An investigation of the suitability of melissopalynology to authenticate Jarrah honey

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ABSTRACT

This study reports on the analysis of eleven Jarrah (*Eucalyptus marginata*) honeys, of which nearly half (n = 5) were re-classified as Blackbutt (*E. patens*) honey on the grounds of the predominant flower pollen identified by melissopalynology. Based on a comprehensive analysis of the honeys' physico- and phytochemical characteristics and antioxidant activity data, taking into account pH, electrical conductivity, refractive index and Brix values as well as moisture content, individual fructose and glucose content and derived fructose to glucose ratio alongside total phenolic content and antioxidant activity determined by the DPPH assay, no statistically significant difference was found amongst the eleven honeys classified by pollen analysis into two honey groups, 'Jarrah' or 'Blackbutt'. This study therefore draws into question the value of melissopalynology as an analysis tool to authenticate Jarrah honey.

1. Introduction

As a honey's nectar source is the main determinant of its commercial and therapeutic value, authentication of its floral origin is an important quality control aspect. Pollen analysis (also known as melissopalynology) has been used for many years for the determination of the botanical origin of honey, especially in Europe (Stefan Bogdanov et al., 1999; Louveaux et al., 1970; Sniderman et al., 2018). It records the type and relative quantity of different pollen grains found in honey by microscopic analysis with the dominant pollen being considered as a marker for the honey's nectar source (Iurlina et al., 2009; Jerković et al., 2009). However, while melissopalynology might be an adequate approach for many European honeys, there are cases, in particular when honey is not harvested from areas with limited floral diversity (e.g. from agricultural monocultures where bees are used for pollination services or from tree plantations) but from areas of rich natural diversity (e.g. natural bushlands or pristine forests) where the technique seems to fail the industry and where strong criticism has thus been voiced. A recent study by Sniderman et al. (2018) has demonstrated that pollen analysis is not suitable for the authentication of Australian eucalyptus honeys or generally honeys derived from pristine natural sources where the flowering seasons of different floral species might overlap.

It must also be highlighted that honey is produced from flower nectar, not from flower pollen (Robertson, 2019); hence, flower pollen can be considered a natural honey contaminant (Fernandez and Ortiz, 1994). Bees collect flower pollen to complement their nutritional needs (T'ai & Cane, 2000; Zarchin et al., 2017); at times the flower pollen they collect might therefore be from a different botanical source than the nectar they collect and use to produce honey. In cases where there is not much botanical diversity (e.g. monocultures, plantations) the plants visited by bees to collect nectar might happen to be the same as the plants from which nectar is collected, but the more choice bees have (e.g. when foraging in naturally diverse areas) the more refined their foraging might be – they might collect pollen to meet their protein needs but might visit potentially different flowers to collect nectar for honey production, which acts as a main source of carbohydrates. In this light, in particular in an Australian context where honeys are often harvested from areas rich in botanical diversity, melissopalynology is a controversial method for honey authentication.

Furthermore, Molan (1998) pointed out that honey, which has been filtered with diatomaceous earth, has no pollen left that could be used for its authentication. Also, honey produced from secretions of

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extrafloral nectaries (a major source of honey produced from cotton plants (*Gossypium hirsutum*), castor-oil plants (*Ricinus communis*) and rubber trees (*Hevea brasiliensis*)) does not contain any pollen to identify its floral origin. Similarly, in Rewarewa (*Knightia excelsa*) and Tawari (*Lxerba brexioides*) trees endemic to New Zealand, bees are impeded from pollen access during nectar collection (Li, 2017), leading to an undercount of the respective pollen in the honey produced from these botanical sources. Moreover, thyme honey often has a low total pollen count as only some thyme flowers produce nectar and pollen, whereas others produce nectar only (Li, 2017). Even for the well-researched Mānuka honey (derived from *Leptospermum scoparium*), the limitations of melissopalynology have been demonstrated as Mānuka pollen appear visually to be almost identical to Kānuka (*Kunzea ericoides*) when investigated under a light microscope (Stephens et al., 2010).

Taking all of the above into account, the value of melissopalynology for honey authentication might be called into question as it might not always act as a suitable tool to authenticate the nectar source of the honey; indeed, it can be argued that pollen analysis might be more suited to the confirmation of the honey's geographical origin or provenance (Louveaux et al., 1970; Newstrom-Lloyd, 2017; Newstrom-Lloyd et al., 2017) and also the time of its collection as pollen grains, as natural honey contaminants, may provide evidence for many (but not necessarily all) of the floral sources the bees visited (Robertson, 2019).

In order to determine if the findings of pollen analysis, in a specific Australian context, are in alignment with honey chemistry and thus in this instance an adequate predictor of a honey's floral origin, this study investigated the chemical characteristics as well as antioxidant properties of eleven honey samples considered by a beekeeper to be Jarrah (*Eucalyptus marginata*) honey. Subsequent melissopalynological analysis carried out by a commercial laboratory reclassified five honey samples as Blackbutt (*Eucalyptus patens*) honey and one as multifloral honey.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents used in this study and their suppliers: Fructose, Maltose, Aniline, Vanillin, Folin and Ciocalteu's Phenol Reagent 2N (Sigma-Aldrich, St. Louis, MO, USA), 4,5,7-Trihydroxyflavanone (Alfa Aesar, England, UK), 2,2-Diphenyl-1-picrylhydrazyl (DPPH*) (Fluka AG, Buchs SG, Switzerland), Anhydrous sodium sulfate and Silica gel 60 F254 HPTLC glass plates (20 cm × 10 cm) (Merck KGaA, Darmstadt, Germany), Boric acid (Pharma Scope, Welshpool, WA, Australia), Glucose, Sucrose, Sodium carbonate anhydrous (Chem-Supply Pty Ltd., St. Gillman, SA, Australia), Gallic acid, Diphenylamine, Phosphoric acid (Ajax Finechem Pvt Ltd., Sydney, Australia).

Solvents used in this study and their suppliers: Methanol (Scharlau, Barcelona, Spain), Dichloromethane (Merck KGaA, Darmstadt, Germany), Ethanol, Ethyl acetate and Formic acid (Ajax Finechem Pvt Ltd., Sydney, Australia), 1-Butanol (Chem-Supply Pty Ltd., St. Gillman, SA, Australia), 2-Propanol and Toluene (Asia Pacific Specialty Chemicals Ltd, Sydney, Australia).

2.2. Honey samples

Eleven honey samples were collected (Jan 2020 to Feb 2020) and identified by the beekeeper as Jarrah honey based on the geographical location of the hives, the predominant species flowering at the hive sites and the honey's organoleptic characteristics. Subsequent pollen analysis, however, provided a different assessment, reclassifying the predominant nectar source of over half of the honeys (Table 1). All eleven honeys were therefore subjected to a comprehensive analysis of their physico- and phytochemical characteristics and antioxidant activity. The effect of filtration removing pollen grains present in the samples was also investigated. The chosen filter size (0.45 μm, Filtropur S, Sarstedt AG & Co. KG, Nümbrecht, Germany) ensured the effective removal of

Table 1
Beekeeper assessment and pollen analysis results.

Sample Code	Major Pollen content (%)		Predominant Nectar Source	
	Jarrah (*Eucalyptus marginata*)	Blackbutt (*Eucalyptus patens*)	Based on pollen analysis	Beekeeper's assessment
S1	76	13	Jarrah	Jarrah
S2	75	11	Jarrah	Jarrah
S3	53	41	Jarrah	Jarrah
S4	79	10	Jarrah	Jarrah
S5	76	17	Jarrah	Jarrah
S6	38	46	Mixed flora	Jarrah
S7	32	56	Blackbutt	Jarrah
S8	29	60	Blackbutt	Jarrah
S9	27	67	Blackbutt	Jarrah
S10	28	63	Blackbutt	Jarrah
S11	20	70	Blackbutt	Jarrah

the pollen given that *E. marginata* pollen grains are generally between 15.5 and 21.0 μm and those of *E. patens* between 19.0 and 23.0 μm (Pickett and Newsome, 1997). The samples were stored in glass jars at room temperature in the dark for the duration of the study.

2.3. Determination of physico-chemical properties

Key physico-chemical characteristics such as pH, refractive index, moisture content, colour and fructose to glucose ratio were obtained by standard analytical methodologies. In brief, the pH of each honey was measured by dissolving 1 g of honey in 7.5 ml of carbon-dioxide free water (Stephan Bogdanov, Martin, & Lullmann, 2002) and the resulting pH of the solution was determined with a calibrated pH meter (Thermo Scientific™ Orion™ 3-Star Benchtop pH Meter). The electrical conductivity of a 20% (*w/v*) honey solution was measured at 22 °C using an Electrical Conductometer (Eutech PC 2700 - Eutech Instruments) and expressed as milliSiemens per centimetre (mS/cm) (Adaškevičiūtė et al., 2019). Refractive index and Brix value were determined simultaneously by spreading a sample of each honey over the entire surface of the reading window of a digital refractometer (HI96801, Hanna Instruments, Rhode Island, USA). The honey samples' moisture content was derived from the respective Brix reading as 100% - Brix. Colour was determined by dissolving each honey in sterile distilled water to 50% (w/v) before measuring the optical density (OD) at both 450 nm and 720 nm (Sindi et al., 2019) (MolecularDevices SpectraMax 190 Microplate Reader). The difference between the two OD measurements was multiplied by 1000 and expressed as milli-absorbance units (mAU). Colour values were determined for all honeys both before and after passing through a 0.7 μm glass fibre filter.

2.4. Determination of total phenolics content and antioxidant properties

The antioxidant property of each honey sample was determined in triplicate by recording total phenolics content as well as antioxidant activity measured in a DPPH assay. In brief, the total phenolic content of 200 μL of aqueous 20% (*w/v*) honey solution was determined colorimetrically after treatment with 1 ml of Folin-Ciocalteu reagent followed, after 5 min, by the addition of 800 μL of Na_2CO_3. The absorbance was taken at 760 nm (Cary 60 UV–Vis Spectrophotometer, Agilent Technologies, CA, United States) after 2 h incubation in the dark at ambient temperature. The sample's total phenolics content was expressed as mg Gallic acid equivalent (GAE) per 100 g of honey from the mean of the three measurements (Liberato Mda et al., 2011). For the DPPH assay, 10 μL of a 20% (*w/v*) aqueous honey solution were mixed with 190 μL of 0.130 mM methanolic solution of DPPH* reagent and 100 μL aqueous 100 mM $NaC_2H_3O_2$ buffer (pH 5.5) and the absorbance was measured after 120 min at 520 nm using a BMG Labtech POLARstar Optima Microplate Reader. The sample's radical scavenging activity, expressed

as % DDPH* inhibition, was calculated as the mean of triplicate samples and expressed as μmol Trolox equivalent (TE) per kg of honey (Karabagias et al., 2017).

2.5. High-performance thin layer chromatography (HPTLC) analyses

2.5.1. Organic extract analysis by HPTLC

High Performance Thin Layer Chromatography (HTPLC) derived fingerprints of the honeys' organic extracts with and also without prior filtration (0.45 μm, Filtropur S, Sarstedt AG & Co. KG, Nümbrecht, Germany) to remove any residual pollen grains, were obtained. The organic extracts of the samples were analysed as described by Locher et al. (Locher et al., 2017; Locher et al., 2018). In brief, approximately 1 g of each honey was dissolved in 2 mL of de-ionised water. Duplicate samples were prepared, and half the samples were filtered (0.45 μm, Filtropur S, Sarstedt AG & Co. KG, Nümbrecht, Germany) prior to extraction, the other half were extracted without this filtration step. The extraction was carried out either with Dichloromethane (DCM) only or with a mixture of Acetonitrile (ACN):Dichloromethane (1:1; v/v). The organic solutions were dried with Na_2SO_4 anhydrous, and the resulting extracts following solvent evaporation were stored at 4 °C. Prior to HPTLC fingerprinting, the extracts were reconstituted in 100 μL Dichloromethane (for DCM extracts) or Methanol (for ACN:DCM extracts). For the HPTLC analysis, glass plates (20 × 10 cm, Silica gel 60 F_{254}) were prepared by applying the organic honey extracts (5 μL) as 8 mm bands at 8 mm from the lower edge of the HPTLC plate at a rate of 150 nLs^{-1} using a semi-automated HPTLC application device (Linomat 5, CAMAG). The chromatographic separation for each plate was performed at ambient temperature in a saturated (33% relative humidity) automated development chamber (ADC2, CAMAG) to a distance of 70 mm using a mixture of Toluene: Ethyl acetate: Formic acid (6:5:1, $v/v/v$ for DCM extracts and 1:6:1, $v/v/v$ for ACN:DCM extracts) as mobile phase. The obtained chromatographic results were documented using a HPTLC imaging device (TLC Visualizer 2, CAMAG) under 254 nm and 366 nm, respectively. All chromatographic images were digitally processed and analysed using a specialised HPTLC software (visionCATS, CAMAG) which was also used to control the individual instrumentation modules.

After initial documentation of the chromatographic results the plate was then derivatised with 3.0 mL of Vanillin-Sulfuric acid reagent using a TLC derivatiser (CAMAG Derivatiser) and heated for 3 min at 115 °C using a CAMAG TLC Plate Heater III. The plate was cooled to room temperature and analysed again with the HPTLC imaging device under white light and at 366 nm.

2.5.2. Sugar analysis by HPTLC

The detection and quantification of major sugars in the honey samples was carried out as described by Islam et al. (Islam, Sostaric, Lim, Hammer and Locher, 2020a; 2020b). In brief, aqueous methanolic honey solutions (1 mg/mL) were prepared and 2 μL of the solutions were applied as 8 mm bands at 8 mm from the lower edge of the HPTLC plate (glass plates 20 × 10 cm, silica gel 60 F254) at a rate of 50 nLs^{-1} using a semi-automated HPTLC application device (Linomat 5, CAMAG). Standard solutions of glucose and fructose (both at 250 μg/mL) were also applied at 1 μL, 2 μL, 3 μL and 4 μL. Chromatographic separation was performed at ambient temperature in a saturated (33% relative humidity) automated development chamber (ADC2, CAMAG) to a distance of 85 mm using 1-Butanol:2-Propanol:Boric acid (5.0 mg/mL in water) in a ratio of 3:5:1 ($v/v/v$) as mobile phase. The plate was derivatised with 2.0 mL of Aniline-Diphenylamine-Phosphoric acid reagent (CAMAG Derivatiser) and the derivatised plate was heated for 10 min at 115 °C (CAMAG TLC Plate Heater III), then cooled to room temperature and analysed with the HPTLC imaging device under transmission white (T white) light. The chromatographic images were digitally processed and analysed using a specialised HPTLC software (visionCATS, CAMAG) which was also used to control the individual instrumentation modules.

2.6. Statistical analysis

All experiments were performed in triplicates, and the obtained quantitative results were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was performed, where a p-value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Physico-chemical properties of analysed honeys

Table 2a presents the pH, electrical conductivity, refractive index, Brix value, moisture content, as well as colour readings (prior and after filtration through a 0.7 μm glass fibre filter) for all 11 honeys. Honey is acidic in nature with its pH typically ranging from 3.42 to 6.10 (Abu--Tarboush et al., 1993; Aljohar et al., 2018). All the samples analysed (S1 – S11) were also acidic and their pH was found to be within a narrow range between 5.15 and 5.66. Electrical Conductivity (EC) is strongly correlated with the total elemental content in honey and typically ranges between 0.1 and 3 mS/cm (Bogdanov et al., 2002). The samples analysed in this study (S1 – S11) had EC readings of 1.39–1.60 mS/cm. The samples' moisture content was found to be between 17.10 and 18.00%, which are all below the maximum acceptable moisture content of 20% set by the Codex Alimentarius for Honey (Alimentarius, 2017). The samples' relatively consistent moisture content correlates to the total sugar content captured in Brix values ranging from 82.0 to 82.9 and associated refractive indices of between 1.496 and 1.498. The colour value before filtration for the analysed samples ranged from 403.70 to 538.70 mAU and after filtration from 295.70 to 368.10 mAU.

Although the beekeeper's assessment was that all of the samples were derived from the same nectar source and should be classified as Jarrah (*E. marginata*) honey, melissopalynology painted a different picture, grouping them into two distinct groups, 5 Jarrah (*E. marginata*) honeys (Samples #S1-5) and 5 Blackbutt (*E. patens*) honeys (Samples #S7-11) with one of the honeys (Sample #S6) being classified as multifloral. Table 2b present descriptive statistics (average/min/max) of the various physico-chemical characteristics for all 11 honey samples (Group A), as well as for the 5 samples classified as Jarrah honey (Group J) and the 5 samples reclassified as Blackbutt honey (Group B) based on pollen analysis.

As can be seen from the data presented in Table 2b, a reduction in colour was noted for all samples after filtration, which indicates that pollen grains and other particulate matter contribute to the colour reading of the honey samples. Statistical analysis (one-way ANOVA) demonstrated that there is no statistically significant difference between Group J and Group B in pH (p = 0.1807), electrical conductivity (p = 0.9248), refractive index (p = N/A), Brix (p = 0.4250), colour before (p = 0.0613) or after filtration (p = 0.3668). Thus, it can be concluded that the two honey groups as identified by melissopalynology had very similar pH, electrical conductivity, refractive index and Brix values as well as very similar colour readings before and after filtration.

3.2. Total phenolic content and antioxidant properties

A similar picture emerges from the analysis of the honey samples' total phenolics content and their antioxidant activity measured in the DPPH assay prior to and after filtration (0.45 μm) to remove any pollen grains. Table 3a presents the data for all samples collated from these analyses. Across all samples (S1 – S11), the total phenolics content ranged between 34.75 and 38.80 mg GAE per 100 g honey for the unfiltered samples and between 30.49 and 34.40 mg GAE per 100 g after filtration. This range is remarkably narrow, considering that the antioxidant activity of a large number of Western Australian honeys (n = 451) was found to range between 7.39 and 75.56 mg GAE per 100 g of honey (unpublished data). With respect to their antioxidant activity, the samples were found to have antioxidant activity values ranging from

Table 2a

a Physico-chemical characteristics of the honey samples.

Sample Code	pH	Electrical Conductivity (mS/cm)	Refractive Index (nD20)	Brix	Moisture Content (%)	Colour Before Filtration (mAU)	Colour After Filtration (mAU)
S1	5.35	1.41	1.498	82.7	17.3	470.7	329.9
S2	5.15	1.42	1.497	82.6	17.4	538.7	368.1
S3	5.66	1.60	1.496	82.0	18.0	484.1	347.3
S4	5.29	1.39	1.498	82.9	17.1	528.4	351.8
S5	5.34	1.43	1.497	82.4	17.6	483.7	325.1
S6	5.38	1.48	1.498	82.8	17.2	469.4	318.3
S7	5.54	1.57	1.497	82.6	17.4	486.8	355.0
S8	5.49	1.56	1.498	82.7	17.3	484.9	346.7
S9	5.54	1.60	1.498	82.7	17.3	468.1	352.0
S10	5.41	1.44	1.497	82.6	17.4	403.7	295.7
S11	5.45	1.52	1.498	82.8	17.2	433.6	299.3

Table 2b

Descriptive statistics (mean / min / max) of the various physico-chemical data

	pH	Electrical Conductivity (mS/cm)	Refractive Index (nD20)	Brix	Moisture Content (%)	Colour Before Filtration (mAU)	Colour After Filtration (mAU)
Group A							
average	5.42	1.49	1.50	82.62	17.38	477.46	335.38
min	5.15	1.39	1.50	82.00	17.10	403.70	295.70
max	5.66	1.60	1.50	82.90	18.00	538.70	368.10
Group J							
average	5.36	1.45	1.50	82.52	17.48	501.12	344.44
min	5.15	1.39	1.50	82.00	17.10	470.70	325.10
max	5.66	1.60	1.50	82.90	18.00	538.70	368.10
Group B							
average	5.49	1.54	1.50	82.68	17.32	455.42	329.74
min	5.41	1.44	1.50	82.60	17.20	403.70	295.70
max	5.54	1.60	1.50	82.80	17.40	486.80	355.00

Group A – all honey samples.
Group J – samples classified as Jarrah honey based on pollen analysis.
Group B – samples classified as Blackbutt honeys based on pollen analysis.

Table 3a

Total phenolics content and antioxidant activities of the honey samples.

Sample Code	Total phenolics content (mg GAE/100g)		DPPH Total Antioxidant Activity (µmol TE/kg)	
	Non-Filtered	Filtered	Non-Filtered	Filtered
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
S1	35.67 ± 0.28	32.68 ± 0.39	2808.57 ± 41.98	2243.51 ± 98.26
S2	36.67 ± 1.12	31.81 ± 0.74	3034.82 ± 72.92	2367.59 ± 149.50
S3	35.73 ± 0.62	32.22 ± 1.54	2911.87 ± 181.80	2265.06 ± 188.11
S4	34.75 ± 0.76	30.49 ± 0.59	2875.54 ± 114.14	2343.70 ± 60.34
S5	36.22 ± 0.58	32.62 ± 1.00	2630.88 ± 126.56	2385.69 ± 145.40
S6	38.67 ± 0.48	32.72 ± 1.83	3042.50 ± 46.89	2306.06 ± 61.62
S7	37.09 ± 0.34	33.29 ± 1.33	2899.46 ± 177.71	2384.12 ± 155.58
S8	38.80 ± 2.19	34.40 ± 1.52	2885.92 ± 154.93	2551.60 ± 127.99
S9	37.82 ± 0.15	33.83 ± 1.06	2475.98 ± 89.20	2367.63 ± 196.59
S10	35.49 ± 0.70	31.30 ± 1.39	2338.17 ± 64.52	2248.97 ± 216.60
S11	35.75 ± 1.23	30.84 ± 0.60	2458.41 ± 73.20	2298.73 ± 26.08

2338.17 to 3042.50 µmol TE/kg honey prior to filtration and between 2243.51 and 2551.60 µmol TE/kg for the filtered samples. Again, this demonstrates a rather consistent antioxidant activity across the 11 samples in light of variations between 248.26 and 5407.76 µmol TE/kg

seen across Western Australian honeys (n = 451, unpublished data). Table 3b shows descriptive statistics (average/min/max) for the data generated when all samples are analysed together (Group A) as well as for those samples classified, based on pollen analysis, as Jarrah honey (Group J) and for those samples classified as Blackbutt honeys (Group B).

Across all honeys a drop in antioxidant activity and total phenolics content can be noticed after filtration, which indicates that pollen grains present in these honeys contribute to the overall antioxidant activity (Wilczyńska, 2014), but this effect is similar in both groups and therefore does not seem to be greatly influenced by the specific type of pollen

Table 3b

Descriptive statistics (mean / min / max) for total phenolics content and DPPH antioxidant activity

	Total phenolics content (mg GAE/100g)		DPPH total antioxidant (µmol TE/kg)	
	Non-Filtered	Filtered	Non-Filtered	Filtered
Group A				
average	36.61	32.38	2760.19	2342.06
min	34.75	30.49	2338.17	2243.51
max	38.80	34.40	3042.50	2551.60
Group J				
average	35.81	31.96	2852.34	2321.11
min	34.75	30.49	2630.88	2243.51
max	36.67	32.68	3034.82	2385.69
Group B				
average	36.99	32.73	2611.59	2370.21
min	35.49	30.84	2338.17	2248.97
max	38.80	34.40	2899.46	2551.60

Group A – all honey samples.
Group J – samples classified as Jarrah honey based on pollen analysis.
Group B – samples classified as Blackbutt honeys based on pollen analysis.

that is predominately present in the samples. While slight differences recorded in individual total phenolics contents and the DPPH assay data can be assumed to be reflective of natural variations between the samples, statistical analysis (one-way ANOVA) found no statistically significant differences between Group J and Group B with respect to total phenolics content of non-filtered (p = 0.1292) and filtered (p = 0.3716) samples, nor in the DPPH total antioxidant activity of those samples that were filtered (p = 0.1117) or not filtered (p = 0.4271) prior to the assay.

3.3. HPTLC analysis of organic honey extracts

HPTLC analysis is able to capture very rich information. HPTLC banding patterns, capturing Rf value, colour and intensity of each band that is obtained for a honey's organic extract at 254 nm and 366 nm prior to, as well as at 366 nm and white light after derivatisation, are representative of the honey's nectar source (Locher et al., 2017, 2018). Samples S1 – S11, independent of their classification based on pollen analysis, present a very coherent banding pattern in the HPTLC analysis of their organic extracts, independent of the type of mobile phase used (Fig. 1 a-d and Fig. 3 a-d) and it can therefore be concluded that all eleven HPTLC fingerprints are very similar (Fig. 5 and Material S1) except for very minor variations reflective of a natural product.

In samples S2 and S4 a bright blue band (Rf 0.46, Fig. 1c and Rf 0.68, Fig. 3c) can be observed at white light after derivatisation, which is considered a marker band of *Banksia sessilis* nectar (Islam et al., 2021). This bright blue band persists after filtration (Rf 0.46, Fig. 2c and Rf 0.68, Fig. 4c) to remove pollen grains, which indicates that bees also collected some *Banksia sessilis* nectar in the production of the two honeys. Interestingly, a slight contribution of the pollen present in the samples can also be noted when comparing the respective fingerprints prior (Fig. 1 a-d, Fig. 3 a-d) and after filtration (Fig. 2 a-d, Fig. 4 a-d). In particular in the more polar regions of the fingerprint (Rf 0.09–0.12) two dark blue bands can be noted at 366 nm after derivatisation (Figs. 1d, 3d), which disappear in the filtered samples (Figs. 2d, 4d). This finding is in line with the antioxidant analysis and also the colour measurement of

the honey samples where a drop in readings can be noted upon filtration, indicating a small contribution of the pollen grains. However, as can be seen in the respective HPTLC fingerprints, their overall contribution is relatively small and, more importantly to the focus of this study, it can be concluded that after filtration, thus after the removal of the very characteristic that has led to the samples being classified into two distinct groupings, no differences in the individual HPTLC fingerprints can be noted. This, again, highlights that despite the samples being considered to represent two distinctly different honeys according to pollen analysis, they appear chemically equivalent.

3.4. Sugar analysis by HPTLC

Honeys of different floral origins tend to differ in their individual sugar profile which in turn can have an impact on physico-chemical characteristics (e.g. osmolarity) and influence their crystallisation behaviour. Jarrah honey, for example, is known to have a relatively high fructose to glucose ratio and for this reason does not tend to crystallise (Escuredo et al., 2014; Gleiter et al., 2006; Laos et al., 2011; Smanalieva and Senge, 2009). Individual glucose and fructose contents as well as the derived fructose to glucose ratios have been determined (Table 4a). For the analysed samples (S1 – S11), fructose contents ranged from 41.81 to 44.2 g/100g honey and glucose contents from 23.27 to 25.39 g/100g. Associated fructose: glucose ratios were found to be between 1.67 and 1.84.

Across all samples, and also when analysed within the two major groups, very similar glucose and fructose contents were found with the respective fructose to glucose ratios indicating fructose as the dominant sugar (Table 4b). The derived values all fall within the typical fructose to glucose ratio reported for Jarrah honey (Manning, 2011). This finding is also not surprising considering that none of the samples crystallised during the duration of the study as crystallisation is commonly associated with higher glucose levels. Statistical analysis (one-way ANOVA) confirmed that there is no statistically significant difference between Group J and Group B in their fructose (p = 0.0727) or glucose (p =
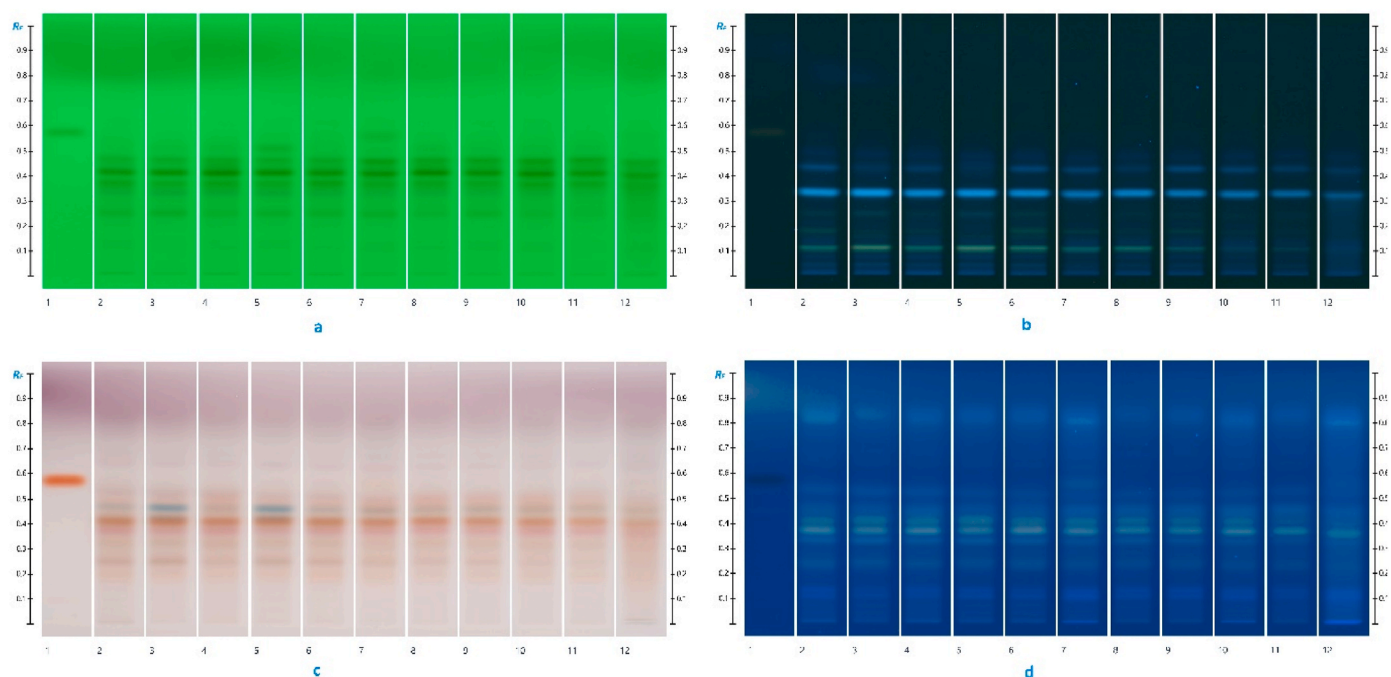

Fig. 1. HPTLC Images taken prior to derivatisation at (a) 254 nm and (b) 366 nm; after derivatisation with vanillin reagent at (c) white and (d) 366 nm; Track 1—4,5,7-Trihydroxyflavanon, Track 2— S1, Track 3— S2, Track 4— S3, Track 5— S4, Track 6— S5, Track 7— S6, Track 8— S7, Track 9— S8, Track 10— S9, Track 11— S10 and Track 12— S11; 5 μL honey extract respectively; (all samples – prior to filtration; extraction solvent: Dichloromethane; mobile phase: Toluene: Ethyl acetate: Formic acid (6:5:1, v/v/v).
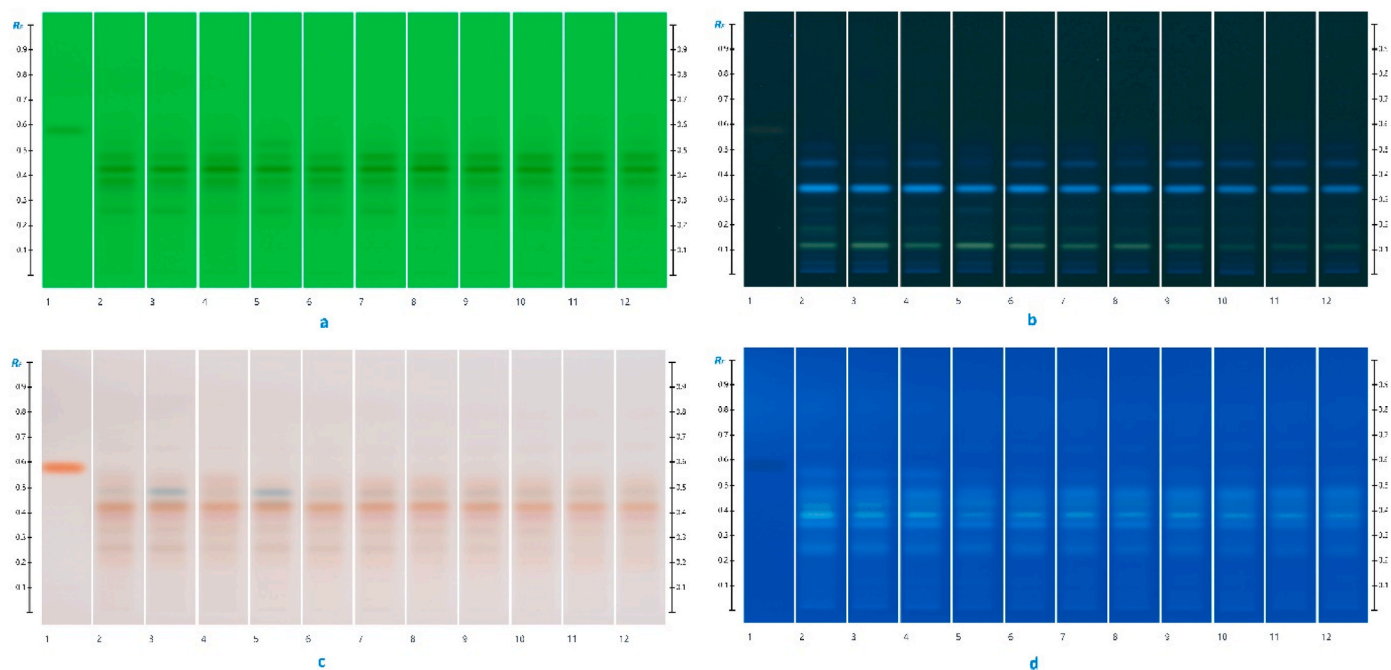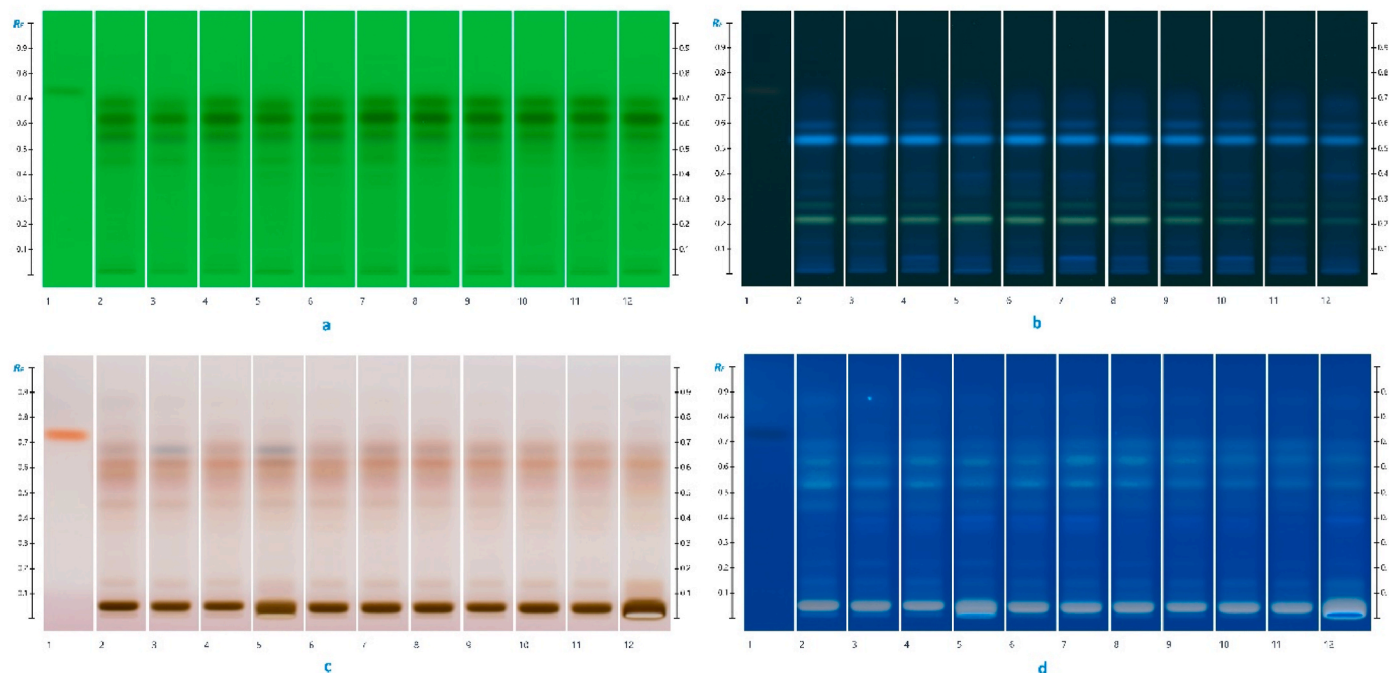
Fig. 2. HPTLC Images taken prior to derivatisation at (a) 254 nm and (b) 366 nm; after derivatisation with vanillin reagent at (c) white and (d) 366 nm; Track 1–4,5,7-Trihydroxyflavanon, Track 2— S1, Track 3— S2, Track 4— S3, Track 5— S4, Track 6— S5, Track 7— S6, Track 8— S7, Track 9— S8, Track 10— S9, Track 11— S10 and Track 12— S11; 5 μL honey extract respectively; (all samples – after filtration (0.45 μm); extraction solvent: Dichloromethane; mobile phase: Toluene: Ethyl acetate: Formic acid (6:5:1, v/v/v)).

Fig. 3. HPTLC Images taken prior to derivatisation at (a) 254 nm and (b) 366 nm; after derivatisation with vanillin reagent at (c) white and (d) 366 nm; Track 1–4,5,7-Trihydroxyflavanon, Track 2— S1, Track 3— S2, Track 4— S3, Track 5— S4, Track 6— S5, Track 7— S6, Track 8— S7, Track 9— S8, Track 10— S9, Track 11— S10 and Track 12— S11; 5 μL honey extract respectively; (all samples – prior to filtration; extraction solvent: Acetonitrile: Dichloromethane (1:1; v/v); mobile Phase: Toluene: Ethyl acetate: Formic acid (1:6:1, v/v/v)).

0.2167) content nor in their respective fructose to glucose ratios ($p = 0.9385$).

4. Conclusion

Although pollen analysis classified five of the eleven investigated samples as Jarrah (*E. marginata*) honey, five as Blackbutt (*E. patens*) honey and one as multifloral, HPTLC fingerprinting of the organic
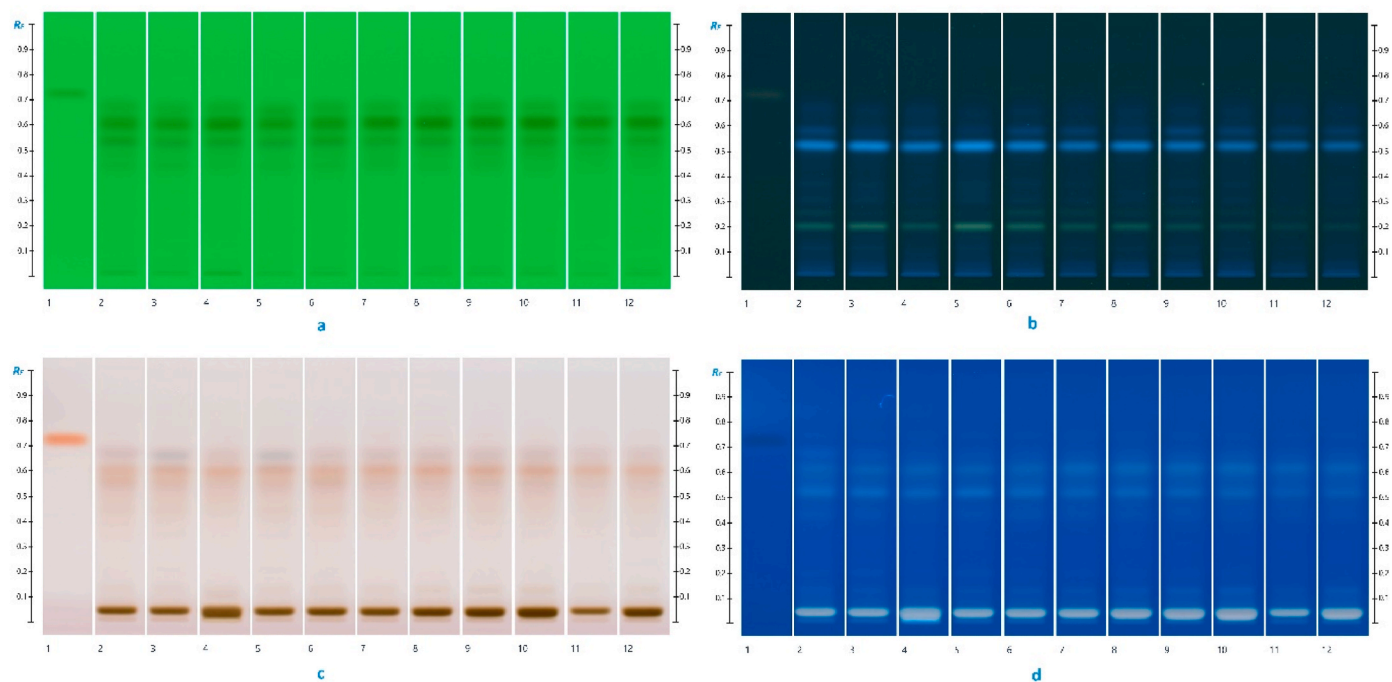
Fig. 4. HPTLC Images taken prior to derivatisation at (a) 254 nm and (b) 366 nm; after derivatisation with vanillin reagent at (c) white and (d) 366 nm; Track 1–4,5,7-Trihydroxyflavanon, Track 2— S1, Track 3— S2, Track 4— S3, Track 5— S4, Track 6— S5, Track 7— S6, Track 8— S7, Track 9— S8, Track 10— S9, Track 11— S10 and Track 12— S11; 5 μL honey extract respectively; (all samples – after filtration (0.45 μm); extraction solvent: Acetonitrile: Dichloromethane (1:1; v/v); mobile phase: Toluene: Ethyl acetate: Formic acid (1:6:1, v/v/v)).
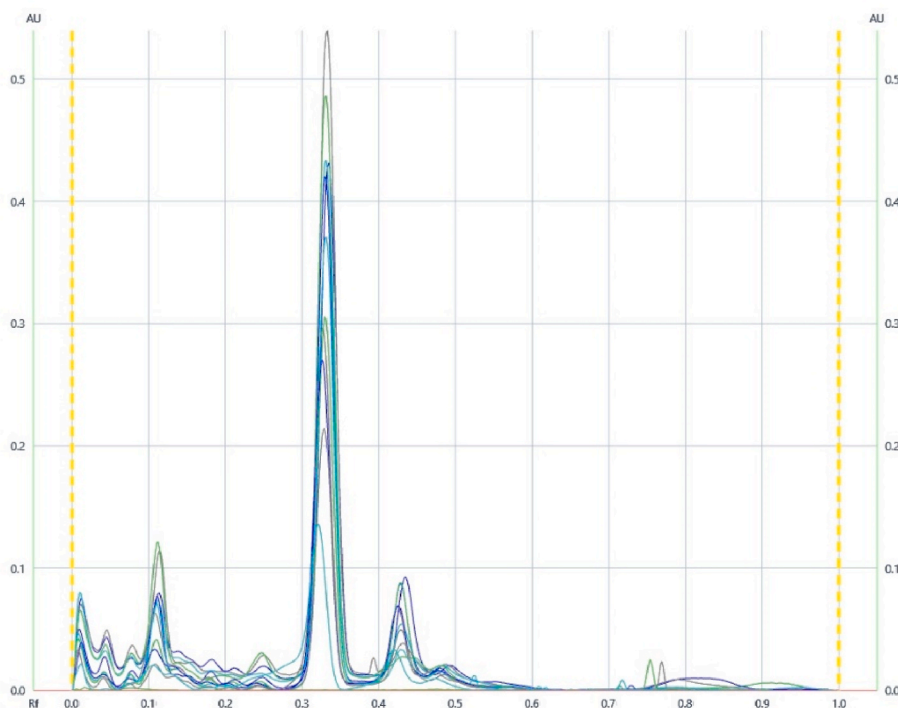
Fig. 5. Overlay of the honey samples' chromatograms obtained at 366 nm (all samples – prior to filtration (0.45 μm); extraction solvent: Dichloromethane; mobile phase: Toluene: Ethyl acetate: Formic acid (6:5:1, v/v/v)).

extracts of these honeys, obtained after extraction with two solvent systems of different polarity, returned almost identical results for all samples. The fingerprints also did not change significantly with filtration, which demonstrates that pollen grains present in the samples play only a limited role in the phytochemical composition of the honey

extracts. The noticeable, though very limited influence of pollen grains on the samples' colour, total phenolics content and antioxidant activity has been captured in this study, but more importantly it could be demonstrated that with, and also without the presence of these pollen grains, there was no statistically significant difference between the

Table 4a

a Fructose and Glucose content, and fructose to glucose ratio (F: G) of honeys.

Sample Code	Fructose (g/100g honey) Mean ± SD	Glucose (g/100g honey) Mean ± SD	Fructose: Glucose
S1	42.13 ± 0.86	23.69 ± 0.35	1.78
S2	42.78 ± 0.49	23.71 ± 0.38	1.80
S3	42.84 ± 0.62	23.87 ± 1.59	1.80
S4	41.81 ± 2.58	23.43 ± 1.29	1.78
S5	42.37 ± 2.55	24.22 ± 1.55	1.75
S6	43.89 ± 1.47	23.92 ± 1.44	1.83
S7	42.73 ± 2.25	23.27 ± 0.98	1.84
S8	43.09 ± 1.76	24.02 ± 1.14	1.79
S9	44.20 ± 0.68	24.33 ± 1.60	1.82
S10	42.32 ± 2.12	25.39 ± 1.51	1.67
S11	43.51 ± 0.93	24.35 ± 0.93	1.79

Descriptive statistics (mean/min/max) were derived for all samples taken together (Group A) as well as for those samples classified, based on pollen analysis, as Jarrah honey (Group J) and for those classified as Blackbutt honeys (Group B) (Table 4b).

Table 4b

Descriptive statistics (mean/min/max) for sugar content.

	Fructose (g/100g honey)	Glucose (g/100g honey)	Fructose: Glucose
Group A			
average	42.88	24.02	1.79
min	41.81	23.27	1.67
max	44.2	25.39	1.84
Group J			
average	42.37	23.78	1.78
min	41.81	23.43	1.75
max	42.84	24.22	1.80
Group B			
average	43.17	24.27	1.78
min	42.32	23.27	1.67
max	44.2	25.39	1.84

Group A – all honey samples.
Group J – samples classified as Jarrah honey based on pollen analysis.
Group B – samples classified as Blackbutt honeys based on pollen analysis.

samples beyond slight natural variations. Neither on the basis of their physico-chemical characteristics (pH, electrical conductivity, refractive index/Brix, moisture content), nor on the basis of their major sugar profile or fructose to glucose ratio, nor in their organic extract composition prior or after filtration could any differences be noted that would justify that the honeys were classified as being of different floral origin. The same picture emerged from the analysis of the samples' antioxidant properties and total phenolics content. The findings of this study therefore draw into question melissopalynology as a suitable tool to authenticate Jarrah honey and support the assessments by others, such as Sniderman et al. (2018), who have already expressed their reservations over pollen analysis as honey authentication tool, specifically in the context of Australian *Eucalyptus* honeys.

It is recommended that future studies continue to explore this issue for larger sample sets and also for honeys from other nectar sources to help establish a better understanding of the role of melissopalynology in the authentication of Australian honeys harvested from pristine and botanically diverse natural areas. The authentication of honey is a high-stake exercise as honeys of different floral origin can yield distinctly different market prices. Thus, analytical methods for honey authentication need to be carefully selected. As demonstrated by the findings of this study, in particular in the context of honeys harvested from diverse, botanically rich areas such as Western Australian Jarrah honey, pollen analysis might not be the most appropriate analytical tool. Rather the chemistry of the honey, which is a direct reflection of its floral nectar source, should be the focus of authentication efforts.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2022.02.014.

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