, S. A., Naqvi, S. A. H., & Dandiya, P. C. (1991). Antibacterial, antifungal, antiamoebic, antiinflammatory and antipyretic studies on propolis bee products. *Journal of Ethnopharmacology*, *35*(1), 77–82. doi:10.1016/0378-8741(91)90135-Z

dos Santos Pereira, A., Pinto, A. C., Nobrega Cardoso, J., Radler de Aquino Neto, F., Freiman de Souza Ramos, M., Dellamora-Ortiz, G. M., & Pereira dos Santos, E. (1998). Application of high temperature high resolution gas chromatography to crude extracts of propolis. *Journal of High Resolution Chromatography*, *21*(7), 396–400. doi:10.1002/(SICI)1521-4168(19980701)21:7<396::AID-JHRC396>3.3.CO;2-F

Frozza, C. O., Garcia, C. S. C., Gambato, G., de Souza, M. D. O., Salvador, M., Moura, S., … Roesch-Ely, M. (2013). Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. *Food and Chemical Toxicology*, *52*, 137–142. doi:10.1016/j.fct.2012.11.013

Gardana, C., Scaglianti, M., Pietta, P., & Simonetti, P. (2007). Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography–tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, *45*(3), 390–399. doi:10.1016/j.jpba.2007.06.022

Gregoris, E., & Stevanato, R. (2010). Correlations between polyphenolic composition and antioxidant activity of Venetian propolis. *Food and Chemical Toxicology*, *48*(1), 76–82. doi:10.1016/j.fct.2009.09.018

Gulcin, I., Bursal, E., Sehitoglu, M. H., Bilsel, M., & Goren, A. C. (2010). Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey. *Food and Chemical Toxicology*, *48*, 2227–2238. doi:10.1016/j.fct.2010.05.053

Hashemi, J. H. (2016). Biological effect of bee propolis: A review. *European Journal of Applied Sciences*, *8*(5), 311–318.

González, M., Gómez, M. I., Tereschuk, M. L., & Molina, A. (2009). Thermal stability of propolis from Tucumán, Argentina. *Journal of Apicultural Research*, *48*(4), 270–278. doi:10.3896/IBRA.1.48.4.07

Hatano, A., Nonaka, T., Yoshino, M., Ahn, M. R., Tazawa, S., Araki, Y., & Kumazawa, S. (2012). Antioxidant activity and phenolic constituents of red propolis from Shandong, China. *Food Science and Technology Research*, *18*(4), 577–584. doi:10.3136/fstr.18.577

Hausen, B. M., & Wollenweber, E. (1988). Propolis allergy: (III). Sensitization studies with minor constituents. *Contact Dermatitis*, *19*(4), 296–303. doi:10.1111/j.1600-0536.1988.tb02931.x

Huang, S., Zhang, C. P., Wang, K., Li, G. Q., & Hu, F. L. (2014). Recent advances in the chemical composition of propolis. *Molecules*, *19*(12), 19610–19632. doi:10.3390/molecules191219610

Isla, M. I., Zampini, I. C., Ordóñez, R. M., Cuello, S., Juárez, B. C., Sayago, J. E., … Maldonado, L. M. (2009). Effect of seasonal variations and collection form on antioxidant activity of propolis from San Juan, Argentina. *Journal of Medicinal Food*, *12*(6), 1334–1342. doi:10.1089/jmf.2008.0286

Kalogeropoulos, N., Konteles, S. J., Troullidou, E., Mourtzinos, I., & Karathanos, V. T. (2009). Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. *Food Chemistry*, *116*(2), 452–461. doi:10.1016/j.foodchem.2009.02.060

Kumazawa, S., Ahn, M. R., Fujimoto, T., & Kato, M. (2010). Radical-scavenging activity and phenolic constituents of propolis from different regions of Argentina. *Natural Product Research*, *24*(9), 804–812. doi:10.1080/14786410802615270

Kumazawa, S., Hamasaka, T., & Nakayama, T. (2004). Antioxidant activity of propolis of various geographic origins. *Food Chemistry*, *84*(3), 329–339. doi:10.1016/S0308-8146(03)00216-4

Lahouel, M., Boulkour, S., Segueni, N., & Fillastre, J. P. (2004). Effet protecteur des flavonoïdes contre la toxicité de la vinblastine, du cyclophosphamide et du paracétamol par inhibition de la peroxydation lipidique et augmentation du glutathion hépatique. *Pathologie Biologie*, *52*(6), 314–322. doi:10.1016/j.patbio.2004.01.001

Lotfy, M. (2006). Biological activity of bee propolis in health and disease. *Asian Pacific Journal of Cancer Prevention*, *7*(1), 22–31.

Marcucci, M. C. (1995). Propolis: Chemical composition, biological properties and therapeutic activity. *Apidologie*, *26*(2), 83–99. doi:10.1051/apido:19950202

Melliou, E., & Chinou, I. (2004). Chemical analysis and antimicrobial activity of Greek propolis. *Planta Medica*, *70*(6), 515–519. doi:10.1055/s-2004-827150

Melliou, E., Stratis, E., & Chinou, I. (2007). Volatile constituents of propolis from various regions of Greece–Antimicrobial activity. *Food Chemistry*, *103*(2), 375–380. doi:10.1016/j.foodchem.2006.07.033

Moura, S. A., Negri, G., Salatino, A., Lima, L. D., Dourado, L. P., Mendes, J. B., … Cara, D. C. (2011). Aqueous extract of Brazilian green propolis: Primary components, evaluation of inflammation and wound healing by using subcutaneous implanted sponges. *Evidence-Based Complementary and Alternative Medicine*, *2011*, 748283. doi:10.1093/ecam/nep112

Nader, M. A., El-Agamy, D. S., & Suddek, G. M. (2010). Protective effects of propolis and thymoquinone on development of atherosclerosis in cholesterol-fed rabbits. *Archives of Pharmacal Research*, *33*(4), 637–643. doi:10.1007/s12272-010-0420-1

Nagai, T., Inoue, R., Inoue, H., & Suzuki, N. (2003). Preparation and antioxidant properties of water extract of propolis. *Food Chemistry*, *80*(1), 29–33. doi:10.1016/S0308-8146(02)00231-5

Negri, G., Salatino, M. L. F., & Salatino, A. (2003). Green propolis**"**: Unreported constituents and a novel compound from chloroform extracts. *Journal of Apicultural Research*, *42*(3), 39–41. doi:10.1080/00218839.2003.11101087

Papay, V., Toth, L., Soltesz, M., Nagy, E., & Litkei, G. (1985). Isolated compounds from Hungarian propolis and populi gemma. *Studies in Organic Chemistry. Flavonoids and Bioflavonoids*, *25*, 233–240.

Pérez-Parada, A., Colazzo, M., Besil, N., Geis-Asteggiante, L., Rey, F., & Heinzen, H. (2011). Determination of coumaphos, chlorpyrifos and ethion residues in propolis tinctures by matrix solid-phase dispersion and gas chromatography coupled to flame photometric and mass spectrometric detection. *Journal of Chromatography A*, *1218*(34), 5852–5857. doi:10.1016/j.chroma.2011.06.097

Popova, M. P., Graikou, K., Chinou, I., & Bankova, V. S. (2010). GC-MS profiling of diterpene compounds in Mediterranean propolis from Greece. *Journal of Agricultural and Food Chemistry*, *58*(5), 3167–3176. doi:10.1021/jf903841k

Popova, M., Trusheva, B., Cutajar, S., Antonova, D., Mifsud, D., Farrugia, C., & Bankova, V. (2012). Identification of the plant origin of the botanical biomarkers of Mediterranean type propolis. *Natural Product Communications*, *7*(5), 1934578X1200700–1934578X1200570. doi:10.1177/1934578X1200700505

Righi, A. A., Alves, T. R., Negri, G., Marques, L. M., Breyer, H., & Salatino, A. (2011). Brazilian red propolis: Unreported substances, antioxidant and antimicrobial activities. *Journal of the Science of Food and Agriculture*, *91*(13), 2363–2370. doi:10.1002/jsfa.4468

Sawah, J. S., & Kav, J. L. S. P. (2010). Apoptosis of human breast cancer cells induced by ethylacetate extracts of propolis. *American Journal of Biochemistry and Biotechnology*, *6*(2), 84–88.

Sforcin, J. M., & Bankova, V. (2011). Propolis: Is there a potential for the development of new drugs? *Journal of Ethnopharmacology*, *133*(2), 253–260. doi:10.1016/j.jep.2010.10.032

Shi, H., Yang, H., Zhang, X., & Yu, L. L. (2012). Identification and quantification of phytochemical composition and anti-inflammatory and radical scavenging properties of methanolic extracts of Chinese propolis. *Journal of Agricultural and Food Chemistry*, *60*(50), 12403–12410. doi:10.1021/jf3042775

Silici, S., & Kutluca, S. (2005). Chemical composition and antibacterial activity of propolis collected by three different races of honey bees in the same region. *Journal of Ethnopharmacology*, *99*(1), 69–73. doi:10.1016/j.jep.2005.01.046

Silva-Carvalho, R., Baltazar, F., & Almeida-Aguiar, C. (2015). Propolis: A complex natural product with a plethora of biological activities that can be explored for drug development. *Evidence-Based Complementary and Alternative Medicine*, *2015*, 1. doi:10.1155/2015/206439

Spinelli, S., Conte, A., Lecce, L., Incoronato, A. L., & Del Nobile, M. A. (2015). Microencapsulated propolis to enhance the antioxidant properties of fresh fish burgers. *Journal of Food Process Engineering*, *38*(6), 527–535. doi:10.1111/jfpe.12183

Volf, I., Ignat, I., Neamtu, M., & Popa, V. I. (2014). Thermal stability, antioxidant activity, and photo-oxidation of natural polyphenols. *Chemical Papers*, *68* (1), 121–129. doi:10.2478/s11696-013-0417-6

Žilius, M., Ramanauskienė, K., Juškaitė, V., & Briedis, V. (2016). Formulation of Propolis phenolic acids containing microemulsions and their biopharmaceutical characterization. *Evidence-Based Complementary and Alternative Medicine*, *2016*, 1. doi:10.1155/2016/8175265