Commercial Propolis Liquid Products: Comparison of Physicochemical Properties and Antioxidant and Antimicrobial Properties

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Summary

Propolis is a resinous substance, which is well-known for its functional properties (e.g. antioxidant, antimicrobial and anti-inflammatory) and collected by honeybees from various plants. Due to its various health beneficial properties, propolis is widely used in many products (e.g. foods, beverages and toothpastes) and liquid propolis extract products are also commercially available as natural healthy supplements. Raw propolis has been broadly investigated, while, there has been much less research on the physicochemical and functional properties of commercial liquid propolis products. This study was thus aimed to evaluate and compare 20 commercial propolis liquid products manufactured in 4 different countries (Australia, China, Korea and New Zealand), in terms of physiochemical properties (e.g. water and ethanol miscibility, colour, pH), chemical composition (e.g. total phenolic and total flavonoid contents) and functional properties (e.g. antioxidant capacity and antimicrobial activity). Besides, all propolis samples were analysed for the detection of heavy metal (e.g. lead, cadmium, and arsenic) and rare earth elements in order to determine the safety and quality of propolis products. Also, the content of salicin in propolis was measured as an indicator of the adulteration of propolis with poplar tree gum.

The visual colour of liquid propolis products varied from dark brown, red to green. Almost all commercial propolis samples analysed in this study were more soluble in ethanol than in water, except a propolis sample containing Tween 20 (emulsifier). Most propolis samples were also acidic with pH < 5, whereas, the Korean propolis samples containing potassium carbonate had alkaline pH values. The analysed total flavonoid (TF) content of 19 propolis products matched their labelled values specified on their product packaging. However, some unexpected results were obtained with the TF content being measured to be higher than the total phenolic (TP) content from 4 Korean propolis samples, in which salicin was also detected. This indicates that those 4 propolis products might have been adulterated with poplar tree gum. In terms of the functional properties of propolis, it was found that their antioxidant activity highly corresponded to the TP and TF contents. On the contrary, there was no linear correlation between TP or TF content and antimicrobial activity of the propolis products. Propolis products showed a greater effect on Gram-positive bacteria (*S. aureus* and *B. cereus*) than the Gram-negative

bacterium (*E. coli*). Among all propolis samples from the different regions, the propolis samples from New Zealand had a relatively higher TP and TF content and also showed a higher antimicrobial activity than the propolis samples from the other countries. Nevertheless, the content of heavy metal elements (As and Pb) detected was relatively much higher in New Zealand propolis products than that from the other countries. On the other hand, liquid propolis products from Australia contained less heavy metal elements and had the lower possibility of adulteration by poplar tree gum and the stable antioxidant and antimicrobial activities, which seemed to be a better choice among the 20 samples studied in this study.

In conclusion, since there is no proper criterion to monitor the quality of propolis, it is necessary to develop a series of indices to evaluate the commercial liquid propolis products, for example, sensory (colour and smell), chemical composition (TP and TF contents), functional properties (antioxidant and antimicrobial activities) and safety properties (heavy metal elements and adulterations).

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Chapter 1. Introduction

Propolis is a well-known resinous substance also called bee glue, which has a dark brown colour and is produced by honeybees from various plants buds or resins (Silici & Kutluca, 2005; Bankova et al., 2019). It is generally used to repair honeycombs and stabilise the moisture and temperature of beehives by honeybees (Zabaiou, Fouache, Trousson, Baron, & Zellagui, 2017; Bankova et al., 2019). In general, propolis comprises of plant resins (50%), beeswax (30%), essences (10%), pollens (5%), and other organic constituents (5%) (Gómez-Caravaca, Gómez-Romero, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2006; Falcão et al., 2010; Huang, Zhang, Wang, Hu, & Li, 2014).

Propolis can be generally classified into two types, such as Brazilian type and European type, according to the botanic origin of propolis (Markham, Mitchell, Wilkins, Daldy, & Lu, 1996; Xu, Luo, Chen, & Fu, 2009). The Brazilian type propolis is basically from the countries located in tropical zone, including Amazon, Brazil, Cuba, Tunisia and so forth (Markham et al., 1996; Xu et al., 2009). The European type is also named as poplar type, as this type of propolis is mainly collected from poplar tree which is widely grown not only in Europe, but also in Africa, China, Korea, New Zealand and other temperate area around the world (Markham et al., 1996; Bankova, de Castro, & Marcucci, 2000).

The chemical composition and some physical characteristics of propolis vary according to the source of plant and the place of region (Silici & Kutluca, 2005; Zabaiou et al., 2017). In terms of chemical constituents, poplar propolis contains large amounts of flavones and flavanones, compared to phenolic acid and their esters (Markham et al., 1996; Bankova et al., 2019). On the other hand, the Brazilian propolis is comprised of a high proportion of *p*-coumaric acid derivatives (Markham et al., 1996; Xu et al., 2009; Huang et al., 2014). However, the core composition of propolis is phenolics, especially flavonoids (Silici & Kutluca, 2005; Viuda-Martos, Ruiz-Navajas, Fernandez-Lopez, & Perez-Alvarez, 2008; Xu et al., 2009; Huang et al., 2014; Oryan, Alemzadeh, & Moshiri, 2018a).

Propolis is renowned for its functional properties, including antioxidant (Toreti, Sato, Pastore, & Park, 2013; Sforcin, 2016), antimicrobial (Kumazawa, Hamasaka, & Nakayama, 2004; Silici & Kutluca, 2005), anti-inflammatory (Kumazawa et al., 2004;

Toreti et al., 2013), and anticancer activity (Kumazawa et al., 2004). Studies have shown a correlation between the phenolics contents and some functional properties of propolis, such as antioxidant property (Zunini et al., 2010). Due to its identified functional properties, propolis has been broadly applied to many products, including foods, beverages, toothpaste and etc. (Archaina, Rivero, Sosa, & Coronel, 2015; Kubiliene et al., 2015; Xavier et al., 2017).

However, raw propolis needs to be purified before being applied to commercial products. Liquid extracts are the most common commercialised products that can be found in the market (Gómez-Caravaca et al., 2006; Gardana, Scaglianti, Pietta, & Simonetti, 2007; Xu et al., 2009). Ethanol, propylene glycol, water, and edible oils have been used as solvents to extract and preserve the bioactive compounds from propolis, as they are non-toxic to human (Hu et al., 2005; Kubiliene et al., 2015; Sforcin, 2016).

Cvek et al. (2008) indicated that propolis tends to be a good source that contains undesirable trace elements and heavy metal elements, as it is from various plants in different region. Some previous studies have shown that the amounts of elements detected in propolis are related to its geographical origin (region) (Bonvehí & Bermejo, 2013; Formicki, Gren, Stawarz, Zysk, & Gal, 2013). Nevertheless, the concentration of metals found in commercial propolis products might also be influenced by process conditions (e.g. types of solvent, extraction procedures, and extraction time) (Tosic, Stojanovic, Mitic, Pavlovic, & Alagic, 2017). However, up to date, there is less research focusing on the element content in commercial propolis products, on which more attention is needed.

By considering food fraud, due to the similar physical and chemical properties of polar tree gum to propolis, some studies reported that low-cost poplar tree gum was mixed with commercial propolis products to reduce the manufacturing cost and make more profit (Zhang, Ping, Wang, Huang, & Hu, 2015). Poplar tree gum is extracted from populous buds, which has the similar colour and chemical compositions to the poplar type propolis (Vardar-Ünlü, Silici, & Ünlü, 2008; Zhang, Zheng, Liu, & Hu, 2011). To detect its adulteration, some phenolic glycosides (e.g. salicin) which is unique in poplar tree gum can be analysed and used as a marker to identify whether propolis products have been adulterated or not (Pearl & Darling, 1971; Zhang et al., 2011; Zhang et al., 2015).

The physical and chemical properties of raw propolis have been broadly studied (Markham et al., 1996; Falcão et al., 2010; Huang et al., 2014; Archaina et al., 2015; do Nascimento et al., 2018), while studies on commercial propolis products are scant. Therefore, this project aimed to focus on investigating and comparing the properties and the quality of liquid commercial propolis products produced in 4 different countries (Australia, China, Korea, and New Zealand) by analysing the physiochemical properties (e.g. water and ethanol miscibility, colour, and pH), chemical composition (e.g. total phenolic and total flavonoid contents), functional properties (e.g. antioxidant and antimicrobial properties), and elements (e.g. heavy metal elements and rare earth elements), and investigating the adulteration (e.g. presence of salicin) of propolis. In this project, 20 different commercial liquid propolis products were used among which 2 were produced in Australia, 2 in China, 10 in Korean, and 6 in New Zealand.

Chapter 2. Literature Review

2.1 Introduction

The word propolis derived from Greek, in which “pro” means “entrance to”, and “polis” stands for “city”, demonstrating its defensive function in hive (Toreti et al., 2013; Zabaiou et al., 2017; Bankova et al., 2019). It is a renowned resinous material also called bee glue, which is collected by honeybees from various plants organs, including buds, saps, resins and other sources (Silici & Kutluca, 2005; Bankova et al., 2019). Due to the physical properties and composition of propolis, it is used to repair the comb and stabilize the temperature and moisture in the hive by bees (Zabaiou et al., 2017; Bankova et al., 2019) and also to cover cracks formed by other invaders (Sforcin, 2016).

Propolis is generally comprised of plant resins (50%), beeswax (30%), aromatic oil (10%), pollens (5%), and other organic constituents (5%) (Huang, Zhang, Wang, Li, & Hu, 2014). The composition of propolis differs from the source of plant and the place of origin (Silici & Kutluca, 2005; Zabaiou et al., 2017), which significantly influence physical, chemical and functional properties of propolis. Generally, the density of propolis ranges from 1.11-1.14 kg/m^3, and its melting point is in the range of 80 to 105ºC (Bogdanov & Bankova, 2011). Propolis can be classified into two groups by their botanic regions, which are European type and Brazilian type (Markham et al., 1996). The European type is also called poplar type, as poplar trees are the dominant plant of propolis in Europe, China, Korea, Africa, Australasia and other temperate area all over the world. The Brazilian type is not only from Brazil but also from Cuba, Amazon, Tunisia and some other countries located in the tropical zone with lack of poplar trees (Markham et al., 1996; Bankova et al., 2000; Xu, Luo, Chen, & Fu, 2009). This means that the chemical substances in propolis may vary from different locations, their main compounds, however, have been identified to be flavonoids, cinnamic acids, terpenes, phenolic acids, and aromatic acids (Silici & Kutluca, 2005; Popova et al., 2007; Huang et al., 2014; Oryan, Alemzadeh, & Moshiri, 2018b).

Due to its chemical compositions, several studies have reported that propolis has various functional properties, including antioxidant (Toreti et al., 2013; Sforcin, 2016), anti-bacteria (Kumazawa et al., 2004; Silici & Kutluca, 2005; Popova et al., 2007) , antiseptic

(Toreti et al., 2013; Sforcin, 2016), anti-inflammatory (Kumazawa et al., 2004; Toreti et al., 2013; Sforcin, 2016), and anticancer (Kumazawa et al., 2004). These properties give rise to the consumption of propolis as a functional ingredient or supplement in some medicines (Viuda-Martos et al., 2008; Toreti et al., 2013), foods, candies, beverages, toothpaste, liquid propolis products, and other commercial products (e.g. cosmetics and animal feeds) (Kumazawa et al., 2004). Over 200 new products containing propolis have been developed and launched in the world market over the past seven years (Agriculture and Agri-Food Canada, 2017). Also, in term of value addition, the products which claimed containing propolis are more expensive than other bee products. Hence, it is essential to find methods to evaluate the quality or the quantity of functional components of propolis products. However, there is no clear standard to evaluate and characterize the chemical and functional properties of those products (Alvarez-Suarez, 2017; Bankova et al., 2019), because of the variability of propolis' chemical substances as mentioned above.

In terms of trace elements in propolis, although the composition is negligible, Cvek et al. (2008) suggested that the raw propolis could be a source of trace elements as well as some heavy metal elements, since it is a collected by honeybees from diverse plants growing in different origins under various conditions. In the same vein, Gong, Luo, Gong, Gao, and Xie (2012), Bonvehí and Bermejo (2013), and Formicki et al. (2013) noted in their research that elements in propolis are related to their geographic region. However, the concentration of metal elements in commercial propolis depends on the type of solvent applyed to extract the raw propolis (Tosic et al., 2017). However, it should be noted that so far, there is limited research analysing elements in commercial propolis from different regions.

With regard to adulteration, due to the scarcity of resources in nature and the high production cost of propolis, some studies indicate that a low cost polar tree gum that has similar chemical composition and functional properties to propolis was mixed in some poplar type propolis products, *viz.* some companies used the cheaper material to adulterate propolis products to make more profit. (Zhang et al., 2011; Zhang et al., 2015). Polar tree gum is the extraction of Populus buds. Although it has similar smell, colour, and chemical compounds, including flavonoids, phenolics and cinnamic acid derivatives, to the polar type propolis (Vardar-Unlu, Silici & Unlu, 2007; Zhang et al., 2011), it also contains some phenolic glycosides, including salicin and its derivatives, which are not found in

propolis, as these compounds have been hydrolysed by enzymes secreted by honey bees during propolis production (Pearl & Darling, 1971; Zhang et al., 2011; Zhang et al., 2015). Therefore, these unique compounds can be selected as an indicator to identify the adulteration of propolis. However, this has not been well investigated.

2.2 Extractions of Propolis

2.2.1 Preparation of Propolis Extracts

Propolis is collected from propolis traps which are plastic nets with small holes through which bees drop propolis (Bankova et al., 2019). Freezing the traps is likely to be a better way to harvest propolis, since it can make propolis hard and brittle (Bogdanov & Bankova, 2011; Kubiliene et al., 2015; Bankova et al., 2019). And then it could be easily grounded into powder.

Figure 2.1 Harvest of propolis from nets (Bogdanov & Bankova, 2011)

2.2.2 Different Extraction Solvents

Crude propolis has to be purified before its commercialisation. The main purpose of the extraction is to purify propolis by removing impurities (e.g. beeswax) and activating the functional components, including polyphenolic fractions (Bankova et al., 2019). Ethanol, propylene glycol, water and some oils have been commerically used as extraction solvents

as they are non-toxic and safe for human consumption (Krell, 1996; Kubiliene et al., 2015; Sforcin, 2016).

Ethanol extracted propolis (EEP)

Ethanol extracted propolis (EEP) tends to be widely used in commercial liquid propolis products, as it is more likely to be purified and contain more functional compounds (Kubiliene et al., 2015; Sforcin, 2016). Krell (1996) indicated that the maximum ratio of propolis-ethanol solution to get a high efficiency of the propolis extraction is 3:10 (w/w). This was supported by Ildenize et al. (2004), Gómez-Caravaca et al. (2006), and Monroy et al. (2017) that the total phenolic (TP) and total flavonoid (TF) contents were the highest when using ethanol as an extraction solvent among non-toxic extraction solvents. Also, 60-80% ethanol was shown to have a higher extraction capacity of the main functional compounds (e.g. flavonoids) (Ildenize et al., 2004; Gómez-Caravaca et al., 2006; Bogdanov & Bankova, 2011; Ramanauskien, Inkeniene, Petrikait, & Briedis, 2013; Monroy et al., 2017), and the best extraction of some phenolic compounds was obtained when using 70% and 95% ethanol solutions (Bogdanov & Bankova, 2011). The ethanol extract of propolis can be used in many applications, including food and cosmetic products (Krell, 1996). However, the use of propolis in food applications is limited by its undesirable smell and taste during consumption. It cannot also be accepted by some people with alcohol intolerance.

Glycol extracted propolis (GEP)

Glycol (propylene glycol, E1520) is often used to extract propolis to avoid some disadvantages associated with EEP and improve the water extraction dissolution (Sforcin, 2016). The extraction process is similar to using ethanol as the solvent, but with a higher temperature and lower ratio (Krell, 1996).

Water extracted propolis (WEP)

Water is the most commonly used sovlent in the food industry. The perparation of WEP may take a few days by extracting raw propolis(Krell, 1996). During the extraction process, because of the low water solubility of some bioactive compounds, heat treatment can be applied to enhace the efficiency of extraction. Also, some compounds cannot be fully soluble in water, therefore, heating the water and adding propylene glycol are used to make better extraction (Kubiliene et al., 2015; Sforcin, 2016). Moreover, ultrasound can also be used to help reduce the time of extraction, thereby more efficient (Sforcin,

2016). However, the yield of TP and TF in WEP is still lower than the former two methods (Gómez-Caravaca et al., 2006; Kubiliene et al., 2015; Ramanauskiene et al., 2013). For water solubility, only less than 10% of propolis' weight can be dissolved (Bankova et al., 2019). It has also been found that WEP has a lower antimicrobial activity against some typical microbes, including *Staphylococcus aureus, Bacillus subtilis,* and *Escherichia coli*, when compaired with EEP and GEP (Ramanauskiene et al., 2013). This could be related to the fact that the solubility of biologically active compounds in water is low (Kubiliene et al., 2015).

Oil extracted propolis (OEP)

Oils (e.g. olive oil) have been tested as an extraction medium for propolis by heating (Krell, 1996). However, a research conducted by Kubiliene et al. (2015) showed that the concentration of TP in OEP was lower than that in WEP and its antimicrobial activity was also lower. Hence, OEP is not a common type of propolis that can be readily commercialised in the market.

2.3 Physical and Chemical Properties

The functional properties of propolis are correlated with its chemical composition and physical properties, which have been widely researched. Some factors, such as botanical regions, plant sources and type of bees, have impacts on the properties of propolis (Bankova et al., 2000; Falcão et al., 2010; Huang et al., 2014).

2.3.1 Physical Characteristics

The colour of raw propolis varies from origins to origins, but dark brown colour is the most common one (Kasote et al., 2017; do Nascimento et al., 2018). The physical status and texture of raw propolis change with temperature. It is sticky and soft when its temperature is between 20°C and 45°C but becomes hard and fragile once the temperature is below 15°C (Krell, 1996). The solid raw propolis turns into liquid at a temperature range from 60°C to 70°C (Krell, 1996).

2.3.2 Chemical Composition

More than 300 chemical substances have been identified in propolis until 2000 (Huang et al., 2014). Some core chemical compounds of propolis are shown in Table 2.1. The

various chemical constituents are mainly due to the different botanical and plant origin. There are many types of propolis according to the geographic origins. For instance, poplar propolis from Europe, North America, Asia (e.g. China and Korea) and Australasia (Australia and New Zealand); green propolis mainly from Brazil; birch propolis from Russia; red propolis from Cuba, Brazil and Mexico; Clusia propolis from Cuba and Venezuela, and pacific propolis from the Pacific region (Okinawa, China, and Indonesia) (Dos Santos et al., 2017). Recognising the origins and sources of propolis could contribute to characterising and standardising the chemical and functional properties of propolis products (Bogdanov & Bankova, 2011). Hence, many studies have been conducted to analyse the chemical compositions of different origins of propolis. As shown in Table 2.2, the principal constituents of propolis are similar but distinctively diverse between different origins and types (Zabaiou et al., 2017; Bankova et al., 2019). This means that isoflavonoids tend to be the largest part in red propolis, but the proportion of phenolic acids seems to be the highest in green propolis. However, there is no admitted criterion for each compound or any origin of propolis. Thus, it is hard to evaluate the quality of propolis products.

Table 2.1 The core chemical compounds found in propolis since 2000 (Huang et al., 2014)

	Chemical Category	Compound	Geographical Origin	Plant Source
1	Flavonoids	Luteolin	Australia, Brazil, Burma, Canada, Chinese, Cuba, Egypt, Greece, Japan, Kenya, Mexico, Nepal, Poland, Portugal, Solomon Island, China (Taiwan)	Populus, Macaranga, Dalbergia
2	Prenylated flavanoned	7-*O*-prenylpino-cembrin	Greece, Japan	/
3	Neo-flavonoids	Cearoin	Nepal	Dalbergia
4	Monoterpenes Sesquiterpenes Diterpenes	Linalool abietic acid	Brazil, Greece, Indonesia, Iran, Malta, Turkey	Ferula Pinaceae Cupressaceae
5	Triterpenes	Lupeol acetate	Burma, Brazil, Cuba, Egypt, Greece	/
6	Phenylpropanoids and esters	*p*-Methoxycinnamic acid	Australia, Brazil, Egypt, Uruguay	Citrus
7	Prenylated phenylpropanoids	3-Prenyl-4-hydroxycinnamic acid	Brazilian Green propolis	Baccharies
8	Stilbenes and prenylated stilbenes	3-Prenylresveratrol	Australis, Brazil, Greece, Indonesia, Kenya	Macaranga
9	Lignans	6-Methoxydiphyllin	Kenya	/
10	Coumarins	Prenylated coumarin suberosin	Iran	/

Table 2.2 Principal constituents of main propolis types from different origins (Zabaiou et al., 2017)

	Propolis type	Geographic origin	Principal constituents
1	Polar Propolis	Europe, North America, Asia (e.g. China, Korea), New Zealand, temperate zone	Flavones, flavanones, cinnamic acids and their esters
2	Green Propolis	Brazil	Prenylated phenolic acids, flavonoids, phenolics
3	Birch Propolis	Russia	Flavones and flavonols (not the same as in Polar type)
4	Red Propolis	Cuba, Brazil, Mexico	Isoflavonoids (isovalvans, pterocarpans)
5	Mediterranean Propolis	Sicily, Greece, Crete, Malta, Turkey, Algeria	Terpenoids, Diterpenes (primarily acids of labdane type)
6	Clusia Propolis	Cuba, Venezuela	Polyprenylated benzophenones
7	Pacific Propolis	Japan, China (Taiwan), Indonesia	prenylated-flavanones

Phenolics

Phenolics are comprised of flavonoids, and phenolic acids and their derivatives, including cinnamic acids, p-coumaric acids, caffeic acids, chicoric acids, and ferulic acids (Huang et al., 2014; Zabaiou et al., 2017). A number of studies have proved that these compounds are associated with antiviral activities of propolis (Huang et al., 2014; Alvarez-Suarez, 2017; Zabaiou et al., 2017). Moreover, phenolics could contribute to minimizing the damage of deoxyribonucleic acid (DNA) by inhibiting the influence in cultured fibroblasts (Darendelioglu, Aykutoglu, Tartik, & Baydas, 2016). That is to say, Brazil green propolis which is rich in phenolics, tend to be responsible for antiviral and inhibitory activities (Huang et al., 2014; Oryan et al., 2018b). Some caffeic acid derivatives (tetradecenyl caffeate) and isoferulic acid derivative (2-methyl-2-butenyl ferulate) in polar propolis were also identified (Huang et al., 2014).

Flavonoids

Flavonoids is part of polyphenols which has a C6-C3-C6 carbon skeleton structure, as shown in Figure 2.2. There are many groups of flavonoids found in propolis, including flavanone, flavonol, flavone, flavan, chalcone, isoflavone, isodihydriflavone, isoflavan, dihydrochalcone, and neoflavonoid, according to different substitution of functional groups in the skeleton (Huang et al., 2014). According to Huang et.al (2014), the pharmacological functions of propolis are attributed to flavonoids which are the main substances of propolis, and 112 flavonoids have been detected in different types of propolis. Inui et al. (2012) analysed the flavonoids compounds and pointed out that these appear to be explicitly linked to the antioxidant and antimicrobial properties of propolis. This view was supported by a study reported from De Almeida et al. (2013) who proved that the extraction of flavonoids from propolis can help heal burn wounds. Similarly, Raghukumar, Seidel, Vali, Watson, and Fearnley (2010) found many prenylated flavanones are associated with potent antimicrobial activity. As mentioned previously, propolis are rich in flavonoids such as in green propolis, red propolis, and birch propolis. Thus, the level of total flavonoid content is suggested to be a quality index to evaluate the quality of propolis (Gardana et al. 2007). Additionally, the grade evaluation of propolis based on the TF content has been applied by the China Government publishing in Chinese regulation of propolis (GB/T 24283-2009) (Lv et al., 2009). (Table 2.3).

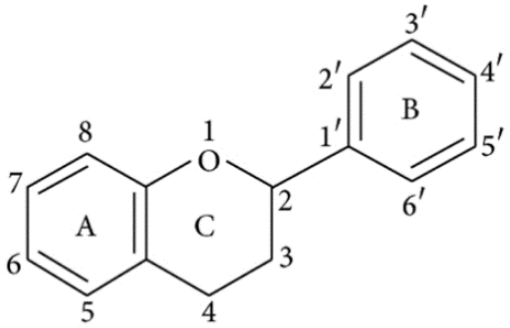

Figure 2.2 Flavonoid skeleton structure (Kumar & Pandey, 2013)

Table 2.3 The relationship of total flavonoid content and quality of propolis

Propolis	TF content	Quality	References
Raw	<11%	Low	Gardana et al., (2007)
	11-14%	Acceptable	
	14-17%	Good	
	>17%	High	
Raw	≥15%	First grade	China National Standard GB/T 24283-2009 edited by Lv et al., (2009)
	≥8%	Second grade	
EEP	≥20%	First grade	
	≥17%	Second grade	

2.4 Functional Properties of Propolis

The functional properties of propolis have been broadly studied (e.g. antioxidant, antimicrobial, anti-inflammatory, and anticancer). Antioxidant and antimicrobial capacities are the two core functional properties that have been widely researched. Literature revealed that the two properties are mainly attributed to the phenolic composition in propolis, especially the flavonoids (Viuda-Martos et al., 2008).

2.4.1 Antioxidant Property

Oxidation reactions could be generated by free radicals that stem from the metabolic processes in human body (Alvarez-Suarez, 2017). Due to the strong oxidation capacity of free redicals, its presence could harm cells and tissues in human body. Some anitoxiants (e.g. vitamin C and E) have the ability quency free radicals by donating hydrogen atom to free radicals and then become antioxidant free radicals. The antioxidant free radicals are however relatively stable through conjugation and electron delocalisation, called resonance stabilization. This phenomenon is referred to as free radical scavenging commonly reported in the study of antioxidant capacity for propolis (Zunini et al., 2010; Socha, Galkowska, Bugaj, & Juszczak, 2015).

Studies have demonstrated the antioxidant capacity of propolis (Toreti et al., 2013; Sforcin, 2016; Alvarez-Suarez, 2017; Bankova et al., 2019), which could help to protect

the human body against free radicals. It has been identified that the main functional propolis compound for antioxidant is flavonoids.

Figure 2.3 Antioxidant effect of quercetin (Alvarez-Suarez, 2017)

Figure 2.3 shows how a free radical is quenched by quercetin, a common compound of flavonoids in propolis, which donates its hydrogen atoms to the free radical, thus reducing the harmful hydroxide radical to water molecules. This means the flavonoid-rich propolis products will be a good natural antioxidant supplement to help treat oxidative stress-related diseases, which is a superior option for consumers as it may be safer than artificial synthetic antioxidants.

2.4.2 Antimicrobial Property

The most renowned properties of propolis are the antimicrobial properties (Alvarez-Suarez, 2017). Propolis is an essential part for honeybees to protect their hives from pathogenic microorganisms and bacteria (Banskota, Tezuka, & Kadota, 2001; Alvarez-Suarez, 2017).

Many researchers claimed that propolis has the antimicrobial activity against both Gram-positive and Gram-negative bacteria, either aerobic or anaerobic types (Alvarez-Suarez, 2017). Two experimental approaches have been mainly used for determining the antimicrobial activity of propolis, which are agar diffusion and broth dilution methods.

These methods determine the inhibitory effect of propolis on the growth of bacteria (etc. *S. aureus*, *B. cereus*, *E. coli*, and *P. aeruginosa*) (Huang et al., 2014; Alvarez-Suarez, 2017; Dos Santos et al., 2017). However, some researchers argue that the bacterial inhibitory effect of propolis tends to be more effective against Gram-positive bacteria than Gram-negative ones (Silici & Kutluca, 2005). Also, the extraction solutions of propolis may influence the bacterial activity (Grange & Davey, 1990). Furthermore, some recent research revealed the antimicrobial activity could defend against bacteria more efficiently than before, such as *Listeria monocytogenes* (Yang, Chang, Chen, & Chou, 2006) although the previous study indicated that propolis was less sensitive to *Listeria spp*. (Grange & Davey, 1990). In other words, the antimicrobial activity of propolis has been explicitly explored with more advanced technology.

2.4.3 Anti-inflammatory Properties

The anti-inflammatory properties of propolis products are extremely valuable in medical area, which could be an effective treatment of skin disease or wound healing (Banskota et al., 2001; Sforcin, 2016). Many factors may interfere with the anti-inflammatory activities of propolis products, including the source and concentration of propolis, and the extraction method of propolis used. To obtain significant anti-inflammatory activities, several experiments have been carried out to determine the functional chemical constituents of propolis in the extraction solutions (Banskota et al., 2001).

2.5 Analyses of Propolis

2.5.1 Chemical Composition

There are various instrumental techniques that have been employed to analyse the chemical composition of propolis both quantitatively and qualitatively, including UV-spectrophotometer, high performance liquid chromatography (HPLC), gas chromatography (GC) and thin layer chromatography (TCL) (Gómez-Caravaca et al., 2006; Falcão et al., 2010).

2.5.2 Determination of Total Phenolics and Total Flavonoids

Spectrophotometry

Spectrophotometry has been broadly used to analyse the contents of total phenolics (TP) and total flavonoids (TF) in propolis (Santos-Buelga & González-Paramás, 2017). Compared to other chromametric means, its advantages are low cost and simple operation (Santos-Buelga & González-Paramás, 2017). Aluminium chloride ($AlCl_3$) reaction is generally used to analyse the content of flavone and flavonol compounds (Chang, Yang, Wen, & Chen, 2002; Pujirahayu, Uslinawaty, & Ritonga, 2014). Another complementary colorimetric method is conducted by 2,4-dinitrophenylhydrazine (DNPH) reaction to determine the concentration of flavanones and dihydroflavonols. Chang et al. (2002) suggested that the cotent of real total flavoniods is close to the sum of the results from the two reactions described above. However, since DNPH reacts with carbonyl groups, it is more likely to overestimate the content of TF due to the interference of other compounds with carbonyl groups, such as saccharides and quercetin (a flavone compound) in the sample. The most common method to evaluate the total phenolic content is Folin-Ciocalteu method (Gómez-Caravaca et al., 2006).

HPLC and GC

Both HPLC and GC can be applied to analyse phenolic compounds. GC is a better option to analyse the volatile composition, while, HPLC is more likely to be employed in non-volatile compounds analysis (Gómez-Caravaca et al., 2006; Santos-Buelga & González-Paramás, 2017). HPLC is therefore more preferable to determine the TF composition, since HPLC has extensive applicability and produces a better result than GC approach (Markham et al., 1996).

2.5.3 Evaluation of Antioxidant Property

The redox reaction is the principle reaction to analyse the antioxidant property of propolis. The methods usually reported in the literature are ferric reducing antioxidant power (FRAP) and 2,2'-diphenyl-1-picrylhydarzyl free radial ($DPPH^{\bullet}$) assays. Antioxidant capacity can be expressed as trolox equivalent antioxidant capacity (TEAC) or half inhibitory concentration (IC_{50}). UV-Vis spectrophotometry is used to evaluate the antioxidant activity by measuring the colour change of the reaction between oxidants and reactants (Archaina et al., 2015; Socha et al., 2015).

2.5.4 Evaluation of Antimicrobial Activity

As mentioned prevously, agar diffusion and broth dilution methods are generally used to determine the antimicrobial activity of propolis (Almeida et al., 2017). The diffusion method involves the incubation of cultures in an agar well or disk on the Muller-Hinton agar plates, and evaluate the strength of antibiotic activity by measuring the diameter of the inhibitory halos (Afrouzan, Tahghighi, Zakeri, & Es-Haghi, 2018; do Nascimento et al., 2018). The broth dillution is cheaper and more sensitive approch than the diffusion methods. This minimum inhibitory concentration (MIC) of antimicrobial activity is determined by incubating a specific concentration of microorganism cultures in the microtiter plates with different concentrations of propolis (Balouiri, Sadiki, & Ibnsouda, 2016). The two common methods have been standardised by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and The Clinical & Laboratory Standards Institute (CLSI). The representative microorganisms used in literature for these tests are *E. coli* (Gram-negative), *S. aureus* and *B. cereus* (Gram-positive) (Almeida et al., 2017; Kasote et al., 2017).

2.5.5 Determination of Elements in Propolis

Heavy metal and rare earth elements

Lead (Pb), arsenic (As), and cadmium (Cd) are the main heavy metal elements, which are generally contained in the air, soil and water (Bonvehí & Bermejo, 2013). Lead is basically from vehicle exhaust which can always be found in the air, while, cadmium is a contaminant from the metal industry. Arsenic is a ubiquitous element in the enviroment, including water, soil and air (Bonvehí & Bermejo, 2013). Since propolis is collected from plants which grow in various places, the contamination of heavy metals could occur along the path from the environment to propolis. It can also be inferred that with different mineral compounds in soil, the possible source of heavy metal contamination in propolis may vary.

There are 14 elments classified as rare earth elements (REE) group, including cerium (Ce), neodymium (Nd), samarium (Sm), terbium (Tb), erbium (Er), praseodymium (Pr), holmium (Ho), thulium (Tm), gadolinium (Gd), dysprosium (Dy), lutetium (Lu), ytterbium (Yb), lanthanum (La) and yttrium (Y) (Germund, 2004). They have been perferably used as indicators of soil in environmental sciences, and always quantified as

a chemically uniform group (Oliveira et al., 2017). This implies that it can be used to trace the geographical origin of propolis by analysing the REE composition. However, so far very few studies have been conducted to determine the elements in propolis.

Elemental analysis technique

Graphite furnace atomic absorption spectrometry (GFAAS) is a traditional method to determine the content of elements in various samples, including propolis (Vardar-Unlu, Silici, & Unlu, 2008; Korn et al., 2013; Aksoy, Atabay, Tirasoglu, Koparan, & Kekillioglu, 2017). However, inductively coupled plasma-optical emission spectrometry (ICP-OES) and inductively coupled plasma-mass spectrometry (ICP-MS) are playing a dominant role in elemental analysis (Vardar-Unlu et al., 2008; Korn et al., 2013; Aksoy et al., 2017; Tosic et al., 2017; Gonzalez-Martin, Revilla, Betances-Salcedo, & Vivar-Quintana, 2018). Compared to GFAAS, ICP-OES and ICP-MS are a multi-element technique to detect elements, which has the lower limit of detection and high accuracy (Tosic et al., 2017). Before the measurement, samples normally need to be digested by acid (e.g. nitric acid, perchloric acid and hydrofluoric acid). Traditional approach for sample digestion is heating by hot plate or hot block, which requires a large amount of acid and long time. Microwave-assisted acid digestion technique has been applied to sample preparation for elemental analysis for the last few decades, which not only simplifies the process but also enhances the efficiency (Korn et al., 2013). In this project, microwave-assisted acid digestion technique and ICP-MS were used to detemine the elements in commercial liquid propolis products.

2.5.6 Identification of Adulteration of Propolis

It has been reported that the extract of populus buds (e.g. poplar tree gum) has been used to adulterate propolis, since it has the physical properties and chemical compositions similar to propolis (Zhang et al., 2011; Zhang et al., 2015). Salicin belongs to the class of organic compounds known as phenolic glycosides, which has been found in the barks, leaves and twigs of poplar trees (Pearl & Darling, 1971; Palo, 1984; Clausen et al., 1989; Zhang et al., 2011). Zhang et al. (2011) noted that the phenolic glycosides would be hydrolysed during the process of collecting propolis by honeybees. This means that phenolic glycosides can be used as a marker compound to evaluate the adulteration of propolis with poplar tree gum.

Technically, HPLC is the general technique used to determine TP andTF as well as indentifying phenolic glycosides (Gómez-Caravaca et al., 2006; Santos-Buelga & González-Paramás, 2017; Zhang et al., 2011). However, mass spectrometry (MS) is more preferable in analysing organic compounds, since it has higher selectivity and sensivity than other detectors (Stobiecki, 2000; Cuyckens & Claeys, 2004; Medana, Carbone, Aigotti, Baiocchi, & Appendino, 2008). Forthermore, the combination of high performance liquid chromatography with mass spectrometry (HPLC-MS) has been applied to determine phenolics and flavonoids for many years. It indicates HPLC-MS is available to be also employed to identify salicin in propolis. Both HPLC and HPLC-MS were used to determine the concentration of salicin in this project.

2.6 Conclusions

Various studies reported the different extraction methods of propolis and the basic chemical compounds of propolis from many different geographic origins, and showed the functional properties of propolis. Spectrophotometry, HPLC, HPLC-MS, DPPH$^{\bullet}$ assay, well/disk diffusion method and broth dilution test are generally ujsed to analyse the chemical and functional properties of propolis. Also, ICP-OES and ICP-MS are the main techinque used to determine the content of metal elements. Salicin can be used as a marker compound to investigate the adulteration of propolis, since it only exists in popolar tree gum not in propolis. In addition, HPLC is also commonly employed to detect the presence of salicin in propolis.Although some studies have been carried out to determine the the physicochemical and funcitonal properties of propolis, there are still some knowldege gaps that need to be investigated. In addition, no studies have been performed to determine and compare differences in the chemical composition, physical properties and functional activities between commercially available propolis liquid products manufactured from different countries.

Chapter 3. Commercial Propolis Liquid Products: Comparison of Physical and Chemical Properties

Abstract

The physical and chemical properties of 20 commercial propolis liquid products manufactured in 4 countries (Australia, China, Korea and New Zealand) were investigated in this study. The pH of propolis samples ranged from 3.55 ± 0.07 to 9.37 ± 0.03, and the visual colour of samples varied from brown, red to green. The miscibility of most samples tended to be better in ethanol than in water, except some samples added with an emulsifier (Tween 20). The content of total phenolic (TP) in propolis products ranged from 16.35 ± 0.28 mg/mL to 70.22 ± 0.47 mg/mL, and total flavonoid (TF) content was from 6.56 ± 0.37 mg/mL to 58.97 ± 0.59 mg/mL. However, some unexpected results were obtained with the TF content being higher than the TP content in some propolis samples which needs to be further investigated.

3.1 Introduction

Propolis is a resinous substance also called bee glue, which has dark colour collected by honeybees from various plants buds or resins (Silici & Kutluca, 2005; Bankova et al., 2019). The chemical composition and physical characteristics of propolis vary depending on the plant sources and geographical regions of propolis (Silici & Kutluca, 2005; Zabaiou, Fouache, Trousson, Baron, Zellagui, et al., 2017). According to the composition differences among regions, propolis is generally classified into two main types, which are Brazilian type and European type (poplar type) (Xu et al., 2009). In terms of chemical constituent, poplar propolis contains high amounts of flavones and flavanones compared to phenolic acid and their esters (Markham et al., 1996; Bankova et al., 2019). On the other hand, Brazilian propolis comprises of a high proportion of *p*-coumaric acid derivatives (Markham et al., 1996; Xu et al., 2009; Huang et al., 2014). However, the core chemical compounds identified from propolis are phenolics especially flavonoids (Silici & Kutluca, 2005; Xu et al., 2009; Huang et al., 2014; Oryan et al., 2018a).

Before commercialisation, crude raw propolis has to be extracted and it has been shown that various solvents have been used as an extraction medium, including ethanol, propylene glycol (often referred to as glycol), water and/or oil (Kubiliene et al., 2015; Sforcin, 2016). Ethanol and glycol extractions are however more commonly used to extract propolis in order to produce commercial liquid propolis products due to the relatively high yield of extraction (Kubiliene et al., 2015; Sforcin, 2016). Consequently, the most common propolis liquid products on the market are an ethanol or glycol base. Traditionally, crude propolis is soaked in a solvent, commonly ethonal, to extract bioactive compounds and remove the beewax (Krell, 1996). Then the liquid propolis is filtered. o achieve an optimal yield, the soaking could be around two weeks.

Due to the various functional properties (e.g. antioxidant and antimicrobial), propolis is broadly applied to many products, such as food, beverage, toothpaste and dietary supplement (Archaina et al., 2015; Kubiliene et al., 2015; Xavier et al., 2017). Propolis are usually characterised for the contents of total phenolic (TP) and total flavonoid (TF) (Santos-Buelga & González-Paramás, 2017). For the evaluation of TP content, Folin-Ciocalteu method is one of the most widely used methods (Gómez-Caravaca et al., 2006; Papotti, Bertelli, Bortolotti, & Plessi, 2012). Aluminium chloride ($AlCl_3$) assay is one of the most commonly used methods for the determination of TF content which is based on the reaction between the carbonyl and hydroxyl groups of flavonoid and aluminium ion (Al^{3+}) (Gómez-Caravaca et al., 2006; Chang et al., 2002).

Studies on the physical and chemical properties of raw propolis have been reported (Markham et al., 1996; Falcão et al., 2010; Huang et al., 2014; Archaina et al., 2015; do Nascimento et al., 2018). However, there has been scant research on the physicochemical properties of commercially available propolis liquid products. Therefore, the objective of this study was to analyse and compare 20 different commercial liquid propolis products (e.g. ethanol or glycol-based poplar type) obtained from 4 different countries (Australia, China, Korea and New Zealand). The propolis samples were analysed for the determination of water and ethanol miscibility, colour, pH and, TP and TF contents.

3.2 Materials and Methods

3.2.1 Materials

The reagents and standards used to determine the chemical properties of propolis are listed in Table 3.1.

Table 3.1 Reagents and standards for determination of chemical properties of commercial propolis liquid products

Name	Concentration/Grade	Supplier
Folin-Ciocalteu Phenol's Reagent	2 mol/L	Merck
Gallic acid	≥ 98.0%	Merck
Ethanol (Absolute)	> 99.8 % HPLC	Thermo Fisher
Methanol (Anhydrous)	> 99.8 % HPLC	Thermo Fisher
Potassium acetate (CH_3COOK)	≥ 99 %	Scharlau
Quercetin	> 95 % HPLC Grade	Sigma-Aldrich
Sodium carbonate (Na_2CO_3)	≥ 99.8	Univar
Aluminium trichloride ($AlCl_3$)	99.99 %	Sigma-Aldrich

3.2.2 Commercial Propolis Samples

Twenty commercial liquid propolis products were investigated in this project, which were manufactured from four different countries including Australia, China, Korea and New Zealand. Details of samples are designated as S1 to S20 respectively which are shown in Table 3.2.

Table 3.2 Product information about 20 different commercial propolis liquid products used in this study

Sample code	Country	Propolis liquid base	Labelled concentration	Source of propolis
S1	Australia	GEP[A]	Equal to propolis 400mg/ml	Australia
S2		GEP[A]	Equal to propolis 300mg/ml	
S3	Korea	EEP[B]	/	China
S4			/	
S5			/	
S6			/	Australia, Brazil, China
S7	China	GEP[A]	TF 40mg/ml	China
S8			TF 32.4mg/ml	
S9	New Zealand	EEP[B]	TF 30 mg/ml	New Zealand
S10		GEP[A]	TF 30 mg/ml	
S11			TF 15 mg/ml	
S12		GEP[A]	TF 30 mg/ml	
S13		EEP[B]	TF 30 mg/ml	
S14		GEP[A]	TF 30 mg/ml	
S15	Korea	WEEP[C]	100[E]	China
S16		EEP[B]	200[E]	
S17		EEP[B]	100[E]	Australia
S18		EEP[B]	100[E]	Korea
S19		SEEP[D]	100[E]	Brazil
S20		WEEP[C]	100[E]	Australia, Brazil, China

[A] GEP represents glycol-based propolis; Sample code was highlighted in blue colour.
[B] EEP represents ethanol extracted propolis; Sample code was highlighted in orange colour.
[C] WEEP represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20; Sample code was highlighted in green colour.
[D] SEEP represents water-soluble ethanol extracted propolis containing Tween 20; Sample code was highlighted in purple colour.
[E]100 means that the TF content in the product is at least 1% (w/v), and 200 means 2% (w/v);

3.3 Analyses of Physicochemical Properties

3.3.1 Miscibility

All propolis samples were analysed for their miscibility with water and ethanol (>99.8%), respectively. Briefly, 1 mL of each propolis sample was added into 9 mL of distilled water or ethanol in a glass test tube. The mixture of water and propolis was thoroughly mixed by shaking and then visually observed for their appearance and the formation of a homogeneous solution.

3.3.2 Colour

The colour of all original samples and 10 times ethanolic dilutions was analysed using a colour spectrophotometer (CM-5, Konica Minolta). The prepared ethanolic dilutions were filtered through 20 μm filter to remove any precipitates before measurements. Data were collected by SpectraMagic NX ver. 2.6 software. The colour measurement was based on CIE L*a*b* and L*C*h colour space systems with illuminate D65 at 10° view angle. Prior to the measurement of sample colour, the colour spectrophotometer was calibrated using both zero calibration plate (CM-A204) and white calibration (100%) with distilled water.

3.3.3 pH

All propolis samples were analysed using a pH meter (PB-11, Sartorius) for the determination of pH at room temperature (20ºC) directly without any dilution. Before measurement, the pH meter was calibrated by the buffer solutions of pH 4 and 7, respectively.

3.3.4 Total Phenol (TP) Content

Sample preparation

The content of total phenol (TP) in the propolis samples was analysed in this study according to the Folin-Ciocalteu method reported by Papotti et al. (2012) with some modifications. All samples were diluted with methanol in triplicate before the measurement. Sample 6 specified as S6 in Table 3.2 was diluted to a 100-fold with methanol, while the other 19 samples were diluted to a 200-fold with methanol. Also, the Folin-Ciocalteu reagent was also diluted to a 10-fold with water. All the diluted propolis samples (0.2 mL) were then mixed with the Folin-Ciocalteu reagent dilution (5 mL). A 4 mL of 5 % aqueous sodium carbonate (Na_2CO_3) solution was added into the mixtures after incubation for 3 minutes and well shaken.

A series of different concentrations of gallic acid (100, 200, 250, 400 and 500 mg/L) were used to construct a standard curve using the same reagents as samples for the determination of the TP content in the propolis samples. All the standards and samples prepared were incubated for 1 hr under the dark condition and ambient temperature, and then measured for their absorbance at 760 nm using a spectrophotometer (UV-1601,

Shimadzu). The spectrophotometer was set to zero using an absolute methanol solution as the blank solvent. The TP contents of triplicate samples measured were averaged and expressed as mg of gallic acid equivalent (GE) per mL of propolis (mg GE/mL).

3.3.5 Total Flavonoid (TF) Content

Sample preparation

Aluminium chloride method reported by Chang et al. (2002) was modified to analyse the TF content of propolis samples. Sample dilutions were prepared in triplicate before the measurement. Sample 6 was 100 times diluted with absolute methanol, and the other 19 samples were diluted to 200-fold with absolute methanol. The diluted samples (0.5 mL) were mixed with 0.1 mL of aluminium trichloride ($AlCl_3$) solution (2% w/v), 0.1 mL of potassium acetate (CH_3COOK) solution (1 mol/L) and 4.3 mL of absolute methanol.

Standard curve preparation

Quercetin was chosen as a standard to express TF in the propolis samples. A series of concentration applied to construct the standard curve, including 50, 75, 100, 150, 200, and 300 mg/L. All standards were added with the same reagents as samples.

The absorbance was used at 415 nm, and the measurements of all the standards and samples were made against absolute methanol solution as the blank by UV-1601 spectrophotometer after incubation for 30 minutes at room temperature and under dark condition. Triplicate samples were measured, and the results were recorded as mg of quercetin per mL of propolis (mg QE/mL).

3.3.6 Statistical Data Analysis

All experiments were conducted in triplicates for each product. The results were presented as mean value +/- standard deviation (SD). The results of TF and TP were analysed by the analysis of variance (ANOVA) using IBM SPSS software (Ver. 24) to evaluate the difference between samples at 95% confidence level. Results of post-hoc Tukey's test were used to compare the mean differences of the TF and TP contents between propolis samples.

3.4 Results and Discussion

3.4.1 Colour Measurement

The colour of the liquid propolis products was observed visually, before conducting the instrumental colour measurement. Figure 3.1 illustrates the colour varies from brown, red, to green. Both Australian (S1 and S2) and Chinese (S7 and S8) propolis showed a dark brown colour. For the propolis products produced in Korea, the visual colours were observed to be different from dark reddish (e.g. S15 and S20), dark green (S19) and light brown (S18).

The colour differences between samples could be due to difference in the crude raw propolis initially used for the extraction of propolis. As mentioned in Table 3.2, the Korean propolis products were not only extracted from Korean local raw propolis but also made from the raw propolis stock obtained from other countries, such as Australia, Brazil and China. Although an overall brown hue was also observed from all the New Zealand (NZ) samples (S9 – S14), the intensity of their colours varied from light to dark brown as shown in Figure 3.1. The difference in colour intensity and darkness seemed to be related to the TP and TF contents in S9 - S14 as shown in Table 3.5. For example, among the NZ propolis samples, S12 was very dark and its TP and TF contents were the highest while S11 was light and its TP and TF contents were the lowest. In other words, the higher TP or TF in the NZ samples was related to a dark and non-vivid brown colour. This colour phenomenon from NZ made propolis liquid products indicates that phenolic compounds including flavonoids are the dominant colour pigment in propolis.

After 10-fold dilution with ethanol, the visual colour of 20 propolis samples was much lighter as expected. As Figure 3.2 illustrates that the colour of diluted propolis samples also varied from brown, red, orange and yellow. Propolis from New Zealand (S9-S14) were generally lighter than other products, presenting yellow and reddish-orange colour. The dilution of S3 (Korean propolis) and S8 (Chinese propolis) showed still dark brown, but Australian propolis showed reddish colour after dilution. The bright yellow colour was found in dilutions of two New Zealand propolis products (S9 and S11) and one Korean propolis (S19). The colour of Australia (S1, S2) and New Zealand (S9-S11) propolis product dilutions showed a correlation with their TP contents, which was darker with higher concentration of TP content (Table 3.5).

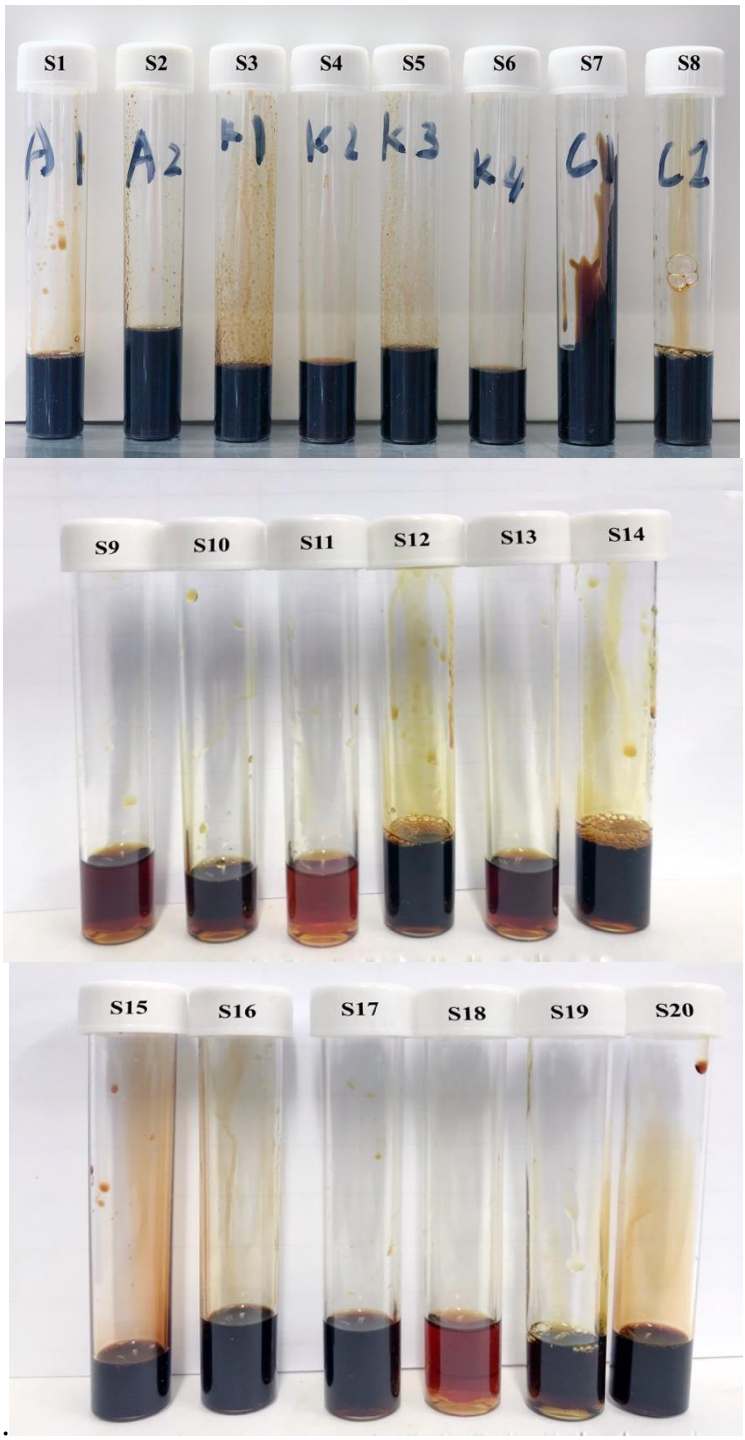

Figure 3.1 Visual appearance (e.g. colour) of 20 different propolis liquid samples

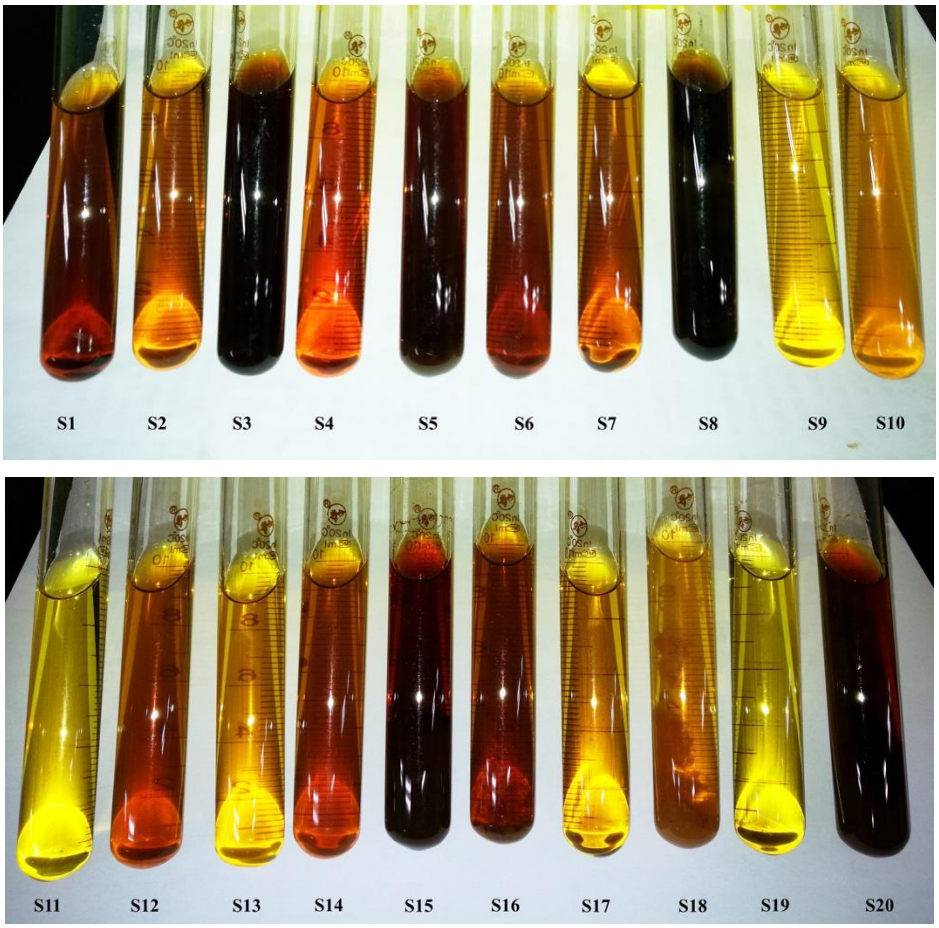

Figure 3.2 Appearance of 20 different propolis liquid samples after mixing with ethanol at 1:9 ratio (v/v)

The instrumental measurement of colour was only analysed from the propolis samples diluted 10-fold in ethanol. The reasons were that for certain stock propolis liquid samples, especially Australian and Chinese ones (S1 and S2; S7 and S8), the measured CIE values tended to zero (very dark black) due to the remarkable colour intensity, which resulted in the lack of comparison for colour. And most samples were also not miscible with water, which is discussed later in this chapter.

The results of CIE L*, a*, b*, C* and h are shown in Table 3.3. Firstly, there was no trend of colour appearance or difference that could be related to differences in the type of extraction solvent applied for producing the commercialised liquid propolis products. The lightness L* values ranged from 31.34 to 80.14. Among the 20 samples diluted with ethanol, 14 samples were overall appeared to be dark, showing as L* less than 50, while the other 6 samples (S1, S2, S4, S5, S6 and S8) had a higher lightness value, especially

S8 sample's L* value (80.14 ± 0.69). It should be mentioned that unlike the observed dark colour of S8 sample visually as shown in Figure 3.2, its lightness L* value was measured to be highest.

For the a*, most of the samples were reddish with positive a* values, except three samples (S15, S16 and S20) with negative a* values ranging from -0.16 to -0.07 which indicate their colour has slightly greenish tint but not pronounced as its value was close to zero. For the b* values, all samples had positive b* values, indicating the colours of the propolis liquids were yellowish. By combining the measured a* and b* values, it can be concluded that most propolis samples had a brown colour mixed with red and yellow, except for 3 samples (S15, S16 and S20) that were yellowish with a small green tint. As a result, the latter 3 samples had the hue angle values higher than 90 (117.86, 154.97 and 153.88) as shown in Table 3.3. It was not easy to correlate between the visual colour observation and the instrumental colour measurement as indicated above using S8 sample as an example.

Table 3.3 CIE L*, a*, b*, C* and h values of propolis liquid samples' colour

Countries	Sample	L*	a*	b*	C*	h
Australia	S1	64.69 ± 0.74	18.36 ± 0.22	49.07 ± 0.51	52.39 ± 0.40	69.48 ± 0.42
	S2	75.06 ± 0.12	11.46 ± 0.33	58.63 ± 0.30	59.68 ± 0.12	79.30 ± 0.99
Korea	S3	45.55 ± 0.33	30.19 ± 0.65	24.68 ± 0.65	39.16 ± 0.65	39.00 ± 0.58
	S4	70.45 ± 0.22	25.92 ± 0.81	63.92 ± 0.55	68.96 ± 0.21	67.69 ± 0.40
	S5	54.77 ± 0.54	27.58 ± 0.27	40.20 ± 0.59	48.97 ± 0.71	55.30 ± 0.28
	S6	68.71± 0.47	16.57 ± 0.26	54.13 ± 0.48	56.61 ± 0.38	72.98 ± 0.39
China	S7	42.53 ± 0.06	18.86 ± 0.05	19.01 ± 0.14	26.77 ± 0.13	45.23 ± 0.15
	S8	80.14 ± 0.69	3.56 ± 0.24	54.13 ± 0.37	55.92 ± 0.59	86.28 ± 0.18
NZ	S9	40.98 ± 0.30	28.96 ± 0.23	16.44 ± 0.11	33.05 ± 0.17	30.27 ± 0.44
	S10	34.50 ± 0.33	12.29 ± 0.12	4.66 ± 0.23	13.47 ± 0.16	21.33 ± 0.22
	S11	48.57 ± 0.19	35.49 ± 0.33	29.38 ± 0.18	46.31 ± 0.11	39.57 ± 0.28
	S12	32.06 ± 0.15	0.61 ± 0.06	0.46 ± 0.06	0.90 ± 0.07	36.92 ± 0.63
	S13	35.12 ± 0.31	15.30 ± 0.46	5.38 ± 0.28	13.21 ± 0.29	23.77 ± 0.91
	S14	32.27 ± 0.42	1.29 ± 0.31	0.73 ± 0.24	1.49 ± 0.36	27.89 ± 0.58
Korea	S15	31.34 ± 0.09	**-0.07 ± 0.07**	0.46 ± 0.05	0.26 ± 0.06	117.86 ± 0.80
	S16	31.38 ± 0.15	**-0.16 ± 0.05**	0.06 ± 0.02	0.17 ± 0.04	154.97 ± 0.70
	S17	31.47 ± 0.16	0.19 ± 0.02	0.23 ± 0.11	0.23 ± 0.07	49.83 ± 0.28
	S18	45.94 ± 0.32	31.60 ± 0.40	24.03 ± 0.47	39.89 ± 0.32	36.59 ± 0.25
	S19	33.28 ± 0.57	3.36 ± 0.19	2.13 ± 0.18	3.68 ± 0.13	31.67 ± 0.41
	S20	31.56 ± 0.21	**-0.11 ± 0.02**	0.19 ± 0.12	0.20 ± 0.09	153.88 ± 0.88

Blue colour represents glycol-based propolis;
Orange colour represents ethanol extracted propolis;
Green colour represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20;
Purple colour represents water-soluble ethanol extracted propolis containing Tween 20.

3.4.2 Miscibility of Propolis Liquid Products

The results of miscibility of all the samples with ethanol or water at 1:9 ratios are shown in Figures 3.2 and 3.3, respectively. Before mixing, all the original propolis liquid samples were homogenous solutions as shown in Figure 3.1. However, when mixed with water, most samples, except for 3 samples (S15, S19 and S20), became turbid forming aggregates and precipitates, resulting in phase separation. The reason for those 3 samples remaining as a clear, uniform dispersion without any aggregation could be due to the fact

that the products were further processed by evaporating ethanol and adding Tween 20 to make the products. For the GEP (glycol-based propolis) samples as indicated in Table 3.2, when they were added into water, relatively larger pieces of aggregates were formed due to some coagulation, leading to precipitation. This phenomenon was observed clearly from S1 sample as shown in Figure 3.3. On the other hand, EEP (ethanol extract propolis) samples seemed to form smaller pieces of aggregates which were suspended in the mixtures (e.g. S9 sample). The results indicate the significant poor water miscibility of propolis liquid products which could be a barrier that negatively influences the further application of propolis liquids in the food industry, for example, to fortify beverage with propolis as a functional ingredient.

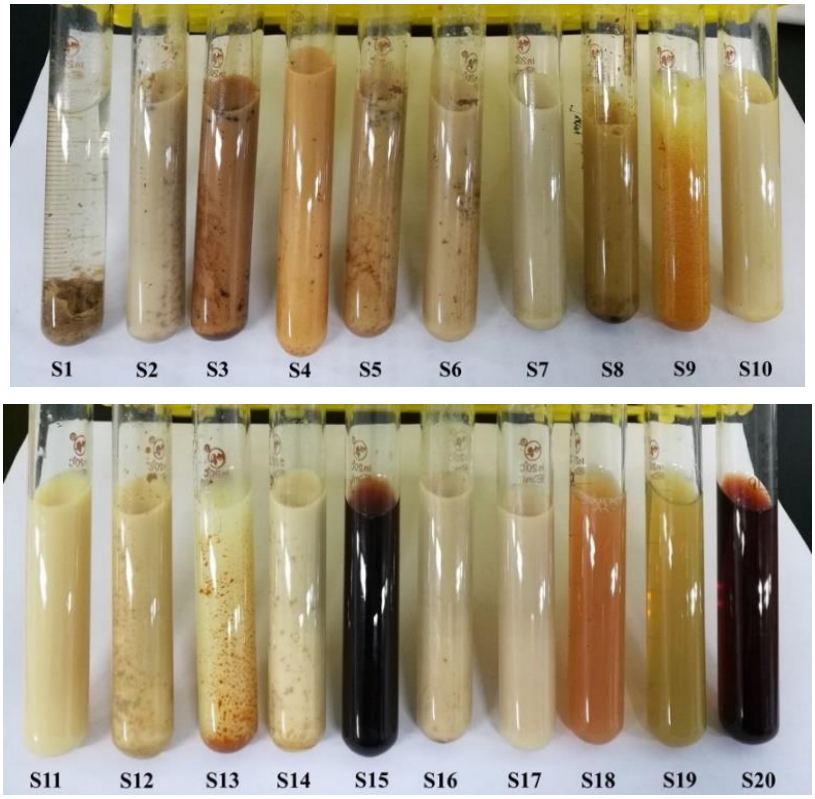

Figure 3.3 Appearance of 20 different propolis liquid samples after mixing with water at 1:9 ratio (v/v)

A totally opposite phenomenon was observed when the propolis liquid products were mixed with ethanol. In their 10-fold ethanolic dilutions, all the GEP and EEP samples were fully miscible with ethanol forming homogeneous and stable solutions. However, the two WEEP samples (S15 and S20) had significant sedimentation although this is not seen clearly in Figure 3.2. The precipitant was believed to be K_2CO_3. The reason is that firstly, the SEEP (S19) was fully dissolved in ethanol. The only difference between the

WEEP and SEEP samples is that the WEEP contains K_2CO_3 while the SEEP does not according to descriptions specified on their product labels. Secondly, to confirm whether the precipitant was K_2CO_3, several drops of 6 M of HCl was added to the precipitant. It was found clearly that bubbles were forming.

3.4.3 pH of Propolis Liquid Samples

The pH values of all the 20 samples are shown in Table 3.4. The pH of all commercial propolis liquids (EEPs and GEPs) except for those WEEP and SEEP samples (S15, S19 and S20) were acidic ranging from 3.55 ± 0.07 to 5.54 ± 0.01. Pujirahayu et al. (2014) who also investigated commercialised propolis liquids in their study showed some similar results although the pH values of propolis were 6.3 ± 0.1 in ethanol solvent and 5.4 ± 0.2 in propylene glycol solvent in their study.

On the other hand, the SEEP and WEEPs had higher pH values. The SEEP (S19) sample had its pH close to neutral with 6.77 ± 0.07. The WEEP samples (S15 and S20) were alkaline with their pH values being 8.61 ± 0.06 and 9.37 ± 0.03, respectively, which could be attributed to the presence of K_2CO_3.

Table 3.4 The pH of 20 different propolis liquid samples including extraction medium used

Countries	Sample code	Extraction solvent	pH
Australia	S1	Glycol	3.55 ± 0.07
	S2	Glycol	5.37 ± 0.03
Korea	S3	Ethanol	4.74 ± 0.03
	S4	Ethanol	4.65 ± 0.02
	S5	Ethanol	5.41 ± 0.03
	S6	Ethanol	5.50 ± 0.01
China	S7	Glycol	4.36 ± 0.04
	S8	Glycol	4.79 ± 0.04
NZ	S9	Ethanol	4.88 ± 0.02
	S10	Glycol	4.67 ± 0.04
	S11	Glycol	4.77 ± 0.03
	S12	Glycol	4.45 ± 0.01
	S13	Ethanol	4.86 ± 0.02
	S14	Glycol	4.55 ± 0.02
Korea	S15	**Ethanol (contains potassium carbonate and Tween 20)**	**8.61 ± 0.06**
	S16	Ethanol	5.16 ± 0.07
	S17	Ethanol	5.54 ± 0.01
	S18	Ethanol	4.61 ± 0.04
	S19	Ethanol (contains Tween 20)	6.77 ± 0.07
	S20	**Ethanol (contains potassium carbonate and Tween 20)**	**9.37 ± 0.03**

Blue colour represents glycol-based propolis;
Orange colour represents ethanol extracted propolis;
Green colour represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20;
Purple colour represents water-soluble ethanol extracted propolis containing Tween 20;

3.4.4 Total Phenolic and Total Flavonoid Contents in Propolis

The original data and standard curve for the determination of total phenolic (TP) and total flavonoid (TF) contents are presented in the Appendix 3. The results of TP and TF contents are shown in Table 3.5. The TF content between the propolis products ranged from 6.56 ± 0.37 QE mg/mL (S15) to 58.97 ± 0.59 QE mg/mL (S12). The analysed results could not be evaluated for their accuracy as the exact concentration of TF or TP was not clearly specified on their product packages. Nevertheless, when compared to the TF content claimed by the companies, the TF content of most samples was higher than its content labelled on their product packages, except for S8, in which the identified TF content was 27.19 ± 0.76 mg QE/mL which was less than the claimed 32.4 mg/mL.

Although it is not sure, the result of the Chinese propolis liquid product (S8) being measured to be lower in the TF content may be due to different standards chosen and used to represent the TF content. In this experiment, quercetin was used as a standard whereas rutin is the legal standard mentioned the China regulation to represent the TF content in propolis (China propolis standard).

With regards to the TP content, as shown in Table 3.5, among all the samples, the TP content of S12 was the highest (70.22 ± 0.47 GE mg/mL) while it was the lowest (16.35 ± 0.28 GE mg/mL) for S4, followed by S15 (17.39 ± 0.37 GE mg/mL), S18 (22.00 ± 0.42 GE mg/mL), S17 (24.03 ± 0.42 GE mg/mL), and S20 (25.35 ± 0.40 GE mg/mL), which had the TP content being lower than 30 GE mg/mL. It should be pointed that the commercial propolis liquids are generally made by extraction and dilution with suitable carrier solvent, such as ethanol and propylene glycol, with a certain confidential ratio. Hence, neither the analysed TP nor TF values can reveal or correlate back to the TP or TF content in their raw propolis. Thus, the results of TP or TF cannot be compared directly with any literature which studied based on raw propolis. Also, as aforesaid, there is few research which focused on the commercial propolis liquid products. Furthermore, commercial products could also be manufactured by mixing propolis from different sources (e.g. various countries and botanic areas). For instance, S20 was informed to be made by blending propolis from Australia, Brazil and China.

Table 3.5 The results of TP and TF contents in samples

Countries	Sample code	TP (mg GE/mL)	TF (mg QE/mL)	Labelled content	TF/TP (%)
Australia	S1	40.50 ± 0.45	29.82 ± 0.27	Equal to propolis 400mg/ml	73.63 ± 1.49
	S2	31.44 ± 0.51	22.86 ± 0.24	Equal to propolis 300mg/ml	72.70 ± 1.84
Korea	S3	31.17 ± 0.63	21.80 ± 0.64	/	69.93 ± 2.60
	S4	16.35 ± 0.28	6.56 ± 0.37	/	40.14 ± 2.48
	S5	**31.35 ± 0.35**	**35.08 ± 0.60**	/	**111.92 ± 4.43**
	S6	25.35 ± 0.05	18.97 ± 0.52	/	74.82 ± 3.02
China	S7	**48.99 ± 0.31**	**51.29 ± 0.12**	TF 40mg/ml	**104.70 ± 2.25**
	S8	48.87 ± 0.49	**27.19 ± 0.76**	**TF 32.4mg/ml**	55.63 ± 1.68
NZ	S9	53.60 ± 0.53	41.30 ± 0.65	TF 30 mg/ml	77.05 ± 1.64
	S10	64.85 ± 0.72	53.87 ± 0.82	TF 30 mg/ml	83.06 ± 1.66
	S11	35.95 ± 0.49	24.74 ± 0.22	TF 15 mg/ml	68.83 ± 1.46
	S12	70.22 ± 0.47	58.97 ± 0.59	TF 30 mg/ml	83.97 ± 1.31
	S13	68.29 ± 0.62	54.86 ± 0.42	TF 30 mg/ml	80.34 ± 1.12
	S14	68.11 ± 0.47	54.63 ± 0.14	TF 30 mg/ml	80.21 ± 0.97
Korea	S15	**17.39 ± 0.37**	**20.84 ± 0.37**	1% (w/v)	**119.86 ± 8.53**
	S16	**37.36 ± 0.47**	**54.09 ± 0.50**	2% (w/v)	**144.79 ± 5.77**
	S17	**24.03 ± 0.42**	**29.68 ± 0.40**	1% (w/v)	**123.52 ± 6.57**
	S18	22.00 ± 0.49	12.86 ± 0.19	1% (w/v)	58.43 ± 1.77
	S19	35.04 ± 0.60	27.72 ± 0.25	1% (w/v)	79.10 ± 1.92
	S20	**25.35 ± 0.40**	**24.43 ± 0.16**	1% (w/v)	**96.39 ± 3.72**

Blue colour represents glycol-based propolis;
Orange colour represents ethanol extracted propolis;
Green colour represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20;
Purple colour represents water-soluble ethanol extracted propolis containing Tween 20.

Based on the content of TP shown in Figure 3.4, the 20 samples can be statistically identified into 11 groups with the TP content increasing from letters a to k at 95% confidence level (CL). Similarly, the content of TF in the 20 products could be divided into 12 groups, as shown in Figure 3.5, with increasing with the marked letter from a to l.

The samples in the same group means that there was no significant statistical difference of TP or TF at 95% CL by ANOVA test.

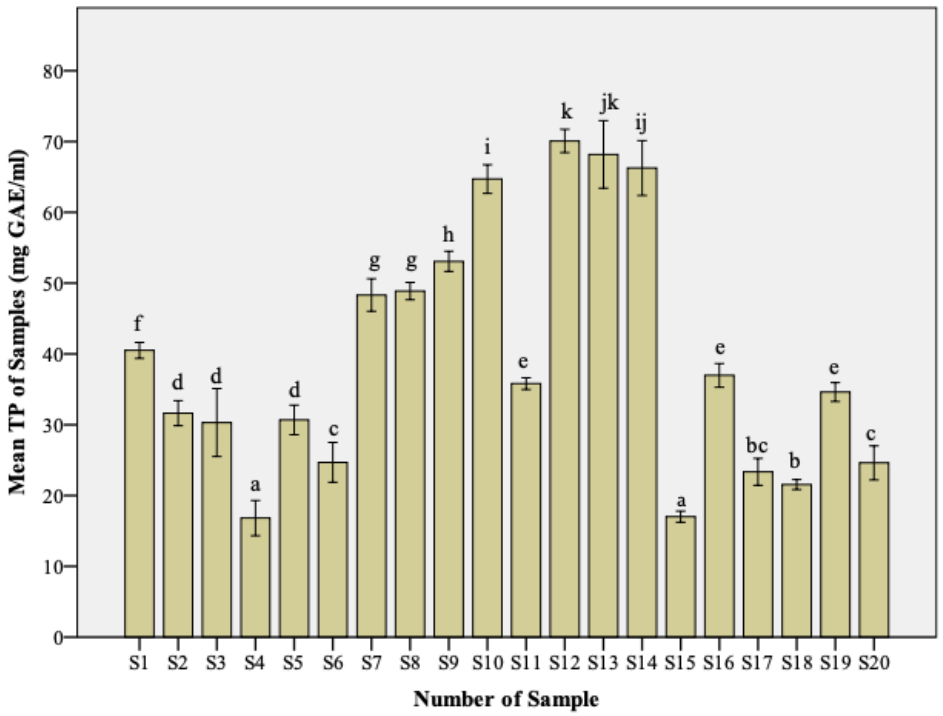

Figure 3.4 Total phenolic (TP) content of propolis samples. The significant difference is shown by different letters according to Tukey's HSD test at 95% CL

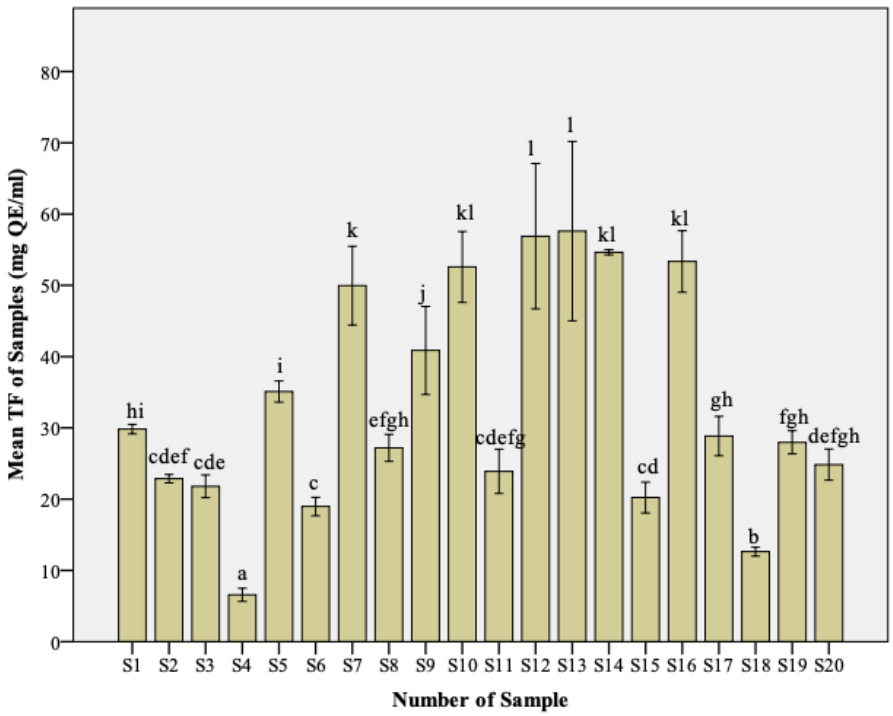

Figure 3.5 Total flavonoid content (TF) of samples. The significant difference is shown by different letters according to Tukey's HSD test at 95% CL

However, the ratios of TF to TP which were greater than 100% were unexpected which was observed from some samples (S5, S7, S15, S16, S17 and S20) as shown in Table 3.5. For instance, the ratio was 144.79 ± 5.77 % for S16. It should be mentioned that flavonoids are defined as a type of polyphenol compounds that have a special C6-C3-C6 skeleton structure whereas polyphenols are a subgroup of phenolics (Lee and Wong, 2014). Hence, the TF content should not be greater than the corresponding TP content. This phenomenon has not been indicated in the literature. Although it is not sure, the TF/TP ratio of > 100% observed from some samples could be due to adulteration of propolis by poplar tree gum. According to Zhang et al. (2015), among 66 commercial propolis products they analysed, 44 samples were identified to be adulterated by poplar tree gum, as the cost of poplar tree gum is only around one-tenth of propolis but it has the functional properties similar to propolis. In terms of safety, it is believed that there is no harm by replacing propolis with poplar tree gum. The results of the suspected adulteration by adding poplar tree gum into some propolis products will be discussed in Chapter 5.

3.5 Conclusions

The chemical composition of TP and TF and physical properties (miscibility, colour and pH) of 20 commercially available propolis liquid products were analysed in this study. Visually, most samples had a brownish or yellowish colour in appearance with some variations in colour hue, lightness and intensity. The pH of most samples was acidic ranging from 3.55 ± 0.07 to 6.77 ± 0.07, while 2 propolis samples (S15 and S20) containing potassium carbonate were alkaline with pH 8.61± 0.06 and 9.37± 0.03, respectively. In terms of water or ethanol miscibility, the majority of propolis samples were not miscible with water. However, 3 samples (S15, S19 and S20) which contained an emulsifier, Tween 20, showed high water solubility. On the other hand, almost all samples were well mixed with ethanol although some small floccules formed in S8, S12 and S14 (GEP samples), and larger amount of sediments separated out in the samples containing potassium carbonate (S15 and S20).

The TF content of almost all samples ranged from 20 to 59 mg QE/mL and seemed to agree reasonably with their labelled content even though the content of TF in S8 was slightly lower than its value stated on the product package. Among all the samples, S4

had the lowest contents of TP and TF that were far less than the contents of TP and TF in other samples. It also confirmed that a higher TF content corresponded to a higher TP content in poplar type propolis. However, the TF contents of 6 samples (S5, S7, S15, S16, S17 and S20) were higher than their TP content which was unexpected. This might be resulting from the adulteration of propolis by poplar tree gum.

Chapter 4. Commercial Propolis Liquid Products: Comparison of Functional Properties

Abstract

The antioxidant and antimicrobial capabilities of 20 liquid propolis products from 4 countries (Australia, China, Korea and New Zealand) were investigated in this chapter. In terms of antioxidant property, the IC_{50} value of products ranged from 0.24 ± 0.02% to 0.93 ± 0.03%, except samples with alkaline pH (S15 and S20). Two Korean propolis samples (S15 and S20) with high pH levels had higher IC_{50} values (4.34 ± 0.43% and 1.78 ± 0.06%) indicating their antioxidant activity being lower than the other propolis samples. For the antimicrobial activity, Gram-positive bacteria (*S. aureus* and *B. cereus*) were more sensitive to all propolis products than Gram-negative bacteria (*E. coli*). Among all propolis samples from different countries, New Zealand products had both relatively higher antioxidant and antimicrobial activities.

4.1 Introduction

Propolis is natural substance of honeybee product, which is also named bee glue. It is a resin-like material gathered from various organs from plants, which can contribute to the different chemical compositions in propolis (Xavier et al., 2017; Afrouzan et al., 2018; Liben, Atlabachew, & Abebe, 2018; Bankova et al., 2019). These compounds vary from the region, source of plant, even seasons for collecting and species of honeybees (Markham et al., 1996; Silici & Kutluca, 2005; Zabaiou, Fouache, Trousson, Baron, Zellagui, et al., 2017; Bankova et al., 2019). The main compounds, such as phenolics and flavonoids, are associated with the functional properties of propolis (Huang et al., 2014; Oryan et al., 2018a). Various functional properties have been widely studied, including antioxidant (Kumazawa et al., 2004; Zunini et al., 2010; Toreti, Sato, Pastore, & Park, 2013; Sforcin, 2016), antimicrobial (Silici & Kutluca, 2005; Viuda-Martos et al., 2008; Al-Ani, Zimmermann, Reichling, & Wink, 2018) and anticancer (Kumazawa et al., 2004).

The assay of antioxidant capacity can be conducted by DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) assay (Archaina et al., 2015; Socha et al., 2015). The principle of this

approach is the redox reaction between free radicals and reductant, which is evaluated by measuring the change of absorbance by UV-spectrophotometry at 517 nm (Xu et al., 2009; Socha et al., 2015). The antioxidant property of samples measured as the radical scavenging activity (RSA %) is calculated and expressed as the half inhibitory concentration (IC_{50}).

For the antimicrobial activity, broth dilution and agar well diffusion are the most common approaches (Almeida et al., 2017). For the agar well diffusion method, according to Afrouzan et al. (2018) and do Nascimento et al. (2018), the antimicrobial activity is evaluated by measuring the diameter of the inhibitory halo on the agar plate around the agar well after a standard incubation. The strength of the antimicrobial property evaluated by the broth dilution approach can be applied to determine the minimum inhibitory concentration (MIC) of each propolis product by incubating different concentrations in microtiter plates. Previous studies showed that Gram-positive bacteria are more sensitive to propolis than Gram-negative bacteria (Alvarez-Suarez, 2017; Kasote et al., 2017).

The antioxidant and antimicrobial activity of 20 different commercial propolis liquid products obtained from 4 different countries as described in Chapter 3 were analysed and compared in this chapter. The antioxidant capacity was evaluated by the DPPH$^{\bullet}$ assay. The strength of the antioxidant capacity was ranked by the RSA%. Both agars well diffusion and broth dilution methods were used to assess the antimicrobial activity of the propolis samples. The evaluation of strength for the property was expressed as diameter of inhibitory halo and MIC value, respectively.

4.2 Materials and Methods

4.2.1 Commercial Propolis Samples

Twenty commercial liquid propolis products, which were manufactured in 4 different countries (Australia, China, Korea and New Zealand) as shown in Chapter 3 (Table 3.1) were analysed for their antioxidant and antimicrobial properties.

4.2.2 Determination of Antioxidant Property

The antioxidant property of propolis samples was analysed by the DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) assay reported by Xavier et al. (2017) with some modifications. DPPH

was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and used as a free radical reagent to determine the antioxidant activity of propolis samples by measuring their free radical scavenging capacity. Initially, all propolis samples were diluted to a series of different concentrations with absolute methanol. The concentrations of each sample after dilution are shown in Table 4.1 which were based on the total phenolic (TP) content of each propolis samples analysed in Chapter 3 (Table 3.5). The diluted samples (0.1 mL) were then mixed with 2.9 mL of $DPPH^{\bullet}$ (0.05 mg/mL in absolute methanol) followed by incubation in the dark for 30 minutes at room temperature. A mixture of 0.1 mL of absolute methanol and 2.9 mL $DPPH^{\bullet}$ was used as a control solution. All samples and the control solution were measured for their absorbance at 517 nm against absolute methanol as blank using a UV-Visible spectrophotometer (Shimadzu UV-1601). The antioxidant property of samples analysed by determining their radical scavenging activity (RSA%) was expressed as the IC_{50} value which is defined as the half maximal inhibitory concentration (Xavier et al., 2017). This was calculated by the plot of RSA% against the concentration of sample. The equation for RSA% is shown below:

$$\text{RSA\%} = (1 - \frac{A_S}{A_C}) \times 100$$

where Ac represents the absorbance of the control solution and As represents the absorbance of diluted samples.

Table 4.1 Dilutions of propolis samples to different concentrations with methanol for antioxidant analysis

Countries	Sample	Concentration (% v/v)					
Australia	S1	1.00	0.50	0.33	0.25	0.20	0.17
	S2	2.00	1.00	0.50	0.33	0.25	0.17
Korea	S3	4.00	2.00	1.00	0.50	0.33	0.25
	S4	1.00	0.50	0.33	0.25	0.20	0.17
	S5	1.00	0.50	0.33	0.25	0.20	0.17
	S6	2.00	1.00	0.50	0.33	0.25	0.17
China	S7	1.00	0.50	0.33	0.25	0.20	0.17
	S8	1.00	0.50	0.33	0.25	0.20	0.17
NZ	S9	1.00	0.50	0.33	0.25	0.20	0.17
	S10	1.00	0.50	0.33	0.25	0.20	0.17
	S11	1.00	0.50	0.33	0.25	0.20	0.17
	S12	1.00	0.50	0.33	0.25	0.20	0.17
	S13	1.00	0.50	0.33	0.25	0.20	0.17
	S14	1.00	0.50	0.33	0.25	0.20	0.17
Korea	S15	100.00	10.00	5.00	3.33	2.50	2.00
	S16	10.00	5.00	2.00	1.00	0.50	0.33
	S17	10.00	5.00	2.00	1.00	0.50	0.33
	S18	10.00	5.00	2.00	1.00	0.50	0.33
	S19	10.00	5.00	2.00	1.00	0.50	0.33
	S20	10.00	5.00	2.00	1.00	0.50	0.33

Blue colour represents glycol-based propolis;
Orange colour represents ethanol extracted propolis;
Green colour represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20;
Purple colour represents water-soluble ethanol extracted propolis containing Tween 20.

4.2.3 Determination of Antimicrobial Property

Materials, microorganisms and apparatus

The visible spectrophotometer (Novaspec III) from Amersham Biosciences was used to standardise the microbial inoculum. The information of materials and microorganisms which were used to test the antimicrobial property is shown in Table 4.2.

Table 4.2 Materials and microorganisms used to determine the antimicrobial activity of propolis samples

Materials	Supplier
Escherichia coli	NCTC 8196
Bacillus cereus	MU-A44
Staphylococcus aureus	NCTC 4163
Tryptone soy broth (TSB)	ThermoFisher
Mueller Hinton agar (MHA)	ThermoFisher
Mueller Hinton broth (MHB)	ThermoFisher
Resazurin	Certified by Biological Stain Commission
96-well tissue culture plate	BioFil
Plastic petri dish (90×15 mm)	LabServ

Culture media preparation

Tryptone soy broth (TSB), Mueller Hinton agar (MHA), and Mueller Hinton broth (MHB) were rehydrated in RO (reverse osmosis) water in media bottles, respectively, by following the ratios shown in the directions for use of each medium. All culture media were autoclaved for 15 minutes at 121°C for sterilisation before use.

Standard culture suspension preparation

Three bacteria were isolated and cultured on nutrient agar by the microbiology lab at Massey University in Auckland. They were then inoculated into TSB and incubated at appropriate temperatures for 24 hours, including *Escherichia coli* (35°C), *Bacillus cereus* (30°C) and *Staphylococcus aureus* (35°C). Each microbial suspension stock was then standardised to the 0.5 McFarland turbidity (1 to 2×10^8 CFU/mL) by adjusting the ratio of culture suspension and TSB and measuring the absorbance value at 625 nm to be in the range from 0.08 to 0.13 according to the two standard guidelines created by the Clinical and Laboratory Standards Institute (CLSI) (2012) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2017).

Well diffusion method

A well diffusion method recommended by the EUCAST (2017) was used to determine the antimicrobial activity of the propolis samples with some modifications. One mL of the standardised microorganisms of each bacterium (0.5 McFarland) was added into 14 mL melted MHA and well mixed to be diluted into 1×10^7 CFU/mL. Then, all cultures

containing MHA were poured into petri dishes and solidified. After solidification, six wells (6 mm diameter) were made in each petri dish by using a sterile cock borer. Exact 35 µl of stock propolis samples was pipetted into the wells of every petri dish, as well as a positive control (70% ethanol) and a negative control (sterile RO water). All petri dishes were incubated at an optimum temperature for 24 hours (*S. aureus* and *E. coli* at 35°C and *Bacillus cereus* at 30°C), then the zones of inhibition were measured by a Vernier caliper.

Minimum inhibitory concentration (MIC)

The MIC of all propolis samples was analysed by the standard method according to the CLSI (2012) M7-A10 document. The 96 well microplates were used in this study and the procedures of Almeida et al. (2017) were followed with some modifications. The tested microorganisms were prepared as 1×10^6 CFU/mL by mixing 0.1 mL of the 0.5 McFarland standard culture suspension with 14.9 ml of MHB (150-fold dilution) before they were introduced into the well microplates. Accurately 100 µl of 70% ethanol was taken and added into the first row of the plate as a positive control and 100 µl of MHB in the last row of the plate as a negative control. A volume of 200 µl of each diluted sample was pipetted into the second row of the well plate and 100 µl of MHB in the rest of wells of the plate. The serial dilutions of propolis samples were prepared by taking 100 µl of the propolis solution from the previous row of the plate and well mixed with previously added 100 µl of MHB. Then, a 100 µl of the prepared bacterial suspension was introduced into each well of the microplate containing the propolis samples, positive or negative control. The microplates were incubated for 24 hours at 35°C for *S. aureus* and *E. coli* and 30°C for *B. cereus*. Before the visual observation of the results, a 2 to 4 hours secondary incubation was done by adding 40 µl of resazurin solution (100 µg/ml) into each well and incubating for coloration. The growth of bacteria was indicated by colour differences with the blue colour representing no bacterial growth in the well and the pink colour indicating the survival of bacteria (Almeida et al.,2017). Further information is shown in Appendix 5. All propolis samples were analysed in triplicate.

4.3 Results and Discussion

4.3.1 Antioxidant Capacity

The original data, including absorbance, calculated RSA% and regression equations of half inhibitory concentration (IC_{50}), are shown in Appendix 4. The final results of antioxidant capacity of propolis samples were expressed as IC_{50}. The smaller the IC_{50} value the stronger antioxidant activity the propolis liquid. The IC_{50} values measured from all samples are shown in Table 4.3. Also, the results of TP and TF contents analysed in Chapter 3 and reported in Table 3.5 are also shown in Table 4.3.

The results showed that the ranges of IC_{50} for the commercial propolis liquid products were between $0.24 \pm 0.02\%$ and $4.34 \pm 0.43\%$. In general, the IC_{50} values of most samples were less than 0.5% and had the tendency of decreasing with an increase in the TP and TF contents, except some of the Korean products like S16. The S16 sample had the TP content as 37.36 GE mg/mL which was similar to S3 but its IC_{50} was measured to be double ($0.74 \pm 0.11\%$). As the lowest results of IC_{50} means the greatest antioxidant capability, S12, S13 and S14 samples with IC_{50} values around 0.25% can be considered as the propolis product with the strongest antioxidant property. These three propolis liquids were also found to contain the highest TP and TF contents among the 20 samples. On the contrary the lowest contents of TP and TF were in S4 being 16.35 and 6.56%, respectively, whose IC_{50} was relatively higher being $0.93 \pm 0.03\%$ than the other samples. These results were similar to the previous study conducted by Viuda-Martos et al. (2008) which showed that the antioxidant activity of propolis was highly correlated to its phenolic and flavonoid contents. The correlation between antioxidant capacity and TP and TF contents in propolis samples from different countries is demonstrated in Figure 4.1 to compare the propolis antioxidant properties according to their place of production. As can be seen in Figure 4.1, the antioxidant capacity increased with either the TP or TF content of propolis samples regardless of their regions of manufacturing. As a result, the correlation for all samples, except the two samples from China (S7 and S8) with the R^2 values of the regression lines between IC_{50} and TP content being greater than 0.91. Especially the propolis samples from New Zealand and Australia had the R^2 values that were greater than 0.96. Similarly, the correlation between TF and antioxidant capacity for all samples was also very strong being greater than 0.93.

Table 4.3 The results of IC_{50} in 20 propolis samples. The results of TP, TF and pH are derived from Table 3.5 in Chapter 3.

Country	Sample	IC_{50} (%v/v)[a]	TP (mg/mL)	TF (mg/mL)	pH
Australia	**S1**	0.37 ± 0.04	40.50 ± 0.45	29.82 ± 0.27	3.55 ± 0.07
	S2	0.48 ± 0.05	31.44 ± 0.51	22.86 ± 0.24	5.37 ± 0.03
Korea	**S3**	0.37 ± 0.02	31.17 ± 0.63	21.80 ± 0.64	4.74 ± 0.03
	S4	**0.93 ± 0.03**	**16.35 ± 0.28**	**6.56 ± 0.37**	**4.65 ± 0.02**
	S5	0.32 ± 0.02	**31.35 ± 0.35**	**35.08 ± 0.60**	5.41 ± 0.03
	S6	0.49 ± 0.02	25.35 ± 0.05	18.97 ± 0.52	5.50 ± 0.01
China	**S7**	0.40 ± 0.02	**48.99 ± 0.31**	**51.29 ± 0.12**	4.36 ± 0.04
	S8	0.43 ± 0.02	48.87 ± 0.49	27.19 ± 0.76	4.79 ± 0.04
NZ	**S9**	0.45 ± 0.02	53.60 ± 0.53	41.30 ± 0.65	4.88 ± 0.02
	S10	0.39 ± 0.03	64.85 ± 0.72	53.87 ± 0.82	4.67 ± 0.04
	S11	0.79 ± 0.08	35.95 ± 0.49	24.74 ± 0.22	4.77 ± 0.03
	S12	0.25 ± 0.01	70.22 ± 0.47	58.97 ± 0.59	4.45 ± 0.01
	S13	0.26 ± 0.05	68.29 ± 0.62	54.86 ± 0.42	4.86 ± 0.02
	S14	0.24 ± 0.02	68.11 ± 0.47	54.63 ± 0.14	4.55 ± 0.02
Korea	**S15**	**4.34 ± 0.43**	**17.39 ± 0.37**	**20.84 ± 0.37**	**8.61 ± 0.06**
	S16	0.74 ± 0.11	**37.36 ± 0.47**	**54.09 ± 0.50**	5.16 ± 0.07
	S17	1.03 ± 0.10	**24.03 ± 0.42**	**29.68 ± 0.40**	5.54 ± 0.01
	S18	0.58 ± 0.05	22.00 ± 0.49	12.86 ± 0.19	4.61 ± 0.04
	S19	**0.81 ± 0.06**	**35.04 ± 0.60**	**27.72 ± 0.25**	**6.77 ± 0.07**
	S20	**1.78 ± 0.06**	**25.35 ± 0.40**	**24.43 ± 0.16**	**9.37 ± 0.03**

[a] IC_{50} were calculated by SPSS at 95% confidence level.
Blue colour represents glycol-based propolis;
Orange colour represents ethanol extracted propolis;
Green colour represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20;
Purple colour represents water-soluble ethanol extracted propolis containing Tween 20.

However, there were also some unexpected results observed. The TP and TF contents of S15 (17.39% and 20.84%) were similar to those of S4 (16.35% and 6.56%) but its IC_{50} value (4.34% ± 0.43%) was over 4 times higher than S4 (0.93% ± 0.03%). This was much higher than the other samples. Also, the antioxidant capacity of S19 and S20 samples

seemed to be uncorrelated to their TP and TF contents. These results could be due to the pH of the samples (S15, S19 and S20) with pH 8.61, 6.77 and 9.37, respectively, which were higher than the other samples being mostly lower than pH 5.55. The alkaline pH of S15 and S20 was due to the presence of potassium carbonate (K_2CO_3) which was added by the product manufacturer based on the product packaging labels. The alkaline pH seemed to reduce the antioxidant activity of the samples. These results were in agreement with the study of Sun, Mu and Xi (2017). They suggested that the antioxidant capacity of phenolic compounds is more likely to increase at a lower pH (e.g. pH 3, 5 and 7) than an alkaline pH (e.g. pH 8). Similarly, Friedman and Jürgens (2000) reported that phenolic compounds were unstable at high pH due to the transformation of their molecular structures. As a consequence, their ability to sequester free radicals is affected. For instance, some phenolic compounds such as gallic acid would be more negatively charged at a higher pH and lose the ability of hydrogen donation to free radicals (Viuda-Martos et al., 2008). Another possible reason for the relatively higher IC_{50} values observed in those propolis samples could be due to the presence of TWEEN 20 in the products (S15, S19 and S20). According to the study reported by Akhila (2010), once the concentration of TWEEN 20 is above its critical micelle concentration, phenolic compounds would be entrapped by the formation of micelles. Therefore, micelles might play a role as a barrier that prevents the bioactive phenolics in propolis from acting as antioxidant.

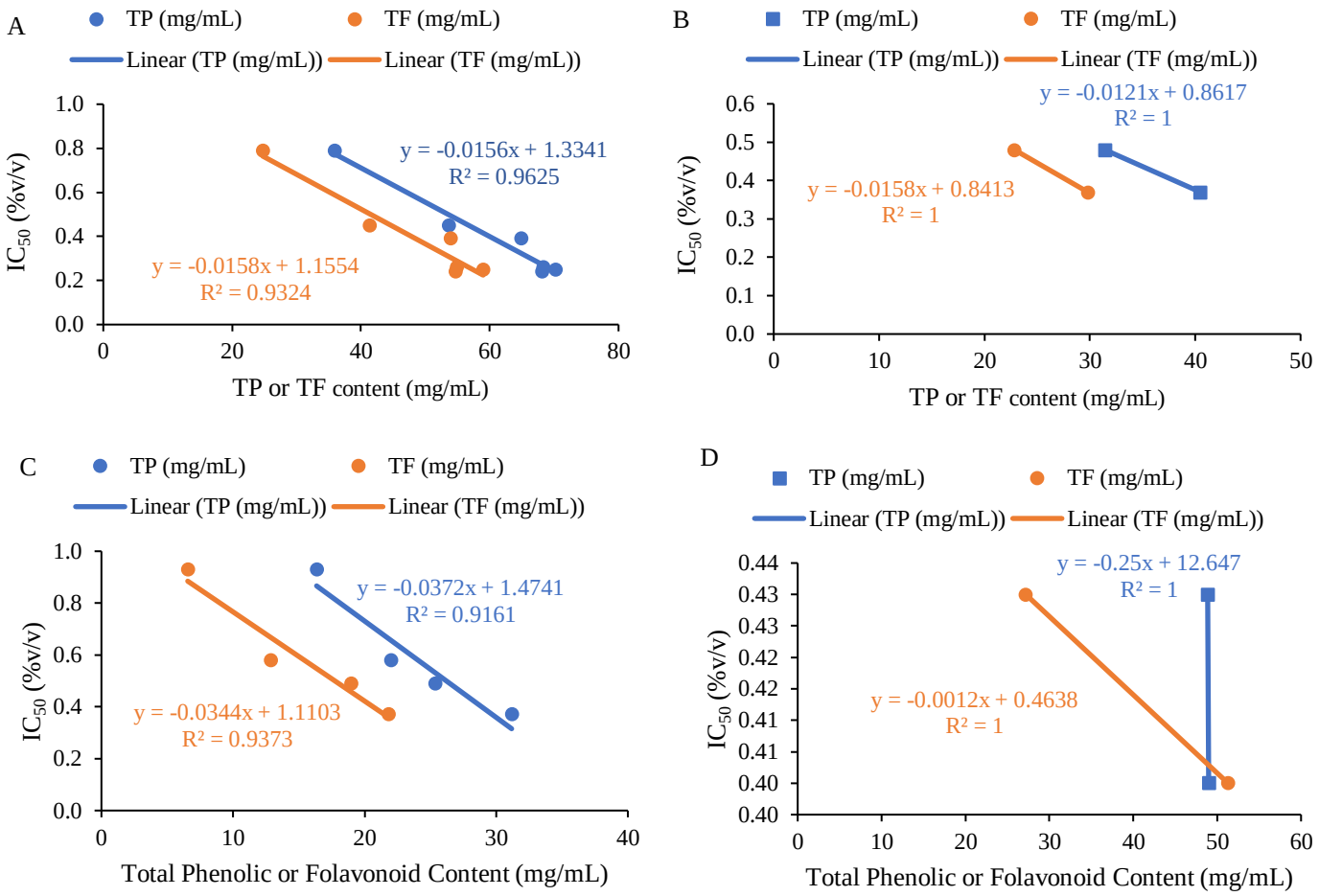

Figure 4.1 Correlation between antioxidant capacity and TP or TF content in (A) New Zealand propolis products (S9-S14); (B) Australian propolis products (S1 and S2); (C) Korean propolis products (S3, S4, S6 and S18); and (D) Chinese propolis products (S7 and S8)

4.3.2 Antimicrobial Activity

The detailed data for antimicrobial activity of 20 propolis samples determined by the agar well diffusion and broth dilution methods are demonstrated in Appendix 5. The greater inhibitory halo and smaller MIC values indicate the stronger antimicrobial activity. In general, as shown in Table 4.4, all propolis samples had greater influence on the Gram-positive bacteria (*S. aureus* and *B. cereus*) than the Gram-negative bacteria (*E. coli*), which was in line with the previous studies reported by some other researchers (Almeida et al., 2017; Kasote et al., 2017; Afrouzan et al., 2018). This means that Gram-positive bacteria are more sensitive to propolis than Gram-negative bacteria.

The results of well diffusion method indicated that the inhibition halos of all propolis samples except S7 for *E. coli* were much smaller being < 5.00 mm than the other two Gram-positive bacteria. Moreover, the inhibition zones of S15 and S20 against *E. coli* were hardly seen by visual observation, which indicated the measure was 0.00 ± 0.00 mm. However, the two samples showed a better inhibitory effect on *S. aureus* (12.78 ± 0.20 mm and 10.78 ± 0.25 mm) and *B. cereus* (8.87 ± 1.04 mm and 9.27 ± 0.38 mm). On the other hand, S19 had the lowest inhibitory effect on the Gram-positive bacteria among the 20 samples, in which the inhibitory zones were less than 4.8 mm.

As shown in Table 4.4, the MIC values of propolis samples (S9 to S14) manufactured in New Zealand ranged from 0.26% to 0.39% against *S. aureus*, 0.10% to 0.33% against *B. cereus*, and 3.13% to 6.25% against *E. coli*. Five (S15, S16, S17, S18, and S20) out of 10 Korean propolis samples (S15, S16, S17, S18, and S20) also had the MIC values less than 0.40% against the Gram-positive bacteria. According to Kasote et al. (2017), the samples with MIC less than 1.00% could be considered as decent antimicrobials. The estimated MIC against *E. coli* was 3.13% for the propolis samples obtained from Australia (S1 and S2) and New Zealand (S9-S14), 12.50% for the samples from China (S7 and S8) and ranging from 6.25% to 25.00% for the samples from Korea (S3-S6, and S15-S20)

However, unexpected results were also found in S2 and S7, which had a large inhibition zone but the high MIC values against the Gram-positive bacteria. This might be attributed to the high viscosity of samples, which tended to form micelles when samples were diluted by MHB before the MIC test. It was indicated that if the bioactive compounds were solubilised within micelles, there would be an adverse effect on antimicrobial

activity of the products (Khunkitti, 2010). This can also explain the low MIC value for S19. As it contained Tween 20 contributing to its high viscosity, which may also form micelles when mixed with MHB.

Table 4.4 Antimicrobial activity of 20 propolis liquid products evaluated by well diffusion and MIC methods

Samples	*S. aureus*		*B. cereus*		*E. coli*	
	Zone of Inhibition (mm)	MIC (%v/v)	Zone of Inhibition (mm)	MIC (%v/v)	Zone of Inhibition (mm)	MIC (%v/v)
S1	8.72 ± 0.74	0.78 ± 0.00	9.70 ± 0.53	1.56 ± 0.00	2.50 ± 0.25	3.13 ± 0.00
S2	10.80 ± 0.10	3.13 ± 0.00	11.63 ± 1.35	2.60 ± 0.90	4.17 ± 0.84	3.13 ± 0.00
S3	9.10 ± 0.82	1.04 ± 0.45	8.32 ± 0.57	0.78 ± 0.00	2.63 ± 0.39	3.13 ± 0.00
S4	12.60 ± 0.33	1.56 ± 0.00	7.82 ± 0.73	2.08 ± 0.90	3.18 ± 0.08	3.13 ± 0.00
S5	9.65 ± 0.48	2.60 ± 0.90	7.73 ± 0.39	3.13 ± 0.00	2.33 ± 0.22	5.21 ± 1.80
S6	10.45 ± 0.05	1.56± 0.00	8.48 ± 0.42	1.56 ± 0.00	2.25 ± 0.25	6.25 ± 0.00
S7	10.28 ± 0.19	2.60 ± 0.90	10.52 ± 0.47	2.08 ± 0.90	7.13 ± 1.12	6.25 ± 0.00
S8	12.48 ± 0.37	1.04 ± 0.45	11.02 ± 0.68	0.78 ± 0.00	1.73 ± 0.92	6.25 ± 0.00
S9	12.72 ± 0.23	0.39 ± 0.00	9.92 ± 0.57	0.20 ± 0.00	4.03 ± 0.74	6.25 ± 0.00
S10	13.68 ± 0.18	0.26 ± 0.11	9.87 ± 0.86	0.10 ± 0.00	3.82 ± 0.91	3.13 ± 0.00
S11	12.65 ± 0.51	0.26 ± 0.11	8.72 ± 0.60	0.13 ± 0.06	4.00 ± 0.66	3.13 ± 0.00
S12	10.98 ± 0.69	0.33 ± 0.11	7.80 ± 0.40	0.16 ± 0.06	2.83 ± 0.65	3.13 ± 0.00
S13	11.55 ± 0.13	0.39 ± 0.00	8.98 ± 0.62	0.33 ± 0.11	3.50 ± 0.23	3.13 ± 0.00
S14	11.45 ± 0.53	0.26 ± 0.11	7.52 ± 0.58	0.20 ± 0.17	2.90 ± 0.29	3.13 ± 0.00
S15	12.78 ± 0.20	0.39 ± 0.00	8.87 ± 1.04	0.20 ± 0.00	0.00 ± 0.00	3.13 ± 0.00
S16	8.93 ± 0.25	0.26 ± 0.11	6.27 ± 0.16	0.13 ± 0.06	0.55 ± 0.32	3.13 ± 0.00
S17	8.85 ± 0.57	0.20 ± 0.00	7.13 ± 0.25	0.13 ± 0.06	0.45 ± 0.14	4.17 ± 1.80
S18	12.65 ± 0.55	0.33 ± 0.11	9.60 ± 1.11	0.10 ± 0.00	3.30 ± 0.32	3.13 ± 0.00
S19	4.78 ± 0.18	5.21 ± 1.80	3.22 ± 0.12	3.13 ± 0.00	1.45 ± 0.29	12.50 ± 0.00
S20	10.78 ± 0.25	0.39 ± 0.00	9.27 ± 0.38	0.10 ± 0.00	0.00 ± 0.00	3.13 ± 0.00

Blue colour represents glycol-based propolis;
Orange colour represents ethanol extracted propolis;
Green colour represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20;
Purple colour represents water-soluble ethanol extracted propolis containing Tween 20.

Unlike the high correlation was observed for the propolis samples between their antioxidant capacity and the TP or TF content, the antimicrobial activity of propolis samples was found to have no relationship with their TP or TF content. As can be seen in Figures 4.2 and 4.3, all the R^2 were less than 0.08, which indicated the correlation between TP or TF content and the antimicrobial property was absent. This was totally different from the effect of phenolics on antioxidant property in which all the phenolic compounds may contribute to but only certain phenolic compounds such as CAPE may have antimicrobial activity (Viuda-Martos et al., 2008). This implies that the antimicrobial activity of propolis is not solely based on or derived directly from the phenolic compounds (Viuda-Martos et al., 2008).

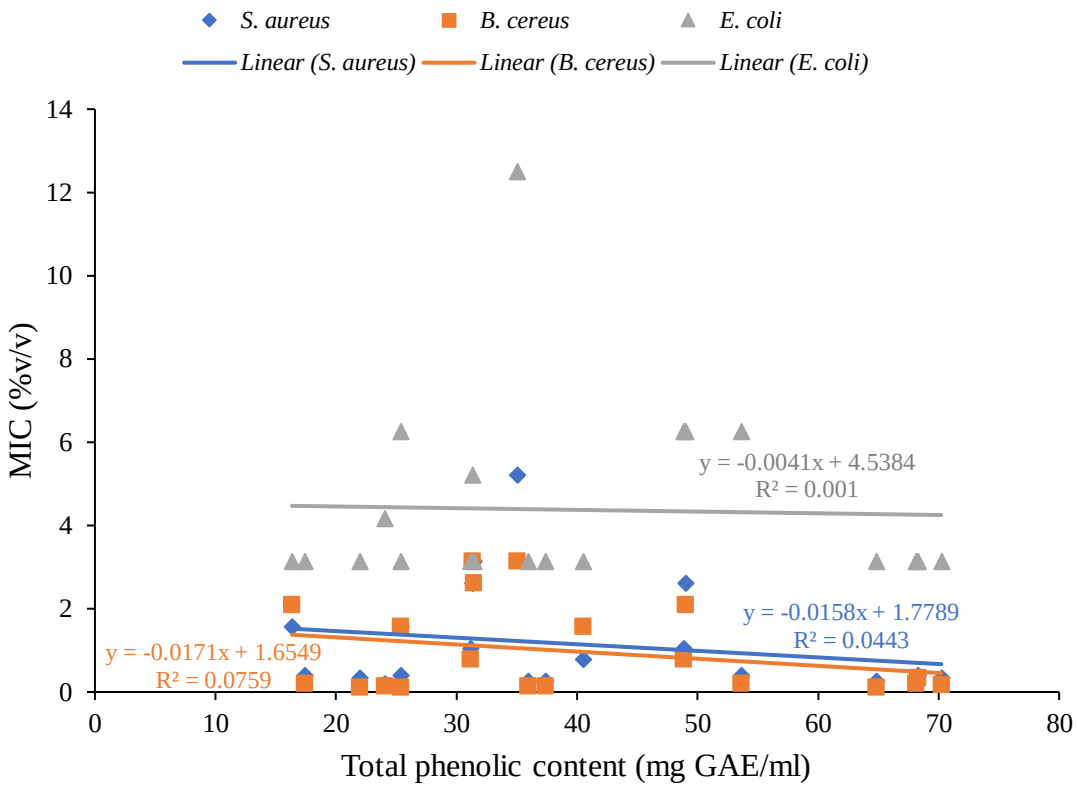

Figure 4.2 Plot of MIC vs TP for 20 commercial liquid propolis samples

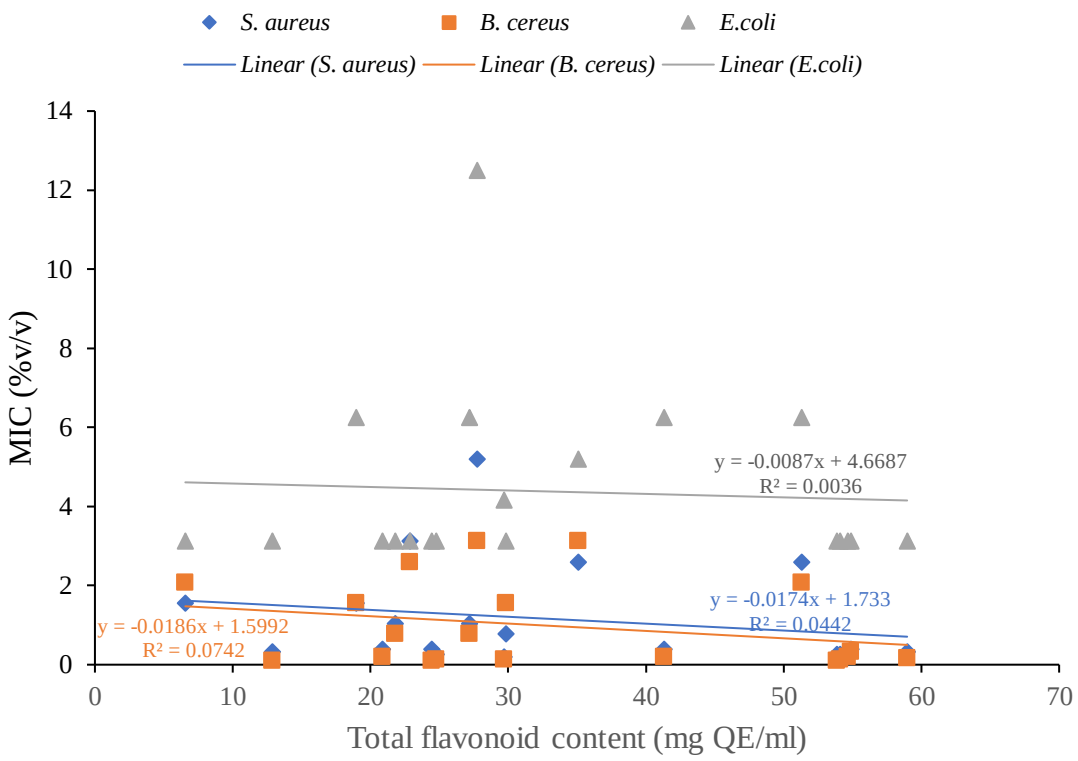

Figure 4.3 Plot of MIC vs TF for 20 commercial liquid propolis samples

4.4 Conclusions

The antioxidant capacity of propolis products was highly correlated to their TP and TF contents as the higher TP and TF contents contributed to the stronger antioxidant activity for the propolis samples. The products from New Zealand (S12-S14) had the highest antioxidant activity among the 20 samples while the lowest antioxidant value of product was from Korea (S15). This was attributed to the alkaline pH (8.61) of the product, as the functional compounds (e.g. phenolics) are more stable in low pH than in high pH.

On the contrary, there was no correlation between TP or TF content and the antimicrobial activity of propolis products because of not all phenolic compounds have antimicrobial activity. All samples showed greater effects on the Gram-positive bacteria (*S. aureus* and *B. cereus*) than the Gram-negative bacterium (*E. coli*). Among 20 samples, Australian and New Zealand propolis products showed a slightly higher antimicrobial activity against *E. coli* than the products from China and Korea. Also, compared to the propolis products from different regions, the products from New Zealand had greater antimicrobial activity.

Chapter 5. Commercial Poplar Type Propolis Liquid Products: Elements and Adulteration in Propolis

Abstract

The quality indices (heavy metal elements, rare earth elements, and salicin content) of 20 commercial liquid propolis products obtained from Australia, China, Korea and New Zealand were investigated and compared. The concentration of elements in propolis varied from the geographic regions. The highest level of arsenic and lead was found in S14 (New Zealand product), and cadmium was the greatest in S8 (Chinese propolis). Also, S8 was found to contain 15 rare earth elements (^{140}Ce, ^{163}Dy, ^{166}Er, ^{157}Gd, ^{165}Ho, ^{139}La, ^{175}Lu, ^{146}Nd, ^{141}Pr, ^{147}Sm, ^{159}Tb, ^{232}Th, ^{169}Tm, ^{89}Y, and ^{172}Yb), and had the highest ^{140}Ce content (36.10 µg/L). The suspected adulteration was found in 7 Korean propolis products in this project. The content of salicin in propolis samples detected by HPLC-MS ranged from 17.10 ± 5.20 mg/L to 209.20 ± 10.28 mg/L, whereas it was detected from 64.43 ± 1.91 mg/L to 589.23 ± 17.01 mg/L by HPLC. Among all samples analysed in this study, Australian propolis seemed to have no adulteration and have lower heavy metal contamination, which could be considered as higher quality of the products.

5.1 Introduction

Propolis is a well-known resinous substance with complex compounds and has been applied to functional foods as a phytochemical ingredient (Cantarelli, Camiña, Pettenati, Marchevsky, & Pellerano, 2011; Bonvehí & Bermejo, 2013). Numerous functional properties of propolis have been broadly studied including antioxidant, antimicrobial, anti-inflammatory and anticancer properties (Bankova et al., 2000; Silici & Kutluca, 2005; Viuda-Martos et al., 2008; Sforcin, 2016; Zabaiou, Fouache, Trousson, Baron, Zellagui, et al., 2017). As a consequence, in recent years there has been a growing increase in numerous commercial applications of propolis (Kumazawa et al., 2004; Toreti, Sato, Pastore, & Park, 2013).

Propolis produced by honeybees derived from diverse plants in different regions can vary in their chemical compositions (e.g. phenolics compounds and flavonoids) (Bankova et al., 2000; Huang et al., 2014) as well as mineral and heavy metal compositions (Gong et

al., 2012; Bonvehí & Bermejo, 2013; Korn et al., 2013). Minimal concentration of heavy metals (As, Cd, Hg, and Pb) were determined in propolis from South Spain by Bonvehí and Bermejo (2013). On the contrary, propolis from certain areas of south Poland was found contaminated with lead (Formicki et al., 2013).

The sources of propolis under different environmental conditions (e.g. air and soil) could contaminate the propolis products (Cvek et al., 2008; Bonvehí & Bermejo, 2013). Besides the contamination of metals into propolis, poplar tree gum has been reported to be added into some commercial propolis products, since it could reduce the cost of propolis and make more profit (Zhang et al., 2015). Poplar tree gum has similar colour, smell, chemical and functional properties to the poplar type propolis (Vardar-Ünlü et al., 2008). Poplar trees are the main source for poplar type propolis which is generally found in non-tropical regions of Asia, Europe and North America (Vardar-Ünlü et al., 2008).

Few studies have attempted to investigate the methods to distinguish the poplar tree gum from propolis (Wu, Sun, Zhao, Li, & Zhou, 2008; Zhang et al., 2011). During the process of propolis production by honeybees, the salivary and enzymatic secretions of honeybees could modify the compounds of resins collected from plants, which results in the distinguished constituents between the propolis and poplar tree gum (Bonvehí, Coll, & Jordà, 1994; Burdock, 1998). For instance, the phenolic glycosides (e.g. salicin and its derivatives) have been investigated as indicators to characterise the poplar tree gum (Pearl & Darling, 1971), since these compounds might be hydrolysed by β-glycosidase during the propolis collection and processing by honey bees, hence not present in natural propolis products (Zhang et al., 2011; Zhang et al., 2015). The presence of salicin can thus be used as a marker for detecting adulteration of propolis by poplar tree gum.

There are few studies on the analysis of commercial propolis products in terms of their mineral contents and heavy metal elements (Gonzalez-Martin et al., 2018). The objectives of this chapter were to identify the metal elements and analyse adulteration of 20 commercial liquid propolis samples manufactured from 4 different countries (Australia, China, Korea and New Zealand) based on the determination of salicin content.

5.2 Materials and Methods

5.2.1 Commercial Propolis Samples

Twenty commercial liquid propolis products described in Chapter 3 (Table 3.1) were also used in this study.

5.2.2 Determination of Metal Elements

Reagents and apparatus

The reagents and standards used to analyse metal elements in propolis are shown in Table 5.1. Besides, microwave digestion system MARS 6 from CEM company and inductively coupled plasma-mass spectrometry (ICP-MS) ICAP-Q from Thermo Fisher Scientific company located in China were used in this experiment.

Table 5.1 Reagents and standards for determination of metal elements

Name	Concentration/Grade	Company
Ultrapure water	18.2 MΩcm	Milli-Q, Millipore Corp
Nitric acid	Trace Metal grade (70%)	Fisher Chemical
Hydrogen peroxide	MOS grade	Merck
Standard solution of Lead (Pb) (GBW08619)	1000 μg/mL (1% HNO_3)	National Institute Metrology
Standard solution of Arsenic (As) (GBW08611)	1000 μg/mL (1% HNO_3)	National Institute Metrology
Standard solution of Cadmium (Cd) (GBW08612)	1000 μg/mL (1% HNO_3)	National Institute Metrology
Standard solution of Rare earth elements (Ce, Nd, Sm, Tb, Er, Pr, Ho, Tm, Gd, Dy, Lu, Yb, La, Y) GBW(E) 082428	10 μg/mL (1% HNO_3)	National Institute Metrology

Sample preparation

The experimental method reported by Gonzalez-Martin et al. (2018) was used with some modifications. Before putting in the microwave digestion instrument, around 0.5 gram of propolis sample was weighed into a Teflon digestion tube, then 6 mL of nitric acid and 2 mL of hydrogen peroxide were added into the same tube. The weight of each sample used is shown in Appendix 7, and the digestion program used is illustrated in Table 5.2. After the digestion, the cooled digested solution was transferred into a 25 mL volumetric flask

followed by adding ultrapure water to the mark. All glassware used were washed with nitric acid before use, and all samples were analysed in triplicate. Also, 6 mL nitric acid and 2 mL hydrogen peroxide were used as control (blank).

Table 5.2 Digestion program for propolis samples

Step	Ramp (min)	Hold (min)	Temperature (°C)	Power (KW)
1	15:00	10:00	120	600
2	15:00	20:00	180	600

Standard curve preparation

The stock standard solutions of lead (Pb), arsenic (As) and cadmium (Cd) were prepared by diluting the standard stock solutions shown in Table 5.1 from 1000 μg/mL to 10 μg/mL with 1% nitric acid. Then, the standard solutions of the three elements were stepwise diluted for the preparation of a standard curve for each metal from 10 μg/mL to 20 μg/L, 10 μg/L, 5 μg/L, 2 μg/L, and 1μg/L, respectively, with 1% nitric acid. Besides, the standard stock solution of rare earth elements also shown in Table 5.1 was diluted initially from 10 μg/mL to 1 μg/mL with 1% nitric acid, then the stock solution was stepwise further diluted to a series of standard solutions of rare earth elements (2 μg/L, 1 μg/L, 0.5 μg/L and 0.1 μg/L) with 1% nitric acid to create as a standard curve.

Measurements

All elements were determined by ICP-MS, which is one of the most significant advanced techniques for metal elements analysis (Gonzalez-Martin et al., 2018). ^{208}Pb, ^{75}As, ^{111}Cd, ^{140}Ce, ^{163}Dy, ^{166}Er, ^{157}Gd, ^{165}Ho, ^{139}La, ^{175}Lu, ^{146}Nd, ^{141}Pr, ^{147}Sm, ^{159}Tb, ^{232}Th, ^{169}Tm, ^{89}Y, and ^{172}Yb were chosen as the target elements. The limits of detection (LOD) for each element can be expressed as the standard deviation (SD) values obtained from ten independent analyses of the experimental blanks. The LOD was 0.5 μg/L for ^{75}As, and ^{111}Cd, 5 μg/L for ^{208}Pb, 0.9 μg/L for ^{140}Ce, 0.3 μg/L for ^{163}Dy, 0.2 μg/L for ^{166}Er, 0.5 μg/L for ^{157}Gd, 0.1 μg/L for ^{165}Ho, 1.4 μg/L for ^{139}La, 0.1 μg/L for ^{175}Lu, 0.8 μg/L for ^{146}Nd, 0.7 μg/L for ^{141}Pr, 0.5 μg/L for ^{147}Sm, 0.2 μg/L for ^{159}Tb, 0.5 μg/L for ^{232}Th, 0.1 μg/L for ^{169}Tm, 1.1 μg/L for ^{89}Y, and 0.2 μg/L for ^{172}Yb, respectively.

5.2.3 Determination of Salicin for Adulteration of Propolis

The phenolic glycosides (e.g. salicin and its derivatives) have been investigated as indicators to characterise the poplar tree gum (Pearl & Darling, 1971). Since these compounds might be hydrolysed by β-glycosidase during propolis collection and processing by honeybees (Zhang et al., 2011; Zhang et al., 2015), the presence of salicin in propolis can thus be used as a marker for detecting poplar tree gum adulterated in propolis.

Reagents

The reagents and standards used to analyse salicin in propolis are shown in Table 5.3.

Table 5.3 Reagents and standards for determination of salicin in propolis

Name	Concentration/Grade	Company
Ultrapure water	18.2 MΩcm	Milli-Q, Millipore Corp
Acetonitrile	HPLC grade (>99.9%)	Fisher Chemical
Methanol	HPLC grade (>99.9%)	Fisher Chemical
Ethanol	HPLC grade (>99.9%)	Fisher Chemical
Phosphoric acid	HPLC grade (>85%)	Fisher Chemical
Membrane Filter	0.45 μm acrodisc syringe filter	Waters
Salicin standard	≥ 98%	Sigma-Aldrich

High performance liquid chromatography (HPLC)

Sample preparation

All propolis samples were prepared in duplicate. Two grams of each sample were weighed and diluted into 50 mL by adding 75% ethanol in the volumetric flask. All diluted samples were filtered through a 0.45 μm membrane filter after well mixing.

Standard curve

Ten milligrams of salicin were weighed and diluted into a 10 mL volumetric flask with absolute methanol to make a 1 mg/mL stock standard solution. Then, a series of dilutions were made by stepwise diluting the stock solution to 0.06, 0.04, 0.02, 0.01 and 0.005 mg/mL, respectively, for constructing a standard curve.

Measurement

High performance liquid chromatography (HPLC) equipped with C18 column (4.6 mm × 250 mm × 5 μm) and UV-detector (Shimadzu LC-20AD) was used in the experiment. The temperature and detection wavelength used were 30°C and 213 nm, respectively. The limit of detection (LOD) was 65 mg/L, which was calculated by the SD values of ten independent analyses of the experimental blanks. The HPLC conditions for analysis are shown in Table 5.4.

Table 5.4 The conditions used for salicin determination in propolis by HPLC

Time (min)	Mobile Phase A% (Acetonitrile)	Mobile Phase B% (0.5% aqueous phosphoric acid)	Flow Rate (mL/min)
0.0	5	95	0.8
16.0	5	95	0.8
16.1	100	0	0.8
19.0	100	0	0.8
19.1	5	95	0.8
20.0	5	95	0.8

High performance liquid chromatography-mass spectrometry (HPLC-MS)

Sample preparation

All propolis samples were prepared in triplicate. One gram of each sample was weighed and diluted into 100mL by 75% ethanol in the volumetric flask. All sample dilutions were filtered through a 0.45 μm membrane filter after well mixing.

Standard curve

Ten milligrams of salicin were weighed and diluted into 10 mL volumetric flask with methanol to make a 1 mg/mL stock standard solution. Then, 100 μL of the stock solution were piped into 10 mL volumetric flask and diluted with methanol to prepare the working standard solution (10 mg/L). The working standard solution was stepwise diluted to 3.0, 2.0, 1.0, 0.8, 0. 6, 0.4, 0.2 and 0.1 mg/L to create the standard curve for salicin.

Measurement

High performance liquid chromatography-mass spectrometry (HPLC-MS) equipped with TQD-detector (Waters Co.) and ACQUITY BEN C18 column (2.1 mm × 50 mm × 1.7 μm) was used at 30°C. The LOD was 10 mg/L, which was calculated by the SD values of ten independent analyses of the experimental blanks. The conditions for analysis are shown in Tables 5.5 and 5.6.

Table 5.5 The conditions for salicin determination for HPLC part of HPLC-MS

Time (min)	Mobile Phase A% (Acetonitrile)	Mobile Phase B% (0.5% aqueous phosphoric acid)	Flow Rate (mL/min)
0.0	5	95	0.3
2.1	95	5	0.3
2.5	5	95	0.3
3.5	5	95	0.3

Table 5.6 The conditions for salicin determination for MS part of HPLC-MS

Parent (m/z)	Daughter (m/z)	Cone (V)	Collision (V)
309	184	48	18
309	147	48	20
309	23	48	22

5.3 Results and Discussion

5.3.1 Heavy Metal and Rare Earth Elements in Propolis

Microwave digestion and ICP-MS approach were used in this experiment. The original data are shown in Appendix 7. The quantities of heavy metals (As, Cd, and Pb) and rare earth elements in propolis samples were expressed in mg/L, as demonstrated in Tables 5.7 and 5.8.

It was found that the quantities of cadmium were much lower in all the commercial liquid propolis products than the other two heavy metal elements (arsenic and lead). The level

of cadmium in 15 out of 20 propolis samples was lower than the LOD (0.0005 mg/L), which indicated that these liquid propolis products were generally free from cadmium contamination. Although cadmium was detected in S3, S5, S7 and S16, the quantities were really low (0.0033 ± 0.0001 mg/L, 0.0013 ± 0.0001 mg/L, 0.0043 ± 0.0002 mg/L, and 0.0007 ± 0.0001 mg/L, respectively). However, S8 was an exception and the concentration of cadmium in S8 (Chinese propolis sample) was extremely higher than all other samples (0.9195 ± 0.0005 mg/L). The content of total arsenic in all samples ranged from 0.0058 ± 0.0002 mg/L to 0.4350 ± 0.0027 mg/L, except S19 whose total arsenic content was lower than its LOD. Besides, lead was found in all the liquid propolis samples, and the concentration of lead in all propolis products ranged from 0.0194 ± 0.0009 mg/L to 0.7253 ± 0.0024 mg/L. The highest level of total arsenic (0.4350 ± 0.0027 mg/L) and lead (0.7253± 0.0024 mg/L) was in the same propolis sample which was S14 (New Zealand propolis). As shown in Table 5.7, the mean concentration of lead (0.293 mg/L) in New Zealand propolis samples was generally higher than the propolis samples from other countries (0.066 mg/L for Australian propolis, 0.189 mg/L for Chinese propolis and 0.078 mg/L for Korean propolis). The contamination of heavy metals in Australian (S1 and S2) and Korean samples (S3-S6, and S15-S20) was less than the liquid propolis products produced from the other two countries (China and New Zealand).

Although it is not sure, these differences could be attributed to the geographic region, collecting method, processing approach and surrounding environment (Gong et al., 2012; Bonvehí & Bermejo, 2013). Lead is ubiquitous in the environment (Bogdanov, 2006) and generally comes from industrial pollution plus vehicle exhaust which contaminate air, pollen and propolis (Bonvehí & Bermejo, 2013). The contamination of lead in propolis is likely to be due to adsorption by plants from polluted air and the environment, and then collected by honeybees. The results obtained in this study were in agreement with some previous studies, which illustrated that the mean concentration of Pb in propolis was ranging from 0.023 to 0.843 mg/L (Roman & Popiela-Pleban, 2012; Aksoy et al., 2017; Gonzalez-Martin et al., 2018). Unlike lead, cadmium is from the metal industry and can be transferred from soil to plants, but can hardly be transported by air (Bogdanov, 2006). This indicates that cadmium contamination in the propolis products is originated from the geographic location of the raw propolis. For instance, the raw propolis of S8 (Chinese product), which was found to contain an exceptionally high amount of cadmium

compared to the other samples, might be from an industrial area which could lead to its contamination.

In the aspect of safety of the propolis for consumption, according to the European Commission (2006), the maximum levels for lead and cadmium in foodstuffs are 1.5 mg/L and 1.0 mg/L, respectively. It is worth noting that the lead and cadmium contents of all samples were below the limitations set by the European Commission (2006). Therefore, it could be considered that all the propolis liquid products used in this study were safe for consumption, except for S8 sample with 0.9195 ± 0.0005 mg/L cadmium which was almost close to 1.0 mg/L limit. However, there is no proper criterion to regulate the contamination limits for commercial propolis products. Based on the results shown in Table 5.7, the risk of arsenic and cadmium contamination in propolis seemed to be low. Although arsenic was detected in 95% of samples except for S19, only the inorganic compounds with trivalent arsenic (As^{+3}) are known to be highly toxic to human and organic compounds are non-toxic (Jain & Ali, 2000; Bonvehí & Bermejo, 2013).

As shown in Table 5.8, the fifteen rare earth elements were much lower than those three heavy metals in propolis samples, and Tb, Tm, Yb and Lu were below their LOD in all 20 samples. On the other hand, Ce was detected in 12 out of 20 samples, ranging from 0.963 µg/L to 3.160 µg/L, and Nd and Sm were found in 25% of samples with low levels (from 0.935 µg/L to 1.230 µg/L and 0.574 µg/L to 1.296 µg/L), except S8 (36.097 µg/L, 13.421 µg/L and 6.324 µg/L for Ce, Nd and Sm, respectively). This is supported by a previous research which indicated that Ce is abundant in the environment (Germund, 2004).

Table 5.7 The concentration of heavy metal elements in propolis

Countries	Sample	^{75}As (mg/L)	^{111}Cd (mg/L)	^{208}Pb (mg/L)
Australia	S1	0.0216 ± 0.0005	≤ 0.0005	0.1074 ± 0.0020
	S2	0.0211 ± 0.0004	≤ 0.0005	0.0251 ± 0.0005
Korea	S3	0.1032 ± 0.0003	0.0033 ± 0.0001	0.2265 ± 0.0006
	S4	0.0399 ± 0.0005	≤ 0.0005	0.0671 ± 0.0016
	S5	0.0702 ± 0.0010	0.0013 ± 0.0001	0.2621 ± 0.0020
	S6	0.0242 ± 0.0006	≤ 0.0005	0.0683 ± 0.0010
China	S7	0.1933 ± 0.0014	0.0043 ± 0.0002	0.2316 ± 0.0004
	S8	0.0415 ± 0.0018	**0.9195 ± 0.0005**	0.1459 ± 0.0020
NZ	S9	0.0665 ± 0.0006	≤ 0.0005	0.0904 ± 0.0016
	S10	0.2410 ± 0.0002	≤ 0.0005	0.1370 ± 0.0012
	S11	0.0496 ± 0.0018	≤ 0.0005	0.1057 ± 0.0016
	S12	0.2837 ± 0.0001	≤ 0.0005	0.2919 ± 0.0020
	S13	0.0963 ± 0.0010	≤ 0.0005	0.4066 ± 0.0016
	S14	**0.4350 ± 0.0027**	≤ 0.0005	**0.7253 ± 0.0024**
Korea	S15	0.0162 ± 0.0009	≤ 0.0005	0.0519 ± 0.0001
	S16	0.0170 ± 0.0007	0.0007 ± 0.0001	0.0378 ± 0.0018
	S17	0.0136 ± 0.0003	≤ 0.0005	0.0282 ± 0.0006
	S18	0.0058 ± 0.0002	≤ 0.0005	0.0194 ± 0.0009
	S19	≤ 0.0005	≤ 0.0005	0.0805 ± 0.0004
	S20	0.0109 ± 0.0003	≤ 0.0005	0.0948 ± 0.0004

Blue colour represents glycol-based propolis;
Orange colour represents ethanol extracted propolis;
Green colour represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20;
Purple colour represents water-soluble ethanol extracted propolis containing Tween 20.

It is worth noting that the detected quantities of rare earth elements in S8 (Chinese propolis) was obviously higher than in the other samples, which also contained the highest amount of cadmium content among the tested samples. This might result from the environment contamination to the plants and the collected part of the plants, as suggested by Cao, Chen, Gu, and Wang (2000) and Germund (2004) that its rare concentrations in plants are in the order: root > leaf > stem > grain

Table 5.8 The concentration of rare earth elements (μg/L) in propolis samples

Sample	^{89}Y (μg/L)	^{139}La (μg/L)	^{140}Ce (μg/L)	^{141}Pr (μg/L)	^{146}Nd (μg/L)	^{147}Sm (μg/L)	^{157}Gd (μg/L)	^{159}Tb (μg/L)	^{163}Dy (μg/L)	^{165}Ho (μg/L)	^{166}Er (μg/L)	^{169}Tm (μg/L)	^{172}Yb (μg/L)	^{175}Lu (μg/L)	^{232}Th (μg/L)
S1	≤1.1	≤1.4	1.856	≤0.7	1.001	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S2	≤1.1	≤1.4	≤0.9	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S3	1.125	≤1.4	0.967	≤0.7	1.230	0.574	≤0.5	≤0.2	0.371	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S4	≤1.1	≤1.4	1.626	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S5	1.567	≤1.4	≤0.9	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	0.327	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S6	≤1.1	8.033	≤0.9	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	0.876
S7	≤1.1	≤1.4	1.590	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S8	6.583	≤1.4	36.097	1.564	13.421	6.324	2.530	≤0.2	1.582	0.388	1.056	≤0.1	≤0.2	≤0.1	7.098
S9	≤1.1	≤1.4	≤0.9	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S10	≤1.1	≤1.4	≤0.9	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S11	≤1.1	≤1.4	≤0.9	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S12	≤1.1	≤1.4	≤0.9	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S13	≤1.1	≤1.4	0.945	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S14	≤1.1	≤1.4	≤0.9	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S15	≤1.1	≤1.4	3.160	≤0.7	1.194	1.296	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S16	≤1.1	≤1.4	1.365	≤0.7	0.935	0.926	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S17	≤1.1	≤1.4	0.963	≤0.7	≤0.8	0.575	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S18	≤1.1	≤1.4	2.920	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S19	≤1.1	≤1.4	1.936	≤0.7	≤0.8	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5
S20	≤1.1	≤1.4	2.759	≤0.7	1.052	≤0.5	≤0.5	≤0.2	≤0.3	≤0.1	≤0.2	≤0.1	≤0.2	≤0.1	≤0.5

Blue colour represents glycol-based propolis;
Orange colour represents ethanol extracted propolis;
Green colour represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20;
Purple colour represents water-soluble ethanol extracted propolis containing Tween 20.

5.3.2 Salicin Determination in Propolis

HPLC and HPLC-MS were used to determine the adulteration of poplar tree gum in propolis by analysing the presence and content of salicin in propolis. The final results of salicin determination expressed in mg/L are shown in Table 5.9. The original data are shown in Appendix 7.

Salicin was found in 7 out of 20 samples (S3, S5, S6, S15, S16, S18 and S20) by HPLC-MS while 9 samples were found to contain salicin by HPLC (S2, S3, S5, S6, S15, S16, S17, S18 and S20). Moreover, the detected quantities of salicin analysed by HPLC-MS were nearly half of the values detected by HPLC. The salicin content ranged from 17.10 ± 5.20 mg/L to 209.20 ± 10.28 mg/L detected by HPLC-MS, and from 64.43 ± 1.91 mg/L to 589.23 ± 17.01 mg/L by HPLC. Besides, the salicin content in S2 and S17 was below the LOD analysed by HPLC-MS while it was found by HPLC. The salicin was undetectable in propolis samples from China and New Zealand whereas it was found in Korean propolis by both HPLC and HPLC-MS methods.

These differences might be attributed to the different qualitative and quantitative methods of the equipment. The retention time was generally used to determine the existence of target component by HPLC. On the other hand, both retention time and the ion ratio of quantitative ions to qualitative ions were applied to the qualitative method of HPLC-MS (Agüero et al., 2011). Thereby, HPLC-MS could be more accurate than HPLC (Agüero et al., 2011).

Moreover, as shown in Figure 5.1, there was another peak near the retention time of salicin using the same separated program of HPLC (Zhang et al., 2011) which might have influenced the calculation of the salicin content. This could be more likely to result in the higher concentration of salicin determined by HPLC. Therefore, the data from HPLC-MS seemed more reliable than HPLC (Agüero et al., 2011). The commercial propolis products from Korea (S3, S5, S6, S15, S16, S18 and S20) seemed to have the highest incidence of adulteration with poplar tree gum among all samples. Zhang et al (2013) reported that the liquid propolis products had the highest incidence of adulteration with salicin, and salicin was detected in 80% of the commercial liquid propolis samples they analysed (mean concentration 160 mg/L).

Table 5.9 Salicin in propolis samples determined by HPLC and HPLC-MS

Countries	Sample	Salicin (mg/L) HPLC-MS	Salicin (mg/L) HPLC
Australia	S1	≤10	≤60
	S2	≤10	75.00 ± 0.00
Korea	S3	157.87 ± 3.50	262.50 ± 1.77
	S4	≤10	≤60
	S5	83.33 ± 2.65	125.00 ± 0.00
	S6	35.67 ± 0.74	75.00 ± 0.00
China	S7	≤10	≤60
	S8	≤10	≤60
NZ	S9	≤10	≤60
	S10	≤10	≤60
	S11	≤10	≤60
	S12	≤10	≤60
	S13	≤10	≤60
	S14	≤10	≤60
Korea	S15	209.20 ± 10.28	589.23 ± 17.01
	S16	17.10 ± 5.20	64.43 ± 1.91
	S17	≤10	79.23 ± 3.77
	S18	205.50 ± 2.88	368.88 ± 10.92
	S19	≤10	≤60
	S20	204.97 ± 0.55	382.65 ± 4.17

Sample code in blue colour represents glycol-based propolis;
Sample code in orange colour represents ethanol extracted propolis;
Sample code in green colour represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20;
Sample code in purple colour represents water-soluble ethanol extracted propolis containing Tween 20.

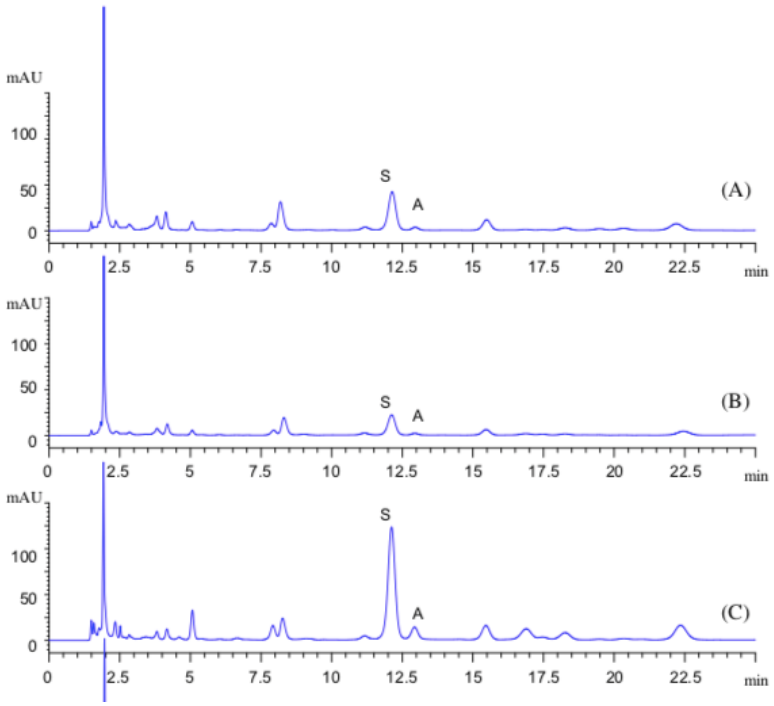

Figure 5.1 The chromatogram of salicin detected by HPLC (Zhang et al., 2011). S- Salicin; A- Unknown Compound

5.4 Conclusions

The quality of 20 commercial liquid propolis samples from 4 countries was investigated in this chapter based on the determination of heavy metal elements, rare earth elements, and adulteration (salicin content) index.

The variation of concentrations of heavy metal elements in the commercial liquid propolis products depended on various conditions, including geographic region, surrounding environment, collection method of the raw propolis, and processing technique of extraction. As shown from the results, there were fewer heavy metal elements detected in Australian and Korean products. It was worth noting that the highest arsenic and lead contents were detected in New Zealand propolis (S14) whereas Chinese propolis (S8) had the greatest amount of cadmium. Hence, further research may be needed to analyse the elements in the soil that raw propolis was collected to confirm whether it was due to contamination from natural environment or a manufacturing production line in a factory.

For rare earth elements, 11 out of 15 elements were detected in S8 (Chinese propolis) which also had the highest cadmium content. This may be from the environment, for instance, soil. Also, Ce was more common than other rare earth elements, which was

generally found in propolis samples in this project, probably due to its abundance in the environment.

Salicin was found in 7 out of 10 Korean propolis products (S3, S5, S6, S15, S16, S18 and S20), which indicated that the products might have been adulterated by poplar tree gum. It seemed that the propolis products from the other three countries were not adulterated as their salicin contents were below the LOD.

Overall, Australian propolis was unlikely to be adulterated and had lower heavy metal contaminations, which indicated the higher quality of the products.

Chapter 6. Overall Conclusions and Recommendations

6.1 Conclusions

Twenty liquid commercial propolis products from 4 countries (Australia, China, Korea and New Zealand) were investigated in this project. The physiochemical properties (e.g. colour, pH), chemical compositions (e.g. total phenolic and total flavonoid contents) and functional properties (e.g. antioxidant capability and antimicrobial activity) as well as three heavy metal elements (e.g. lead, cadmium, and arsenic), rare earth elements, and the adulteration (e.g. salicin) of these products were evaluated and compared.

The majority of the propolis products showed a brown colour with some red and green colour tint. Most samples were insoluble in water forming aggregates and precipitates, except for Korean samples (S15, S19 and S20) containing Tween 20 that were water-soluble. On the other hand, all propolis samples were miscible with ethanol although there was some flocculation formed in the mixture of propolis products, such as S8, S12 and S14 (GEP samples) and larger amount of sediments in S15 and S20 containing potassium carbonate.

Most propolis samples were acidic and had pH 4-5, whereas, some Korean propolis samples with potassium carbonate had an alkaline pH value. This might have influenced the determination of total phenolic (TP) content in products, as previous studies showed that the phenolic compounds were unstable in high pH solutions. Hence, samples containing added potassium carbonate (S15 and S20) had a higher pH and lower TP. The total flavonoid (TF) content of all samples met the level of TF specified on their product labels, except a sample from China (S8) which was slightly lower than the labelled value. Moreover, the TF content of six samples from Korea (S5, S7, S15, S16, S17 and S20) was higher than their TP content, which might be attributed to the presence of unexpected substances in the products, for instance, poplar tree gum extract.

The antioxidant capacity of propolis products was highly related to TP and TF contents in the samples. The highest antioxidant activity was shown in New Zealand products (S12-S14), whose TP and TF contents were also relatively high among 20 samples. However, S15 from Korea had the lowest antioxidant capacity and a lower TP content.

On the contrary, there was no linear correlation between TP or TF content and antimicrobial activity of the samples, as many studies indicated that there was no antimicrobial activity shown in some of the phenolic compounds. The propolis samples showed greater effects on Gram-positive bacteria (*S. aureus* and *B. cereus*) than Gram-negative bacteria (*E. coli*). Among all samples, propolis from New Zealand showed the higher antimicrobial activity than the propolis samples from the other countries.

However, the content of heavy metal elements (As and Pb) in New Zealand was higher than the propolis products originated from the other countries. Also, the sample S8 (Chinese propolis) contained the highest concentration of cadmium and various rare earth elements. Compared to the propolis from New Zealand and China, the Korean and Australian propolis had a lower elements concentration.

Salicin was detected in 70% of the Korean propolis samples (S3, S5, S6, S15, S16, S18 and S20). As mentioned previously, the TF content of S5, S7, S15, S16, S17 and S20 was higher than their TP. These results also indicated that poplar tree gum was more likely to be adulterated in S5, S15, S16 and S20 (Korean propolis products).

Overall, although the antioxidant capacity and antimicrobial activity were high in New Zealand propolis, the heavy metal elements were fairly high. Similarly, one of Chinese propolis samples contained a higher cadmium content, which may pose threat to human health. As to Korean samples, the detection of salicin indicating adulteration tended to be the main concern of commercial propolis products. Among all regions, liquid commercial propolis from Australia had the fewer heavy metal element contamination and lower possibility of adulteration with stable antioxidant and antimicrobial activity.

6.2 Recommendations

The physicochemical and functional properties of 20 commercial liquid propolis products from 4 countries were investigated. Some more further studies may need to be carried out as following:

1. The influence of pH on the composition and solubility of propolis could be investigated more.
2. Propolis is generally from plant. However, the elements in commercial propolis might be influenced by not only the environment but also some human factors.

For instance, the contamination during the extraction process of propolis. It is also important to investigate the elements in raw propolis (i.e. before and after extraction).

3. The reasons for the high heavy metal elements detected in New Zealand propolis need to be investigated to determine the source of heavy metal contamination.

4. There is no standard and regulation for monitoring and determining the quality of propolis. This needs to be developed and established.

5. Poplar tree gum extract is known to be similar to propolis. Hence, it would be useful to investigate the two products by analysing and comparing their physicochemical and functional properties.

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Appendix 1. Results of Colour Measurement

Table A1.1 Results of propolis samples in CIE L* a* b*

Sample	L*			a*			b*		
S1	63.83	65.12	65.11	18.62	18.26	18.21	48.48	49.35	49.37
S2	77.08	75.17	74.93	11.03	11.57	11.73	58.97	58.49	58.43
S3	45.79	49.18	45.69	29.48	32.35	30.74	23.39	31.22	25.42
S4	70.31	70.71	72.34	26.50	26.27	25.00	63.41	63.86	65.50
S5	54.72	55.34	57.26	27.82	27.63	27.28	39.71	40.03	42.85
S6	68.29	69.22	68.62	16.79	16.28	16.63	53.77	54.67	53.94
S7	42.54	42.47	42.58	18.80	18.87	18.90	18.89	18.98	19.16
S8	80.79	80.23	79.41	3.29	3.65	3.74	53.72	54.25	56.42
S9	41.10	40.64	41.21	29.22	28.88	28.78	16.45	16.55	16.33
S10	34.35	34.27	34.87	12.21	12.23	12.43	4.81	4.77	4.39
S11	48.76	48.56	48.38	35.87	35.33	35.27	29.56	29.21	29.36
S12	31.92	32.05	32.22	0.56	0.61	0.67	0.41	0.53	0.44
S13	35.34	35.25	34.77	14.79	15.69	15.41	5.63	5.44	5.08
S14	32.24	32.71	31.87	1.00	1.61	1.27	0.45	0.83	0.90
S15	31.33	31.25	31.43	-0.09	0.00	-0.13	0.43	0.52	0.44
S16	31.53	31.38	31.23	-0.17	-0.11	-0.21	0.08	0.05	0.06
S17	31.32	31.47	31.63	0.19	0.17	0.21	0.24	0.12	0.33
S18	45.58	46.03	46.21	32.02	31.55	31.23	23.92	24.55	23.63
S19	33.90	33.17	32.78	3.28	3.57	3.22	2.34	2.03	2.02
S20	31.69	31.67	31.32	-0.11	-0.12	-0.09	0.29	0.06	0.21

Table A1.2 Results of propolis samples in CIE L* C* h*

Sample	L*			C*			h		
S1	63.83	65.12	65.11	51.93	52.62	52.62	69.00	69.70	69.75
S2	77.08	75.17	74.93	59.81	59.62	59.60	80.44	78.81	78.65
S3	45.79	49.18	45.69	37.63	44.96	39.89	38.44	43.97	39.59
S4	70.31	70.71	72.34	68.72	69.05	70.11	67.31	67.64	69.11
S5	54.72	55.34	57.26	48.48	48.64	50.79	54.98	55.39	57.52
S6	68.29	69.22	68.62	56.33	57.04	56.45	72.66	73.42	72.87
S7	42.54	42.47	42.58	26.65	26.76	26.91	45.13	45.17	45.40
S8	80.79	80.23	79.41	53.82	54.38	56.55	86.49	86.15	86.21
S9	41.10	40.64	41.21	33.21	33.06	32.88	30.77	29.95	30.08
S10	34.35	34.27	34.87	13.39	13.37	13.65	21.44	21.48	21.08
S11	48.76	48.56	48.38	46.23	46.44	46.27	39.45	39.36	39.89
S12	31.92	32.05	32.22	0.88	0.97	0.84	37.64	36.45	36.68
S13	35.34	35.25	34.77	13.35	12.88	13.40	23.23	24.82	23.25
S14	32.24	32.71	31.87	1.10	1.81	1.55	28.55	27.46	27.66
S15	31.33	31.25	31.43	0.21	0.33	0.24	117.26	118.77	117.54
S16	31.53	31.38	31.23	0.18	0.21	0.13	154.57	155.78	154.55
S17	31.32	31.47	31.63	0.30	0.23	0.17	50.08	49.52	49.89
S18	45.58	46.03	46.21	39.97	40.17	39.54	36.76	36.30	36.70
S19	33.90	33.17	32.78	3.55	3.68	3.80	31.35	31.53	32.14
S20	31.69	31.67	31.32	0.30	0.14	0.15	154.53	152.88	154.23

Appendix 2. Results of pH

Table A2.1 pH of 20 propolis samples

Samples	pH		
S1	3.62	3.53	3.49
S2	5.39	5.37	5.34
S3	4.71	4.75	4.76
S4	4.63	4.67	4.65
S5	5.38	5.41	5.43
S6	5.49	5.51	5.49
S7	4.40	4.35	4.32
S8	4.83	4.79	4.76
S9	4.86	4.89	4.89
S10	4.71	4.66	4.64
S11	4.80	4.77	4.75
S12	4.45	4.46	4.44
S13	4.88	4.84	4.85
S14	4.57	4.53	4.55
S15	8.55	8.63	8.66
S16	5.23	5.15	5.09
S17	5.55	5.53	5.53
S18	4.65	4.61	4.58
S19	6.85	6.73	6.72
S20	9.40	9.37	9.35

Table A2.2 Tukey HSD test of pH between samples

Tukey HSD[a]

Number of Sample	N	Subset for alpha = 0.05															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	3	3.5467															
7	3		4.3567														
12	3		4.4500	4.4500													
14	3			4.5500	4.5500												
18	3				4.6133	4.6133											
4	3				4.6500	4.6500	4.6500										
10	3					4.6700	4.6700	4.6700									
3	3						4.7400	4.7400	4.7400								
11	3							4.7733	4.7733	4.7733							
8	3								4.7933	4.7933							
13	3									4.8567							
9	3									4.8800							
16	3										5.1567						
2	3											5.3667					
5	3											5.4067	5.4067				
6	3												5.4967	5.4967			
17	3													5.5367			
19	3														6.7667		
15	3															8.6133	
20	3																9.3733
Sig.		1.000	.263	.172	.172	.938	.318	.136	.963	.107	1.000	.998	.318	.998	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 3. Results of Total Phenolic (TP) and Total Flavonoid (TF)

A3.1 Standard curve of TP content

Table A3.1 Absorbance of gallic acid for standard curve (TP).

Concentration (mg/L)	Absorbance		
100	0.279	0.281	0.278
200	0.534	0.538	0.529
300	0.642	0.633	0.652
400	0.979	0.968	0.988
500	1.208	1.204	1.211

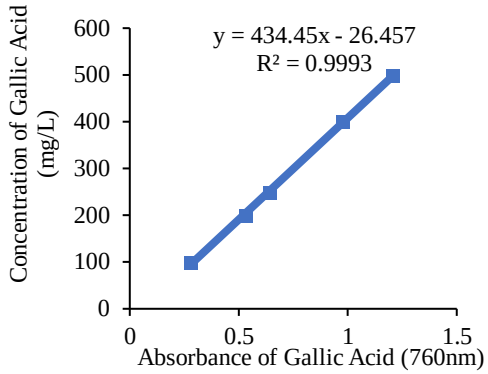


Figure A3.1 Standard curve of TP content.

Table A3.2 Absorbance of samples for TP detection

Sample	Dilution	Absorbance		
S1	200-fold	0.521	0.530	0.530
S2	200-fold	0.416	0.427	0.425
S3	200-fold	0.415	0.416	0.428
S4	100-fold	0.430	0.441	0.440
S5	200-fold	0.426	0.418	0.421
S6	200-fold	0.353	0.353	0.352
S7	200-fold	0.628	0.625	0.621
S8	200-fold	0.617	0.628	0.625
S9	200-fold	0.679	0.684	0.671
S10	200-fold	0.817	0.806	0.801
S11	200-fold	0.479	0.477	0.469
S12	200-fold	0.875	0.865	0.867
S13	200-fold	0.852	0.838	0.850
S14	200-fold	0.843	0.841	0.851
S15	200-fold	0.257	0.266	0.259
S16	200-fold	0.495	0.485	0.493
S17	200-fold	0.334	0.335	0.342
S18	200-fold	0.320	0.310	0.311
S19	200-fold	0.467	0.456	0.470
S20	200-fold	0.353	0.357	0.348

Table A3.3 Tukey HSD test of TP between samples [ab]

Number of Sample	N	Subset for alpha = 0.05										
		1	2	3	4	5	6	7	8	9	10	11
S4	3	16.818										
S15	3	17.008										
S18	3		21.560									
S17	3		23.352	23.352								
S20	3			24.614								
S6	3			24.686								
S3	3				30.305							
S5	3				30.681							
S2	3				31.637							
S19	3					34.629						
S11	3					35.804						
S16	3					36.979						
S1	3						40.500					
S7	3							48.320				
S8	3							48.871				
S9	3								53.073			
S10	3									64.702		
S14	3									66.259	66.259	
S13	3										68.168	68.168
S12	3											70.077
Sig.		1.000	.739	.970	.971	.288	1.000	1.000	1.000	.893	.642	.642

[a] Means for groups in homogeneous subsets are displayed.
[b] Uses Harmonic Mean Sample Size = 3.000.

A3.2 Standard curve of TF

Table A3.4 Absorbance of quercetin for standard curve.

Concentration (mg/L)	Absorbance		
50	0.246	0.254	0.238
75	0.367	0.374	0.360
100	0.498	0.503	0.494
150	0.768	0.773	0.761
200	1.058	1.074	1.043
300	1.589	1.577	1.597

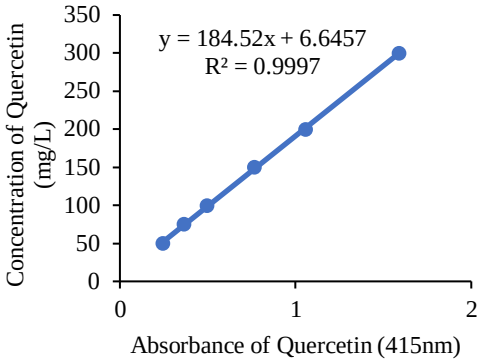


Figure A3.2 Standard curve of TF content.

Table A3.5 Absorbance of samples for TF detection.

Sample	Dilution	Absorbance		
S1	200-fold	0.764	0.778	0.774
S2	200-fold	0.576	0.586	0.588
S3	200-fold	0.540	0.550	0.574
S4	200-fold	0.301	0.317	0.341
S5	400-fold	0.432	0.438	0.448
S6	200-fold	0.462	0.489	0.483
S7	200-fold	1.358	1.355	1.350
S8	200-fold	0.677	0.715	0.710
S9	200-fold	1.101	1.066	1.081
S10	400-fold	0.699	0.681	0.701
S11	200-fold	0.640	0.630	0.632
S12	400-fold	0.755	0.763	0.771
S13	400-fold	0.701	0.713	0.709
S14	400-fold	0.705	0.705	0.702
S15	200-fold	0.520	0.538	0.529
S16	400-fold	0.688	0.702	0.697
S17	200-fold	0.779	0.769	0.757
S18	200-fold	0.317	0.307	0.313
S19	200-fold	0.708	0.715	0.721
S20	200-fold	0.625	0.631	0.625

Table A3.6 Tukey HSD test of TF between samples[ab]

Number of Sample	N	Subset for alpha = 0.05											
		1	2	3	4	5	6	7	8	9	10	11	12
S4	3	6.563											
S18	3		12.629										
S6	3			18.969									
S15	3			20.224	20.224								
S3	3			21.798	21.798	21.798							
S2	3			22.856	22.856	22.856	22.856						
S11	3			23.898	23.898	23.898	23.898	23.898					
S20	3				24.847	24.847	24.847	24.847	24.847				
S8	3					27.186	27.186	27.186	27.186				
S19	3						27.964	27.964	27.964				
S17	3							28.852	28.852				
S1	3								29.819	29.819			
S5	3									35.084			
S9	3										40.864		
S7	3											49.956	
S10	3											52.587	52.587
S16	3											53.351	53.351
S14	3											54.631	54.631
S12	3												56.901
S13	3												57.603
Sig.		1.000	1.000	.159	.239	.080	.123	.154	.150	.097	1.000	.224	.141

[a] Means for groups in homogeneous subsets are displayed.

[b] Uses Harmonic Mean Sample Size = 3.000.

Appendix 4. Results of DPPH Assay

Table A4.1 Absorbance and calculated RSA% of sample 1

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=0.830			Ac=0.856			Ac=0.833		
1.00	0.125	0.122	0.122	84.94	85.30	85.30	85.40	85.75	85.75	84.99	85.35	85.35
0.50	0.309	0.303	0.303	62.77	63.49	63.49	63.90	64.60	64.60	62.91	63.63	63.63
0.33	0.474	0.464	0.425	42.89	44.10	48.80	44.63	45.79	50.35	43.10	44.30	48.98
0.25	0.555	0.516	0.542	33.13	37.83	34.70	35.16	39.72	36.68	33.37	38.06	34.93
0.20	0.579	0.542	0.579	30.24	34.70	30.24	32.36	36.68	32.36	30.49	34.93	30.49
0.17	0.620	0.615	0.624	25.30	25.90	24.82	27.57	28.15	27.10	25.57	26.17	25.09

Table A4.2 Absorbance and calculated RSA% of sample 2

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=0.830			Ac=0.856			Ac=0.833		
2.00	0.121	0.117	0.128	85.42	85.90	84.58	85.86	86.33	85.05	85.47	85.95	84.63
1.00	0.13	0.123	0.13	84.34	85.18	84.34	84.81	85.63	84.81	84.39	85.23	84.39
0.50	0.355	0.379	0.366	57.23	54.34	55.90	58.53	55.72	57.24	57.38	54.50	56.06
0.33	0.517	0.498	0.512	37.71	40.00	38.31	39.60	41.82	40.19	37.94	40.22	38.54
0.25	0.602	0.565	0.586	27.47	31.93	29.40	29.67	34.00	31.54	27.73	32.17	29.65
0.20	0.617	0.635	0.628	25.66	23.49	24.34	27.92	25.82	26.64	25.93	23.77	24.61

Table A4.3 Absorbance and calculated RSA% of sample 3

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=0.830			Ac=0.856			Ac=0.833		
1.00	0.140	0.135	0.146	83.13	83.73	82.41	83.64	84.23	82.94	83.19	83.79	82.47
0.50	0.240	0.246	0.244	71.08	70.36	70.60	71.96	71.26	71.50	71.19	70.47	70.71
0.33	0.383	0.392	0.394	53.86	52.77	52.53	55.26	54.21	53.97	54.02	52.94	52.70
0.25	0.469	0.516	0.497	43.49	37.83	40.12	45.21	39.72	41.94	43.70	38.06	40.34
0.20	0.533	0.554	0.547	35.78	33.25	34.10	37.73	35.28	36.10	36.01	33.49	34.33
0.17	0.584	0.607	0.596	29.64	26.87	28.19	31.78	29.09	30.37	29.89	27.13	28.45

Table A4.4 Absorbance and calculated RSA% of sample 4

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=0.830			Ac=0.856			Ac=0.833		
2.00	0.130	0.133	0.134	84.34	83.98	83.86	84.81	84.46	84.35	84.39	84.03	83.91
1.00	0.294	0.342	0.319	64.58	58.80	61.57	65.65	60.05	62.73	64.71	58.94	61.70
0.50	0.525	0.531	0.523	36.75	36.02	36.99	38.67	37.97	38.90	36.97	36.25	37.21
0.33	0.615	0.625	0.619	25.90	24.70	25.42	28.15	26.99	27.69	26.17	24.97	25.69
0.25	0.660	0.710	0.666	20.48	14.46	19.76	22.90	17.06	22.20	20.77	14.77	20.05

Table A4.5 Absorbance and calculated RSA% of sample 5

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=0.830			Ac=0.856			Ac=0.833		
1.00	0.140	0.134	0.144	83.13	83.86	82.65	83.64	84.35	83.18	83.19	83.91	82.71
0.50	0.202	0.248	0.225	75.66	70.12	72.89	76.40	71.03	73.71	75.75	70.23	72.99
0.33	0.371	0.412	0.391	55.30	50.36	52.89	56.66	51.87	54.32	55.46	50.54	53.06
0.25	0.472	0.512	0.487	43.13	38.31	41.33	44.86	40.19	43.11	43.34	38.54	41.54
0.20	0.547	0.556	0.558	34.10	33.01	32.77	36.10	35.05	34.81	34.33	33.25	33.01
0.17	0.582	0.600	0.597	29.88	27.71	28.07	32.01	29.91	30.26	30.13	27.97	28.33

Table A4.6 Absorbance and calculated RSA% of sample 6

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=0.830			Ac=0.856			Ac=0.833		
2.00	0.123	0.121	0.127	85.18	85.42	84.70	85.63	85.86	85.16	85.23	85.47	84.75
1.00	0.126	0.144	0.136	84.82	82.65	83.61	85.28	83.18	84.11	84.87	82.71	83.67
0.50	0.365	0.392	0.379	56.02	52.77	54.34	57.36	54.21	55.72	56.18	52.94	54.50
0.33	0.488	0.522	0.509	41.20	37.11	38.67	42.99	39.02	40.54	41.42	37.33	38.90
0.25	0.573	0.590	0.590	30.96	28.92	28.92	33.06	31.07	31.07	31.21	29.17	29.17

Table A4.7 Absorbance and calculated RSA% of sample 7

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=0.830			Ac=0.856			Ac=0.833		
1.00	0.151	0.147	0.152	81.81	82.29	81.69	82.36	82.83	82.24	81.87	82.35	81.75
0.50	0.242	0.30 8	0.274	70.84	62.89	66.99	71.73	64.02	67.99	70.95	63.03	67.11
0.33	0.395	0.426	0.409	52.41	48.67	50.72	53.86	50.23	52.22	52.58	48.86	50.90
0.25	0.488	0.526	0.510	41.20	36.63	38.55	42.99	38.55	40.42	41.42	36.85	38.78
0.20	0.554	0.563	0.560	33.25	32.17	32.53	35.28	34.23	34.58	33.49	32.41	32.77
0.17	0.585	0.619	0.604	29.52	25.42	27.23	31.66	27.69	29.44	29.77	25.69	27.49

Table A4.8 Absorbance and calculated RSA% of sample 8

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=0.830			Ac=0.856			Ac=0.833		
1.00	0.125	0.122	0.122	84.94	85.30	85.30	85.40	85.75	85.75	84.99	85.35	85.35
0.50	0.309	0.303	0.303	62.77	63.49	63.49	63.90	64.60	64.60	62.91	63.63	63.63
0.33	0.474	0.464	0.425	42.89	44.10	48.80	44.63	45.79	50.35	43.10	44.30	48.98
0.25	0.555	0.516	0.542	33.13	37.83	34.70	35.16	39.72	36.68	33.37	38.06	34.93
0.20	0.579	0.542	0.579	30.24	34.70	30.24	32.36	36.68	32.36	30.49	34.93	30.49
0.17	0.620	0.615	0.624	25.30	25.90	24.82	27.57	28.15	27.10	25.57	26.17	25.09

Table A4.9 Absorbance and calculated RSA% of sample 9

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
1.00	0.158	0.154	0.154	88.49	88.78	88.78	88.43	88.73	88.73	88.33	88.63	88.63
0.50	0.468	0.463	0.455	65.91	66.28	66.86	65.74	66.11	66.69	65.44	65.81	66.40
0.33	0.773	0.757	0.766	43.70	44.87	44.21	43.41	44.58	43.92	42.91	44.09	43.43
0.25	0.918	0.913	0.914	33.14	33.50	33.43	32.80	33.16	33.09	32.20	32.57	32.50
0.20	1.044	1.038	1.026	23.96	24.40	25.27	23.57	24.01	24.89	22.90	23.34	24.22
0.17	1.032	1.054	1.054	24.84	23.23	23.23	24.45	22.84	22.84	23.78	22.16	22.16

Table A4.10 Absorbance and calculated RSA% of sample 10

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
1.00	0.155	0.155	0.158	88.71	88.71	88.49	88.65	88.65	88.43	88.55	88.55	88.33
0.50	0.473	0.459	0.462	65.55	66.57	66.35	65.37	66.40	66.18	65.07	66.10	65.88
0.33	0.638	0.633	0.619	53.53	53.90	54.92	53.29	53.66	54.69	52.88	53.25	54.28
0.25	0.793	0.785	0.788	42.24	42.83	42.61	41.95	42.53	42.31	41.43	42.02	41.80
0.20	0.993	0.982	0.988	27.68	28.48	28.04	27.31	28.11	27.67	26.66	27.47	27.03
0.17	0.965	0.973	0.975	29.72	29.13	28.99	29.36	28.77	28.62	28.73	28.14	27.99

Table A4.11 Absorbance and calculated RSA% of sample 11

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
10.00	0.135	0.141	0.137	90.17	89.73	90.02	90.12	89.68	89.97	90.03	89.59	89.88
5.00	0.146	0.144	0.149	89.37	89.51	89.15	89.31	89.46	89.09	89.22	89.36	89.00
2.00	0.157	0.144	0.153	88.57	89.51	88.86	88.51	89.46	88.80	88.40	89.36	88.70
1.00	0.358	0.344	0.345	73.93	74.95	74.87	73.79	74.82	74.74	73.56	74.59	74.52
0.50	0.853	0.864	0.848	37.87	37.07	38.24	37.55	36.75	37.92	37.00	36.19	37.37
0.33	1.024	1.037	1.036	25.42	24.47	24.54	25.04	24.08	24.16	24.37	23.41	23.49

Table A4.12 Absorbance and calculated RSA% of sample 12

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
1.00	0.158	0.153	0.156	88.49	88.86	88.64	88.43	88.80	88.58	88.33	88.70	88.48
0.50	0.192	0.183	0.187	86.02	86.67	86.38	85.94	86.60	86.31	85.82	86.48	86.19
0.33	0.456	0.472	0.46	66.79	65.62	66.50	66.62	65.45	66.33	66.32	65.14	66.03
0.25	0.655	0.667	0.672	52.29	51.42	51.06	52.05	51.17	50.81	51.62	50.74	50.37
0.20	0.834	0.827	0.829	39.26	39.77	39.62	38.95	39.46	39.31	38.40	38.92	38.77
0.17	0.875	0.887	0.894	36.27	35.40	34.89	35.94	35.07	34.55	35.38	34.49	33.97

Table A4.13 Absorbance and calculated RSA% of sample 13

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
1.00	0.159	0.158	0.156	88.42	88.49	88.64	88.36	88.43	88.58	88.26	88.33	88.48
0.50	0.253	0.245	0.251	81.57	82.16	81.72	81.48	82.06	81.63	81.31	81.91	81.46
0.33	0.583	0.597	0.588	57.54	56.52	57.17	57.32	56.30	56.95	56.94	55.91	56.57
0.25	0.841	0.835	0.839	38.75	39.18	38.89	38.43	38.87	38.58	37.89	38.33	38.04
0.20	0.958	0.943	0.949	30.23	31.32	30.88	29.87	30.97	30.53	29.25	30.35	29.91
0.17	1.047	1.036	1.045	23.74	24.54	23.89	23.35	24.16	23.50	22.67	23.49	22.82

Table A4.14 Absorbance and calculated RSA% of sample 14

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
1.00	0.151	0.143	0.134	89.00	89.58	90.24	88.95	89.53	90.19	88.85	89.44	90.10
0.50	0.182	0.188	0.189	86.74	86.31	86.23	86.68	86.24	86.16	86.56	86.12	86.04
0.33	0.443	0.489	0.471	67.73	64.38	65.70	67.57	64.20	65.52	67.28	63.88	65.21
0.25	0.721	0.735	0.715	47.49	46.47	47.92	47.22	46.19	47.66	46.75	45.72	47.19
0.20	0.734	0.731	0.736	46.54	46.76	46.39	46.27	46.49	46.12	45.79	46.01	45.64
0.17	0.838	0.839	0.840	38.97	38.89	38.82	38.65	38.58	38.51	38.11	38.04	37.96

Table A4.15 Absorbance and calculated RSA% of sample 15

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
100.00	1.964	1.830	1.873	-	-33.28	-36.42	-43.78	-33.97	-37.12	-45.05	-35.16	-38.33
10.00	0.677	0.683	0.681	50.69	50.25	50.40	50.44	50.00	50.15	50.00	49.56	49.70
5.00	0.622	0.632	0.616	54.70	53.97	55.13	54.47	53.73	54.90	54.06	53.32	54.51
3.33	0.773	0.779	0.780	43.70	43.26	43.19	43.41	42.97	42.90	42.91	42.47	42.39
2.50	0.847	0.841	0.832	38.31	38.75	39.40	37.99	38.43	39.09	37.44	37.89	38.55
2.00	0.962	0.951	0.968	29.93	30.74	29.50	29.58	30.38	29.14	28.95	29.76	28.51

Table A4.16 Absorbance and calculated RSA% of sample 16

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
10.00	0.297	0.302	0.303	78.37	78.00	77.93	78.26	77.89	77.82	78.06	77.70	77.62
5.00	0.211	0.203	0.206	84.63	85.21	85.00	84.55	85.14	84.92	84.42	85.01	84.79
2.00	0.196	0.194	0.198	85.72	85.87	85.58	85.65	85.80	85.51	85.52	85.67	85.38
1.00	0.365	0.377	0.365	73.42	72.54	73.42	73.28	72.40	73.28	73.04	72.16	73.04
0.50	0.766	0.745	0.752	44.21	45.74	45.23	43.92	45.46	44.95	43.43	44.98	44.46
0.33	1.007	1.012	1.009	26.66	26.29	26.51	26.28	25.92	26.13	25.63	25.26	25.48

Table A4.17 Absorbance and calculated RSA% of sample 17

Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
10.00	0.214	0.209	0.211	84.75	85.10	84.96	84.50	84.87	84.72	84.77	85.12	84.98
5.00	0.192	0.194	0.192	86.32	86.17	86.32	86.10	85.95	86.10	86.33	86.19	86.33
2.00	0.213	0.208	0.211	84.82	85.17	84.96	84.58	84.94	84.72	84.84	85.20	84.98
1.00	0.563	0.566	0.550	59.87	59.66	60.80	59.23	59.02	60.17	59.93	59.72	60.85
0.50	0.984	0.995	0.988	29.86	29.08	29.58	28.75	27.95	28.46	29.96	29.18	29.68
0.33	1.127	1.124	1.143	19.67	19.89	18.53	18.39	18.61	17.23	19.79	20.00	18.65

Table A4.18 Absorbance and calculated RSA% of sample 18

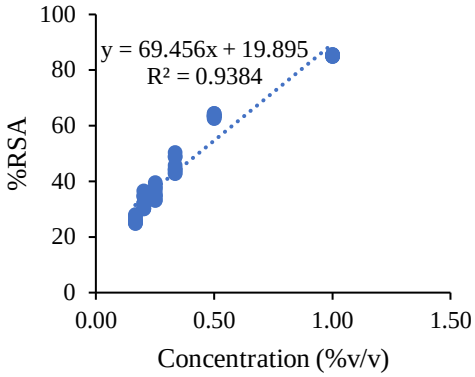
Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
10.00	0.145	0.151	0.147	89.44	89.00	89.29	89.39	88.95	89.24	89.29	88.85	89.14
5.00	0.161	0.158	0.159	88.27	88.49	88.42	88.21	88.43	88.36	88.11	88.33	88.26
2.00	0.314	0.319	0.328	77.13	76.77	76.11	77.01	76.65	75.99	76.81	76.44	75.78
1.00	0.656	0.647	0.643	52.22	52.88	53.17	51.98	52.64	52.93	51.55	52.22	52.51
0.50	0.789	0.796	0.789	42.53	42.02	42.53	42.24	41.73	42.24	41.73	41.21	41.73
0.33	1.183	1.185	1.179	13.84	13.69	14.13	13.40	13.25	13.69	12.63	12.48	12.92

Table A4.19 Absorbance and calculated RSA% of sample 19

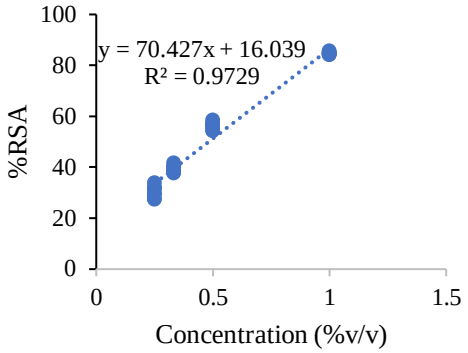
Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
10.00	0.147	0.145	0.143	89.29	89.44	89.58	89.24	89.39	89.53	89.14	89.29	89.44
5.00	0.146	0.146	0.144	89.37	89.37	89.51	89.31	89.31	89.46	89.22	89.22	89.36
2.00	0.163	0.159	0.162	88.13	88.42	88.20	88.07	88.36	88.14	87.96	88.26	88.04
1.00	0.459	0.457	0.458	66.57	66.72	66.64	66.40	66.54	66.47	66.10	66.25	66.17
0.50	0.815	0.823	0.815	40.64	40.06	40.64	40.34	39.75	40.34	39.81	39.22	39.81
0.33	1.003	1.018	1.006	26.95	25.86	26.73	26.57	25.48	26.35	25.92	24.82	25.70

Table A4.20 Absorbance and calculated RSA% of sample 20

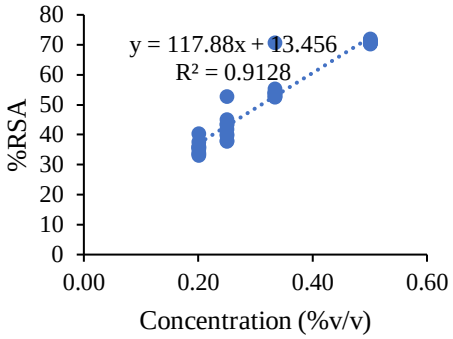
Concentration (% v/v)	Absorbance (As)			RSA%								
				Ac=1.373			Ac=1.366			Ac=1.354		
10.00	0.640	0.632	0.647	54.38	54.95	53.88	53.66	54.24	53.15	54.45	55.02	53.95
5.00	0.528	0.526	0.536	62.37	62.51	61.80	61.77	61.91	61.19	62.42	62.56	61.85
2.00	0.630	0.627	0.632	55.10	55.31	54.95	54.38	54.60	54.24	55.16	55.37	55.02
1.00	0.884	0.892	0.895	36.99	36.42	36.21	35.99	35.41	35.19	37.08	36.51	36.30
0.50	1.118	1.107	1.104	20.31	21.10	21.31	19.04	19.84	20.06	20.43	21.21	21.42
0.33	1.189	1.178	1.202	15.25	16.06	14.33	13.90	14.72	12.96	15.37	16.18	14.45



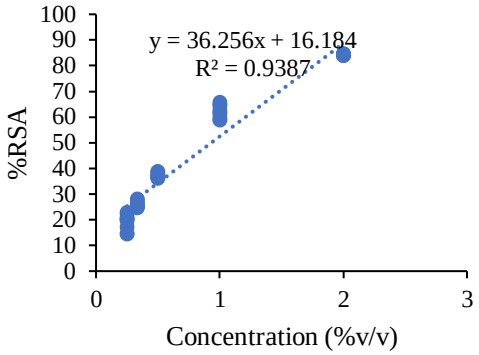
S1



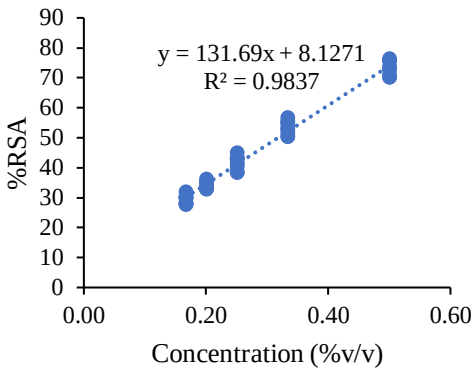
S2



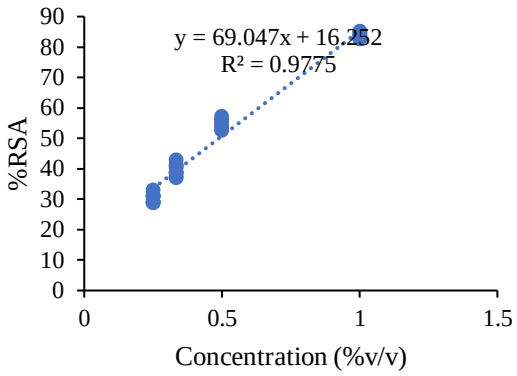
S3



S4

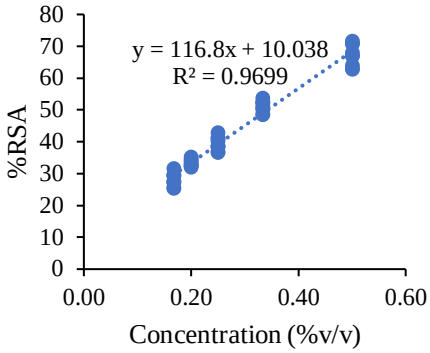


S5

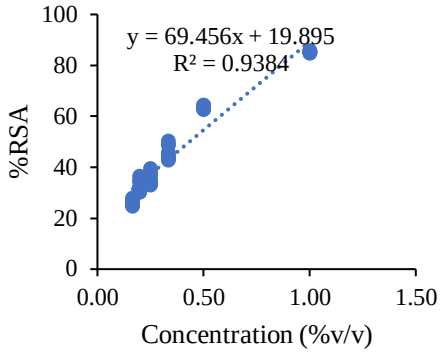


S6

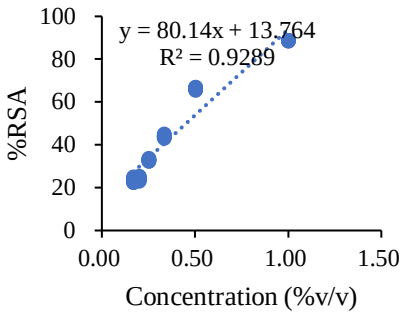
Figure A4.1 Plot of RSA% versus concentration of sample 1-6



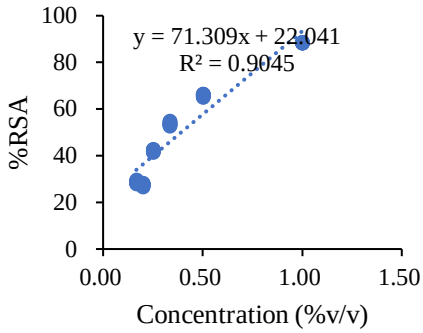
S7



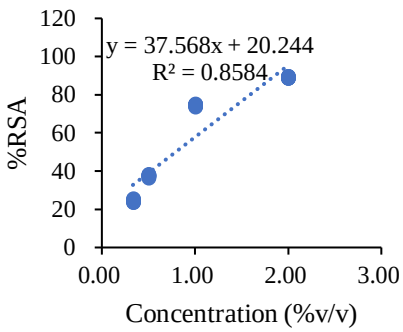
S8



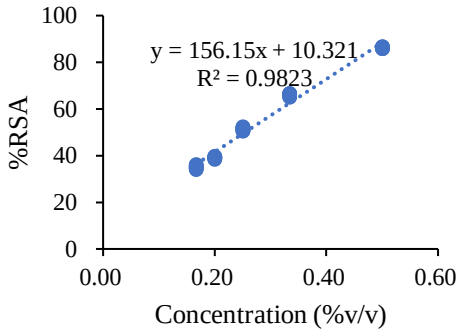
S9



S10



S11



S12

Figure A4.2 Plot of RSA% versus concentration of sample 6-12

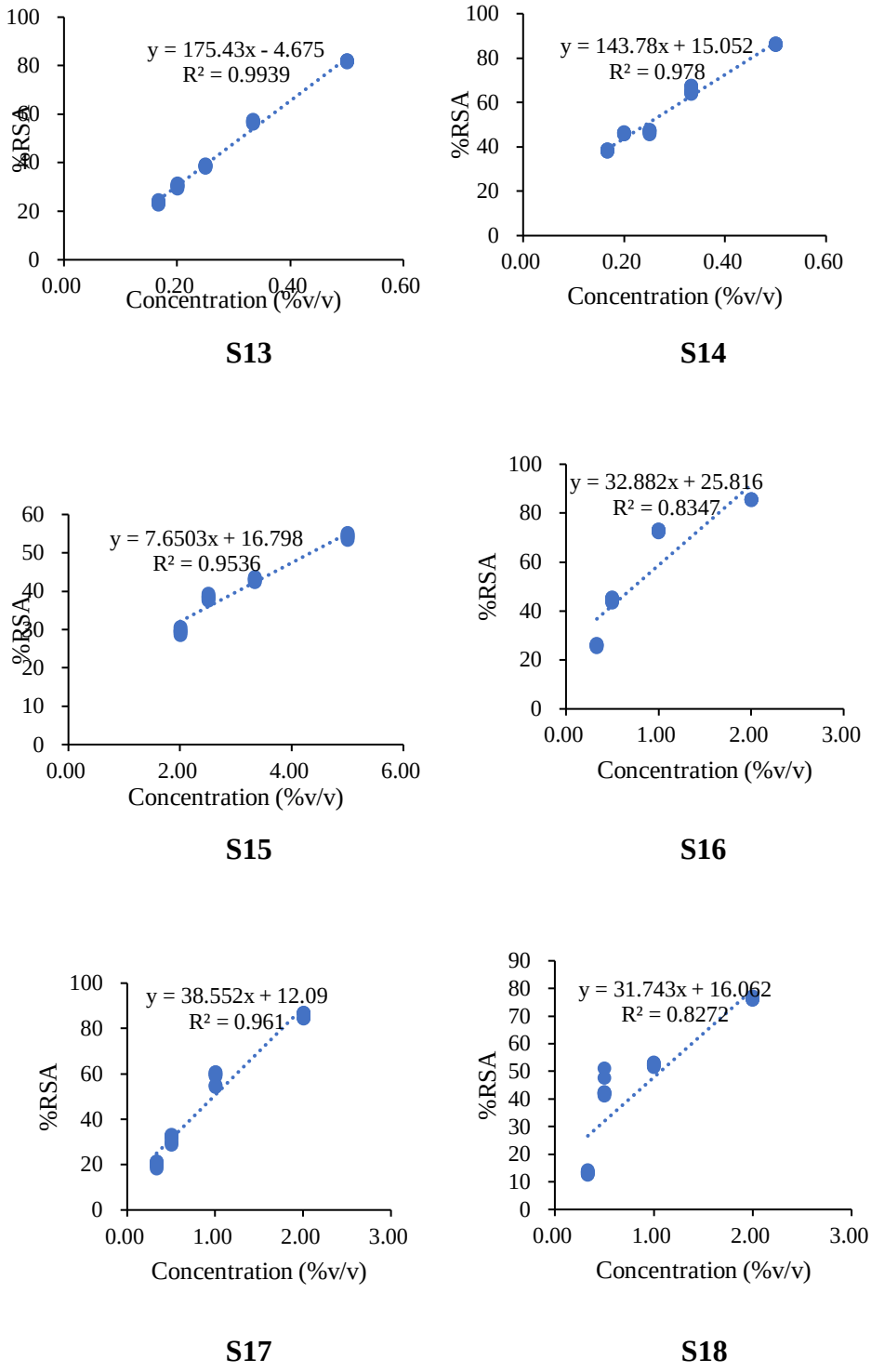
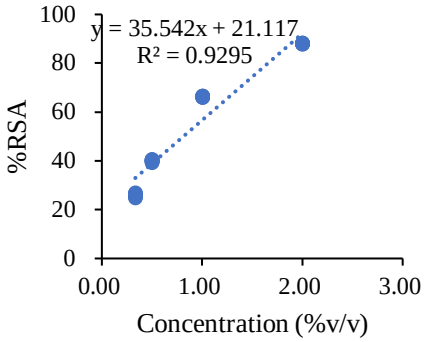
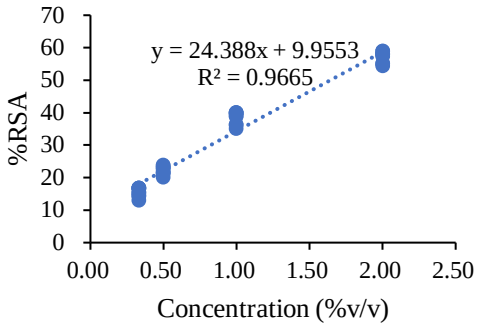


Figure A4.3 Plot of RSA% versus concentration of sample 13-18





S19 **S20**

Figure A4.4 Plot of RSA% versus concentration of sample19- 20

Appendix 5. Results of Antimicrobial Properties

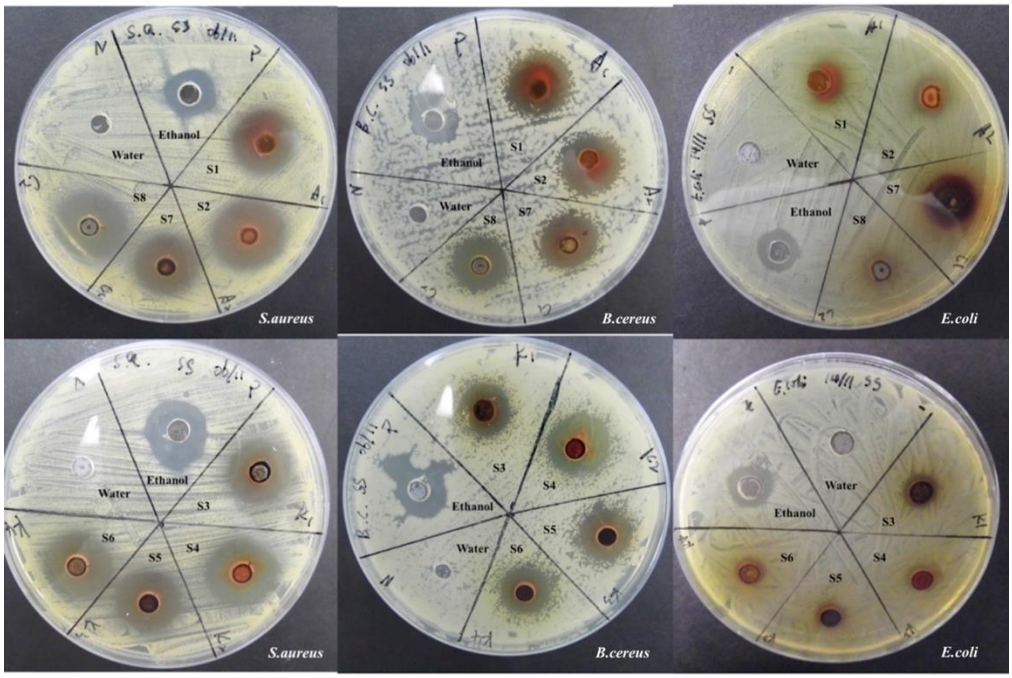


Figure A5.1 Well diffusion S1-S8 (*S. aureus. B. cereus, and E. coli* from left to right)

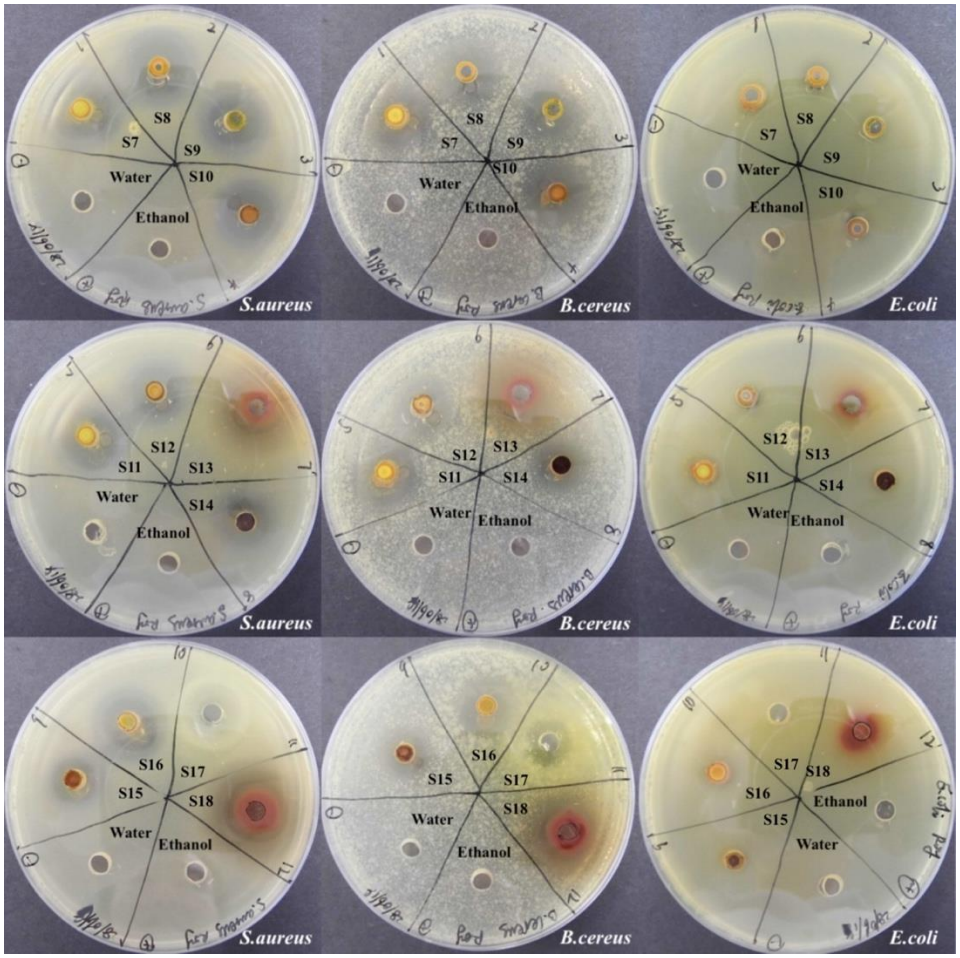


Figure A5.2 Well diffusion S9-S20 (*S. aureus. B. cereus, and E. coli* from left to right)

Table A5.1 Inhibition zone containing the diameter (6mm) of well

Sample	Well diffusion (mm)								
	S. aureus			*E. coli*			*B. cereus*		
S1	13.90	14.90	15.35	8.65	8.70	8.15	15.15	15.74	16.20
S2	16.70	16.90	16.80	11.35	9.70	9.45	19.15	17.20	16.55
S3	14.15	15.60	15.55	9.15	8.55	8.20	14.15	14.95	13.85
S4	18.65	18.25	18.90	9.30	9.15	9.10	13.05	14.50	13.90
S5	15.10	15.95	15.90	8.60	8.35	8.05	13.85	14.05	13.30
S6	16.40	16.50	16.45	8.40	8.45	7.90	14.95	14.15	14.35
S7	16.20	16.50	16.15	13.85	14.00	11.55	17.05	16.35	16.15
S8	18.20	18.90	18.35	9.00	7.35	6.85	17.75	16.40	16.90
S9	18.95	18.70	18.50	11.00	9.20	9.90	15.75	15.45	16.55
S10	19.50	19.85	19.70	10.65	8.55	10.25	15.50	15.25	16.85
S11	18.10	19.10	18.75	10.65	9.10	10.25	14.65	14.15	15.35
S12	16.80	17.75	16.40	9.75	8.40	8.35	13.80	14.20	13.40
S13	17.40	17.60	17.65	9.80	9.45	9.25	14.60	15.70	14.65
S14	18.05	17.25	17.05	9.25	8.55	8.90	12.95	13.50	14.10
S15	18.90	18.55	18.90	6.00	6.00	6.00	13.95	14.65	16.00
S16	15.10	14.65	15.05	6.70	6.10	6.85	12.15	12.20	12.45
S17	15.50	14.60	14.45	6.65	6.35	6.35	13.40	13.10	12.90
S18	19.20	18.65	18.10	9.05	9.10	9.75	14.40	16.60	15.80
S19	10.95	10.80	10.60	7.75	7.05	7.55	9.15	9.35	9.15
S20	16.55	17.05	16.75	6.00	6.00	6.00	15.10	15.70	15.00

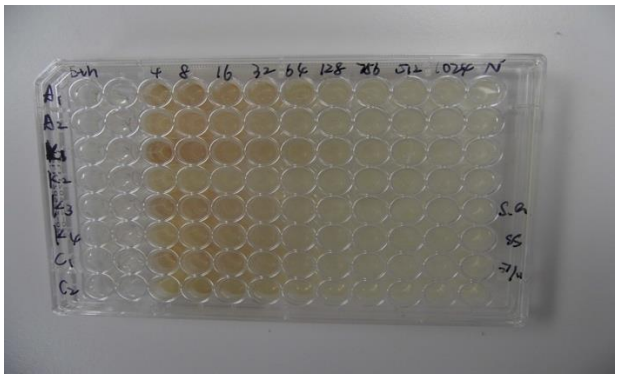


Figure A5.3 Sample 1-8 with different dilutions for investigating MIC of *S. aureus*, after first incubation

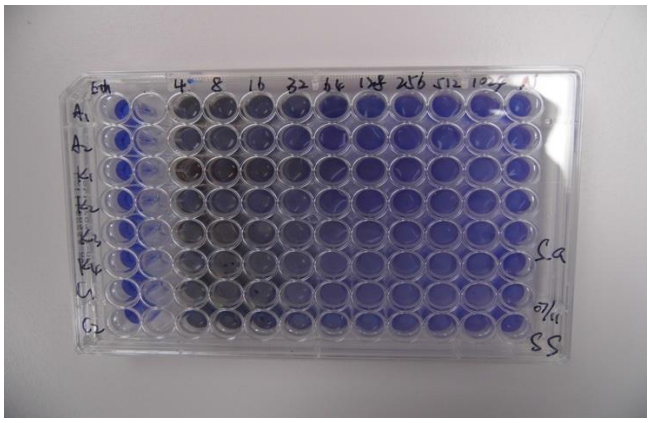


Figure A5.4 Sample 1-8 with different dilutions for investigating MIC of *S. aureus*, adding resazurin

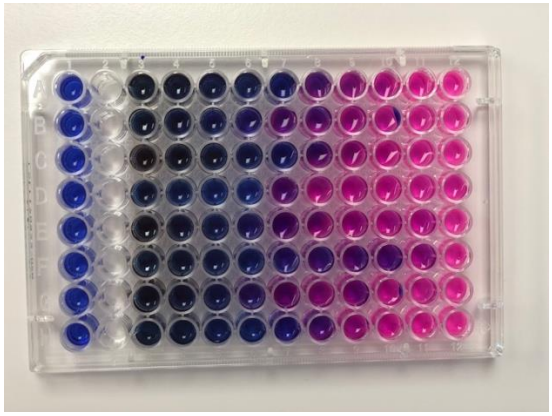


Figure A5.5 Sample 1-8 with different dilutions for investigating MIC of *S. aureus*, adding resazurin and after secondary incubation

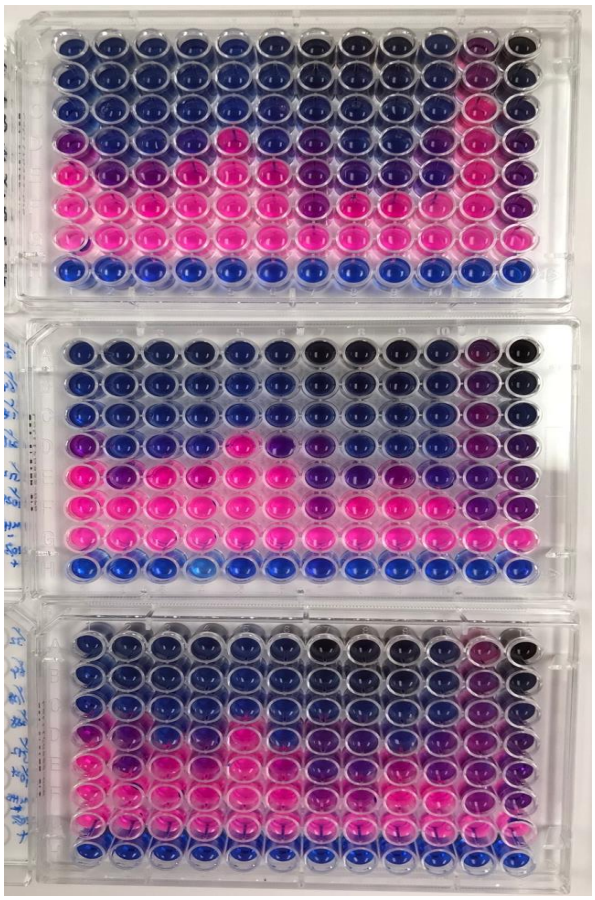


Figure A5.6 Samples 9-20 with different dilutions for investigating MIC of *S. aureus*, adding resazurin and after secondary incubation

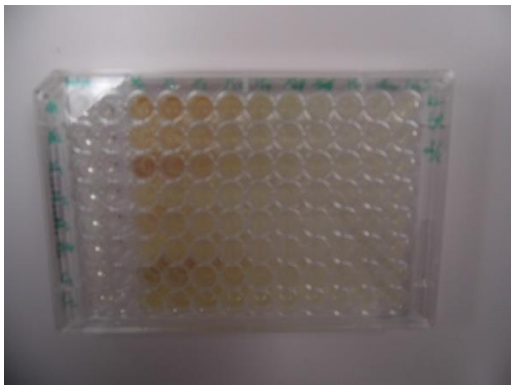


Figure A5.7 Samples 1-8 with different dilutions for investigating MIC of *E. coli*, after first incubation

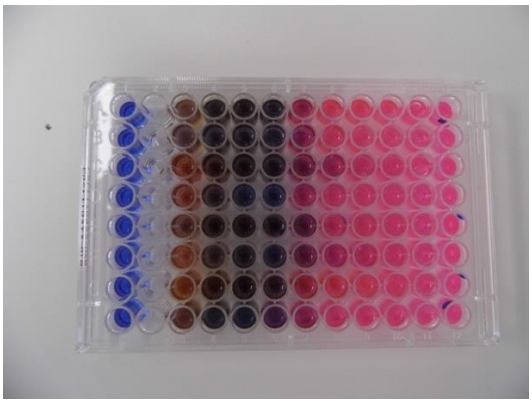


Figure A5.8 Samples 1-8 with different dilutions for investigating MIC of *E. coli*, adding resazurin and after secondary incubation

Figure A5.9 Samples 9-20 with different dilutions for investigating MIC of *E. coli*, adding resazurin and after secondary incubation

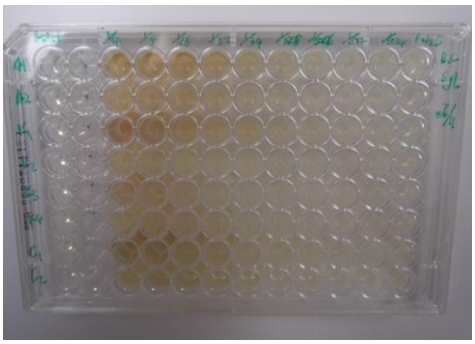


Figure A5.10 Samples 1-8 with different dilutions for investigating MIC of *B. cereus*, after first incubation

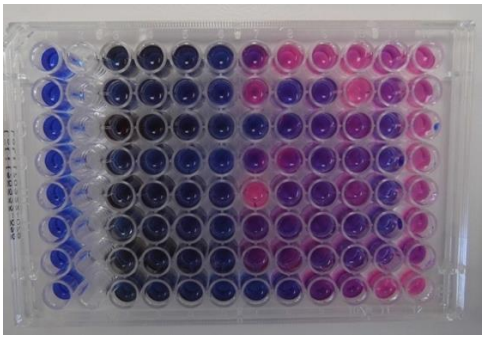


Figure A5.11 Samples 1-8 with different dilutions for investigating MIC of *B. cereus*, adding resazurin and after secondary incubation

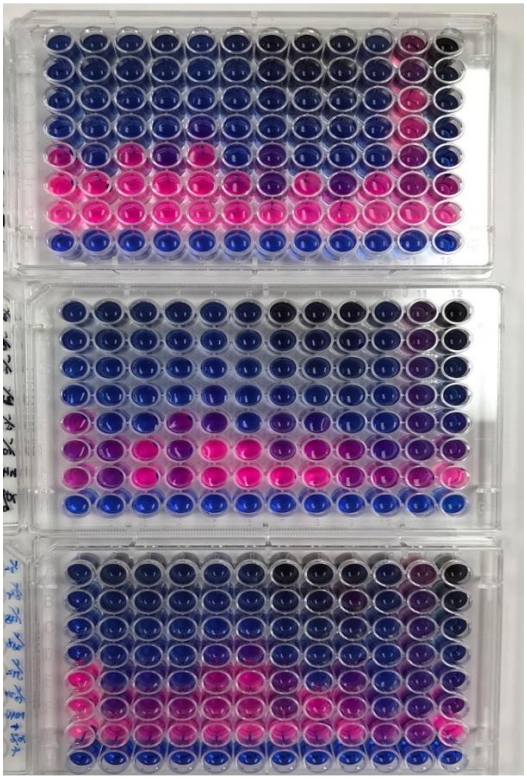


Figure A5.12 Samples 9-20 with different dilutions for investigating MIC of *B. cereus*, adding resazurin and after secondary incubation

Table A5.2 MIC for *S. aureus*

Samples	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
MIC (%v/v)	1.56	6.25	1.56	3.13	6.25	3.13	6.25	3.13	0.78	0.39	0.39	0.78	0.78	0.39	0.78	0.39	0.39	0.78	12.50	0.78
	1.56	6.25	1.56	3.13	6.25	3.13	6.25	1.56	0.78	0.39	0.78	0.39	0.78	0.78	0.78	0.39	0.39	0.39	12.50	0.78
	1.56	6.25	3.13	3.13	3.13	3.13	3.13	1.56	0.78	0.78	0.39	0.78	0.78	0.39	0.78	0.78	0.39	0.78	6.25	0.78

Table A5.3 MIC for *E. coli*

Samples	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
MIC (%v/v)	6.25	6.25	6.25	6.25	6.25	12.5	12.5	12.5	12.5	6.25	6.25	6.25	6.25	6.25	6.25	6.25	12.5	6.25	25.00	6.25
	6.25	6.25	6.25	6.25	12.5	12.5	12.5	12.5	12.5	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	25.00	6.25
	6.25	6.25	6.25	6.25	12.5	12.5	12.5	12.5	12.5	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	25.00	6.25

Table A5.4 MIC for *B. cereus*

Samples	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
MIC (%v/v)	3.13	3.13	1.56	3.13	6.25	3.13	3.13	1.56	0.39	0.20	0.39	0.39	0.78	0.20	0.39	0.20	0.20	0.20	6.25	0.20
	3.13	6.25	1.56	3.13	6.25	3.13	3.13	1.56	0.39	0.20	0.20	0.39	0.39	0.20	0.39	0.39	0.20	0.20	6.25	0.20
	3.13	6.25	1.56	6.25	6.25	3.13	6.25	1.56	0.39	0.20	0.20	0.20	0.78	0.78	0.39	0.20	0.39	0.20	6.25	0.20

Appendix 6. Results of Salicin

Table A6.1 The number and information of samples in this experiment

Sample	Numbers in this	Country	Brand	Propolis liquid base
S1	Au1	Australia	Blossom	GEP[A]
S2	Au2		Healthy Care	GEP[A]
S3	K1	Korea	Seoul Propolis	EEP[B]
S4	K2			
S5	K3			
S6	K4			
S7	baihua	China	Baihua	GEP[A]
S8	mifengtang		Beehall	
S9	NZ1	New Zealand	Comvita	EEP[B]
S10	NZ2			GEP[A]
S11	NZ3			
S12	NZ4		Manuka Health	GEP[A]
S13	NZ5		Arataki Honey	EEP[B]
S14	NZ6		NZ Health	GEP[A]
S15	S7	Korea	Seoul Propolis	WEEP[C]
S16	S8			EEP[B]
S17	S9			EEP[B]
S18	S10			EEP[B]
S19	S11			SEEP[D]
S20	S12			WEEP[C]

[A] GEEP represents glycol-based propolis; Sample code was highlighted in blue colour;
[B] EEP represents ethanol extracted propolis; Sample code was highlighted in orange colour;
[C] WEEP represents water-soluble base by evaporating ethanol after extraction, and it contains potassium carbonate and Tween 20; Sample code was highlighted in green colour;
[D] SEEP represents water-soluble ethanol extracted propolis containing Tween 20; Sample code was highlighted in purple colour;
[E]100 means that the TF content in the product is at least 1% (w/v), and 200 means 2% (w/v).

Dataset: Untitled

Last Altered: Wednesday, January 23, 2019 18:11:26 China Standard Time
Printed: Wednesday, January 23, 2019 18:12:14 China Standard Time

Method: C:\Dailywork\salicin.PRO\MethDB\salincin.mdb 23 Jan 2019 16:48:03
Calibration: C:\Dailywork\salicin.PRO\CurveDB\20190103-3ppm.cdb 23 Jan 2019 17:14:36

Compound name: salicin

Name	Sample Text	Type	Std....	Pred....	RT	Quan Trace	Sec.Trace	Ion Rat...	Ion Ratio	Area	Conc.
2019010433	0.1ug/mL	Standard	0.100	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.941	199.652	0.095
2019010434	0.2ug/mL	Standard	0.200	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.751	276.776	0.225
2019010435	0.4ug/mL	Standard	0.400	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.715	411.168	0.437
2019010436	0.6ug/mL	Standard	0.600	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.895	564.700	0.645
2019010437	0.8ug/mL	Standard	0.800	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.773	612.633	0.738
2019010438	1ug/mL	Standard	1.000	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.844	805.045	1.016
2019010439	2ug/mL	Standard	2.000	1.24	1.23	309.01 > 184...	309.01 > 14...	1.490	2.094	1329.552	1.726
2019010440	3ug/mL	Standard	3.000	1.24	1.23	309.01 > 184...	309.01 > 14...	1.490	1.792	2441.904	3.531

Compound name: salicin
Correlation coefficient: r = 0.999279, r^2 = 0.998558
Calibration curve: 1019.17 * x + 205.909
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

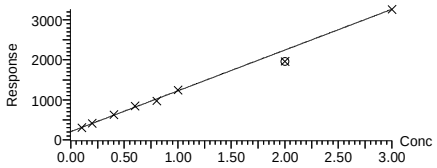


Figure A6.1 Standard curve of salicin by HPLC-MS

Dataset: C:\Dailywork\salicin.PRO\Qld\20190103-3ppm.qld

Last Altered: Saturday, January 26, 2019 12:28:03 China Standard Time
Printed: Saturday, January 26, 2019 12:29:14 China Standard Time

Method: C:\Dailywork\salicin.PRO\MethDB\salincin.mdb 23 Jan 2019 16:48:03
Calibration: C:\Dailywork\salicin.PRO\CurveDB\20190103-3ppm.cdb 23 Jan 2019 17:14:36

Compound name: salicin

Name	Sample Text	Type	Std....	Pred....	RT	Quan Trace	Sec.Trace	Ion Rat...	Ion Ratio	Area	Conc.
2019010374	Au1-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010375	Au1-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010376	Au1-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010377	Au2-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010378	Au2-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010379	Au2-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010380	S7-1	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.620	1475.819	2.140
2019010381	S7-2	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.822	1555.978	2.162
2019010382	S7-3	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.986	1474.934	1.974
2019010383	S8-1	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.728	265.347	0.209
2019010387	S8-2	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	2.220	219.948	0.111
2019010388	S8-3	Analyte	/	1.24	1.23	309.01 > 184...	309.01 > 14...	1.490	1.580	252.036	0.202
2019010389	S9-1	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.961	120.184	0.000
2019010390	S9-2	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.081	98.655	0.000
2019010391	S9-3	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.907	100.921	0.000
2019010392	S10-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010393	S10-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010394	S10-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010395	S11-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010396	S11-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010397	blank	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010398	A1+0.5ug/mL-1	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	2.058	438.852	0.438
2019010399	blank	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010400	S11-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010401	S12-1	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.892	1499.211	2.046
2019010402	S12-2	Analyte	/	1.24	1.23	309.01 > 184...	309.01 > 14...	1.490	2.024	1540.635	2.056
2019010403	S12-3	Analyte	/	1.24	1.23	309.01 > 184...	309.01 > 14...	1.490	1.817	1478.367	2.047

Compound name: salicin

Name	Sample Text	Type	Std....	Pred....	RT	Quan Trace	Sec.Trace	Ion Rat...	Ion Ratio	Area	Conc.
2019010332	K1-1	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.944	1221.555	1.613
2019010333	K1-2	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.669	1135.983	1.580
2019010334	K1-3	Analyte	/	1.24	1.23	309.01 > 184...	309.01 > 14...	1.490	1.886	1162.121	1.543
2019010338	K2-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010339	K2-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010340	K2-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010341	K3-1	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.839	665.361	0.806
2019010342	K3-2	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	2.069	712.576	0.835
2019010343	K3-3	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	2.182	741.556	0.859
2019010344	K4-1	Analyte	/	1.24	1.23	309.01 > 184...	309.01 > 14...	1.490	1.835	365.081	0.351
2019010345	K4-2	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.890	370.360	0.354
2019010346	K4-3	Analyte	/	1.24	1.24	309.01 > 184...	309.01 > 14...	1.490	1.947	381.745	0.365
2019010347	Nz1-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010351	Nz1-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010352	Nz1-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010353	Nz2-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010354	Nz2-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010355	Nz2-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010356	Nz3-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010357	Nz3-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010358	Nz3-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010359	Nz4-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010363	Nz4-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010364	Nz4-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010365	Nz5-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010366	Nz5-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010367	Nz5-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010368	Nz6-1	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010369	Nz6-2	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/
2019010370	Nz6-3	Analyte	/	1.24	/	309.01 > 184...	309.01 > 14...	1.490	/	/	/

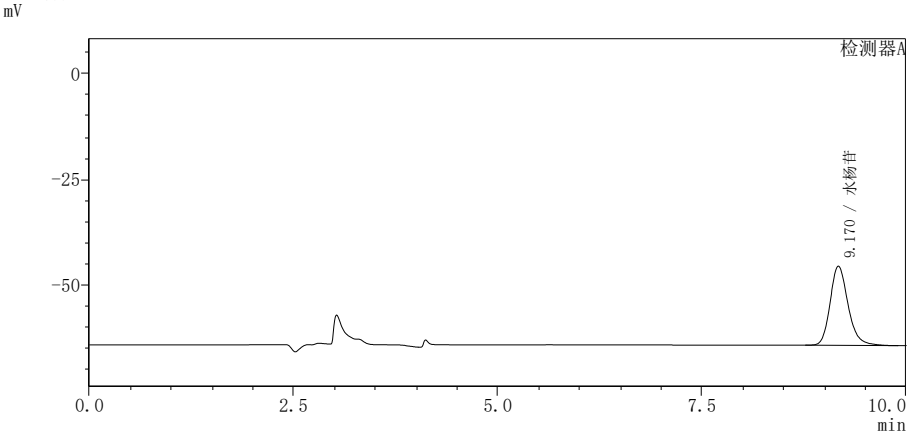
Figure A6.2 The concentration of salicin in samples by HPLC-MS

SHIMADZU LabSolutions 分析报告

〈样品信息〉

数据文件名 : std-0.02.lcd
方法文件名 : 水杨苷-数据处理.lcb.lcm
样品类型 : 标准
分析日期 : 2019/1/16 15:42:22

〈色谱图〉

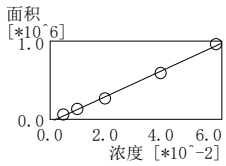


检测器A 213nm

峰号	化合物名	保留时间	浓度	浓度单位	面积	高度
1	水杨苷	9.170	0.019	mg/mL	286940	18798
总计					286940	18798

校准曲线

化合物名 : 水杨苷
定量计算法 : 外标法
校准曲线公式 : f(x)=1.67400e+007*x-24923.
相关系数(R)=0.9984000
拟合度(R^2)=0.9968026(1.869667e+009!=--)
剩余值的平方和(RSS)=1.869667e+009
平均 RF 1.538956e+007
RF标准偏差 8.472205e+005
RF相对标准偏差RSD 5.505165
校准曲线类型 : 直线
原点 : 未过原点



浓度(比率)	面积
0.005	76924
0.01	150167
0.02	286940
0.04	621421
0.06	999831

检测人： 复核人：

Figure A6.3 Standard curve of salicin by HPLC

Appendix 7. Results of Metal Elements and Rare Earth Elements

Table A7.1 Original weight of samples for digestion

Samples	Weight (g)		
	1	2	3
S1	0.5462	0.5217	0.6009
S2	0.6015	0.6963	0.6308
S3	0.5720	0.5488	0.6051
S4	0.6504	0.6730	0.6437
S5	0.6650	0.6284	0.6229
S6	0.6364	0.6225	0.6651
S7	0.6608	0.6676	0.7007
S8	0.3626	0.3918	0.4022
S9	0.7565	0.6906	0.7352
S10	0.7814	0.6460	0.8152
S11	0.6734	0.7385	0.7997
S12	0.5798	0.6333	0.6284
S13	0.7006	0.7252	0.6576
S14	0.5554	0.6360	0.6479
S15	0.5778	0.6137	0.5444
S16	0.5970	0.5791	0.6543
S17	0.6264	0.5984	0.5970
S18	0.6338	0.6325	0.6301
S19	0.6427	0.5155	0.4242
S20	0.5528	0.5799	0.6077

Table A7.2 The detected concentration of heavy metal elements by ICP-MS

Sample	^{75}As (µg/L)			^{111}Cd (µg/L)			^{208}Pb (µg/L)		
S1	0.597	0.556	0.633	0.061	0.067	0.062	2.511	2.339	2.664
S2	0.624	0.712	0.638	0.056	0.035	0.027	0.709	0.829	0.743
S3	2.474	2.374	2.619	0.193	0.188	0.195	5.287	5.103	5.589
S4	1.167	1.181	1.133	0.032	0.036	0.075	1.852	1.968	1.808
S5	1.982	1.854	1.888	0.147	0.149	0.148	7.121	6.646	6.672
S6	0.727	0.703	0.777	0.025	0.089	0.081	1.883	1.794	1.923
S7	5.186	5.184	5.516	0.174	0.185	0.182	6.196	6.247	6.545
S8	0.653	0.746	0.712	13.393	14.476	14.863	2.209	2.318	2.415
S9	2.117	1.972	2.058	0.019	0.024	0.018	2.820	2.663	2.749
S10	7.650	6.339	7.979	0.064	0.058	0.063	4.423	3.619	4.599
S11	1.443	1.530	1.762	0.015	0.027	0.043	3.012	3.225	3.455
S12	6.697	7.303	7.242	0.069	0.041	0.044	6.869	7.566	7.414
S13	2.795	2.896	2.680	0.043	0.065	0.042	11.494	11.875	10.858
S14	9.719	11.251	11.384	0.051	0.060	0.075	16.188	18.542	18.981
S15	0.447	0.476	0.393	0.041	0.026	0.025	1.265	1.337	1.191
S16	0.472	0.474	0.492	0.079	0.081	0.087	0.971	0.898	1.099
S17	0.398	0.399	0.387	0.046	0.051	0.043	0.787	0.726	0.738
S18	0.214	0.216	0.204	0.025	0.011	0.027	0.529	0.564	0.568
S19	0.079	0.061	0.043	0.006	0.008	0.008	2.123	1.725	1.435
S20	0.299	0.316	0.339	0.019	0.019	0.015	2.168	2.268	2.358

Table A7.3 The detected concentration of rare earth elements by ICP-MS

Sample	89Y (μg/L)	^{139}La (μg/L)	^{140}Ce (μg/L)	^{141}Pr (μg/L)	^{146}Nd (μg/L)	^{147}Sm (μg/L)	^{157}Gd (μg/L)	^{159}Tb (μg/L)	^{163}Dy (μg/L)	^{165}Ho (μg/L)	^{166}Er (μg/L)	^{169}Tm (μg/L)	^{172}Yb (μg/L)	^{175}Lu (μg/L)	^{232}Th (μg/L)
	-0.009	0.194	0.049	-0.023	0.020	0.021	0.008	-0.028	0.007	0.031	0.009	-0.026	-0.003	0.032	0.015
S1	-0.010	0.181	0.049	-0.022	0.021	0.021	0.008	-0.028	0.006	0.031	0.009	-0.026	-0.004	0.032	0.016
	-0.007	0.184	0.056	-0.023	0.026	0.022	0.009	-0.028	0.007	0.031	0.009	-0.026	-0.004	0.032	0.017
	-0.013	0.152	0.027	-0.027	0.006	0.018	0.006	-0.028	0.005	0.031	0.009	-0.026	-0.004	0.032	0.014
S2	-0.015	0.182	0.037	-0.025	0.008	0.019	0.007	-0.028	0.005	0.031	0.008	-0.026	-0.004	0.032	0.013
	-0.012	0.163	0.033	-0.026	0.007	0.029	0.006	-0.028	0.011	0.031	0.008	-0.026	-0.004	0.032	0.012
	0.023	0.253	0.035	-0.009	0.023	0.034	0.003	-0.026	0.013	0.032	0.012	-0.026	0.000	0.032	0.018
S3	0.023	0.239	0.027	-0.018	0.029	0.035	0.005	-0.027	0.018	0.032	0.011	-0.026	-0.002	0.032	0.018
	0.032	0.269	0.035	-0.025	0.033	0.024	0.007	-0.028	0.006	0.031	0.009	-0.026	-0.004	0.032	0.022
	-0.016	0.076	0.054	-0.027	0.003	0.020	0.007	-0.029	0.005	0.031	0.008	-0.026	-0.005	0.032	0.012
S4	-0.014	0.074	0.055	-0.026	0.008	0.020	0.007	-0.028	0.005	0.031	0.008	-0.026	-0.004	0.032	0.014
	-0.009	0.072	0.049	-0.024	0.008	0.021	0.009	-0.028	0.006	0.031	0.009	-0.026	-0.004	0.032	0.014
	0.040	0.083	0.029	-0.025	0.014	0.025	0.014	-0.027	0.012	0.033	0.012	-0.026	0.002	0.033	0.016
S5	0.041	0.089	0.025	-0.025	0.012	0.026	0.012	-0.027	0.013	0.032	0.013	-0.025	0.001	0.033	0.016
	0.039	0.087	0.023	-0.025	0.015	0.024	0.012	-0.027	0.012	0.033	0.013	-0.026	0.002	0.033	0.018
	0.000	0.481	0.021	-0.027	0.001	0.016	0.004	-0.023	0.002	0.036	0.003	-0.024	0.008	0.034	0.076
S6	-0.020	0.475	0.019	-0.028	0.001	0.019	0.004	-0.029	0.004	0.031	0.008	-0.026	-0.005	0.032	0.011
	-0.018	0.484	0.021	-0.027	0.003	0.020	0.005	-0.028	0.004	0.031	0.008	-0.026	-0.004	0.032	0.013
	-0.003	0.047	0.056	-0.024	0.014	0.019	0.009	-0.028	0.007	0.032	0.010	-0.026	-0.003	0.032	0.025
S7	-0.009	0.045	0.048	-0.026	0.008	0.019	0.008	-0.028	0.007	0.031	0.009	-0.026	-0.004	0.032	0.019
	-0.010	0.120	0.055	-0.015	0.037	0.019	0.010	-0.028	0.006	0.031	0.010	-0.026	-0.003	0.032	0.020
	0.101	0.237	0.513	0.027	0.205	0.116	0.041	-0.024	0.029	0.036	0.022	-0.024	0.007	0.034	0.126
S8	0.101	0.245	0.561	0.021	0.202	0.113	0.045	-0.021	0.028	0.038	0.025	-0.024	0.012	0.034	0.124
	0.102	0.266	0.551	0.024	0.213	0.117	0.043	-0.021	0.028	0.037	0.026	-0.024	0.011	0.034	0.110
	-0.016	0.234	0.025	-0.026	0.001	0.019	0.006	-0.028	0.005	0.031	0.008	-0.026	-0.004	0.032	0.012
S9	-0.020	0.208	0.026	-0.028	0.001	0.018	0.006	-0.029	0.004	0.031	0.007	-0.026	-0.005	0.032	0.011
	-0.017	0.216	0.024	-0.027	0.001	0.019	0.006	-0.029	0.005	0.031	0.008	-0.026	-0.004	0.032	0.012
	-0.004	0.045	0.017	-0.022	0.025	0.024	0.010	-0.028	0.007	0.032	0.009	-0.026	-0.004	0.032	0.019
S10	-0.020	0.046	0.018	-0.028	0.004	0.019	0.008	-0.029	0.004	0.031	0.008	-0.026	-0.005	0.032	0.011
	-0.015	0.058	0.023	-0.026	0.009	0.019	0.006	-0.028	0.005	0.031	0.008	-0.026	-0.004	0.032	0.011

Sample	89Y (µg/L)	^{139}La (µg/L)	^{140}Ce (µg/L)	^{141}Pr (µg/L)	^{146}Nd (µg/L)	^{147}Sm (µg/L)	^{157}Gd (µg/L)	^{159}Tb (µg/L)	^{163}Dy (µg/L)	^{165}Ho (µg/L)	^{166}Er (µg/L)	^{169}Tm (µg/L)	^{172}Yb (µg/L)	^{175}Lu (µg/L)	^{232}Th (µg/L)
	-0.019	0.164	0.020	-0.027	0.002	0.018	0.006	-0.028	0.004	0.031	0.008	-0.026	-0.005	0.032	0.012
S11	-0.019	0.175	0.022	-0.027	0.005	0.018	0.008	-0.029	0.004	0.031	0.008	-0.026	-0.005	0.032	0.011
	-0.012	0.163	0.026	-0.025	0.012	0.020	0.007	-0.028	0.006	0.031	0.009	-0.026	-0.004	0.032	0.013
	-0.011	0.126	0.027	-0.026	0.008	0.019	0.009	-0.028	0.006	0.031	0.009	-0.026	-0.003	0.032	0.015
S12	-0.019	0.136	0.026	-0.027	0.007	0.018	0.006	-0.028	0.005	0.031	0.008	-0.026	-0.005	0.032	0.013
	-0.013	0.138	0.031	-0.026	0.009	0.019	0.006	-0.028	0.005	0.031	0.009	-0.026	-0.004	0.032	0.013
	-0.015	0.112	0.015	-0.027	0.004	0.017	0.006	-0.028	0.005	0.031	0.008	-0.026	-0.004	0.032	0.012
S13	-0.007	0.117	0.033	-0.025	0.001	0.019	0.008	-0.028	0.006	0.031	0.009	-0.026	-0.003	0.032	0.014
	-0.004	0.111	0.059	-0.022	0.003	0.021	0.013	-0.028	0.007	0.032	0.009	-0.026	-0.003	0.032	0.018
	-0.017	0.032	0.029	-0.027	0.002	0.019	0.007	-0.028	0.005	0.031	0.008	-0.026	-0.005	0.032	0.011
S14	-0.013	0.037	0.032	-0.026	0.008	0.020	0.007	-0.028	0.005	0.031	0.008	-0.026	-0.003	0.032	0.013
	-0.014	0.044	0.034	-0.026	0.005	0.018	0.005	-0.029	0.004	0.031	0.008	-0.026	-0.005	0.032	0.010
	-0.005	0.074	0.097	-0.017	0.038	0.060	0.009	-0.028	0.007	0.032	0.010	-0.026	-0.003	0.032	0.018
S15	-0.008	0.058	0.077	-0.021	0.025	0.044	0.008	-0.028	0.007	0.032	0.010	-0.026	-0.004	0.032	0.017
	-0.012	0.051	0.075	-0.022	0.020	0.040	0.008	-0.028	0.007	0.032	0.009	-0.026	-0.003	0.032	0.017
	-0.010	0.051	0.046	-0.020	0.032	0.048	0.008	-0.028	0.007	0.032	0.009	-0.026	-0.004	0.032	0.016
S16	-0.011	0.060	0.062	-0.020	0.026	0.040	0.008	-0.028	0.007	0.031	0.009	-0.026	-0.004	0.032	0.017
	-0.023	0.032	0.019	-0.026	0.009	0.033	0.005	-0.029	0.004	0.031	0.008	-0.026	-0.005	0.032	0.011
	-0.010	0.044	0.030	-0.024	0.017	0.034	0.007	-0.028	0.006	0.031	0.009	-0.026	-0.004	0.032	0.012
S17	-0.015	0.043	0.034	-0.024	0.016	0.031	0.006	-0.028	0.005	0.031	0.008	-0.026	-0.004	0.032	0.013
	-0.019	0.040	0.036	-0.024	0.013	0.031	0.006	-0.028	0.005	0.031	0.008	-0.026	-0.004	0.032	0.014
	-0.013	0.069	0.032	-0.024	0.012	0.026	0.008	-0.028	0.006	0.032	0.009	-0.025	-0.004	0.033	0.012
S18	-0.007	0.044	0.038	-0.024	0.017	0.027	0.008	-0.028	0.006	0.032	0.009	-0.026	-0.003	0.032	0.012
	0.008	0.150	0.181	-0.022	0.024	0.025	0.009	-0.028	0.007	0.032	0.010	-0.026	-0.003	0.032	0.015
	-0.019	0.049	0.026	-0.026	0.008	0.023	0.006	-0.028	0.005	0.031	0.008	-0.026	-0.004	0.032	0.012
S19	-0.006	0.053	0.061	-0.024	0.015	0.025	0.008	-0.028	0.006	0.031	0.009	-0.026	-0.004	0.032	0.014
	-0.007	0.052	0.056	-0.024	0.013	0.024	0.006	-0.028	0.006	0.031	0.009	-0.026	-0.004	0.032	0.013
	-0.005	0.057	0.080	-0.017	0.039	0.026	0.011	-0.028	0.008	0.032	0.010	-0.026	-0.003	0.032	0.020
S20	-0.009	0.064	0.077	-0.023	0.017	0.025	0.008	-0.028	0.007	0.031	0.010	-0.026	-0.003	0.032	0.019
	-0.010	0.051	0.064	-0.023	0.016	0.022	0.008	-0.028	0.007	0.032	0.009	-0.026	-0.003	0.032	0.016

Concentrations

1/31/2019 1:45:15 PM

Analysis index: 19 Analysis label: 5 Analysis started at: 1/31/2019 10:36:30 AM

Category	73Ge (KED)	75As (KED)	111Cd (KED)	208Pb (KED)	209Bi (KED)
Concentration average	103.677 %	0.746 ppb	14.476 ppb	2.318 ppb	115.742 %
Intensity average	39,877 cps	1,882 cps	120,805 cps	256,413 cps	4,659,962 cps

Analysis index: 20 Analysis label: 6 Analysis started at: 1/31/2019 10:38:10 AM

Category	73Ge (KED)	75As (KED)	111Cd (KED)	208Pb (KED)	209Bi (KED)
Concentration average	104.541 %	0.672 ppb	14.663 ppb	3.215 ppb	116.691 %
Intensity average	40,209 cps	1,720 cps	123,418 cps	354,812 cps	4,698,196 cps

Analysis index: 21 Analysis label: 7 Analysis started at: 1/31/2019 10:39:37 AM

Category	73Ge (KED)	75As (KED)	111Cd (KED)	208Pb (KED)	209Bi (KED)
Concentration average	98.513 %	0.447 ppb	0.041 ppb	2.665 ppb	104.737 %
Intensity average	37,891 cps	1,104 cps	373 cps	265,443 cps	4,216,888 cps

Analysis index: 22 Analysis label: 8 Analysis started at: 1/31/2019 10:40:59 AM

Category	73Ge (KED)	75As (KED)	111Cd (KED)	208Pb (KED)	209Bi (KED)
Concentration average	100.397 %	0.476 ppb	0.026 ppb	1.337 ppb	106.665 %
Intensity average	38,615 cps	1,192 cps	261 cps	140,147 cps	4,294,529 cps

Analysis index: 23 Analysis label: 9 Analysis started at: 1/31/2019 10:42:16 AM

Category	73Ge (KED)	75As (KED)	111Cd (KED)	208Pb (KED)	209Bi (KED)
Concentration average	100.507 %	0.393 ppb	0.025 ppb	3.391 ppb	111.239 %
Intensity average	38,658 cps	999 cps	250 cps	356,261 cps	4,478,680 cps

Analysis index: 24 Analysis label: 10 Analysis started at: 1/31/2019 10:43:42 AM

Category	73Ge (KED)	75As (KED)	111Cd (KED)	208Pb (KED)	209Bi (KED)
Concentration average	108.149 %	0.472 ppb	0.079 ppb	15.271 ppb	143.590 %
Intensity average	41,597 cps	1,276 cps	743 cps	2,027,340 cps	5,781,173 cps

Analysis index: 25 Analysis label: 11 Analysis started at: 1/31/2019 10:45:09 AM

Category	73Ge (KED)	75As (KED)	111Cd (KED)	208Pb (KED)	209Bi (KED)
Concentration average	114.692 %	0.484 ppb	0.081 ppb	0.880 ppb	151.231 %
Intensity average	44,113 cps	1,385 cps	810 cps	135,257 cps	6,088,842 cps

Figure A7.1 The concentration of standards (^{111}Cd, ^{75}As, ^{208}Pb) by ICP-MS

Concentrations

1/31/2019 1:47:48 PM

Concentrations:

Analysis index: 1 Analysis label: 0 Analysis started at: 1/28/2019 3:22:23 PM

Category	89Y	103Rh	139La	140Ce
Concentration average	-0.024 ppb	100.000 %	0.023 ppb	0.006 ppb
Intensity average	1,231 cps	229,212,745 cps	1,638 cps	2,269 cps

141Pr	146Nd	147Sm	157Gd	159Tb
-0.029 ppb	-0.003 ppb	0.019 ppb	0.004 ppb	-0.027 ppb
386 cps	261 cps	162 cps	73 cps	451 cps

163Dy	165Ho	166Er	169Tm	172Yb
0.003 ppb	0.031 ppb	0.007 ppb	-0.026 ppb	-0.005 ppb
46 cps	42 cps	57 cps	59 cps	33 cps

175Lu	232Th
0.032 ppb	0.008 ppb
42 cps	269 cps

Analysis index: 2 Analysis label: 0.1ppb Analysis started at: 1/28/2019 3:24:37 PM

Category	89Y	103Rh	139La	140Ce
Concentration average	0.077 ppb	98.743 %	0.113 ppb	0.101 ppb
Intensity average	16,291 cps	226,331,599 cps	16,997 cps	16,978 cps

141Pr	146Nd	147Sm	157Gd	159Tb
0.072 ppb	0.088 ppb	0.112 ppb	0.103 ppb	0.074 ppb
18,454 cps	3,247 cps	2,834 cps	3,307 cps	18,419 cps

163Dy	165Ho	166Er	169Tm	172Yb
0.092 ppb	0.118 ppb	0.103 ppb	0.076 ppb	0.087 ppb
4,214 cps	17,268 cps	6,162 cps	18,788 cps	3,913 cps

175Lu	232Th
0.120 ppb	0.102 ppb
17,116 cps	13,404 cps

Analysis index: 3 Analysis label: 0.5ppb Analysis started at: 1/28/2019 3:26:41 PM

Category	89Y	103Rh	139La	140Ce
Concentration average	0.485 ppb	104.260 %	0.464 ppb	0.459 ppb
Intensity average	82,673 cps	238,977,221 cps	82,694 cps	77,554 cps

141Pr	146Nd	147Sm	157Gd	159Tb
0.489 ppb	0.497 ppb	0.470 ppb	0.455 ppb	0.491 ppb
98,750 cps	17,811 cps	14,137 cps	15,836 cps	99,326 cps

163Dy	165Ho	166Er	169Tm	172Yb
0.490 ppb	0.465 ppb	0.461 ppb	0.488 ppb	0.500 ppb
24,403 cps	91,550 cps	30,858 cps	100,271 cps	22,685 cps

175Lu	232Th
0.462 ppb	0.449 ppb
89,202 cps	66,334 cps

Analysis index: 4 Analysis label: 1ppb Analysis started at: 1/28/2019 3:28:44 PM

Category	89Y	103Rh	139La	140Ce
Concentration average	1.114 ppb	99.359 %	0.983 ppb	1.047 ppb
Intensity average	174,418 cps	227,742,516 cps	169,160 cps	166,303 cps

141Pr	146Nd	147Sm	157Gd	159Tb
1.126 ppb	1.033 ppb	0.985 ppb	1.053 ppb	1.117 ppb
209,265 cps	34,960 cps	28,559 cps	34,875 cps	208,374 cps

163Dy	165Ho	166Er	169Tm	172Yb
1.024 ppb	0.956 ppb	1.037 ppb	1.116 ppb	1.035 ppb
48,804 cps	185,205 cps	66,385 cps	212,120 cps	44,467 cps

175Lu	232Th
0.956 ppb	1.056 ppb
181,851 cps	149,340 cps

Concentrations

1/31/2019 1:47:48 PM

Analysis index: 5 Analysis label: 2ppb Analysis started at: 1/28/2019 3:31:12 PM

Category	89Y	103Rh	139La	140Ce
Concentration average	1.948 ppb	100.685 %	2.017 ppb	1.987 ppb
Intensity average	306,052 cps	230,782,410 cps	355,442 cps	319,684 cps

141Pr	146Nd	147Sm	157Gd	159Tb
1.941 ppb	1.985 ppb	2.014 ppb	1.984 ppb	1.945 ppb
361,763 cps	67,817 cps	59,854 cps	66,828 cps	364,725 cps

163Dy	165Ho	166Er	169Tm	172Yb
1.991 ppb	2.030 ppb	1.991 ppb	1.946 ppb	1.983 ppb
96,256 cps	406,848 cps	129,950 cps	371,507 cps	86,102 cps

175Lu	232Th
2.031 ppb	1.985 ppb
400,439 cps	285,909 cps

Analysis index: 7 Analysis label: kb-1 Analysis started at: 1/28/2019 3:35:15 PM

Category	89Y	103Rh	139La	140Ce
Concentration average	-0.020 ppb	105.994 %	6.551 ppb	0.021 ppb
Intensity average	1,863 cps	242,950,797 cps	1,217,093 cps	4,953 cps

141Pr	146Nd	147Sm	157Gd	159Tb
-0.027 ppb	0.002 ppb	0.018 ppb	0.007 ppb	-0.028 ppb
774 cps	443 cps	136 cps	178 cps	160 cps

163Dy	165Ho	166Er	169Tm	172Yb
0.004 ppb	0.031 ppb	0.008 ppb	-0.026 ppb	-0.005 ppb
101 cps	132 cps	80 cps	98 cps	46 cps

175Lu	232Th
0.032 ppb	0.015 ppb
93 cps	1,317 cps

Analysis index: 8 Analysis label: kb-2 Analysis started at: 1/28/2019 3:38:39 PM

Category	89Y	103Rh	139La	140Ce
Concentration average	-0.018 ppb	108.863 %	10.630 ppb	0.030 ppb
Intensity average	2,271 cps	249,527,820 cps	2,036,781 cps	6,653 cps

141Pr	146Nd	147Sm	157Gd	159Tb
-0.026 ppb	0.008 ppb	0.019 ppb	0.007 ppb	-0.028 ppb
1,032 cps	693 cps	166 cps	198 cps	134 cps

163Dy	165Ho	166Er	169Tm	172Yb
0.004 ppb	0.031 ppb	0.008 ppb	-0.026 ppb	-0.005 ppb
127 cps	118 cps	103 cps	93 cps	58 cps

175Lu	232Th
0.032 ppb	0.013 ppb
52 cps	1,067 cps

Analysis index: 9 Analysis label: kb-3 Analysis started at: 1/28/2019 3:40:22 PM

Category	89Y	103Rh	139La	140Ce
Concentration average	-0.022 ppb	100.128 %	0.274 ppb	0.010 ppb
Intensity average	1,439 cps	229,506,465 cps	45,833 cps	2,920 cps

141Pr	146Nd	147Sm	157Gd	159Tb
-0.029 ppb	-0.002 ppb	0.018 ppb	0.004 ppb	-0.029 ppb
432 cps	294 cps	124 cps	84 cps	81 cps

163Dy	165Ho	166Er	169Tm	172Yb
0.004 ppb	0.031 ppb	0.008 ppb	-0.026 ppb	-0.005 ppb
80 cps	92 cps	70 cps	52 cps	39 cps

175Lu	232Th
0.032 ppb	0.011 ppb
42 cps	628 cps

Figure A7.2 The concentration of standards (Rare earth elements) by ICP-MS