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Comparative Study of the Extraction Methods for the Instrumental Analysis of Bee Propolis

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Abstract

As a natural resinous substance collected by honeybees from buds and exudates of trees, propolis is used by bees as a glue, general-purpose sealer, and draught extruder for beehives. In this paper, different extraction methods were employed to compare their efficiency in the extraction of bee propolis samples. The methods employed using ethanol as a solvent were the following: soaking method, ultrasonication method, and microwave method. Gas chromatography-mass spectrometry (GC-MS) and spectroscopic methods such as absorbance and fluorescence were utilized to determine the amount of phenolic compounds extracted and compare each extraction's efficiency method. Results showed samples obtained from ultrasonication and microwave methods gave the highest yields. Both methods can be performed within a short time in comparison to the soaking method.

Keywords: propolis, extraction, ultrasonication, microwave

Introduction

Propolis is a product of the beehive along with honey, pollen, and wax. It is a natural and resinous substance collected by bees from exudates of buds, leaves, branches, and barks of trees. It has also been known as bee glues since propolis is used by the honey bees to seal cracks and block holes in the hive. It has been used since ancient times and recently gained popularity in health foods and cosmetic products because of its well-known biological activities. Several studies have reported its antioxidant, antifungal, anti-inflammatory, and antibacterial activities (Camuri, Costa, Siuiti Ito, & Moreira Pazin, 2018). Although its natural properties are due to the phenolics and flavonoids, the main composition of propolis are the following: balsamic substances (50%), waxes (30%), essential and aromatic oils (10%), pollen (5%), and others (5%). Its chemical composition is also dependent on some factors such as bee species, geographical locations, and time of collection (Silici & Kutluca, 2005).

Since raw propolis cannot be used as crude material, it must be purified by solvent extraction. The main purpose of solvent extraction is to recover materials that possess the biological activities mentioned above. There are different solvent extraction methods used to remove the active components in propolis. Among these are traditional methods such as maceration and Soxhlet extractions. Maceration is the traditional soaking method where a suitable solvent is used to dissolve the propolis component without producing heat, thereby making this suitable for heat-stable substances (Khacha-ananda, Tragoolpua, Chantawannakul, & Tragoolpua, 2013). However, this method is time-consuming, requiring 1 to 10 days. On the other hand, Soxhlet extraction made use of specialized glassware and involved heating to evaporate the solvent to extract the sample and then collect the condensed extract. It made use of the solvent reflux and siphon principle. Although it is efficient in solvent use and extraction, it is not useful for temperature-sensitive chemicals.

Recently, modern extraction methods, such as ultrasonic extraction (sonication), microwave-assisted extraction, and supercritical fluid extraction, have been used. Sonication made use of sound energy to break the cell membranes, disrupt the cell wall structure, and accelerate the diffusion of a solvent through membranes. Microwave-assisted extraction, on the other hand, made use of microwaves that can easily penetrate the sample pores, causing the solvent trapped in the pores to heat evenly and rapidly. Supercritical extraction employs CO_2 at its supercritical condition (Idrus et al., 2018). These newer methods have higher extraction yields and shorter extraction times in comparison to the traditional method. In the case of microwave-assisted extraction and supercritical fluid, less solvent is utilized (Trusheva, Trunkova, & Bankova, 2007).

In this study, phenolic compounds were extracted from propolis sample utilizing different extraction methods reported in the literature. The main objective of this study is to compare different extraction methods such as ultrasonication and microwave-assisted extraction with the traditional soaking (maceration) method. The effectiveness of each extraction was made by analyzing the extracts using GC-MS and absorbance and fluorescence spectroscopy.

Materials and Methods

Propolis sample was obtained from a bee farm in Sorsogon, Philippines. This was transported to the United States and then frozen until analysis. The propolis sample was then pulverized using mortar and pestle, and 1.0 g of sample was mixed with 10.0 mL ethanol and extracted using different methods such as soaking, ultrasonication, and use of microwave (Figure 1). Each extraction method was done three times.

Extraction Using Different Methods

For the soaking method, the propolis sample was soaked in ethanol in a 20 mL vial at room temperature (25 °C). Two different extraction times were used: 24 h and 48 h, and the mixtures were stored in the dark. For the ultrasonication method, the propolis samples in a 20 mL vial were extracted using an 80 W ultrasonic bath (Fisher Scientific FS20H). Two different extraction times were also used: 30 min and 60 min. For the microwave-assisted extraction (MAE) protocol, the propolis-solvent mixture was placed in a 50 mL beaker and microwaved for a total of 10 s (2 x 5 s power on and 10 s off in between) using a standard 700W household microwave (Samsung). The resulting mixtures from the different extraction methods were then filtered using Whatman® UNIFLO® syringe filter with 0.45 µm pore size. The collected extracts placed in a 4 mL dram vial and stored below 0°C in the dark were analyzed using gas chromatography-mass spectrometry and spectroscopic methods: absorbance and fluorescence.

Furthermore, 1.0 mL of extract from each extraction method was used to determine the percentage of materials extracted. These were evaporated to dryness at room temperature until a constant weight was recorded. The percentage of the dry extracts was determined from the means of three replicates.

Absorbance-Fluorescence Spectroscopy

The propolis sample was first removed from frozen storage and allow to reach room temperature before analysis. For the spectroscopic methods, the collected and filtered extract (5.0 µL) was diluted with ethanol in a 5-mL volumetric flask, and the resulting mixture was analyzed for absorbance and fluorescence.

Each extract was placed in a quartz cuvette (3 mL) and was used for all spectroscopic analysis. A JASCO v-570 spectrophotometer (Easton, MD) was used to obtain the absorbance of the different extracts. For the emission measurements, a FluoroMax-4 Spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) using 1 nm slits was used. The emission intensity was obtained at two different excitation wavelengths for each sample: 290 nm and 330 nm. Pure ethanol was used as a blank, and its absorbance and emission spectra were also obtained. All spectroscopic measurements were performed in triplicate, and all readings from each extraction method were averaged for comparison purposes.

Gas Chromatography-Mass Spectrometry (GC-MS)

A 5 µL pure extract was used in each scan. A Hewlett-Packard gas chromatograph 6890 series linked to a Hewlett-Packard 5973 mass selective detector with 30 µm x 250 µm x 0.28 µm HP5-MS column was used for GC-MS analysis. The total analysis run is 36 minutes long. With

an injector temperature of 110 °C, a temperature program of 110 °C held for two min and then ramped to 280 °C at a rate of 10 °C/min, and a 15-min hold at 280 °C was applied. With helium as the carrier gas, a flow rate of 1.5 mL/min was used. Upon completion, peaks were identified through their MS spectra using the database of the system (NIST Mass Spectral Library).

Statistical Analysis

Experimental data (absorbance and emission signals in certain wavelengths and percent yield) were evaluated using student's t-test ($p < 0.05$) to compare the difference between spectra.

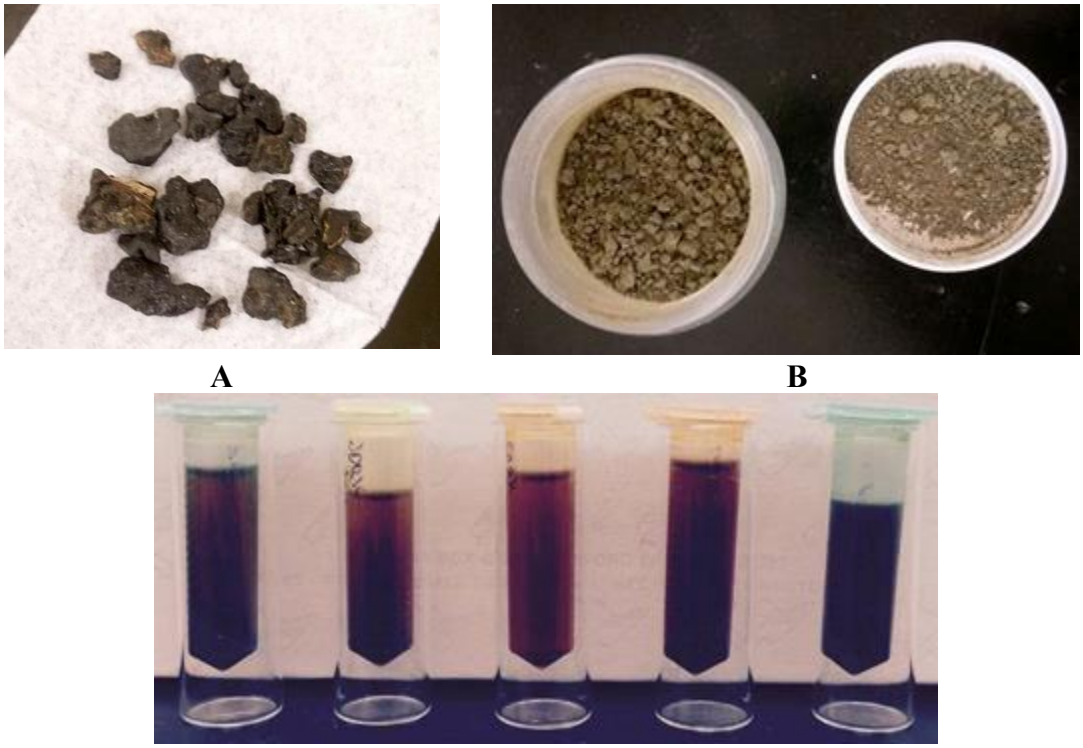

Figure 1. Bee propolis at different stages of sample preparation. A) raw sample B) ground sample and extracts obtained using different methods.

Results and Discussion

The extraction of materials, especially bioactive compounds from propolis, is the first step in its use in the nutraceutical industry. In this process, the solvent plays a very important role. For this study, pure ethanol was utilized as solvent since it is non-toxic and has the advantage of being a highly efficient solvent of polyphenols from propolis. This solvent is usually used at a varying concentration to extract propolis. However, since one of the instruments used is GC-MS, pure ethanol was used as a solvent. Also, a 1:10 (mass: volume) ratio of sample to solvent was used in

all extraction methods. This was the optimum amount reported in one study (Trusheva et al., 2007), where ratios larger than were necessarily leading only to solvent and energy losses.

The efficiency of different methods (soaking, ultrasonication, and microwave) to extract materials from propolis was compared in this study. This was done by analyzing the extracts using instrumental methods. Among these are electronic absorbance and emission-steady state fluorescence. Both techniques are useful for quantitative purposes. Absorption spectroscopy is based on the absorption of energy by molecules at a specific wavelength. This technique can be utilized to characterize the absorption and transmission of materials in propolis samples. This has also been used for bee products like propolis because of its non-destructive nature (Maldonado et al., 2020).

Figure 2 shows the absorbance spectra of extracts obtained from different methods. It is not surprising that all extracts have the same profile as they all came from the same batch of samples. This also suggests that all extracts have a homogenous chemical composition. At least four major peaks (at 220, 230, 295, and 330 nm) and a minor peak at 380 nm regions were observed in all samples. Phenolic compounds such as flavonoids can be found from 290-400 nm regions, and based on the absorbance, the extracting solvent used was able to recover these phenolic compounds (Tomazzoli et al., 2015). It has also been reported that the visible spectra of the propolis can be related to typical polyphenol spectra that have a broad band centered between 280-330 nm (Catalin Mot et al., 2011) that is similar to the obtained absorbance profile

Extract from the microwave method has the highest absorbance based on the peak at 290 nm, and at 330 nm region followed both by sonication for 60 min and soaking for two days. Soaking propolis samples for one day has the lowest absorbance at the two peak regions, followed by sonication for 30 min. Statistical analysis showed that the absorbance obtained from the microwave extract is significantly different from the other extracts. It was also determined that there are no significant differences in absorbance for extracts from both sonication and soaking. Absorbance results confirmed that the longer the extraction time, the more materials that can be extracted. Both soaking and sonication yield higher absorbance at a longer period, significantly different from those obtained at a shorter period.

On the other hand, fluorescence spectroscopy is used for fluorescent materials. Although some phenolic compounds fluoresce, this technique is seldom used in propolis samples. It is used to analyze the Brazilian green propolis containing Artepillin C, a cinnamic acid derivative that presents two prenylated groups (Barbosa da Silva Cunha et al., 2006; Camuri et al., 2018). This method was also used as a detection method in HPLC to analyze coumarin derivatives in propolis (Hrobonova, Lehotay, Cizmarik, & Sadecka, 2013). Lately, fluorescence microscopy was used to characterize propolis from Brunei (Abdullah et al., 2019).

The results from absorbance, however, did not translate to the same trend in fluorescence. Using an excitation wavelength of 290 nm (Figure 3), the extract from the microwave showed the lowest emission intensity. Soaking for two days exhibited the highest intensity, followed by sonication for 60 min. Statistical analysis showed a significant difference among all extracts except for the ones obtained from sonication and soaking at a shorter period. On the other hand, for excitation at 330 nm, the microwave extraction yield extracts with the highest intensity followed

by soaking and sonication both of shorter times. All spectra from different extracts are significantly different from one another.

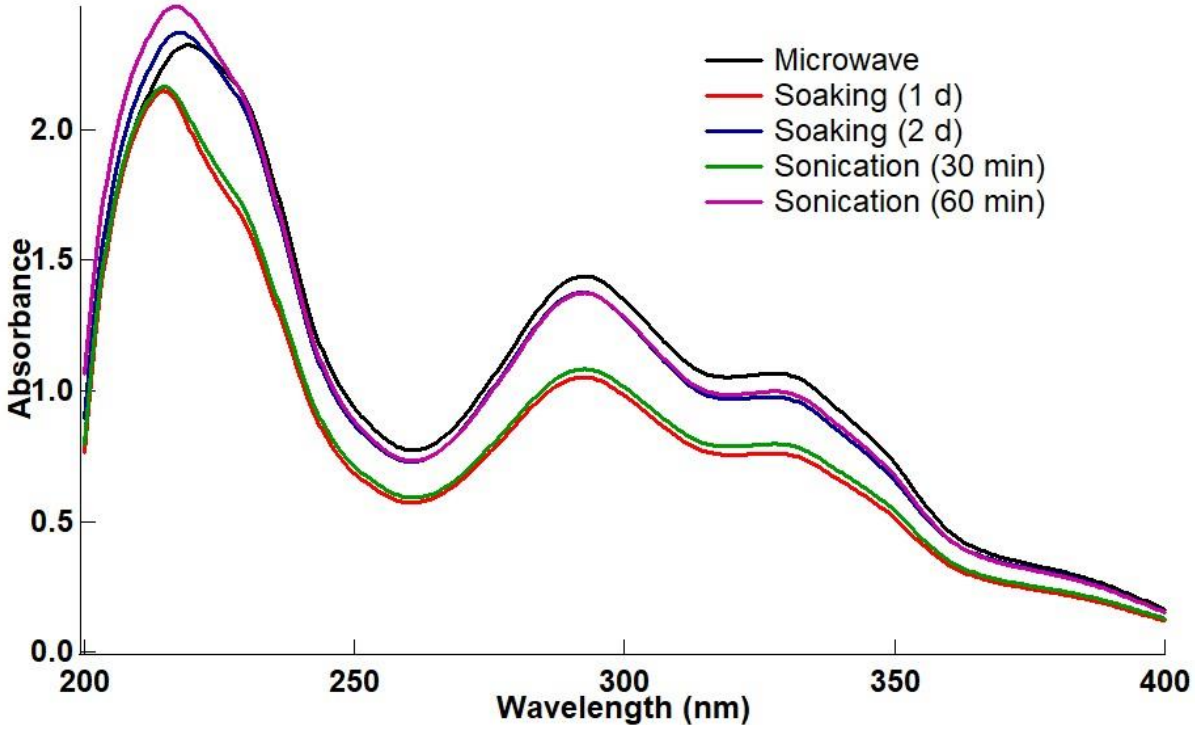

Figure 2. Absorbance of extracts obtained using different methods.

Results acquired using GC-MS showed total ion chromatograms (Figure 4) of the same profile for all extracts obtained using different extraction methods, with the highest peak observed around 25.19 min. The microwave extraction method exhibited the highest relative abundance, with the chromatograms showing a relative abundance of approximately 5.2 x 10^6. Ultrasonic extraction showed the highest peak with a relative abundance of 4.9 x 10^6 for 60 minutes and 4.0 x 10^6 at 30 minutes. The soaking method exhibited a relative abundance of around 4.7 x 10^6 for two days and 4.5 x 10^6 for one day. Analyzing the propolis component, the highest peak was found to be amyrin, a pentacyclic triterpene commonly found in propolis (Yam-Puc et al., 2019). Unfortunately, the other peaks present were not identified as MS spectra analysis showed match results lower than 50 percent. This can only mean that the present library installed in the instrument does not have the chemicals commonly present in propolis samples.

Lastly, in terms of extract yield, microwave extraction gives around 15.5 $\pm$ 0.5% yield, which is significantly higher than that obtained using ultrasonication (8.5 $\pm$ 0.9%) and soaking (7.7 $\pm$ 0.4 %) at a longer period, which is not significantly different. Also, there is no significant difference in terms of exposure time as ultrasonication has 7.9 $\pm$ 0.5% yield while soaking has 7.3 + 0.5%. This yield is consistent with the trends observed in a study by Trusheva et al. (2007) in terms of the extraction methods. However, the percent yield obtained is lower than earlier studies (Trusheva et al. 2007, Khacha-ananda et al., 2013) that utilized the same extraction methods.

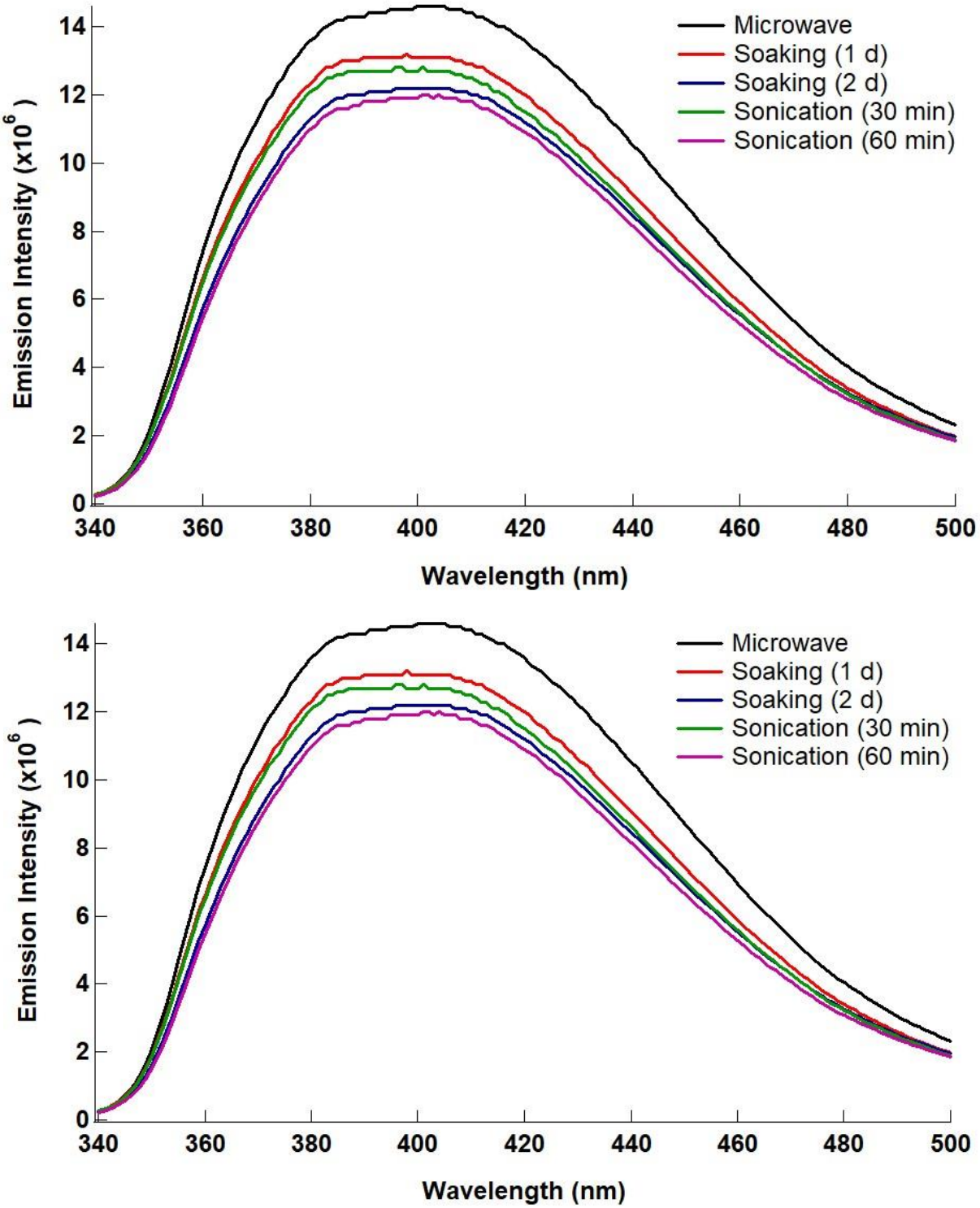

Figure 3. Emission spectra excited at 290 nm (top) and at 330 nm (below) of extracts obtained using different methods.

Results from the instrumental methods used to analyze extracts showed that microwave extraction has the highest signal, except for the emission spectra excited at 290 nm. This was followed by ultrasonication and, lastly, soaking. It is also shown that the longer the time for sonication and soaking, the more materials that can be extracted. This study is very similar to the one conducted by Trusheva et al. (2007) where different extraction methods (maceration, ultrasonication, and microwave) and 70% ethanol as a solvent were used. However, the efficiency of extraction was based on biological compounds in terms of the amount of extract, total flavone and flavonol, amount of flavanones and dihydroxyflavonols, and total phenolics content. Another study (Oroian, Dranca, & Ursachi, 2020), which used these three extracting methods, was also reported and performed the same analysis reported by Trusheva et al. (2007). Both studies reported that ultrasonication is better than microwave extraction and maceration in terms of higher extraction yield and selectivity (Oroian et al., 2020; Trusheva et al., 2007).

Since the extracts were not assayed, similar to those reported in the literature, the GC-MS is the only one that can be used to look for any differences among the extracts. Although major peaks can be found in all extracts (Figure 4), it can be noticed that peaks coming out before 24.00 mins are not as pronounced as that observed in ultrasonication and soaking methods. The peaks are also different in terms of peak height ratio among the three extraction methods. It has also been reported that microwave extraction results in the extraction of a large amount of unwanted waxes hence lower selectivity in terms of extracting bioactive compounds (Trusheva et al., 2007). The extract obtained from microwave extraction that showed the lowest emission signal excited at 290 nm might be due to a smaller amount of fluorescent compounds than the other extracts. It is also possible that the high temperature associated with the high power applied in microwave extraction leads to thermal degradation of fluorescent materials (Hamzah & Leo, 2015) that can be excited at 290 nm.

There have been numerous studies on the advantages of one extraction method over another extraction method. Microwave-assisted extraction set at 106°C, with 80% ethanol as a solvent and an extraction time of 15 min was found to be better than other techniques, such as maceration, heat reflux extraction (HRE), and ultrasound-assisted extraction in terms of shorter extraction time and lower volume of solvent needed (Pellati, Prencipe, Bertelli, & Benvenuti, 2013). Another study showed a higher percentage yield after extraction using maceration (18.1%) compared to sonication (15.7%); however, significantly greater antioxidant activity and flavonoid compounds were found for extract obtained by ultrasonication than those obtained by maceration (Khacha-ananda et al., 2013). Lastly, a combination of extraction methods results in better performance of the propolis extract. Antimicrobial activity against selected bacterial and fungal species showed propolis extract obtained after 1-day and 7-day shaking extraction followed by 20 min of ultrasonication are better than those obtained by just shaking extraction or ultrasonication alone (Pobiega, Krasniewska, Derewiaka, & Gniewosz, 2019).

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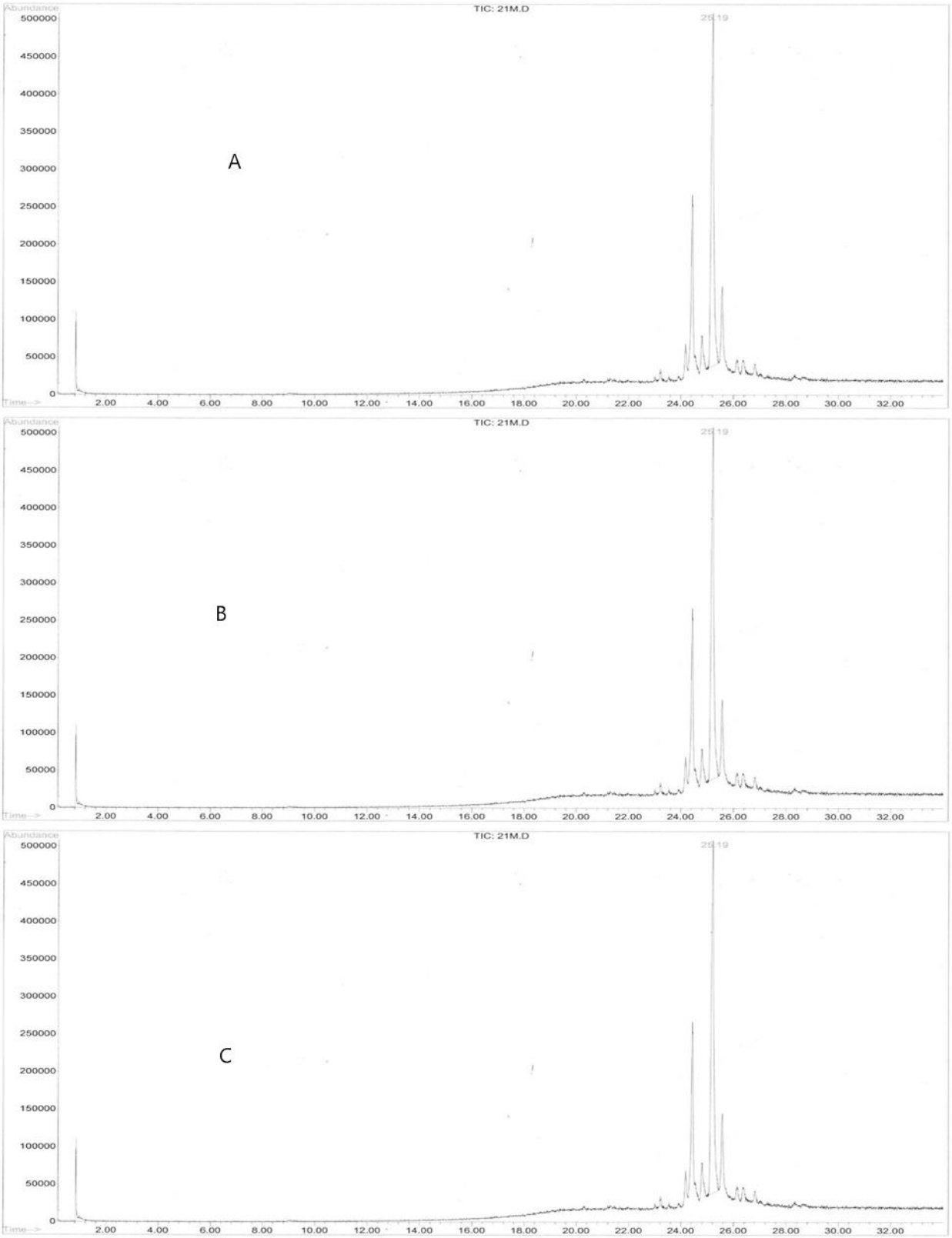

Figure 4. Total ion chromatogram of propolis extracts obtained using A) microwave extraction, B) ultrasonication at 60 mins, and C) soaking for two days.

Although both microwave and ultrasonication may pose potential degradation of materials, both methods showed rapid and better extraction over maceration. Microwave can be a good extraction method as long as the temperature was below 125°C, has a shorter time (15 min), and less solvent (sample to solvent ratio of 1:5 (w/v)) (Hamzah & Leo, 2015). Ultrasonication, on the other hand, has been hailed as the best alternative method to the traditional maceration and soaking method because it has a higher recovery yield, good selectivity of the target compound, less time consuming, and energy-saving method. It can also consider a green process.

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Conclusions

Different extraction methods were used in the sample preparation of bee propolis. The collected extracts were analyzed using different instrumental methods (GC-MS, absorbance, and fluorescence) to determine the performance of each extraction method. Extracts from microwave and ultrasonication methods were found to have higher signals than those obtained from the soaking method. This further confirmed the applicability of two methods as a rapid and improved extraction method over the traditional and time-consuming soaking method.

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List of abbreviations

GC-MS: gas chromatography-mass spectrometry

Compliance with Ethical Standards

Conflict of Interest

The authors have no conflict of interest.

Human and Animal Rights and Informed Consent

This study does not involve any human or animal subjects.

References

Abdullah, N. A., Ja'afar, F., Yasin, H. M., Taha, H., Petalcorin, M. I. R., Mamit, M. H., . . . Usman, A. (2019). Physicochemical analyses, antioxidant, antibacterial, and toxicity of propolis particles produced by stingless bee *Heterotrigona itama* found in Brunei Darussalam. *Heliyon, 5*, e02476.

Barbosa da Silva Cunha, I., Lucia Tiveron Rodrigues, M., Cesar Meurer, E., Bankova, V. S., Cristina Marcucci, M., Nogueira Eberlin, M., & Helena Frankland Sawaya, A. C. (2006). Effect of the maceration time on chemical composition of extracts of Brazilian propolis. *Journal of Apicultural Research, 45*(3), 137-144.

Camuri, I. J., Costa, A. B., Siuiti Ito, A., & Moreira Pazin, W. (2018). Optical absorption and fluorescence spectroscopy studies of Artepillin C, the major component of green propolis. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 198*, 71-77.

Hamzah, N., & Leo, C. P. (2015). Microwave-assisted extraction of Trigona propolis: The effects of processing parameters. *International Journal of Food Engineering, 11*(6), 861-870. doi:10.1515/ijfe-2015-0106

Hrobonova, K., Lehotay, J., Cizmarik, J., & Sadecka, J. (2013). Comparison HPLC and fluorescence spectrometry methods for determination of coumarin derivatives in propolis. *Journal of Liquid Chromatography & Related Technologies, 36*, 486-503.

Idrus, N. F. M., Yian, L. N., Idham, Z., Aris, N. A., Putra, N. R., Aziz, A. H. A., & Yunus, M. A. C. (2018). Mini review: Application of supercritical carbon dioxide in extraction of propolis extract. *Malaysian Journal of Fundamental and Applied Sciences, 14*(4), 387-396.

Khacha-ananda, S., Tragoolpua, K., Chantawannakul, P., & Tragoolpua, Y. (2013). Antioxidant and anti-cancer cell proliferation activity of propolis extracts from two extraction methods. *Asian Pacific Journal of Cancer Prevention, 14*(11), 6991-6995. doi:10.7314/apjcp.2013.14.11.6991

Maldonado, L., Marcinkevicius, K., Borelli, R., Gennari, G., Salomon, V., Isla, M. I., Vera, N. & Borelli, V. (2020). Differentiation of Argentine propolis from different species of bees and geographical origins by UV spectroscopy and chemometric analysis. *Journal of the Saudi Society of Agricultural Sciences, 19*(2), 185-191.

Oroian, M., Dranca, F., & Ursachi, F. (2020). Comparative evaluation of maceration, microwave and ultrasonic-assisted extraction of phenolic compounds from propolis. *Journal of Food Science and Technology, 57*(1), 70-78. doi:10.1007/s13197-019-04031-x

Pellati, F., Prencipe, F. P., Bertelli, D., & Benvenuti, S. (2013). An efficient chemical analysis of phenolic acids and flavonoids in raw propolis by microwave-assisted extraction combined with high-performance liquid chromatography using the fused-core technology. *Journal of Pharmaceutical and Biomedical Analysis, 81-82*, 126-132. doi:10.1016/j.jpba.2013.04.003

Pobiega, K., Krasniewska, K., Derewiaka, D., & Gniewosz, M. (2019). Comparison of the antimicrobbial activity of propolis extracts obtained by means of various extraction methods. *Journal of Food Science and Technology, 56*(12).

Silici, S., & Kutluca, S. (2005). Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. *Journal of Ethnopharmacology, 99*, 69-73.

Tomazzoli, M. M., Pai Neto, R. D., Moresco, R., Westphal, L., Zeggio, A. R. S., Specht, L., Costa, C., Rocha, M. & Maraschin, M. (2015). Discrimination of Brazilian propolis according to the seasoning using chemometrics and machine learning based on UV-Vis scanning data. *Journal of Integrative Bioinformatics, 12*(4), 279.

Trusheva, B., Trunkova, D., & Bankova, V. (2007). Different extraction methods of biologically active components from propolis: a preliminary study. *Chemistry Central Journal, 1*. doi:10.1186/1752-153x-1-13

Yam-Puc, A., Santana-Hernandez, A.A., Yah-Nahuat, P. N., Ramon-Sierra, J. M., Caceres-Farfan, M. R., Borges-Argaez, R. L., & Ortiz-Vazquez, E. (2019). Pentacyclic triterpenes and other constituents in propolis extractfrom Melipona beecheii collected in Yucatan, México. *Revista Brasileira de Farmacognosia 29 (2019) 358–363, 29*, 358-363.