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UPLC/FT-ICR MS-based high-resolution platform for determining the geographical origins of raw propolis samples

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Abstract

In this study, we demonstrate a high-resolution 15 Tesla Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry coupled with a reverse-phase ultra-performance liquid chromatography (RP-UPLC) system for determining the geographical origins of raw propolis samples. The UPLC/FT-ICR MS-based high-resolution platform was validated on the ethanol-extracted propolis (EEP) from various propolis raw materials originating from different countries (i.e., Argentina, Brazil, China, and Korea) to determine the geographical origins of the propolis and the origin-specific key compounds. Based on approximately 8000 molecular features extracted from UPLC/FT-ICR MS datasets, a partial least squares-discriminant analysis (PLS-DA) plot showed distinct separations among propolis samples from four different origins. Key propolis components contributing to the discrimination of Korean propolis from Brazilian and Chinese propolis were identified and classified into five subgroups (i.e., flavonoids, phenols, terpenoids, fatty acids, and others). This analysis revealed the characteristic features of the different propolis samples, and this analytical platform can be further used to determine the geographical origins and to assess the quality of the commercial products.

Keywords: Propolis, Ethanol extract, FT-ICR, Geographical origin, Quality control

Introduction

Propolis, the resinous substance collected by honey bees (*Apis mellifera*) from buds and resins of various plant species, has been used in folk medicine for many years because of its beneficial effects on various symptoms such as wounds, sore throats, and stomach ulcers (Burdock 1998; Huang et al. 2014). Propolis is composed of various inorganic minerals and organic compounds, including vitamins, amino acids, lipids, organic acids, and flavonoids (Huang et al. 2014). Among the chemical components of propolis, phenolic compounds, including flavonoids, are the major constituents and contribute largely to the pharmacological effects of propolis (Banskota et al. 1998).

Propolis is generally classified as poplar- or Baccharis-type according to its botanical origin. The poplar-type propolis, which originates from Populus buds that are primarily found in East Asian countries such as China and Korea, is known to have high phenolic contents because the poplar-type plants have high phenolic contents (Ristivojevic et al. 2015). Baccharis-type propolis originates from *Baccharis dracunculifolia*, which inhabits South American countries (i.e., Brazil, Bolivia and Argentina) (Park et al. 2004). The *Baccharis* spp. is a significant source of tropical Brazilian green propolis, and cinnamic acid is one of the most significant constituents in the Baccharis plants. Typically, artepillin C, a cinnamic acid derivative, is the representative phenolic compound present in Brazilian propolis. In addition, low flavonoid and phenolic contents and a high content of volatile compounds are the relevant characteristics of Baccharis-type plants.

Because the compositional diversity of propolis depends on the habitats of the source plants, propolis samples collected from different origins exhibit different characteristics or biological activities such as antitumor, antibacterial, antiviral, antifungal, anti-inflammatory, and antioxidant activities (Burdock 1998). For pharmaceutical and food

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applications, knowledge of the chemical composition of the propolis raw materials is necessary; however, to date, the quality of propolis has been examined using a subset of the known phenolic constituents. Therefore, more detailed information is required to better understand the complicated substances in propolis and their related functions. Zhou and colleagues reported an HPLC-based determination of the geographical origins of various Chinese propolis (Zhou et al. 2008). Sawaya and coworkers also demonstrated a simple propolis fingerprinting method using easy ambient sonic-spray ionization mass spectrometry (EASI-MS) to characterize their geographical origin (Sawaya et al. 2010). However, these analytical techniques were not suitable for identifying unknown compounds due to its low resolving power and poor mass accuracy. The development of LC/MS-based analytical methods has enabled to more reliably and accurately identify complicated propolis compounds. Pietta et al. showed that the analytical platforms based on atmospheric pressure chemical ionization (APCI) MS and HPLC combined with photodiode array detection can be utilized for reliably identifying a large number of propolis components (Pietta et al. 2002). Gardana and coworkers also applied LC-tandem MS system in order to determine phenolic compounds in different source origins (Gardana et al. 2007).

More recently, high-resolution mass spectrometry has been used to characterize phenolic compounds in propolis with high accuracy. Among various types of high-resolution mass analyzers including Q-Tof and Orbitrap, Fourier transform ion cyclotron resonance (FT-ICR) is mostly considered as a powerful tool to interpret elemental compositions of compounds of interest (Choi et al. 2018). The indisputable resolving power (full width at half maximum, FWHM: > 800,000 at m/z 200) and mass accuracy (< 1 ppm) of FT-ICR enable to identify chemical compositions of extremely complicated mixtures without chromatographic separation. The isotopic fine structure obtained from FT-ICR MS can also be used to determine the elemental formula (Shi et al. 1998; Nakabayashi et al. 2013). da Costa and coworkers showed the direct infusion electrospray ionization (ESI) FT-ICR mass spectrometry-based evaluation of phenolic compounds in plant leaves (da Costa et al. 2016). Gardana and colleagues also used UPLC/MS/MS and high-resolution FT-ICR MS systems to reliably detect propolis allergens in raw propolis materials and commercial formulations (Gardana and Simonetti 2011).

In the present study, ethanol-extracted propolis (EEP) prepared from various propolis samples originating from different countries were analyzed using high-resolution 15 T FT-ICR mass spectrometry coupled with a reverse-phase ultra-high-performance liquid chromatography (RP-UPLC) system to determine the geographical origins of the propolis (Fig. 1). This analysis showed the characteristic features of the different propolis samples and figured out the key molecules discriminating those geographical origins. Furthermore, these results could be further utilized to assess the quality of commercial products.

Materials and methods

Chemicals

Caffeic acid, *p*-coumaric acid, rutin, quercetin, cinnamic acid, kaempferol, chrysin, pinocembrin, caffeic acid phenethyl ester (CAPE), and artepillin C were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Preparation of ethanol-extracted propolis

Each raw propolis material (10 g) was mixed with ethanol (30 mL) and then incubated for 48 h at room temperature with vigorous shaking. The resultant extract was obtained by filtration. The extracts were finally prepared as 1% of the total flavonoid content based on the quercetin, which was determined as described in the literature (Chang et al. 2002). The resultant EEP samples were stored at − 20 °C until analysis.

UPLC analysis

Chromatography was performed on an ACQUITY HSS T3 column (1.8 μm, 2.1 × 100 mm, Waters, Milford, MA) using an ACQUITY UPLC™ system (Waters) by injecting 2 μL of each EEP sample. The column was maintained at 40 °C. The gradient condition of UPLC started at 10% (*v*/*v*) acetonitrile (ACN)/water with 0.1% formic acid and was maintained for 5 min at a flow rate of 300 μL/min; the ACN content was linearly ramped to 45%, where it was maintained at this flow rate for 10 min, then ramped to 90%, where the flow rate was maintained for 3 min. The column was washed with 98% ACN for 4 min and re-equilibrated with 10% ACN for 4 min for the next run. The effluent was monitored at a wavelength of 280 nm.

UPLC/FT-ICR MS analysis

Mass spectrometric analysis of the extracts was performed using a 15 T FT-ICR mass spectrometer (solariX XR^{TM} system, Bruker Daltonics, Billerica, MA) equipped with an electrospray ionization (ESI) source and an ACQUITY UPLC™ system. The UPLC conditions were the same as those previously described. The eluent was introduced into the mass spectrometer to acquire high-resolution MS spectra in positive ion mode within the mass range from m/z 150 to m/z 1000. The MS parameters of the positive ESI mass spectrometer were an ESI voltage of 4.5 kV, a drying gas flow rate of 8.0 L/min, a drying gas temperature of 220 °C, a skimmer

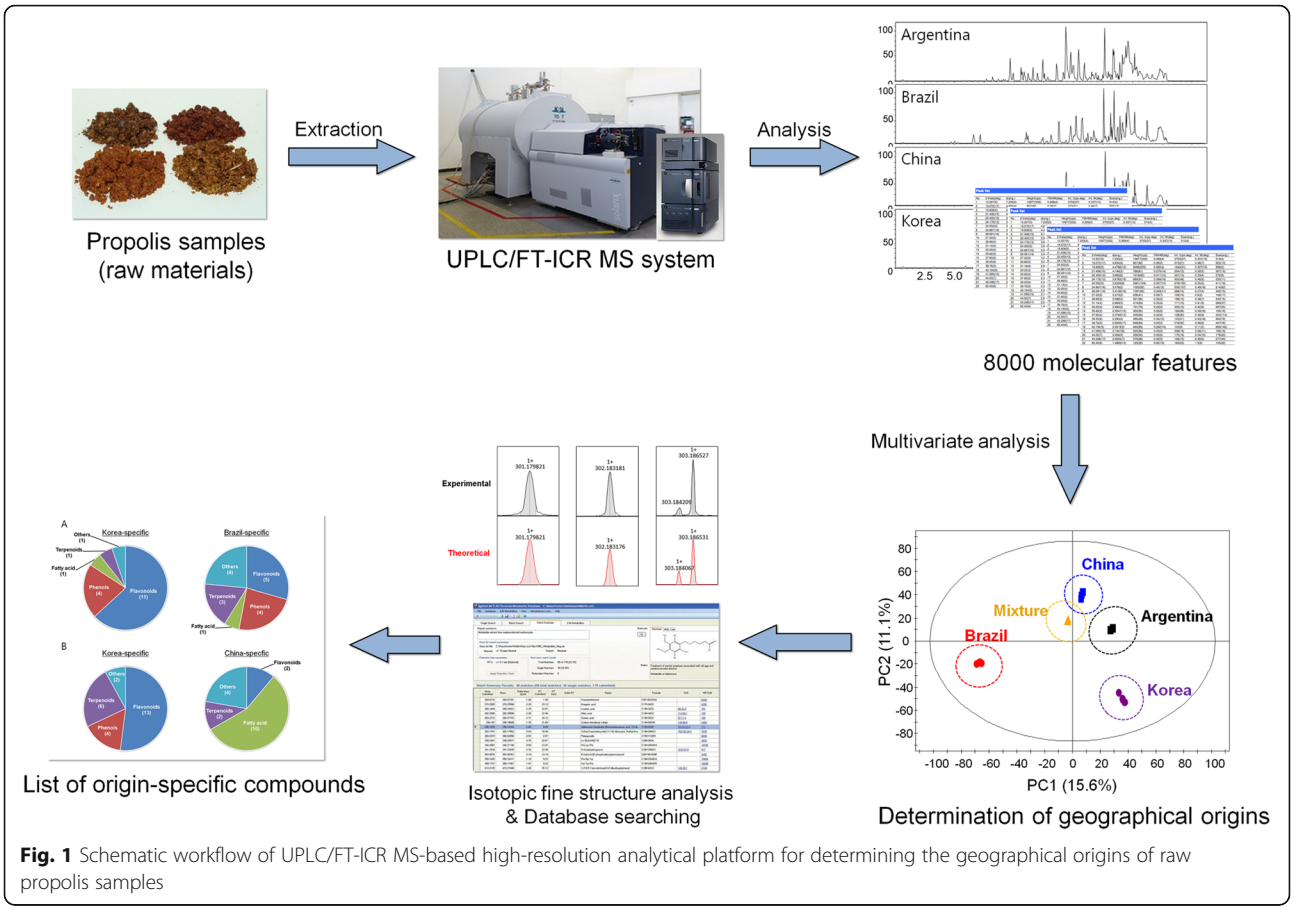

Fig. 1 Schematic workflow of UPLC/FT-ICR MS-based high-resolution analytical platform for determining the geographical origins of raw propolis samples

voltage of 15 V, a collision gas energy of − 3.0 V, an accumulation time of 50 ms, a transient length of 1.398 s, an acquisition size of 4 MB, and a scan number of 1, with a sine-bell apodization window function applied in the time-domain signal. External calibration was performed with quadratic regression using a 100 μg/mL arginine solution. Data acquisition was controlled by the ftmsControl 2.1 and HyStar 4.1 software (Bruker Daltonics), and data processing for selection of molecular features was performed using the DataAnalysis 4.4 program (Bruker Daltonics). Bucketing of the molecular features was processed using ProfileAnalysis 2.2 software (Bruker Daltonics).

Multivariate analyses

The multivariate data analyses such as principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) with Pareto scaling were performed using the SIMCA-P+ 12.0 software (Umetrics, Umeå, Sweden) to discriminate the different origins. Molecular features containing the retention time and mass-to-charge ratio were extracted from the UPLC/FT-ICR MS raw spectra of four propolis samples and one mixture sample to determine key compounds among different propolis samples. The datasets including the UPLC/FT-ICR MS-based molecular features were used for the multivariate analysis. After the PLS-DA analysis, the interesting variables were selected using the variable importance in projection (VIP) scores over 4 for further identification of key propolis components reflecting the origin. The molecular formulae and corresponding compounds of the selected variables were identified via METLIN (Smith et al. 2005), HMDB (Wishart et al. 2007), and other publicly available database searches.

Results and discussion

Quantitation of ten phenolic components in various EEP samples by UPLC analysis

To investigate the chemical composition of propolis originating from different countries, we prepared EEP using raw materials from four different countries (i.e., Argentina, Brazil, China, and Korea). Four EEP samples were analyzed using UPLC with a UV detector (280 nm), and typical chromatograms were obtained. In general, a diode array detector (DAD) has been used to measure a variety of flavonoids or phenolic compounds at a range of wavelengths (i.e., 200–500 nm) because those molecules have typical UV absorption maximum (Harnly et al. 2007;

Pellati et al. 2011; Zhang et al. 2013). Most flavonoids or phenols showed an absorption maximum at around 240–290 or 300–350 nm. However, those phenolic compounds were mostly been detected at a UV wavelength of 280 nm (Seal 2016; Yang et al. 2016), so the UPLC-based detection of the phenolic components in EEP samples was done at a wavelength of 280 nm in this study. As shown in Fig. 2, the chromatographic profiles of EEP samples from Asian countries (China and Korea) were similar (Fig. 2d, e), whereas the UPLC chromatograms of South American propolis (Argentina and Brazil) were apparently distinct from each other (Fig. 2b, c). The UPLC profile of Argentinean propolis appeared more similar to that of Korean propolis.

We selected ten phenolic compounds (i.e., coumaric acid, quercetin, cinnamic acid, kaempferol, chrysin, pinocembrin, CAPE, artepillin C, caffeic acid, and rutin), which are the most well-known constituents of propolis, and quantitated the compounds in the EEP samples using standards of these phenolic compounds. Each concentration of the key components in the EEP samples is shown in Fig. 2f. The major compound in Brazilian propolis was artepillin C, as previously determined, and the concentration of artepillin C in Brazilian propolis was sixfold higher than that in Chinese propolis; no artepillin C was observed in the Argentinean or Korean propolis. In addition to the comparison of the chromatographic profiles of phenolic compounds monitored at the wavelength of 280 nm, we also compared the EEP profiles using UPLC combined with high-resolution FT-ICR MS analysis.

In-depth analysis of EEP samples using high-resolution UPLC/FT-ICR MS

The investigation of key components contributing to the determination of the geographical origins of propolis is necessary for assessing the quality of propolis and for better use of propolis depending on the biological activities of the key components. Total UPLC/FT-ICR MS ion chromatograms were obtained for four EEP samples (Fig. 3a–d). To confirm the analysis of the EEP samples, the identification and intensity-based quantification were carried out from the raw data. The quantitative analysis results for the ten key phenolic species were compared with those obtained by UPLC analysis. The quantitative results via UPLC/FT-ICR MS agreed well with the UPLC data, supporting the reliability of the FT-ICR MS data. The comparative results are summarized in Table 1.

To elucidate the key compounds determining the geographical origins of propolis, the molecular features obtained via UPLC/FT-ICR MS analysis were investigated. Approximately 8000 molecular features, including the retention time and m/z values, were extracted from the LC/FT-ICR MS raw data for the four EEP samples. PLS-DA plots of the four propolis samples showed that the UPLC/FT-ICR data well separate the propolis samples by their geographic origin, while the Chinese and Korean propolis were not well resolved by the UPLC-based PLS-DA model, indicating that the selected quantitative information for the ten phenolic compounds in EEP samples was not sufficient to differentiate the samples' origins (Fig. 3e, f). Given these observations, we speculated that the analysis of a few selected

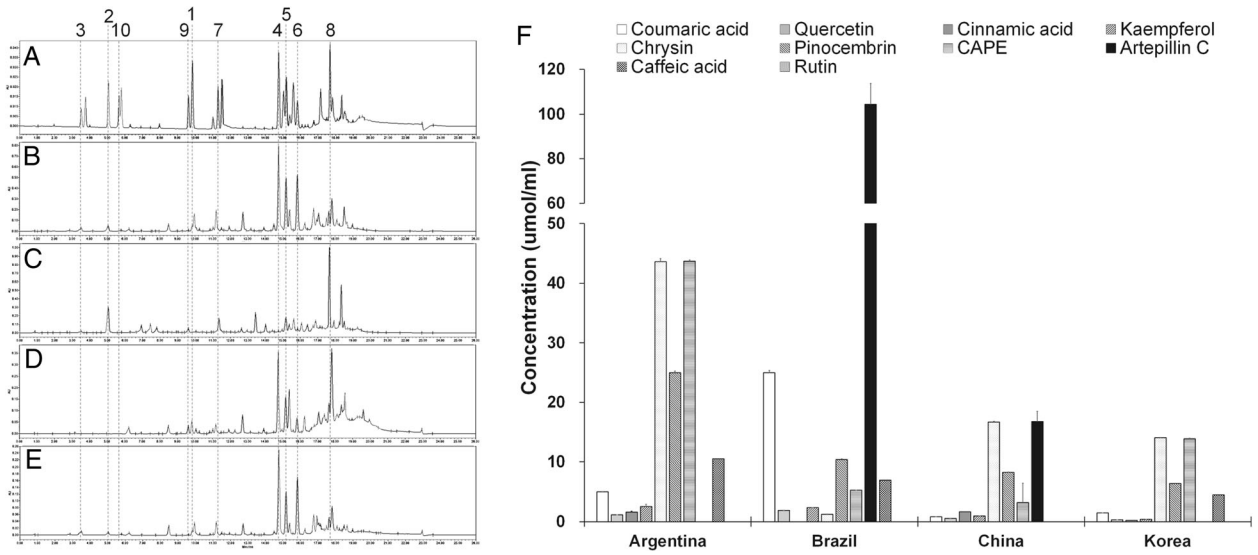

Fig. 2 UPLC chromatograms of EEP samples from South America (**b** Argentina, **c** Brazil) and Asia (**d** China, **e** Korea). **a** The chromatogram obtained from polyphenol standards. The numbers and the corresponding dotted lines indicate each phenolic standard. 1 = cinnamic acid; 2 = coumaric acid; 3 = caffeic acid; 4 = chrysin; 5 = pinocembrin; 6 = CAPE; 7 = kaempferol; 8 = artepillin C; 9 = quercetin; 10 = rutin. **f** Bar graph showing the concentrations of ten phenolic compounds in various EEP samples of different origins

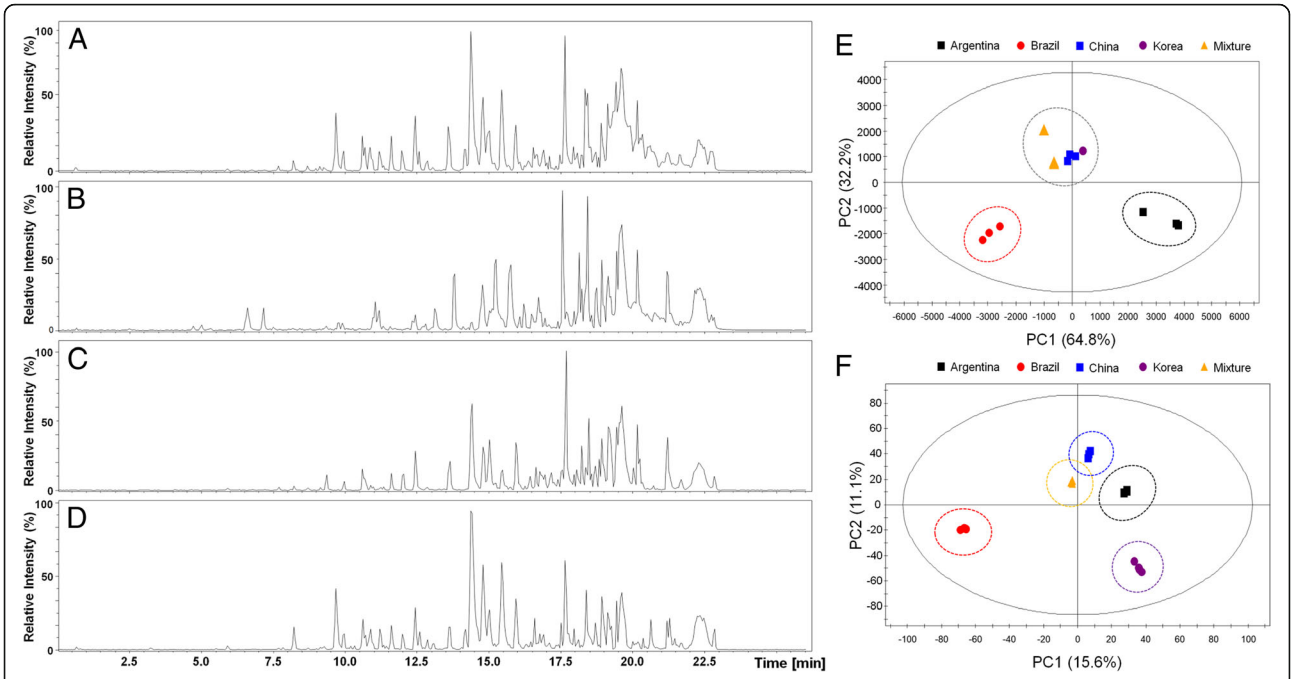

Fig. 3 Total ion chromatograms of EEP samples from **a** Argentina, **b** Brazil, **c** China, and **d** Korea, as obtained by UPLC/FT-ICR MS. PLS-DA models obtained using UPLC and UPLC/FT-ICR MS data. Quantitation data of ten phenolic compounds using UPLC was used for model (**e**), whereas selected key molecular features (VIP > 4, and fold change ≥ 2 or ≤ − 2 or observed only in each sample) obtained by FT-ICR MS analysis were used to generate model (**f**)

constituents in propolis might be insufficient for the quality control or quality assurance of the propolis products and the accurate determination of their source origins. Interestingly, Argentinean propolis differed from the Brazilian propolis, although the two countries are both located in Latin America, whereas China and Korea are in Asia; the Argentinean propolis was not included for further identification of key compounds.

Investigation of key components discriminating Korean propolis from Brazilian and Chinese propolis

As previously mentioned, the chemical composition of South American propolis differs substantially from that of East Asian propolis because of their botanical origins (i.e., poplar- and Baccharis-type plants). As shown in Fig. 3f, we observed good discrimination of Brazilian and Korean propolis; we therefore decided to determine the

Table 1 Comparisons of ten phenolic compounds identified from EEP samples using UPLC and UPLC/FT-ICR MS

No.	Compound	RT (min)	MW	UPLC[a]					UPLC/FT-ICR MS[b]				
				Brazil	China	Korea	K/B	K/C	Brazil	China	Korea	K/B	K/C
1	Cinnamic acid	9.8	148.05	N.D	1.7	0.2	N/A	0.1	N.D	N.D	N.D	N/A	N/A
2	Coumaric acid	4.8	164.05	25	0.8	1.5	0.1	1.9	O	O	O	Unique	Unique
3	Caffeic acid	3.5	180.04	7	N.D	4.5	0.6	N/A	O	O	O	Unique	Unique
4	Chrysin	14.5	254.06	1.2	16.7	14.1	11.8	0.8	O	O	O	Unique	0.8
5	Pinocembrin	14.9	256.07	10.4	8.3	6.4	0.6	0.8	O	O	O	1.1	1.1
6	CAPE	15.6	284.10	5.3	3.2	13.9	2.6	4.3	O	O	O	7.8	4.3
7	Kaempferol	11.0	286.05	2.3	1	0.4	0.2	0.4	O	O	O	-0.4	1.1
8	Artepillin C	17.7	300.17	104.5	16.8	N.D	N/A	N/A	O	O	O	1.8	2.2
9	Quercetin	9.5	302.04	1.9	0.6	0.3	0.2	0.5	O	O	O	0.3	2.0
10	Rutin	5.6	610.15	N.D	N.D	N.D	N/A	N/A	N.D	N.D	N.D	2.2	0.6

N.D not detected, *N/A* not applicable

[a]The values were the concentration of each component (μmoL/mL of EEP extract exhibiting 1% of the total flavonoid quantity) estimated using UPLC

[b]The values were the log2-transformed intensities of each component observed using FT-ICR MS

Table 2 Key compounds discriminating Korean and Brazilian propolis. The key compounds were selected from key molecular features with VIP > 4, and a fold change ≥ 2 or ≤ − 2 or observed only in each sample

No.	RT (min)	*m/z*	Formula	Error (ppm)	VIP	Expected compound	Feature			Class (function)
							Fold change (log2)	Korea only	Brazil only	
1	16.79	161.060	$C_{10}H_8O_2$	0.118	5.69	6-Methylcoumarin	N/A	O		Benzopyrone (flavoring agent)
2	8.26	209.081	$C_{11}H_{12}O_4$	0.053	7.84	Dimethyl caffeic acid	N/A	O		Phenols, caffeic acid derivatives
3	16.75	263.128	$C_{15}H_{18}O_4$	0.236	4.13	Artemisin	N/A	O		Sesquiterpene lactone (drug for malaria)
4	15.48	315.087	$C_{17}H_{14}O_6$	1.098	11.64	Rosinidin	N/A	O		Anthocyanidins
5	12.89	317.066	$C_{16}H_{12}O_7$	0.091	4.79	Rhamnetin	N/A	O		Flavonoids (anti-inflammatory activity)
6	16.90	329.102	$C_{18}H_{16}O_6$	0.352	4.62	Ophiopogonanone A	N/A	O		Homoisoflavonoids (antioxidant activity) (Wang et al. 2017)
7	10.83	338.139	$C_{20}H_{19}NO_4$	0.053	4.02	Columbamine	N/A	O		Alkaloids (antidiabetic agent)
8	14.45	343.118	$C_{19}H_{18}O_6$	0.691	5.27	Tetramethoxyflavone	N/A	O		Flavonoid (anticancer activity)
9	15.49	285.112	$C_{17}H_{16}O_4$	0.039	5.03	CAPE	7.8			Phenols, caffeic acid derivatives
10	12.48	285.075	$C_{16}H_{12}O_5$	0.025	7.18	Genkwanin	7.3			Flavonoids (anti-inflammatory activity)
11	18.43	295.227	$C_{18}H_{30}O_3$	0.698	11.48	Kamlolenic acid	4.9			Fatty acids, Kamala oil's glyceride
12	14.43	255.065	$C_{15}H_{10}O_4$	0.020	17.12	Chrysin	4.6			Flavonoids
13	17.98	357.133	$C_{20}H_{20}O_6$	0.319	6.89	(2S)-5,7,3′,4′-tetrahydroxy-6-(1,1-dimethylallyl) flavanone	4.2			Flavonoids (anticancer activity)
14	10.66	269.081	$C_{16}H_{12}O_4$	0.033	4.11	Strobochrysin	3.9			Flavonoids (anticancer activity)
15	17.69	269.081	$C_{16}H_{12}O_4$	0.331	5.89	Tectochrysin	3.7			Flavonoids (antibacterial activity)
16	9.99	317.066	$C_{16}H_{12}O_7$	0.003	4.83	Quercetin 3-methyl ether	3.7			Flavonoids (antioxidant activity)
17	10.90	273.076	$C_{15}H_{12}O_5$	-0.029	4.70	Butein	2.8			Chalconoids (anti-inflammatory activity)
18	14.83	257.081	$C_{15}H_{12}O_4$	0.144	10.44	Pinocembrin	1.1			Flavonoids (anticancer activity)
19	19.30	268.264	$C_{17}H_{33}NO$	0.276	4.52	(5-heptyl-6-methyloctahydroindolizin-8-yl) methanol	-1.7			Semiochemicals
20	20.27	310.310	$C_{20}H_{39}NO$	0.045	5.89	N-Hexadecanoylpyrrolidine	-2.0			Alkaloids
21	15.25	301.071	$C_{16}H_{12}O_6$	0.086	8.27	Kaempferide	-3.4			Flavonoids (antibacterial activity)
22	15.75	331.081	$C_{17}H_{14}O_7$	0.103	7.87	Quercetin 3,7′-dimethyl ether	-3.5			Flavonoids (vasorelaxant activity)
23	18.26	215.107	$C_{14}H_{14}O_2$	0.558	4.60	Dihydropinosylvin	N/A		O	Phenols (antifungal activity)
24	16.24	231.102	$C_{14}H_{14}O_3$	0.169	4.25	Aucuparin	N/A		O	Benzenoid (phytoalexin)
25	13.18	233.117	$C_{14}H_{16}O_3$	0.133	7.79	Pterosin E	N/A		O	Indanones (anti-inflammatory activity)
26	12.39	249.112	$C_{14}H_{16}O_4$	0.072	4.08	Prenyl caffeate	N/A		O	Caffeic acid derivatives (flavoring agent)
27	16.96	271.097	$C_{16}H_{14}O_4$	0.181	4.68	Haginin B	N/A		O	Flavonoids
28	18.78	273.258	$C_{20}H_{32}$	0.285	9.26	Casbene	N/A		O	Diterpenoids (antifungal activity) (Sitton and West 1975)

Table 2 Key compounds discriminating Korean and Brazilian propolis. The key compounds were selected from key molecular features with VIP > 4, and a fold change ≥ 2 or ≤ − 2 or observed only in each sample *(Continued)*

No.	RT (min)	*m/z*	Formula	Error (ppm)	VIP	Expected compound	Feature			Class (function)
							Fold change (log2)	Korea only	Brazil only	
29	17.59	301.180	$C_{19}H_{24}O_3$	0.000	17.75	Artepillin C	N/A		O	Cinnamic acid derivatives (anti-inflammatory activity)
30	12.48	311.128	$C_{19}H_{18}O_4$	0.077	5.04	Moracin N	N/A		O	Flavonoids
31	17.81	321.243	$C_{20}H_{32}O_3$	0.386	4.27	Hydroxyeicosatetraenoic acid (HETE)	N/A		O	Unsaturated fatty acids
32	9.86	363.180	$C_{20}H_{26}O_6$	0.080	4.51	Gibberellin A36	N/A		O	Diterpenoids (plant hormones) (Stowe and Yamaki 1959)
33	15.13	391.212	$C_{22}H_{30}O_6$	0.427	6.68	Neoquassin	N/A		O	Quassinoids (insecticidal activity) (Dou et al. 2008)
34	7.21	517.134	$C_{25}H_{24}O_{12}$	0.184	5.04	Apigenin 7-(2″,3″-diacetylglucoside)	N/A		O	Flavonoids (anti-cancer effect)
35	19.74	527.337	$C_{32}H_{46}O_6$	0.759	4.27	(24E)-3alpha-acetoxy-15alpha-hydroxy-23oxo-7,9(11),24-lanostatrien-26-oic acid	N/A		O	Triterpenes

No. 1 to 18: key compounds with a fold change of ≥ 2 and no. 19 to 35: key compounds with a fold change of ≤ − 2
Numbers in italic represent the compounds observed only in each sample

Table 3 Key compounds discriminating Korean and Chinese propolis. The key compounds were selected from key molecular features with VIP > 4, and a fold change ≥ 2 or ≤ − 2 or observed only in each sample

No.	RT (min)	*m/z*	Formula	Error (ppm)	VIP	Expected compound	Feature			Class (function)
							Fold change (log2)	Korea only	China only	
1	16.60	283.097	$C_{17}H_{14}O_4$	0.247	4.38	5,7-Dimethoxyisoflavone	N/A	O		Flavonoids (anabolic and anti-catabolic compound)
2	16.86	285.185	$C_{19}H_{24}O_2$	0.228	5.22	Alpha-terpinyl cinnamate	N/A	O		Cinnamic acid derivatives (flavoring agent)
3	17.89	315.196	$C_{20}H_{26}O_3$	0.216	4.71	Lanugone A	N/A	O		Terpenes
4	10.83	338.139	$C_{20}H_{19}NO_4$	0.053	5.01	Ficine	N/A	O		Flavonoidal alkaloid
5	15.60	403.118	$C_{24}H_{18}O_6$	0.275	4.72	Calomelanol I	N/A	O		Flavonoids (antioxidant activity)
6	13.10	421.128	$C_{24}H_{20}O_7$	0.085	4.70	5-O-Methylhoslundin	N/A	O		Flavonoids (antioxidant activity)
7	18.67	453.337	$C_{30}H_{44}O_3$	0.490	4.11	Tyromycic acid	N/A	O		Triterpenoids
8	18.62	475.378	$C_{30}H_{50}O_4$	0.587	4.90	Soyasapogenol A	N/A	O		Triterpenoids (antibacterial activity) (Rupasinghe et al. 2003)
9	20.67	489.394	$C_{31}H_{52}O_4$	0.366	9.99	1α-hydroxy-2β-(4-hydroxybutoxy) vitamin D3	N/A	O		Vitamin D_3
10	19.12	491.373	$C_{30}H_{50}O_5$	0.611	4.04	Meliantriol	N/A	O		Triterpenoids (insecticidal activity)
11	18.36	501.321	$C_{30}H_{44}O_6$	0.578	7.88	Ganolucidic acid D	N/A	O		Terpenoids
12	18.77	503.337	$C_{30}H_{46}O_6$	0.723	7.35	Esculentic acid	N/A	O		Triterpenoids
13	14.50	563.168	$C_{28}H_{31}ClO_{10}$	0.783	7.75	Physalin H	N/A	O		Physalins
14	16.79	161.060	$C_{10}H_8O_2$	0.000	7.05	6-Methylcoumarin	6.2			Benzopyrone (flavoring agent)
15	12.62	329.102	$C_{18}H_{16}O_6$	− 0.079	5.80	Alnetin	5.0			Flavonoids (antifungal activity)
16	12.48	285.075	$C_{16}H_{12}O_5$	0.088	6.81	Genkwanin	4.5			Flavonoids (anticancer activity)
17	14.22	249.112	$C_{14}H_{16}O_4$	− 0.012	8.24	Pyriculol	4.4			Phenylpropanoids (phytotoxin)
18	14.71	285.076	$C_{16}H_{12}O_5$	0.203	4.04	Acacetin	2.9			Flavonoids (anti-inflammatory activity)
19	14.45	343.118	$C_{19}H_{18}O_6$	0.344	6.04	Tetramethoxyflavone	2.7			Flavonoids (anticancer activity)
20	15.48	315.087	$C_{17}H_{14}O_6$	0.133	13.06	Rosinidin	2.4			Anthocyanidins
21	8.26	209.081	$C_{11}H_{12}O_4$	− 0.033	8.44	Dimethylcaffeic acid	2.1			Phenols, caffeic acid derivatives
22	12.89	317.066	$C_{16}H_{12}O_7$	− 0.035	5.18	Rhamnetin	2.0			Flavonoids (anti-inflammatory activity)
23	15.97	285.076	$C_{16}H_{12}O_5$	0.158	7.14	8-C-ethylgalangin	1.6			Flavonoids (anti-inflammatory activity)
24	17.98	357.133	$C_{20}H_{20}O_6$	0.381	6.51	Kievitone	1.4			Flavonoids (phytoalexin)
25	14.83	257.081	$C_{15}H_{12}O_4$	0.086	12.54	Pinocembrin	1.1			Flavonoids (anticancer activity)
26	14.38	315.253	$C_{18}H_{34}O_4$	0.184	4.78	Octadecanedioic acid	− 1.1			Long-chain fatty acids
27	16.94	643.419	$C_{36}H_{60}O_8$	0.575	4.09	Fasciculic acid A	− 1.2			Triterpenoids (calmodulin antagonist activity)
28	15.03	293.042	$C_{15}H_{10}O_5$	0.198	4.03	Apigenin	− 1.5			Flavonoids (anticancer activity)
29	18.45	299.258	$C_{18}H_{34}O_3$	0.478	4.65	Ricinoleic acid	− 1.5			Unsaturated fatty acids (anti-inflammatory activity) (Vieira et al. 2000)

Table 3 Key compounds discriminating Korean and Chinese propolis. The key compounds were selected from key molecular features with VIP > 4, and a fold change ≥ 2 or ≤ − 2 or observed only in each sample *(Continued)*

No.	RT (min)	*m/z*	Formula	Error (ppm)	VIP	Expected compound	Feature			Class (function)
							Fold change (log2)	Korea only	China only	
30	18.68	297.243	$C_{18}H_{32}O_3$	0.273	5.80	Dimorphecolic acid	− 1.8			Fatty acids (antibacterial activity) (Mundt et al. 2003)
31	20.27	310.310	$C_{20}H_{39}NO$	0.055	8.75	Oleoyl ethyl amide	− 2.4			Ethanol amide lipids (analgesic activity)
32	19.51	323.258	$C_{20}H_{34}O_3$	0.121	13.73	(±)5-6E,8Z,11Z-eicosatrienoic acid (HETrE)	− 2.9			Unsaturated fatty acids
33	18.86	339.253	$C_{20}H_{34}O_4$	0.363	9.83	Aphidicolin	− 3.1			Tetracyclic diterpene antibiotics (Antiviral activity)
34	17.52	463.139	$C_{26}H_{22}O_8$	0.555	5.17	Artomunoxanthentrione epoxide	− 3.2			a potential biomarker
35	19.68	325.274	$C_{20}H_{36}O_3$	0.357	6.86	Alchornoic acid	− 3.7			Fatty acids
36	18.08	341.269	$C_{20}H_{36}O_4$	0.325	7.18	Thromboxanoic acid skeleton	− 3.7			Fatty acids
37	19.86	399.290	$C_{26}H_{38}O_3$	0.073	5.54	Ximaosteroid C	− 3.9			Steroids
38	19.68	606.546	$C_{38}H_{71}NO_4$	0.659	6.36	Cer(d16:2(4E,6E)/22:1(13Z)(2OH))	− 4.3			Fatty acids
39	9.39	287.091	$C_{16}H_{14}O_5$	0.066	6.66	Gummiferol	N/A		O	Polyacetylenic diepoxides (anticancer activity)
40	18.21	321.242	$C_{20}H_{32}O_3$	0.159	4.01	15-Hydroxyeicosatetraenoic acid (HETE)	N/A		O	Eicosanoids
41	17.83	343.284	$C_{20}H_{38}O_4$	0.303	6.51	Eicosanedioic acid	N/A		O	Eicosanoids
42	17.27	389.102	$C_{23}H_{16}O_6$	0.239	4.12	Pamoate	N/A		O	Naphthoic acid derivative
43	16.93	421.128	$C_{24}H_{20}O_7$	0.468	7.29	Glabratephrin	N/A		O	Flavonoids (antifeedant activity)

key propolis components discriminating Korean and Brazilian propolis. In addition, the molecular features obtained from Korean and Chinese propolis samples were investigated to figure out the key molecules that differentiate these two Asian propolis varieties. To determine the key components distinguishing the different propolis samples among 8164 molecular features obtained from UPLC/FT-ICR MS analysis, approximately 100 features with VIP values greater than 4.0 and fold changes above or below 2 or fold changes observed only in each sample were selected for further investigation.

Finally, we obtained 35 and 43 key components discriminating Korean and Brazilian propolis and Korean and Chinese propolis, respectively. All the significant compounds contributing to the geographical determination of Korean and Brazilian propolis are summarized in Table 2, while the key compounds discriminating Korean and Chinese propolis are listed in Table 3. Identification of the chemical formulae was possible from the accurate molecular masses and their experimental isotopic fine structures (IFSs) of all metabolites. Prediction of the compound was made by searching METLIN and HMDB databases. The extracted ion chromatograms, the corresponding mass spectra, and IFSs of the proposed compounds were summarized in the Additional file 1. Then, the proposed key compounds could be divided into five subgroups based on their chemical class (i.e., flavonoids, phenols, terpenoids, fatty acids, and others). The pie charts of the chemical classes of the key compounds discriminating Korean and Brazilian propolis showed that the flavonoids were the most abundant (61%), followed by phenols (22%), terpenoids (6%), others (6%), and fatty acids (5%) in Korean propolis; meanwhile, the most frequently assigned class was flavonoids (29%) and others (24%), followed by phenols (23%), terpenoids (18%), and fatty acids (6%) in Brazilian propolis (Fig. 4a). Key propolis classes for differentiating Korean and Chinese propolis were divided by flavonoids (52%), terpenoids (24%), phenols (16%), and others (8%) in Korean propolis, whereas Brazil-specific compounds were classified by fatty acids (56%), others (22%), flavonoids (11%), and terpenoids (11%) (Fig. 4b).

In particular, flavonoid species seemed a most significant contributor differentiating Korean propolis from Brazilian and Chinese propolis. In Brazilian propolis, di- and tri-terpenoids including Gibberellins, a kind of plant hormones that affect plant growth and developments (Hedden and Sponsel 2015), were significantly observed. Those key components would strongly reflect their botanical differences. Between two Asian propolis origins (Korea versus China), flavonoids and terpenoids species were reliably identified as Korea-specific key molecules, but Chinese propolis appears to possess fatty acid class compounds. The unsaturated fatty acids including

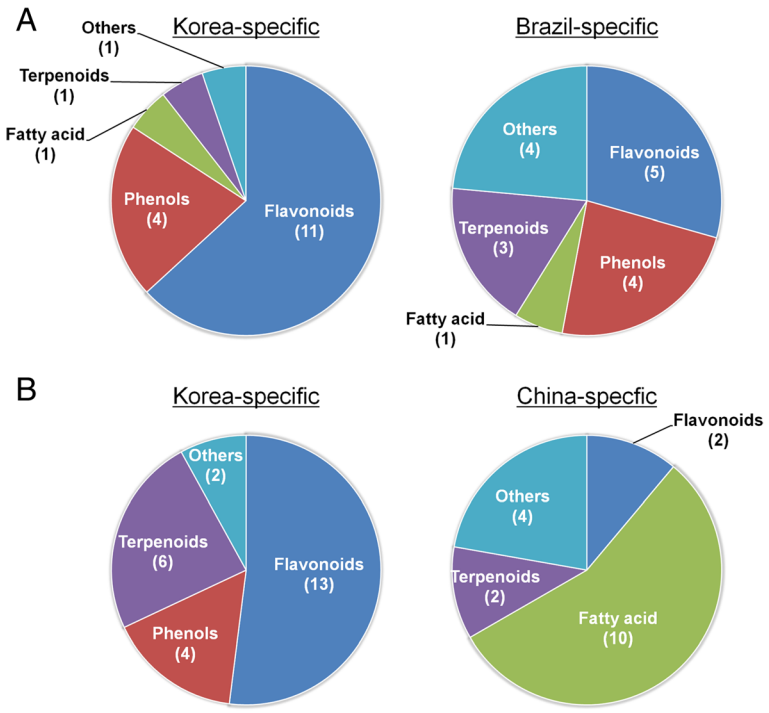

Fig. 4 Pie charts showing compositional differences of key components discriminating **a** Korean and Brazilian propolis and **b** Korean and Chinese propolis

ricinoleic acid and eicosanedioic acid, distinctly found in the Chinese propolis could also be utilized as dietary sources as the unsaturated fatty acids present in propolis are considered to be a good source to the diet (Rebiai et al. 2017).

Conclusions

Here, a high-resolution 15 T FT-ICR MS equipped with UPLC system was introduced to investigate the key phenolic compounds responsible for determining the geographical origin of propolis (i.e., Korea versus Brazil and Korea versus China). We then proposed 16 flavonoids, 8 phenolic compounds, 4 terpenoids, 2 fatty acids, and 5 others in Korean and Brazilian propolis, while 15 flavonoids, 10 fatty acids, 8 terpenoids, 4 phenols, and 6 others were proposed in Korean and Chinese propolis. These key compounds can be used as chemical markers to classify and identify the geographical origins of propolis. In the pharmaceutical and food industries, those key propolis components could play a significant role in distinguishing high-quality propolis from inferior or fake propolis. Moreover, the information of the key propolis constituents can be utilized to verify the effects of propolis in the prevention and treatment of various symptoms and diseases. Further characterization and biological evidence of the key compounds could focus on the evaluation of the compounds for quality assessment of propolis products and for standardization of propolis.

The chemical composition of propolis is strongly linked to vegetation present in the collection area as well as collecting periods and climates (Bankova et al. 2000). However, changes in vegetation on the Chinese continent are as great as changes from southern Argentina to northern Brazil. Yang and coworkers showed that the compositions and concentrations of aroma-active components collected from 23 regions of Chinese continent were significantly different (Yang et al. 2010). Therefore, a more detailed investigation of plant species close to the production area of propolis even in the same country is needed to determine the source origin more accurately. Although the results were obtained with only four different propolis samples from each origin and the key propolis compounds were not confirmed by tandem MS analysis, this UPLC/FT-ICR MS-based high-resolution platform showed the potentials for the comprehensive analysis of highly complicated bioactive compounds.

Furthermore, it could also be used to investigate novel propolis compounds with biological activities and are helpful for the pharmaceutical and food industries, which require an understanding of the chemical composition, botanical origin, and biological properties of propolis.

Additional file

Additional file 1: Extracted ion chromatograms, FT-ICR MS spectra and isotopic fine structures of key compounds discriminating Korean and Brazilian propolis, proposed in Table 2, and discriminating Korean and Chinese propolis, proposed in Table 3. (PDF 1066 kb)

Abbreviations

ACN: Acetonitrile; APCI: Atmospheric pressure chemical ionization; CAPE: Caffeic acid phenethyl ester; EEP: Ethanol-extracted propolis; ESI: Electrospray ionization; FT-ICR: Fourier transform ion cyclotron resonance; IFS: Isotopic fine structures; PCA: Principal component analysis; PLS-DA: Partial least squares-discriminant analysis; RP-UPLC: Reverse-phase ultra-performance liquid chromatography; VIP: Variable importance in projection

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Availability of data and materials

Research data have been provided in the manuscript and supporting information file.

Authors' contributions

CHK and KSJ are involved in research design and execution of the experiments. MYK, SWL, and KSJ are involved in data interpretation and supervision of the studies, and all authors contributed to writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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