Optimisation of Bee Pollen Extraction to Maximise Extractable Antioxidant Constituents

Ivan Lozada Lawag 1,2, Okhee Yoo 2, Lee Yong Lim 2, Katherine Hammer 1,3 and Cornelia Locher 1,2, * 1

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Abstract: This paper presents the findings of a comprehensive review on common bee pollen processing methods which can impact extraction efficiency and lead to differences in measured total phenolic content (TPC) and radical scavenging activity based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) data. This hampers the comparative analysis of bee pollen from different floral sources and geographical locations. Based on the review, an in-depth investigation was carried out to identify the most efficient process to maximise the extraction of components for measurement of TPC, DPPH and FRAP antioxidant activity for two bee pollen samples from western Australia (Jarrah and Marri pollen). Optimisation by Design of Experiment with Multilevel Factorial Analysis (Categorical) modelling was performed. The independent variables included pollen pulverisation, the extraction solvent (70% aqueous ethanol, ethanol, methanol and water) and the extraction process (agitation, maceration, reflux and sonication). The data demonstrate that non-pulverised bee pollen extracted with 70% aqueous ethanol using the agitation extraction method constitute the optimal conditions to maximise the extraction of phenolics and antioxidant principles in these bee pollen samples.

Keywords: bee pollen; Jarrah; Marri; extraction; 2-diphenyl-1-picrylhydrazyl (DPPH); ferric reducing antioxidant power (FRAP); total phenolic content (TPC); optimisation; antioxidant activity; review; multilevel factor analysis (MFA)

1. Introduction

Flower pollen are the reproductive cells found in the stamen of plants which are transferred to the stigma of another plant via pollinating agents, such as bees, other insects, wind and water [1]. Bee pollen, on the other hand, is made by worker honeybees combining flower pollen, nectar and bee salivary constituents, and it is transferred to beehives in the form of pollen baskets attached to the bees’ hind legs [1,2]. Inside the hives, bee pollen is packed into honeycomb cells and covered with a layer of honey and wax to initiate fermentation to generate bee bread, which is the principal source of nutrients for honeybees [2]. Bee pollen, which is also known as apicultural, bee-collected or corbicular pollen, can be harvested for human consumption with the help of pollen traps. These traps are fixed at the entrance of beehives and collect pollen by stripping the pollen baskets from the hind legs of bees on entry to the hives [3].

Bee pollen provides bees with carbohydrates and other necessary nutrients such as proteins, fats, minerals and vitamins [4]. The secondary metabolite profiles of bee pollens vary significantly, reflecting the botanical and geographical origin as well as the climatic conditions, soil type and beekeeper activities [5,6]. The chemical composition of
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Bee pollens typically include 13–55% carbohydrates, 10–40% proteins and 1–13% lipids, alongside minor components such as minerals and trace elements, vitamins, carotenoids, phenolic compounds, flavonoids, sterols and terpenes [6–8]. Some even consider bee pollen to be the only complete food, as it contains all the essential amino acids needed by humans [6]. Bee pollen has been recommended as a natural nutraceutical due to its antimicrobial, antioxidant, anti-inflammatory, antiallergen, anticarcinogenic, antiradiation, antiulcer, hepatoprotective and chemopreventive properties. More recently, bee pollen has been found to modulate gut microbiota to promote gut health [6–8].

The antioxidant activity of bee pollen is mainly associated with the presence of polyphenols in the form of phenolic acids and flavonoids, which exert their antioxidant activity by neutralising free radicals through the donation of electron or hydrogen atoms [9]. This bioactivity has attracted considerable interest in recent years because of its association with anti-inflammatory, anti-cancer and also anti-aging effects [5]. Commonly, the determination of the antioxidant activity of honey and other bee products such as bee pollen involves the use of a range of popular colorimetric assays. These include the measurement of total phenolic content (TPC) [10–14], total flavonoid content (TFC) [13–15], free radical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [4,12–16] or measuring the ferric reducing antioxidant power (FRAP) [4,10,17]. DPPH and FRAP assays, in particular, are widely used to determine the antioxidant activity of plant extracts and food products. These assays use stable redox reagents which are inexpensive and easy to prepare, they are quick and easy to perform, and the results are accurate, highly reproducible, and readily validated [18].

The determination of the antioxidant activity of bee pollen necessitates an extraction step guided by the premise of a high yield and minimal changes to the functional properties of the extract. It is, therefore, necessary to select an appropriate extraction method and solvent, based on sample matrix properties, the chemical properties of the analytes as well as potential matrix–analyte interactions [19]. Further parameters that require consideration are the number of extractions to be performed, the extraction time, the ratio of solvent to raw material, and the extraction pressure and temperature. As the polarity of bioactive polyphenols ranges from very polar to relatively non-polar, the extraction solvent plays a significant role in extraction efficiency [20]. Furthermore, pre-extraction processes such as pulverisation can also affect the extraction efficiency of bioactive principles, as powdered samples have smaller particle sizes and narrower particle size distribution, leading to improved surface contact with extraction solvents [21].

In order to determine an optimal extraction process for bee pollen that yields the maximum extraction of antioxidant constituents, a survey of the literature was carried out. A review of 101 papers published between 2003 and 2021 reporting the antioxidant activity of bee pollen found a wide range of extraction conditions (Table 1). Some, but not all, subjected the samples to pulverisation (31.7%). Extraction solvents also varied, though they were mainly of high polarity, with methanol (21.8%), 70% aqueous ethanol (13.0%), ethanol (10.1%) and water (10.1%) being the more commonly used solvents. As for the extraction process itself, maceration was found to be most widely used (34.8%), followed by sonication (26.1%), agitation (23.1%) and reflux extraction (1.5%).
Table 1. Summary of extraction parameters used to determine the antioxidant activity of bee pollen.

| Author                          | Reference     | Country       | Botanical Origin          | Pre-Extraction | Pulverisation | Extraction Method | Solvent               | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays                      |
|---------------------------------|---------------|---------------|---------------------------|----------------|---------------|------------------|---------------------|--------------------|----------------------|---------------|----------------|------------------|----------------|----------------------------------------|
| Daoud, Ibrahim et al., 2019     | [22]          | Algeria       | *Inula viscosa*           | Stored at 4 °C | None          | Maceration       | 80% Methanol (Aqueous) | 10 g w/ 100 mL | N.I.                 | N.I.          | 24 h          |                  | Dried in vacuo | TPC, TFC, β-carotene bleaching         |
| Machado De-Melo, Estevinho et al., 2016 | [23]          | Brazil        | multifold                 | Stored at −20 °C, some batches were oven dried at 42 °C, another was vacuum lyophilised | Crushed and sieved through a 0.595 mm sieve | Agitation       | 70% Ethanol (Aqueous) | 2 g w/ 25 mL | 70 °C                 | 105 rpm       | 30 min        | N.I.            | Volume was adjusted to 25 mL         | TPC, DPPH, ORAC |
| Kalayçoğlu, Kaygusuz et al., 2017 | [7]            | Turkey        | Chestnuts, Buckwheat, Oak, multiforal | N.I. | Ground in mortar and pestle | Maceration | Water | 0.1 g w/ 5 mL | 80 °C | None                 | 15 min        | N.I.           | Filtered using Whatman #41 | TPC, DPPH |
| Alimoğlu, Guzelmeric et al., 2021 | [24]          | Turkey        | multiforal                | Stored at 20 °C | None          | Agitation and Sonication | 70% Ethanol (Aqueous) | 5 g w/ 50 mL | 40 °C                 | 100 rpm, N.I. | 1 h and 15 min | N.I.            | Filtered using filter paper, dried in vacuo | TPC, TFC, CUPRAC, FRAP |
| Pascoal, Rodrigues et al., 2014 | [25]          | Portugal      | multiforal                | Stored at 20 °C | None          | Maceration       | Methanol | 1.2 w/v | RT | None | 72 h | 2 | Filtered using Whatman #4, dried in vacuo | TPC, TFC, TBARS, DPPH |
| Morais, Moreira et al., 2011    | [26]          | Portugal      | multiforal                | Stored at −20 °C | None          | Maceration       | Methanol | 1.2 w/v | RT | None | 72 h | 2 | N.I. | TPC, DPPH, β-carotene bleaching | |
| Leja, Mareczek et al., 2006     | [27]          | Poland        | multiforal                | Stored at −18 °C | None          | Maceration       | 80% Methanol (Aqueous) | N.I. | N.I.                 | N.I.          | N.I.          | N.I.          | TPC, TFC, anthocyanidins, phenylpropanoids, DPPH, TAA |
Table 1. Cont.

| Author, Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------|---------|-----------------|----------------|---------------|------------------|---------|------------------|------------------------|--------------|----------------|------------------|----------------|-------------------|
| Mărghitaș, Stanciu et al., 2009 [28] | Romania | multifloral | N.I. | None | Maceration and Sonication | Methanol | 2 g w/ 15 mL | RT | None | 1 h and 15 min | 3 | Dried in vacuo | TPC, TFC, DPHH, FRAP, ABTS |
| Thakur and Nanda 2021 [29] | India | Coconut, Coriander, Rapeseed, and multifloral | Stored at −18 °C | Ground (method not indicated) | Maceration and Sonication | 85% Methanol (Aqueous) | 0.15:1 | RT | None | 2 h and 30 min | N.I. | Centrifuged, and Dried in vacuo | TPC, TFC, DPHH, FRAP, ABTS, MCA |
| Machado De-Melo, Estevinho et al., 2018 [30] | Brazil | Mimosa caesalpinifolia, Eucalyptus spp., Rubiaceae, Astrocaryum aculeatismum, Fabaceae, Cocos nucifera, M. verrucosa, Myrcia spp., Alternanthera spp., Asteraceae, Brassica spp., and multifloral | Stored at −4 °C | None | Maceration | Methanol | 1.2 w/v | RT | None | 72 h | 2 | N.I. | TPC, TFC, DPHH, ORAC |
| Machado De-Melo, Estevinho et al., 2018 [30] | Brazil | Mimosa caesalpinifolia, Eucalyptus spp., Rubiaceae, Astrocaryum aculeatismum, Fabaceae, Cocos nucifera, M. verrucosa, Myrcia spp., Alternanthera spp., Asteraceae, Brassica spp., and multifloral | Stored at −4 °C | None | Agitation | 70% Ethanol (Aqueous) | 2 g/25 mL | 70 °C | 105 rpm | 30 min | N.I. | N.I. | TPC, TFC, DPHH, ORAC |
### Table 1. Cont.

| Author                        | Reference                | Country          | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent          | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays   |
|-------------------------------|--------------------------|------------------|------------------|----------------|---------------|------------------|------------------|-------------------|-----------------------|--------------|-----------------|-------------------|------------------|---------------------|
| Kaškonienė, Adaškevičiūtė et al., 2020 | [31]                      | Latvia and Lithuania | multifloral    | Pasteurisation at 95 °C for 20 min and stored at 6–8 °C | None       | Agitation       | 80% Methanol (Aqueous) | 2 g/20 mL       | RT                | 180 rpm            | N.I.           | N.I.            | 7–10 µm paper filter (Labbox), followed by a 0.22 µm polyvinylidene fluoride (PVDF) membrane filter, stored at 4 °C | TPC, TFC, DPPH |
| Wan Omar, Azhar et al., 2016   | [32]                      | Malaysia         | L. terminate    | N.I.            | None          | Sonication      | Methanol         | 10 g/25 mL        | 41 °C                 | N.I.           | 1 h             | N.I.              | Centrifuged, filtered using 0.2 mm filter | DPPH |
| Khongkarat, Ramadhan et al., 2020 | [33]                      | Thailand         | Sunflower (Helianthus annuus L.) | Dried at 40 °C and stored at 25 °C | None       | Agitation       | Methanol         | 140 g/800 mL      | 15 °C                 | 100 rpm        | 18 h            | N.I.             | Centrifuged and dried in vacuo | DPPH |
| Zhang, Wang et al., 2015       | [34]                      | China            | Rape (Brassica campestris L.) | Vacuum dried at 50 °C and stored at −18 °C | Ground (method not indicated) | Maceration       | Water            | 1:20, w/v         | RT                    | None           | 24 h            | N.I.              | Centrifuged, reconstituted to 100 mL | TPC, TFC, ABTS, DPPH, Reducing power |
| Zhang, Wang et al., 2015       | [34]                      | China            | Rape (Brassica campestris L.) | Ground (method not indicated) | Maceration       | 25% Ethanol (Aqueous) | 1:20, w/v         | RT                | None                  | 24 h           | N.I.            | Centrifuged, reconstituted to 100 mL | TPC, TFC, ABTS, DPPH, Reducing power |
| Zhang, Wang et al., 2015       | [34]                      | China            | Rape (Brassica campestris L.) | Vacuum dried at 50 °C and stored at −18 °C | Ground (method not indicated) | Maceration       | 50% Ethanol (Aqueous) | 1:20, w/v         | RT                    | None           | 24 h            | N.I.              | Centrifuged, reconstituted to 100 mL | TPC, TFC, ABTS, DPPH, Reducing power |
Table 1. Cont.

| Author                        | Reference         | Country         | Botanical Origin       | Pre-Extraction Conditions | Pulverisation Method | Extraction Method | Solvent          | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays                  |
|-------------------------------|-------------------|-----------------|------------------------|---------------------------|----------------------|-------------------|------------------|-------------------|-----------------------|--------------|----------------|------------------|-----------------|-----------------------------------|
| Zhang, Wang et al., 2015      | [34]              | China           | Rape (Brassica campestris L.) | Vacuum dried at 50 °C and stored at −18 °C | Ground (method not indicated) | Maceration | 75% Ethanol (Aqueous) | 1:20, w/v | RT | None | 24 h | N.I. | Centrifuged, reconstituted to 100 mL | TPC, TFC, ABTS, DPPH, Reducing power |
| Hemmami, Ben Seghir et al., 2020 | [35]              | Algeria         | multifloral            | Crushed in a commercial blender and homogenised. | Sonication | Methanol | 0.2 g w/2 mL | RT | N.I. | 30 min | N.I. | Centrifuged, filtered using Whatman #1, dried in vacuo, stored in 4 °C in brown bottle | TPC, TFC, TAC |
| Izuta, Narahara et al., 2009  | [36]              | Japan and Spain | Jara pringosa (Cistus ladanifer) and Jara blanca (Cistus albidus) [Spain] | N.I. | None | N.I. | 95% Ethanol (Aqueous) | N.I. | RT | N.I. | N.I. | N.I. | DPPH |
| Zuluaga-Domínguez, Serrato-Bermúdez et al., 2018 | [37]              | Colombia        | Hypochaeris radicata and Brassica sp. | Stored at 2 °C | None | Maceration | 96% Ethanol (Aqueous) | 1 g w/30 mL | N.I. | N.I. | 24 h | N.I. | Filtered using 3 hw filter paper and reconstituted to 100 mL | TPC, TFC, ABTS, FRAP |
| Keskin and Özkök 2020         | [38]              | Turkey          | multifloral            | Dried at different conditions | None | Agitation | Ethanol | 3 g/20 mL | N.I. | N.I. | 12 h | N.I. | Filtered and the final volume was completed to 30 mL | TPC |
Table 1. Cont.

| Author, Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------|---------|------------------|----------------|---------------|-------------------|---------|------------------|------------------------|--------------|----------------|------------------|-----------------|-------------------|
| Vasconcelos, Duarte et al., 2017 | Brazil | multifloral | N.I. | None | Agitation | 70% Ethanol (Aqueous) | 1 g/10 mL | 70 °C | 150 rpm | 30 min | 2 | Centrifuged | TPC, TFC, DPPH, FRAP |
| Suriyatem, Auras et al., 2017 | Thailand | Longan | Stored at 20 °C | None | Maceration | Methanol | 1.2 (w/v) | RT | Shaken by hand twice a day | 72 h | 2 | Filtered using Whatman #4 and dried in vacuo | TPC, DPPH, ABTS |
| Cosmulescu, Trandafir et al., 2015 | Romania | Walnut (Juglans regia L.) | N.I. | None | Sonication and maceration | Methanol | 1 g/10 mL | RT | N.I. | 60 min and 24 h | 1 | Filtered using 0.45 µm membrane filter | TPC, TFC, DPPH |
| Bridi, Atala et al., 2019 | Chile | Brassica rapa and Eschscholzia californica | Stored at −20 °C | None | Sonication | Ethanol | 1 g/10 mL | −20 °C | N.I. | 10 min | 3 | Centrifuged and filtered using Whatman No.1 and reconstituted to 50 mL | TPC, TFC, FRAP, ORAC |
| Lopes, Vasconcelos et al., 2019 | Brazil | multifloral | Stored at 4 °C | None | Maceration | 70% Ethanol (Aqueous) | 1:5 (m/v) | RT | None | 72 h (solvent renewal every 24 h) | 3 | Dried in vacuo | TPC, TFC, FRAP, DPPH, ABTS |
| Lopes, Vasconcelos et al., 2020 | Brazil | multifloral | Stored at 4 °C | None | Maceration | 70% Ethanol (Aqueous) | 1:5 (m/v) | RT | None | 72 h (solvent renewal every 24 h) | 3 | Dried in vacuo, lyophilised | TPC, TFC, FRAP, DPPH, ABTS |
| Futui and Thongwai 2020 | Thailand | multifloral | Stored at −20 °C | Powdered (method not indicated) | Maceration | Water | 1:10 | 45 °C | None | 3 h | 3 | Filtered, dried in vacuo, lyophilised | TPC, TFC, DPPH |
Table 1. Cont.

| Author                      | Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent          | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-----------------------------|-----------|---------|------------------|----------------|---------------|-------------------|------------------|-------------------|----------------------|--------------|----------------|-------------------|----------------|-------------------|
| Futui and Thongwai          | [45]      | Thailand| multifloral      | Stored at −20 °C | Powdered       | Maceration        | 95% Ethanol (Aqueous) | 1:10              | RT                 | None                  | 72 h (solvent renewal every 24 h) | 3             | Filtered, dried in vacuo, lyophilised | TPC, TFC, DPPH |
| Futui and Thongwai          | [45]      | Thailand| multifloral      | Stored at −20 °C | Powdered       | Sonication        | Water             | 1:10              | RT                  | N.I.                    | 30 min       | 2               | Filtered, dried in vacuo, lyophilised | TPC, TFC, DPPH |
| Nurdianah, Ahmad Firdaus    | [46]      | Malaysia| multifloral      | Stored at 4 °C   | None           | Maceration        | Ethanol           | 10 g/100 mL       | RT                  | N.I.                    | 24 h         | 1               | Filtered, dried in vacuo, lyophilised, DPPH |
| Fatrcova-Sramkova, Nozkova  | [47]      | Slovak | Poppy (Papaver somniferum L.), Rape (Brassica napus subsp. napus L.), Sunflower (Helianthus annuus L.). | Stored at −18 °C | Homogenised     | Maceration        | 90% Ethanol (Aqueous) | 5 g/50 mL         | 70 °C               | N.I.                    | 30 min       | N.I.             | DPPH, Reducing Power |
| Sun, Guo et al., 2017       | [48]      | China   | Rape             | Defatted with hexane | None           | Sonication        | 70% Methanol (Aqueous) | N.I.              | RT                 | N.I.                    | 60 min       | N.I.             | TPC, TFC, DPPH, FRAP, ABTS |
| Borycka, Grabek-Lejko et al.| [10]      | Poland  | multifloral      | N.I.             | Ground using mortar and pestle | Agitation        | Water             | 2 g/15 mL         | 70 °C               | N.I.                    | 30 min       | Filtered using Whatman #1 and dried in vacuo | TPC, TFC, DPPH, FRAP, ABTS |
| Borycka, Grabek-Lejko et al.| [10]      | Poland  | multifloral      | N.I.             | Ground using mortar and pestle | Agitation        | 70% Ethanol (Aqueous) | 2 g/15 mL         | 70 °C               | N.I.                    | 30 min       | Filtered using Whatman #1 and dried in vacuo | TPC, TFC, DPPH, FRAP, ABTS |
Table 1. Cont.

| Author                  | Reference          | Country | Botanical Origin | Pre-Extraction | Pulverisation       | Extraction Method | Solvent                  | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------------|--------------------|---------|------------------|----------------|---------------------|--------------------|-------------------------|------------------|-----------------------|--------------|-----------------|-------------------|-------------------|-------------------|
| Borycka, Grabek-Lejko et al., 2016 | [10]               | Poland  | multifloral      | N.I.           | Ground using mortar and pestle | Agitation          | 70% Methanol (Aqueous) | 2 g/15 mL         | 70 °C                 | N.I.          | 30 min          | 1                 | Filtered, using Whatman #1 and dried in vacuo | TPC, TFC, DPPH, FRAP, ABTS |
| Su, Yang et al., 2020   | [49]               | China   | Camellia, Rape, Rose, and Lotus | N.I.         | Mechanical pulverisation | Sonication         | Methanol               | 1:10             | 30 °C                 | 100 W        | 1 h             | 1                 | Filtered, dried in vacuo | TPC, DPPH, RP, ABTS   |
| Uçar, Barlak et al., 2017 | [50]               | Turkey  | multifloral      | N.I.           | Ground (method not indicated) | Agitation          | DMSO                    | 5 g w/20 mL        | 60 °C                 | 150 rpm      | 24 h            | 1                 | Centrifuged      | TPC, TFC, FRAP, ABTS |
| Uçar, Barlak et al., 2017 | [50]               | Turkey  | multifloral      | N.I.           | Ground (method not indicated) | Agitation          | Water                   | 5 g w/20 mL        | 60 °C                 | 150 rpm      | 24 h            | 1                 | Centrifuged      | TPC, TFC, FRAP, ABTS |
| Oroian, Ursachi et al., 2020 | [51]               | Romania | multifloral      | Stored at −20 °C | None               | Sonication         | 80% Methanol (Aqueous) | 1:10–30          | 35 °C, 50 °C, 65 °C | 100 W       | 10–30 min       | 1                 | N.I.             | TPC, TFC          |
| Barbieri, Gabriele et al., 2020 | [52]               | Italy   | multifloral      | Stored at −20 °C | Powdered with mortar and pestle | Agitation          | 95% Ethanol (Aqueous) | 50 mg/mL          | RT                    | N.I.         | 1 h             | N.I.              | N.I.             | TPC, TFC, FRAP |
| Shen, Geng et al., 2019  | [53]               | China   | Schisandra chinensis | Dried at 37 °C | Pulverised (method not indicated) | refluxed           | 70% Ethanol (Aqueous) | 1:15             | Boling Point        | None         | 2 h             | N.I.              | Centrifuged, dried in vacuo, freeze dried | ABTS, FRAP |
| Saral, Yildiz et al., 2016 | [54]               | Turkey  | Castanea sativa L. | N.I.       | None                | Maceration and sonication | Methanol       | 5 g/100 mL         | RT            | N.I.         | 24 h, 3 h        | N.I.              | Filtered, dried in vacuo | TPC, FRAP, DPPH |
| Pérez-Pérez, Vit et al., 2012 | [55]               | Venezuela | multifloral      | N.I.           | Ground in a mortar, frozen | homogenised        | Water                   | 0.1 g/5 mL         | 4 °C                  | N.I.         | N.I.           | N.I.              | Centrifuged      | TPC, ABTS  |
| Author                  | Reference | Country            | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent            | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|------------------------|-----------|--------------------|------------------|----------------|--------------|------------------|-------------------|-------------------|------------------------|---------------|----------------|-------------------|-----------------|-------------------|
| Pérez-Pérez, Vit et al., 2012 | [55]      | Venezuela          | multifloral      | N.I.           | Ground in a mortar, frozen | homogenised      | Methanol          | 0.1 g/5 mL       | 4 °C                   | N.I.          | N.I.           | N.I.              | Centrifuged     | TPC, ABTS         |
| Pérez-Pérez, Vit et al., 2012 | [55]      | Venezuela          | multifloral      | N.I.           | Ground in a mortar, frozen | homogenised      | 95% Ethanol (Aqueous) | 0.1 g/5 mL       | 4 °C                   | N.I.          | N.I.           | N.I.              | Centrifuged     | TPC, ABTS         |
| Daudu 2019             | [56]      | Nigeria            | multifloral      | Oven dried at 40 °C | Ground (method not indicated) | Maceration      | 50% Ethanol (Aqueous) | 0.5 g/5 mL       | N.I.                   | N.I.          | N.I.           | N.I.              | Filtered        | TPC, TFC, NO2, DPPH, TAC, RP, Metal Chelating |
| Daudu 2019             | [56]      | Nigeria            | multifloral      | Oven dried at 40 °C | Ground (method not indicated) | Maceration      | Methanol          | 0.5 g/5 mL       | N.I.                   | N.I.          | N.I.           | N.I.              | Filtered        | TPC, TFC, NO2, DPPH, TAC, RP, Metal Chelating |
| Daudu 2019             | [56]      | Nigeria            | multifloral      | Oven dried at 40 °C | Ground (method not indicated) | Maceration      | Water             | 0.5 g/5 mL       | N.I.                   | N.I.          | N.I.           | N.I.              | Filtered        | TPC, TFC, NO2, DPPH, TAC, RP, Metal Chelating |
| Daudu 2019             | [56]      | Nigeria            | multifloral      | Oven dried at 40 °C | Ground (method not indicated) | Maceration      | Ethanol           | 0.5 g/5 mL       | N.I.                   | N.I.          | N.I.           | N.I.              | Filtered        | TPC, TFC, NO2, DPPH, TAC, RP, Metal Chelating |
| Stanciu, Dezmirian et al., 2016 | [57]      | Romania            | multifloral      | Stored at −18 °C | Ground (method not indicated) | N.I.            | Hexane:dichloromethane 1:1 | N.I.           | N.I.                   | N.I.          | 1 h            | N.I.              | TPC, Total carotenoid, ORAC |
| Stanciu, Dezmirian et al., 2016 | [57]      | Romania            | multifloral      | Stored at −18 °C | Ground (method not indicated) | N.I.            | acetone: water:acetic acid 70:29:5 | N.I.           | N.I.                   | N.I.          | 1 h            | N.I.              | TPC, Total carotenoid, ORAC |
Table 1. Cont.

| Author                      | Reference       | Country      | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method          | Solvent                  | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays                        |
|-----------------------------|-----------------|--------------|------------------|----------------|---------------|-----------------------------|--------------------------|--------------------|----------------------|--------------|----------------|---------------------|-----------------|---------------------------------------|
| Mayda, Özkök et al., 2020   | [58]            | Turkey       | multifloral      | Stored at −18 ºC | None          | Agitation and sonication    | 95% Ethanol (Aqueous)   | 1.5 g / 10 mL       | 40 ºC                | Vortex Mixer | 60 min         | N.I.                 | Filtered through 0.45 µm filter | TPC, TFC, DPPH, ABTS |
| Anjos, Fernandes et al., 2019 | [59]           | Portugal     | multifloral      | Stored at −18 ºC | None          | Agitation                  | 80% Ethanol (Aqueous)   | 11 g / 200 mL       | RT                   | 4 × g        | 24 h           | N.I.                 | Centrifuged, dried in vacuo, freeze dried | TPC, TFC, DPPH, RP |
| Almeida, Reis et al., 2017   | [60]            | Brazil       | multifloral      | N.I.            | None          | Agitation                  | 80% Ethanol (Aqueous)   | 10 g / 100 mL       | 40 ºC                | 150 rpm      | 60 min         | N.I.                 | Filtered, dried in vacuo, freeze dried | TPC, TFC, ABTS, DPPH, FRAP, Coupled oxidation of b-carotene and linoleic acid assay |
| Fatcova-Sramkova, Nozkova et al., 2016 | [61]        | Slovakia     | Helianthus annuus | Stored at 35 ºC | Homogenised (method not indicated) | Maceration     | 90% Ethanol (Aqueous)       | 5 g / 50 mL          | 70 ºC                | N.I.         | 30 min         | N.I.                 | Stored at 5 ºC | TPC, Carotenoids, RP, Flavonoids          |
| Mosic, Trifkovic et al., 2019 | [62]            | Serbia       | multifloral      | N.I.            | None          | Sonication                 | 70% Methanol (Aqueous)  | 1 g / 10 mL         | N.I.                  | N.I.         | 1 h            | N.I.                 | N.I.            | TPC                                       |
| El Ghouizi, Meniy et al., 2020 | [63]           | Morocco      | multifloral      | N.I.            | None          | Agitation                  | 70% Ethanol (Aqueous)   | 2 g / 15 mL         | 70 ºC                | N.I.         | 30 min         | N.I.                 | Filtered using Whatman #5 | TPC, TFC, TAC, RP |
| Rebiai and Lane 2012         | [64]            | Algeria      | Carrot, Rosemary, Eucalyptus, and multifloral | Stored at 4 ºC | Homogenised in blender | Soxhlet       | Methanol                   | 5 g w/ 175 mL       | 70 C               | None          | 2 h            | 1                   | Dried in vacuo | TPC, TFC, TAC |
Table 1. Cont.

| Author, Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|------------------|---------|------------------|----------------|---------------|------------------|---------|------------------|------------------------|--------------|----------------|-------------------|----------------|-------------------|
| Araujo, Chambo et al., 2017 | Brazil | Cocos nucifera, Miconia spp., Spondias spp., Myrcia spp., Eucalyptus spp. | N.I. | None | Agitation | Methanol | 1:1 | N.I. | N.I. | 24 h | 3 | Dried in vacuo | TPC, TFC, DPPH, A BTS |
| Barbara, Machado et al., 2015 | Brazil | multifloral | N.I. | None | Agitation | Methanol | 1:1 | N.I. | N.I. | 24 h | 3 | Dried in vacuo | TPC, TFC |
| Carpes, Mourao et al., 2009 | Brazil | multifloral | Stored at −12 to −15 °C | Crushed using commercial blender | Maceration | 70% Ethanol (Aqueous) | 2 g/15 mL | 70 °C | None | 30 min | N.I. | Filtered and stored | TPC, TFC, DPPH |
| Sartini, Djide et al., 2019 | Indonesia | multifloral | N.I. | Coarse powder (method not indicated) | Maceration | 80% Ethanol (Aqueous) | 100 g/1 L | RT | None | 120 h | N.I. | Dried in vacuo, freeze dried | TPC, TFC, DPPH |
| Le Blanc, Davis et al., 2009 | USA | Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod | N.I. | None | Sonication | Water | 50 mg/mL | 41 °C | None | 90 min | N.I. | N.I. | TPC, Total flavones and flavonol, total flavonones, DPPH, FRAP |
| Le Blanc, Davis et al., 2009 | USA | Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod | N.I. | None | Sonication | Methanol | 50 mg/mL | 41 °C | None | 90 min | N.I. | N.I. | TPC, Total flavones and flavonol, total flavonones, DPPH, FRAP |
| Author                  | Reference | Country | Botanical Origin                                                                 | Pre-Extraction | Pulverisation | Extraction Method | Solvent         | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays                          |
|------------------------|-----------|---------|----------------------------------------------------------------------------------|----------------|---------------|-------------------|----------------|------------------|----------------------|-------------|----------------|-------------------|---------------|---------------------------------------------|
| Le Blanc, Davis et al., 2009 | [1] USA   | USA     | Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod                      | N.I.           | None          | Sonication       | Ethanol         | 50 mg/mL         | 41 °C                | None         | 90 min         | N.I.              | N.I.          | TPC, Total flavones and flavonol, total flavonones, DPPH, FRAP |
| Le Blanc, Davis et al., 2009 | [1] USA   | USA     | Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod                      | N.I.           | None          | Sonication       | Propanol        | 50 mg/mL         | 41 °C                | None         | 90 min         | N.I.              | N.I.          | TPC, Total flavones and flavonol, total flavonones, DPPH, FRAP |
| Le Blanc, Davis et al., 2009 | [1] USA   | USA     | Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod                      | N.I.           | None          | Sonication       | 2-propanol      | 50 mg/mL         | 41 °C                | None         | 90 min         | N.I.              | N.I.          | TPC, Total flavones and flavonol, total flavonones, DPPH, FRAP |
| Le Blanc, Davis et al., 2009 | [1] USA   | USA     | Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod                      | N.I.           | None          | Sonication       | Acetone         | 50 mg/mL         | 41 °C                | None         | 90 min         | N.I.              | N.I.          | TPC, Total flavones and flavonol, total flavonones, DPPH, FRAP |
| Le Blanc, Davis et al., 2009 | [1] USA   | USA     | Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod                      | N.I.           | None          | Sonication       | DMF             | 50 mg/mL         | 41 °C                | None         | 90 min         | N.I.              | N.I.          | TPC, Total flavones and flavonol, total flavonones, DPPH, FRAP |
| Author  | Reference  | Country | Botanical Origin                               | Pre-Extraction | Pulverisation | Extraction Method | Solvent     | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays                  |
|--------|------------|---------|------------------------------------------------|---------------|---------------|------------------|-------------|------------------|-----------------------|--------------|----------------|------------------|----------------|-------------------------------|
| Le Blanc, Davis et al., 2009 | [1]        | USA     | Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod | N.I.          | None          | Sonication      | ACN         | 50 mg/mL         | 41 °C                  | None         | 90 min         | N.I.             | N.I.           | TPC, Total flavones and flavonol, total flavonones, DPPH, FRAP |
| Ceksteryte, Kurtinaitiene et al., 2016 | [12]       | Lithuania | multifloral                                    | Stored at 5–8 °C | None          | Agitation       | 80% Methanol (Aqueous) | 6 g/10 mL       | N.I.           | None                | 5 min          | N.I.             | Centrifuged, dried in vacuo, freeze dried | TPC, DPPH, ABTS, ORAC |
| Özcan, Aljuhaimi et al., 2019 | [69]       | Brazil   | multifloral                                    | Stored at −18 °C | None          | Sonication      | Methanol     | 0.5 g/12 mL       | N.I.                  | None         | 10 min          | N.I.             | Centrifuged, dried in vacuo | TPC, DPPH, Carotenoid, Minerals |
| Zuluaga-Dominguez and Quicazan 2019 | [70]       | Colombia | Hypochaeris spp., and Brassica spp.            | N.I.          | None          | Agitation       | 96% Ethanol (Aqueous) | 1 g/30 mL       | N.I.           | N.I.                | 24 h           | N.I.             | Filtered, volume completed quantitatively to 100 mL | TPC, ABTS, FRAP |
| Duran A 2019 | [71]       | Colombia | multifloral                                    | N.I.          | None          | Agitation       | 96% Ethanol (Aqueous) | 1 g/30 mL       | N.I.           | N.I.                | 24 h           | N.I.             | Filtered, volume completed quantitatively to 100 mL | TPC, TEAC, FRAP |
| Aleksieva, Mladenova et al., 2021 | [72]       | Bulgaria | multifloral                                    | Lyophilised   | None          | Agitation       | Ethanol      | 0.5 g/7.5 mL       | RT                    | N.I.         | 2 h              | N.I.             | filtered       | TPC, TFC, DPPH, TEAC |
| Aleksieva, Mladenova et al., 2021 | [72]       | Bulgaria | multifloral                                    | Lyophilised   | None          | Agitation       | Water        | 0.5 g/7.5 mL       | RT                    | N.I.         | 2 h              | N.I.             | filtered       | TPC, TFC, DPPH, TEAC |
Table 1. Cont.

| Author, Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------|---------|------------------|----------------|--------------|------------------|---------|------------------|------------------------|--------------|----------------|------------------|----------------|-------------------|
| Atsalakis, Chinou et al., 2017 [73] | Greece | Cistus creticus | Stored at −20 °C | None | Maceration | Cyclohexane, Dichloromethane, Butanol and Water | 27.5 g/150 mL | N.I. | None | N.I. | N.I. | N.I. |
| Saral, KiliciArslan et al., 2019 [74] | Turkey | multifloral | Stored at 4 °C | Blended | Maceration | Methanol | N.I. | RT | None | 24 h | N.I. |
| Al-Salem, Al-Yousef et al., 2020 [75] | Saudi Arabia | multifloral | N.I. | None | Maceration | 95% Ethanol (Aqueous) | N.I. | RT | None | 48 h | N.I. |
| Gabriele, Parri et al., 2015 [76] | Italy | multifloral | Stored at −20 °C | None | Agitation | 95% Ethanol (Aqueous) | N.I. | RT | N.I. | 1 h | N.I. |
| Yildiz, Can et al., 2013 [77] | Turkey | multifloral | Dried in 40 °C | Powder (method not indicated) | Sonication | Methanol | 1 g/10 mL | N.I. | None | 3 h | N.I. |
| Rocchetti, Castiglioni et al., 2019 [78] | Italy | multifloral | Stored in the dark at room temperature | Ground (method not indicated) | Agitation | 70% Methanol (Aqueous) | 0.5 g/5 mL | N.I. | Shaking | 5 min | N.I. | Stored at −20 °C |

TPC, TFC, DPPH, ABTS
Catalase (CAT) assay, Vitamin C (ascorbic acid) assay, Glutathione (GSH) assay, Glutathione S-Transferase (GST) activity
TPC, TFC, Total Anthocyanins, Total Carotenoids, FRAP, DPPH
Table 1. Cont.

| Author                  | Reference | Country   | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent          | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction              | Antioxidant Assays          |
|-------------------------|-----------|-----------|------------------|----------------|---------------|-------------------|------------------|-------------------|-----------------------|---------------|-----------------|---------------------|-----------------------------|-----------------------------|
| Yan, Li et al., 2019    | [79]      | China     | Brassica campestris L. | N.I.           | Superfine jet pulverisation Combined low-pressure jet-boiling device | Sonication       | 80% Acetone (Aqueous) | 2 g/15 mL        | N.I.                  | None                  | 30 min            | N.I.                  | Freeze dried and stored at −40 °C | TPC                         |
| Rebiai and Lanez 2013   | [80]      | Algeria   | multifloral      | N.I.           | None          | Maceration        | Methanol         | 5 g/50 mL         | RT                    | None                  | 24 h              | 3                  | Filtered, refrigerated   | TPC, TFC, cyclic voltammetry techniques |
| Cheng, Chen et al., 2019| [81]      | China     | multifloral      | N.I.           | None          | Reflux            | 75% Ethanol (Aqueous) | 1:10              | 75 °C                | None                  | 2 h               | 2                | Centrifuged, dried in vacuo, | DPPH, FRAP                   |
| Muñoz, Velasquez et al., 2020| [82]   | Chile     | Brassica campestris and Galega officinalis | Stored at −18 °C | None | Sonication       | Methanol         | 1 g/7.5 mL        | N.I.                  | 50 Hz                 | 30 min         | 1                | Centrifuged, filtered at 0.45 um, refrigerated | TPC, TFC, FRAP                |
| Yesiltas, Capanoglu et al., 2015| [83]  | Turkey and Spain | multifloral | Stored at −18 °C | Ground (method not indicated) | Maceration and sonication | Methanol         | 2 g/15 mL        | RT                    | N.I.                  | 3 d, 15 min    | 1                | Centrifuged               | TPC, TFC, ABTS, FRAP, DPPH, CUPRAC |
| Mejias and Montenegro 2012| [84]   | Chile     | multifloral      | N.I.           | None          | Suspension        | Water            | N.I.              | N.I.                  | None                  | N.I.            | N.I.              | N.I.                 | TPC, DPPH                   |
| Negri, Teixeira et al., 2011| [85]  | Brazil    | multifloral      | Stored at −18 °C | None          | Maceration        | 70% Methanol (Aqueous) | 1.0 g/75 mL      | RT                    | N.I.                  | 45 min          | N.I.              | Filtered, volume completed quantitatively to 100 mL | TPC, DPPH               |
| Author, Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------|---------|------------------|----------------|---------------|------------------|---------|-----------------|------------------------|---------------|-----------------|------------------|----------------|------------------|
| Campos, Webby et al., 2003 | New Zealand, Portugal | *Salix atrocinera* Brot., *Ranunculus sardous* Crantz, and *Ulex europaeus* L. (Portugal and New Zealand); *Eucalyptus globulus* Labill., *Cistus ladanifer* L., *Echium plantagineum* L., and *Erica australis* L. (Portugal); and *Metrosideros umbellata*, *Ixerba brxoides*, and *Knightia excelsa* (New Zealand) | N.I. | None | Sonication | 50% Ethanol (Aqueous) | N.I. | N.I. | None | N.I. | Centrifuged | DPPH |
| Ulusoy and Kolayli 2014 | Turkey | multifloral | Stored at 4 °C | None | Sonication | Methanol | N.I. | RT | None | 1 h | 3 | Filtered, dried in vacuo | TPC, FRAP, CUPRAC, DPPH |
| Mohdaly, Mahmoud et al., 2015 | Egypt | Maize (*Zea mays*) | Stored at 4 °C | Powdered (method not indicated) | Maceration | Methanol | 10.0 g/100 mL | RT | N.I. | 12 h | 1 | Filtered (Whatman #1), dried in vacuo | DPPH, ABTS |
### Table 1. Cont.

| Author                  | Reference     | Country   | Botanical Origin                                                                 | Pre-Extraction | Pulverisation                   | Extraction Method | Solvent          | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------------|---------------|-----------|---------------------------------------------------------------------------------|----------------|---------------------------------|-------------------|-------------------|--------------------|----------------------|---------------|-----------------|--------------------|------------------|--------------------|
| De-Melo, Estevinho et al., 2018 | [89]          | Brazil    | *Alternanthera, Anadenanthera, Cocos nucifera, Mimosa caesalpiniaefolia, Myrcia, and Mimosa scabrella* | Stored at 4 °C | Crushed using commercial blender | Maceration         | 70% Ethanol (Aqueous) | 2 g/15 mL          | 70 °C                | None          | 30 min          | 1                  | Filtered          | TPC, TFC, DPPH     |
| De-Melo, Estevinho et al., 2018 | [89]          | Brazil    | *Alternanthera, Anadenanthera, Cocos nucifera, Mimosa caesalpiniaefolia, Myrcia, and Mimosa scabrella* | Stored at 4 °C | Crushed using commercial blender | Maceration         | Methanol          | 2 g/15 mL          | 70 °C                | None          | 30 min          | 1                  | Filtered          | TPC, TFC, DPPH     |
| Sahin and Karkar 2019    | [90]          | Turkey    | Chestnut                                                                        | N.I.           | None                            | Sonication         | Ethanol           | 3 g/30 mL          | 65 °C                | None          | 30 h            | 1                  | Filtered          | TPC, FRAP, ABTS, CHROMAC |
| Belina-Aldemita, Schreiner et al., 2020 | [91]          | Philippines | multifloral                                                                     | Under argon at −24 °C, defatted with hexane 3 times | Ground (method not indicated) | Macerated          | Methanol          | 1 g/5 mL           | RT                   | N.I.          | 1 h             | 3                  | Centrifuged, stored at −20 °C | TPC, TFC, and TMAC |
| Belina-Aldemita, Schreiner et al., 2020 | [91]          | Philippines | multifloral                                                                     | Under argon at −24 °C, defatted with hexane 3 times | Ground (method not indicated) | Sonication         | Methanol          | 1 g/5 mL           | RT                   | N.I.          | 1 h             | 3                  | Centrifuged, stored at −20 °C | TPC, TFC, and TMAC |
| Belina-Aldemita, Schreiner et al., 2020 | [91]          | Philippines | multifloral                                                                     | Under argon at −24 °C, defatted with hexane 3 times | Ground (method not indicated) | Maceration          | acidified Methanol solution (methanol and 1 N hydrochloric acid 85:15 v/v). | 1 g/5 mL           | 50 °C                | N.I.          | 30 min          | 3                  | Centrifuged, stored at −20 °C | TPC, TFC, and TMAC |
| Author                        | Reference   | Country    | Botanical Origin                | Pre-Extraction | Pulverisation | Extraction Method | Solvent               | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays                       |
|------------------------------|-------------|------------|---------------------------------|----------------|---------------|-------------------|-----------------------|--------------------|-----------------------|---------------|----------------|------------------|----------------|----------------------------------------|
| Zuluaga-Domínguez, Castro-Mercado et al., 2019 | [92]        | Colombia   | *Hypochaeris* spp., and *Brassica* spp. | Stored at 4 °C | None          | Agitation         | 96% Ethanol (Aqueous) | 1 g/30 mL | N.I.                  | Low speed     | 24 h            | 1                | Filtered using 3 hw filter paper and volume completed quantitatively to 100 mL | TPC, TFC, Total Carotenoids, FRAP, ABTS |
| Bujang, Zakaria et al., 2021 | [93]        | Malaysia   | Maize (*Zea mays* L)             | Stored at −20 °C | Crushed       | Sonication        | 70% Ethanol (Aqueous) | 2 g/15 mL | RT                    | None          | 30 min          | 1                | Centrifuged, filtered using Whatman #2 | TPC, TFC, DPPH |
| Yang, Zhang et al., 2019     | [16]        | China      | Rose                             | N.I.            | Ball milled    | Sonication        | 70% Ethanol (Aqueous) | 50 mg/1 mL | 25 °C                | 600 W and a frequency of 20 kHz | 4 h            | 1                | Filtered          | TFC, DPPH, ORAC, ABTS, In Vivo antioxidant |
| Kaskoniene, Kaskonas et al., 2015 | [94]        | Lithuania  | multifloral                      | N.I.            | None          | Maceration         | 85% Methanol (Aqueous) | N.I.         | RT                    | N.I.          | 24 h            | 3                | Filtered          | TPC, TFC, DPPH |
| Kaskoniene, Katli-Kvičiūtė et al., 2018 | [95]        | Lithuania  | multifloral                      | N.I.            | None          | Maceration         | 85% Methanol (Aqueous) | N.I.         | RT                    | N.I.          | 24 h            | 3                | Filtered          | TPC, TFC, DPPH |
| Khider, Elbanna et al., 2013  | [96]        | Egypt      | Maize (*Zea mays*), clover (*Trifolium alexandrinum*), and Date palm (*Phoenix dactylifera*) | Stored at 4 °C | Powdered (method not indicated) | Maceration | Methanol           | 50 g/500 mL | RT                    | None          | 12 h            | 1                | Filtered using Whatman paper # 5         | DPPH |

*TPC, TFC, DPPH, ORAC, ABTS, In Vivo antioxidant*
| Author, Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------|---------|-----------------|----------------|--------------|------------------|---------|------------------|------------------------|--------------|-----------------|------------------|----------------|------------------|
| Khider, Elbanna et al., 2013 | Egypt | Maize (Zea mays), clover (Trifolium alexandrinum), and date palm (Phoenix dactylifera) | Stored at 4 °C | Powdered (method not indicated) | Maceration | Hexane | 50 g/500 mL | RT | None | 12 h | 1 | Filtered using Whatman paper # 5 | DPPH |
| Canale, Benelli et al., 2016 | Italy | multifold | Stored at −20 °C | None | Sonication and agitation | 80% Methanol (Aqueous) | 0.5 g/15 mL | N.I. and 4 °C | None and N.I. | 30 min and 30 min | 2 | Filtered through 0.45 µm filter | TPC, TFC, Rutin |
| Freire, Lins et al., 2012 | Brazil. | multifold | Dried at 40 °C and then stored in a freezer until use | None | Maceration | 80% Methanol (Aqueous) | N.I. | N.I. | N.I. | N.I. | N.I. | Filtered, dried in vacuo | TPC, DPPH, ABTS, Fe Chelating |
| Kim, Jo et al., 2015 | Korea | multifold | None | Sonication | Ethanol | N.I. | N.I. | N.I. | N.I. | 2 | N.I. | DPPH, TPC |
| Zhang, Yang et al., 2016 | China | Rapeseed (Brassica campestris L.) | N.I. | Powdered (method not indicated) | Sonication | 80% Ethanol (Aqueous) | 50 mL/g | 80 °C | 40 kHz | 30 min | 1 | Filtered through 0.45 µm | TPC, FRAP |
| Zhang, Liu et al., 2020 | China | Rapeseed (Brassica campestris L.) | Extracted with petroleum ether to remove the lipids | Powdered (method not indicated) | Sonication | 80% Ethanol (Aqueous) | 50 mL/g | 80 °C | 40 kHz | 30 min | 1 | Dried in vacuo, lyophilised | DPPH, ABTS, FRAP |
| Castagna, Benelli et al., 2020 | Italy | Chestnut | N.I. | None | Sonication | 80% Methanol (Aqueous) | N.I. | 4 °C | N.I. | 30 min | 1 | Centrifuged and filtered through 0.45 µm filter | TPC, TFC |
| Author                        | Reference | Country     | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method       | Solvent       | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------------------|-----------|-------------|------------------|----------------|---------------|-------------------------|---------------|-------------------|------------------------|---------------|----------------|-------------------|----------------|-------------------|
| Oyarzun, Andia et al., 2020   | [103]     | Chile       | multifloral      | N.I.           | None          | Sonication              | Ethanol       | 1.0 g in 10 mL   | RT                     | 37 kHz frequency and 240 W | 10 min         | 3                | Centrifuged and filtered through Whatman # 1 |
| Asmae, Nawal et al., 2021     | [104]     | Morocco     | multifloral      | Stored at −20 °C | None          | Maceration              | 70% Ethanol (Aqueous) | 1.0 g in 10 mL | RT                     | N.I.         | 1 week         | 1                  | Filtered through Whatman # 1 | TPC, Flavones and Flavonols Content, TAC, DPPH, RP, ABTS |
| Feas, Vazquez-Tato et al., 2012 | [6]       | Portugal    | multifloral      | N.I.           | Ground (method not indicated) | Sonication and maceration | Methanol       | 1:2               | RT                     | N.I.         | 30 min and 2 days | 1                  | Centrifuged, dried in vacuo | TPC, TFC, DPPH, β-Carotene Bleaching |
| Rodriguez-Gonzalez, Ortega-Toro et al., 2018 | [105]     | Colombia    | multifloral      | N.I.           | None          | Microwave Assisted Extraction | Ethanol       | 1 g/10 or 50 mL | Varies                 | 1350 W of power and 60 Hz | 6, 12 and 24 s | 1                | Filtered and stored in −20 °C | TPC, ABTS, FRAP |
| Rodriguez-Gonzalez, Ortega-Toro et al., 2018 | [105]     | Colombia    | multifloral      | N.I.           | None          | Sonication              | Ethanol       | 1 g/10 mL        | N.I.                   | 5 kHz frequency and 250 W  | 15 min         | 1                | Filtered and stored in −20 °C | TPC, ABTS, FRAP |
Table 1. Cont.

| Author, Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------|---------|------------------|----------------|---------------|------------------|---------|------------------|-----------------------|--------------|----------------|------------------|-----------------|------------------|
| Velasquez, Rodriguez et al., 2017 | Chile | multifloral | N.I. | None | Sonication | Water | 1:1 | N.I. | N.I. | 1 h | 5 | Filtered using Whatman #2 dried in vacuo and the dry extract was reconstituted to 10 mL, filtered (EDLAB CA syringe filter 0.45 μm) and stored at −20 ºC. | Total Carotenoid, TPC, FRAP |
| Carpes, de Alencar et al., 2013 | Brazil | multifloral | N.I. | None | Maceration | 70% Ethanol (Aqueous) | 1:1 | 70 ºC | N.I. | 30 min | N.I. | N.I. | TPC, TFC, DPPH, Antioxidant activity by the coupled oxidation of b-carotene and linoleic acid |
| Santa Bárbara, Moreira et al., 2020 | Brazil | multifloral | Oven dried, freeze dried, fresh | None | Agitation | 70% Ethanol (Aqueous) | 10 g/50 mL | RT | N.I. | 45 min | 1 | Filtered, Dried in vacuo | b-Carotene bleaching assay, FRAP, DPPH, TPC, TFC |
| Carpes, Begnini et al., 2007 | Brazil | multifloral | N.I. | Milled | Agitation | 40% Ethanol (Aqueous) | 2.0 g/15 mL | 70 ºC | N.I. | 30 min | 2 | Stored at 5 ºC | Oxidation of b-carotene and linoleic acid, TPC |
Table 1. Cont.

| Author, Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------|---------|------------------|----------------|---------------|-------------------|---------|------------------|----------------------|--------------|------------------|-------------------|----------------|-------------------|
| Carpes, Begnini et al., 2007 | Brazil | multifloral | N.I. | Milled | Agitation | 50% Ethanol (Aqueous) | 2.0 g/15 mL | 70 °C | N.I. | 30 min | 2 | Stored at 5 ºC | Oxidation of -carotene and linoleic acid, TPC |
| Carpes, Begnini et al., 2007 | Brazil | multifloral | N.I. | Milled | Agitation | 60% Ethanol (Aqueous) | 2.0 g/15 mL | 70 °C | N.I. | 30 min | 2 | Stored at 5 ºC | Oxidation of -carotene and linoleic acid, TPC |
| Carpes, Begnini et al., 2007 | Brazil | multifloral | N.I. | Milled | Agitation | 70% Ethanol (Aqueous) | 2.0 g/15 mL | 70 °C | N.I. | 30 min | 2 | Stored at 5 ºC | Oxidation of -carotene and linoleic acid, TPC |
| Carpes, Begnini et al., 2007 | Brazil | multifloral | N.I. | Milled | Agitation | 80% Ethanol (Aqueous) | 2.0 g/15 mL | 70 °C | N.I. | 30 min | 2 | Stored at 5 ºC | Oxidation of -carotene and linoleic acid, TPC |
| Carpes, Begnini et al., 2007 | Brazil | multifloral | N.I. | Milled | Agitation | 90% Ethanol (Aqueous) | 2.0 g/15 mL | 70 °C | N.I. | 30 min | 2 | Stored at 5 ºC | Oxidation of -carotene and linoleic acid, TPC |
| Zou, Hu et al., 2020 | South Korea, China | multifloral | N.I. | None | Maceration | 70% Ethanol (Aqueous) | N.I. | RT | N.I. | 48 h | 3 | Filtered, dried in vacuo | TPC, TFC, DPPH |
| Paradowska, Zielińska et al., 2017 | Poland | Buckwheat, Oilseed Rape | N.I. | None | Sonication | 80% Methanol (Aqueous) | 0.2/10 mL | 25 °C | N.I. | N.I. | 1 | Filtered through a sintered glass filter funnel | TPC, TFC, DPPH, FRAP, ORAC |
| Yildiz, Karahalil et al., 2014 | Turkey | multifloral | N.I. | None | Suspension | Water | N.I. | N.I. | N.I. | N.I. | N.I. | Filtered | TPC, FRAP |
| Silva, Camara et al., 2009 | Brazil | multifloral | N.I. | None | Sonication | Ethanol | N.I. | N.I. | N.I. | N.I. | N.I. | Filtered, dried in vacuo | DPPH |
### Table 1. Cont.

| Author, Reference | Country | Botanical Origin | Pre-Extraction | Pulverisation | Extraction Method | Solvent | Volume of Solvent | Extraction Temperature | Mixing/Power | Extraction Time | No. of Extractions | Post-Extraction | Antioxidant Assays |
|-------------------|---------|------------------|----------------|---------------|------------------|---------|------------------|------------------------|--------------|----------------|------------------|----------------|------------------|
| Dai, Ding et al., 2013 [114] | China | Rape, Rose, Camellia, Herba leonuri and Schizandra | N.I. | None | Sonication | Ethanol | 50 mg/10 mL | N.I. | N.I. | N.I. | N.I. | Filtered, completed to 10 mL | TPC, DPPH |
| Amalia, Diantini et al., 2020 [115] | Indonesia | multifloral | N.I. | None | Suspension | Water | 1:10 | N.I. | N.I. | N.I. | N.I. | Filtered using Whatman® # 41 paper, freeze-dried | DPPH |

Legend: N.I.—not indicated, w/—with, w/v—weight over volume, TPC—total phenolic content, TFC—total flavonoid content, DPPH—2,2-diphenyl-1-picryl-hydrazyl-hydrate assay, ORAC—oxygen radical absorbance capacity, CUPRAC—Cupric reducing antioxidant capacity, FRAP—ferric reducing antioxidant capacity, TBARS—Thiobarbituric acid reactive substances, TAA—total antioxidant activity, ABTS—2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•⁺) radical cation-based assay, MCA—metal chelating activity, TAC—total antioxidant capacity, RP—reducing power, CHROMAC—chromium reducing antioxidant capacity.
It can be assumed that the adopted extraction process for a bee pollen sample, including the sample pre-treatment, extraction method and solvent, impacts the extraction efficiency of antioxidant constituents and consequently on the level of antioxidant activity measured. The objective of this study was to use the Design of Experiment approach to optimise an extraction process for bee pollen with a view to maximising the extraction of antioxidant constituents as measured by the DPPH, FRAP and TPC assays. The independent variables were sample pulverisation, extraction process (agitation, maceration, reflux or sonication) and extraction solvent (methanol, 70% aqueous ethanol, ethanol or water). Two different bee pollen samples from western Australia, Jarrah (Eucalyptus marginata) and Marri (Corymbia calophylla) bee pollen, were used as model bee pollen samples.

2. Materials and Methods

2.1. Chemicals, Reagents and Pollen Samples

The chemicals and reagents were sourced as follows: Folin and Ciocalteu’s phenol reagent 2N, (F9252-1L, Lot No. SHBH4781V), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ ≥ 98.0%, Lot No. BCBW0518, CAS No. 3682-35-7), iron (III) chloride hexahydrate (FeCl₃·6H₂O ACS Reagent 97.0%, Lot No. BCBZ5998, CAS No. 10025-77-1), iron (II) sulphate heptahydrate (FeSO₄·7H₂O ACS reagent, ≥99.0%, Lot No. MKCJ9113, CAS No. 7782-63-0), fructose (C₆H₁₂O₆ ≥ 99.0%, Lot No. SLBZ1343, CAS No. 57-48-7), and maltose (C₁₂H₂₂O₁₁·H₂O BioXtra, ≥99%, Lot No. SLCC4130, CAS No. 6363-53-7) from Sigma Aldrich Truganina, Victoria 3029 Australia.; sodium anhydrous carbonate (Na₂CO₃, LR, B.N. 334280, CAS No. 497-19-8), glucose (D-glucose anhydrous, C₆H₁₂O₆ 99.0%, Batch No. 324472, CAS No. 57-50-1), ethanol (CH₃CH₂OH, B.N. 19758725, 67-56-1) from Scharlau, Barcelona, Spain.

Jarrah (Eucalyptus marginata) and Marri (Corymbia calophylla) pollen, both harvested in October 2019, were purchased from Davies Apiaries in Oldbury, 6121, WA, Australia.

2.2. Extract Preparation

The bee pollen samples had already been dried, processed, and packed commercially, and no further treatment was undertaken prior to their analysis. To obtain pulverised samples (75–150 µm), the crude pollen grains were milled for 5 min using a commercial grinder (Breville Coffee Grinder Model BCG200). An amount of 0.5 g of pollen samples (crude, and non-pulverised) were separately extracted using ethanol, methanol, deionised water, and 70% ethanol in water, and the following extraction procedures:

Agitation. Agitation extraction was performed based on a protocol reported by Aleksieva et al. [72] with minor modifications. The extraction was carried out over 2 h in 7 mL of solvent using a hotplate magnetic stirrer (LLG Uni stirrer 3, John Morris Group) operating at a speed of 1500 rpm, with the temperature set at 40 °C. After 2 h, the solvent was decanted, and was replaced with fresh solvent and the extraction process was repeated two more times for the sample.

Maceration. Maceration extraction was performed based on a protocol reported by Kaškonienė et al. [95] with minor modifications. The bee pollen sample was macerated in 7 mL of extraction solvent at room temperature (25 °C) at a speed of 160 rpm shaking (Memmert Shaker Bath, Model WNB 22) over three days and solvent changes every 24 h.
Reflux. Reflux extraction was performed using an Electromantle reflux set-up and the protocol reported by Cheng et al. [81] with slight modifications. The method employed 21 mL of solvent and an extraction temperature determined by the boiling point of the respective solvent. Extraction was performed once over 2 h for each sample.

Sonication. Sonication extraction was performed following a procedure developed by Yan et al. [79] with slight modifications. The extraction was performed with 21 mL of solvent using a probe sonicator (Sonics Vibra Cell Model VCX130) operating at 130 Watts and 20 kHz. The amplitude was set at 100% and the extraction process was carried out once for 30 min on an ice bath.

Following extraction, the solvent was filtered (Whatman #4 filter paper) and, if the extraction process was repeated, filtrates were combined, and made up to 25 mL with the respective solvent. The resulting solutions were stored at \(-85^\circ\)C until further analysis. Every extraction process was carried out in triplicate for each pollen sample, and the responses combined to determine the mean. Table 2 summarises the independent variables studied along with their corresponding abbreviations.

### Table 2. Independent variables used in the Design of Experiments to optimise the extraction of bee pollen for antioxidant evaluation.

| Factors                  | Tested Conditions | Abbreviation |
|--------------------------|-------------------|--------------|
| Pulverisation            | Crude, non-pulverised | -            |
|                          | Pulverised         | +            |
| Solvent                  | 70% Ethanol        | E70:30       |
|                          | Ethanol            | EtOH         |
|                          | Methanol           | MtOH         |
|                          | Water              | H2O          |
| Extraction Process       | Agitation          | A            |
|                          | Maceration          | M            |
|                          | Reflux             | R            |
|                          | Sonication         | S            |

2.3. Determination of Total Phenolic Content

A sugar solution was used as a blank in order to account for the potential sugar matrix interference in the assay. The sugar solution was prepared by diluting 2 g of a sugar stock solution (21.625 g of fructose, 18.125 g of glucose, 1.000 g of maltose, 0.750 g of sucrose and 8.500 g of water) to 5 mL (40%) with deionised water. The solution was stored under refrigeration and used within a week. A dilute Folin–Ciocalteu reagent was prepared by mixing 1 mL of Folin–Ciocalteu reagent with 30 mL deionised water. A 0.75% anhydrous sodium carbonate solution was prepared by mixing 0.1875 g Na2CO3 in 25 mL water. A 2 mg/mL gallic acid stock solution was prepared by dissolving 200 mg of gallic acid in 100 mL deionised water and standards ranging in concentration from 0.18 mg/mL to 0.06 mg/mL were prepared by diluting the stock with water.

The TPC assay was performed based on the methodology described by Liberato et al. [116] with minor modifications. In brief, for the analysis, 200 µL of pollen extract or 100 µL of gallic acid standard spiked with 100 µL of sugar solution were placed in a test tube followed by the addition of 1 mL of the diluted Folin–Ciocalteu reagent. The mixture was allowed to react for 5 min before 800 µL of Na2CO3 was added. The mixture was kept in the dark for 2 h before the absorbance was measured at 760 nm (Carry 60 Bio UV–Vis spectrophotometer) using 100 µL of water spiked with 100 µL of sugar solution along with other TPC reagents as a blank. The analysis was carried out in triplicate and the mean results for each sample were obtained. The antioxidant activity was expressed as mg of gallic acid equivalent (GAE) per g of pollen.

\[
\text{TPC Value of Sample (mg)Gallic Acid} = \frac{(\Delta \text{Abs} - \text{intercept})}{\text{slope}} \times \text{Dilution Factor} \quad (1)
\]
2.4. Determination of Antioxidant Activity using Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP reagent was prepared by mixing in proportions of 1:1:10 (v/v/v) 10 mM TPTZ (0.31 g dissolved in 100 mL of 40 mM HCl), 20 mM FeCl$_3$·6H$_2$O (0.5406 g dissolved in 100 mL of deionised water) and 300 mM acetate buffer pH 3.6 (3.1 g C$_2$H$_3$NaO$_2$, 16.00 mL of glacial acetic acid dissolved in 1000 mL of deionised water). The reagent was freshly prepared and incubated at 37 °C prior to each test. For the standard curve, a 2 mM stock solution of FeSO$_4$·7H$_2$O was prepared by dissolving 55.6 mg of FeSO$_4$·7H$_2$O in 100 mL of deionised water. Standards ranging in concentration from 1200 µM to 200 µM were prepared prior to each experiment, stored on ice, and used within 2 h. The standard at 600 µM was used as a positive control in each experiment.

The FRAP assay, which is based on the reduction of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe(III)-TPTZ] to ferrous complex at low pH followed by a spectrophotometric analysis, was performed according to the protocol described by Almeida et al. [60] with minor modifications. In brief, 20 µL of pollen extract or standards were mixed with 180 µL of FRAP reagent in a 96-well microplate, incubated at 37 °C for 30 min and the absorbance of the reaction mixture was determined at 620 nm (BMG Labtech POLARstar Optima Microplate Reader). The mean of triplicate analysis results was calculated and the FRAP activity was determined on the interpolation of the standard curve and expressed as µmol Fe$^{2+}$ equivalent (Fe$^{2+}$E)/g FW of pollen.

\[
\text{FRAP Value of Sample (µM)Fe (II)} = \frac{(\Delta \text{Abs} - \text{intercept})}{\text{slope}} \times \text{Dilution Factor (2)}
\]

2.5. Determination of Antioxidant Activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

This colorimetric assay utilises 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, which are purple in colour; the colour decays in the presence of antioxidant agents, seen in a change in absorbance at 517 nm. The DPPH reaction mixture was made up of 0.130 mM DPPH reagent (5.1262 mg of DPPH in 100 mL of methanol), 100 mM NaC$_2$H$_3$O$_2$ buffer pH 5.5 (7.355 g of NaC$_2$H$_3$O$_2$ and 0.621 g of HC$_2$H$_3$O$_2$) and bee pollen extract. Trolox in a concentration range of 600–100 µM (aqueous, pH adjusted to pH 7.0 to completely solubilize in water) was used as the standard, with the 400 µM standard acting as a positive control throughout all tests.

The DPPH assay adopted in this experiment is based on the protocol described by Karabagias et al. [117] with minor modifications. In brief, 10 µL of bee pollen extract or Trolox standards were placed in a 96-well microplate, followed by the addition of 100 µL of NaC$_2$H$_3$O$_2$ buffer and 190 µL of 0.130 mM methanolic DPPH solution. The reaction mixture was kept in the dark for 120 min before the absorbance was measured at 520 nm using the microplate reader. The mean radical scavenging activity of triplicate samples was expressed as the Trolox equivalent (TE), calculated by a linear regression analysis, and then expressed as µmol Trolox equivalent per g of pollen.

\[
\text{DPPH Value of Sample (µM) Trolox} = \frac{(\Delta \text{Abs} - \text{intercept})}{\text{slope}} \times \text{Dilution Factor (3)}
\]

2.6. Statistical Analysis

Statistical analysis of the data was carried out using Design Expert 12 (StatEase Inc., Minneapolis, MN, USA) and two-way t-test or one-way ANOVA was analysed using Graphpad Prism 9 (GraphPad Software, San Diego, CA, USA). Pareto plots of regression coefficients of the independent variables were generated using Microsoft Excel. The signal to noise ratio was set at 5 times the standard deviation of observations for each response ($n= 3$). The model was developed based on the regression analysis of the statistical significance of variables, and the model coefficient was significant when the $F$ value was larger than the critical $F$ value ($p < 0.05$). The relative influence of factors was identified by
comparing the magnitude of regression coefficients. Correlations between responses were established using Spearman regression analysis. When significant interactions between factors were identified, a two-way *t*-test or a one-way analysis of variance with Tukey’s post hoc comparison was used to identify differences between the groups, and the statistical significance was set at *p* > 0.05.

3. Results

A thorough literature review of 101 published articles on the most widely used conditions for extracting antioxidant principles from bee pollen (Table 1) indicated a lack of a consistent approach. Researchers used a variety of antioxidant assays and analysis standards, and also, the manner by which the results were expressed varied greatly, making it difficult to conduct comparative analyses of findings across research groups. Furthermore, there appears to be no agreed protocol to guide the extraction process itself, which is required to establish baseline data for bee pollen of Australian origin in order to compare their antioxidant activity with other bee pollen samples. The gaps in information form the basis for this study, which aimed to optimise the extraction conditions for bee pollen collected in western Australia to enable the maximum extraction of antioxidant constituents as measured by the TPC, DPPH and FRAP assays. The Design of Experiment approach examined three independent variables: sample pulverisation, extraction solvent, and the extraction process. The two pollen types provided sample diversity to enable the development of a generalised extraction protocol that may be adopted for all types of bee pollen collected in western Australian and beyond.

3.1. Analysis of the Optimisation Process

A multilevel factorial design was implemented using Design Expert 12 (StatEase Inc., Minneapolis, MN, USA), with sample pulverisation, extraction solvent, and the extraction process as independent variables. The conditions selected for each variable were based on the popularity of use, as reflected in the literature review (Table 1). The responses (dependent variables) measured were the TPC, DPPH, and FRAP antioxidant activities. As summarised in Table 3 (the full data set is available as Supplementary Material, Table S1), the extraction variables for the multilevel factorial design (categorical) were selected at two levels for pulverisation (A, crude = −, pulverised = +), four levels for extraction solvent (B, 70% ethanol: 30% = E70:30, ethanol = EtOH, water = H₂O, and methanol = MtOH), and four levels for the extraction process (C, agitation = A, maceration = M, reflux = R, and sonication = S). The total runs consisted of 32 experimental points, and each point was triplicated. The sequence of the experiments was randomised, where the random numbers were generated by the Design Expert 12 software.

Pulverisation (Variable A) did not show any significant effect on the TPC, DPPH and FRAP data for the bee pollen (*p* value > 0.05) and was therefore removed from further analysis. Both solvent type (Variable B) and the extraction process (Variable C) were found to have significant impacts on the TPC, DPPH and FRAP antioxidant activity of bee pollen (*p* value < 0.05). A significant interaction between the solvent type and extraction process (Variables B and C) was observed for the TPC and FRAP responses (*p* value < 0.05), whereas the interaction of these variables did not influence the DPPH antioxidant activity.

However, the larger coefficients obtained for extraction solvent relative to those for the extraction process indicate that the selection of solvent has a dominant effect on all three responses based on the tabulated regression coefficients (see Table 4), also seen in Pareto charts (Figures 1–3).
Table 3. Multilevel factorial experimental design to optimise the extraction of antioxidant components in bee pollen. Experiments were conducted with 3 independent variables and the responses comprised of antioxidant activity measured by the TPC, DPPH and FRAP assays.

| Run | Independent Variables | Dependent Variables |
|-----|-----------------------|---------------------|
|     | Pulverisation Solvent Extraction | TPC | DPPH | FRAP | DPPH/FRAP Ratio |
| 1   | –                     | E70:30 A            | 20.86 ± 4.07 | 320.11 ± 27.00 | 342.28 ± 55.57 | 1.07 |
| 2   | +                     | E70:30 A            | 20.85 ± 2.67 | 331.86 ± 43.20 | 357.36 ± 35.65 | 1.08 |
| 3   | –                     | E70:30 M            | 19.99 ± 1.08 | 267.92 ± 39.77 | 396.39 ± 14.23 | 1.48 |
| 4   | +                     | E70:30 M            | 19.42 ± 2.13 | 291.42 ± 56.06 | 349.19 ± 90.21 | 1.20 |
| 5   | –                     | E70:30 R            | 21.37 ± 1.70 | 309.28 ± 32.62 | 326.54 ± 34.19 | 1.17 |
| 6   | +                     | E70:30 R            | 20.46 ± 1.33 | 300.81 ± 48.42 | 328.85 ± 40.44 | 1.16 |
| 7   | –                     | E70:30 S            | 18.80 ± 2.20 | 266.37 ± 56.46 | 326.61 ± 62.16 | 1.23 |
| 8   | +                     | E70:30 S            | 19.68 ± 2.87 | 296.61 ± 55.53 | 338.49 ± 59.04 | 1.14 |
| 9   | –                     | EtOH A              | 16.61 ± 2.15 | 298.92 ± 14.55 | 286.19 ± 29.22 | 0.88 |
| 10  | +                     | EtOH A              | 16.92 ± 3.23 | 297.37 ± 15.99 | 289.85 ± 45.37 | 0.97 |
| 11  | –                     | EtOH M              | 17.03 ± 2.24 | 244.52 ± 55.49 | 302.95 ± 33.92 | 1.24 |
| 12  | +                     | EtOH M              | 16.84 ± 1.72 | 236.25 ± 52.65 | 297.27 ± 33.84 | 1.28 |
| 13  | –                     | EtOH R              | 19.35 ± 1.08 | 285.28 ± 32.63 | 323.98 ± 29.19 | 1.36 |
| 14  | +                     | EtOH R              | 18.84 ± 2.00 | 262.40 ± 37.24 | 310.51 ± 32.89 | 1.25 |
| 15  | –                     | EtOH S              | 15.67 ± 2.24 | 231.12 ± 52.88 | 269.77 ± 24.22 | 1.17 |
| 16  | +                     | EtOH S              | 16.57 ± 2.16 | 251.47 ± 37.37 | 286.61 ± 31.22 | 1.14 |
| 17  | –                     | H2O A               | 3.50 ± 0.52  | 34.67 ± 10.93  | 45.06 ± 12.36  | 1.30 |
| 18  | +                     | H2O A               | 3.68 ± 0.18  | 34.22 ± 6.14   | 44.05 ± 12.66  | 1.29 |
| 19  | –                     | H2O M               | 3.58 ± 0.55  | 20.91 ± 3.80   | 44.42 ± 6.33   | 2.13 |
| 20  | +                     | H2O M               | 3.34 ± 0.30  | 26.04 ± 10.26  | 43.01 ± 8.44   | 1.65 |
| 21  | –                     | H2O R               | 5.07 ± 1.45  | 49.21 ± 13.94  | 83.66 ± 23.00  | 2.83 |
| 22  | +                     | H2O R               | 4.98 ± 1.69  | 49.9 ± 20.24   | 80.89 ± 27.60  | 2.14 |
| 23  | –                     | H2O S               | 4.26 ± 0.29  | 31.72 ± 10.94  | 55.64 ± 9.42   | 1.25 |
| 24  | +                     | H2O S               | 4.35 ± 0.49  | 34.55 ± 4.53   | 56.99 ± 6.81   | 1.65 |
| 25  | –                     | MtOH A              | 19.55 ± 2.22 | 311.92 ± 33.70 | 196.83 ± 43.71 | 0.63 |
| 26  | +                     | MtOH A              | 19.25 ± 3.26 | 298.15 ± 47.53 | 237.14 ± 18.04 | 0.80 |
| 27  | –                     | MtOH M              | 19.17 ± 3.29 | 267.20 ± 62.25 | 221.76 ± 66.73 | 0.83 |
| 28  | +                     | MtOH M              | 18.58 ± 2.90 | 236.85 ± 46.00 | 268.84 ± 38.98 | 1.14 |
| 29  | –                     | MtOH R              | 21.15 ± 3.20 | 253.10 ± 25.66 | 254.02 ± 30.52 | 1.06 |
| 30  | +                     | MtOH R              | 20.00 ± 3.41 | 261.09 ± 41.84 | 270.53 ± 14.49 | 1.11 |
| 31  | –                     | MtOH S              | 17.85 ± 2.21 | 254.80 ± 65.14 | 216.45 ± 26.87 | 0.85 |
| 32  | +                     | MtOH S              | 17.95 ± 2.21 | 261.78 ± 70.17 | 241.74 ± 19.54 | 0.92 |

The relationships between the independent variables and TPC, DPPH and FRAP assays are further illustrated in three-dimensional graphs (Figures 4–6). Figure 4 shows the results for the TPC assay of the bee pollen samples. The highest responses were observed when a solvent of 70% ethanol (E70:30) was coupled with reflux (R) or agitation (A) as the extraction process (p value < 0.05). These conditions may therefore represent optimal parameters for the extraction of the bee pollen samples for the TPC assay. For the DPPH antioxidant activity (Figure 5), the extraction solvent of 70% ethanol (E70:30) coupled with agitation (A) were the best extracting conditions for the bee pollen samples (p value < 0.001). Figure 6 shows that the extraction solvent of 70% ethanol (E70:30) coupled with maceration (M) produced the highest FRAP activity (p value < 0.05), and may therefore be considered as the combination of solvent type and the extraction process of choice for the FRAP assay of the bee pollen samples.
Table 4. Summary of regression coefficients. A[n] = pulverisation; B[n] = solvent type; C[n] = extraction process; AB[n] = interaction of pulverisation and solvent, B[n]C[n] = interaction of solvent type and extraction process. * = significant p-value < 0.05.

| Term     | TPC   | DPPH  | FRAP  |
|----------|-------|-------|-------|
| Intercept| 3.76  | 13.71 | 14.64 |
| A        | -     | -     | 0.1503|
| B[1]     | 0.7323*| 3.36* | 3.93* |
| B[2]     | 0.3897*| 2.26* | 2.47* |
| B[3]     | -1.74*| -8.15*| -7.18*|
| C[1]     | -0.0195*| 0.9483*| -0.4800*|
| C[2]     | -0.0691*| -0.4900*| 0.0891*|
| C[3]     | 0.1706*| -0.2780*| 0.6496*|
| AB[1]    | -     | -     | -0.2061|
| AB[2]    | -     | -     | -0.0267|
| AB[3]    | -     | -     | -0.1826|
| B[1]C[1] | 0.0937*| -     | 0.6036*|
| B[2]C[1] | -0.0348*| -     | -0.0579*|
| B[3]C[1] | -0.1043*| -     | -0.3087*|
| B[1]C[2] | 0.0177*| -     | 0.6213*|
| B[2]C[2] | 0.0365*| -     | 0.1917*|
| B[3]C[2] | -0.0886*| -     | -0.9382*|
| B[1]C[3] | -0.0886*| -     | -1.14* |
| B[2]C[3] | 0.0498*| -     | 0.0415*|
| B[3]C[3] | 0.0529*| -     | 0.9468*|

Figure 1. Pareto chart of regression coefficients for TPC assay data. X-axis indicates the different factors (B[n] = solvent type, C[n] = extraction process, and B[n]C[n] = interaction of solvent type and extraction process) while the values in the left y-axis indicate the coefficient values for the corresponding factors. Black bars indicate positive effect, while white bars indicate negative effect on TPC.
Figure 1. Pareto chart of regression coefficients for TPC assay of the bee pollen samples. The relationships between the independent variables and TPC, DPPH and FRAP activity. These conditions were the best extracting conditions for the bee pollen samples.

Figure 2. Pareto chart of regression coefficients for DPPH antioxidant activity data. X-axis indicates the different factors (B[n] = solvent type and C[n] = extraction process,) while the values in the left y-axis indicate the coefficient values for the corresponding factors. Black bars indicate positive effect, while white bars indicate negative effect on DPPH antioxidant activity.

Figure 3. Pareto chart of regression coefficients for FRAP antioxidant activity data. X-axis indicates the different factors (A[n] = pulverisation, B[n] = solvent type, C[n] = extraction process, AB[n] = interaction of pulverisation and solvent, B[n]C[n] = interaction of solvent type and extraction process) while the values in the left x-axis indicate the coefficient values for the corresponding factors. Black bars indicate positive effect, while white bars indicate negative effect on DPPH antioxidant activity.
**Figure 4.** Interactive effects of extraction solvent and extraction method on the TPC assay for bee pollen samples. TPC values were expressed as mg gallic acid equivalent per gram pollen, B: extraction solvent (70% ethanol:30% = E70:30, ethanol = EtOH, water = H2O, and methanol = MtOH) and C: extraction process (agitation = A, maceration = M, reflux = R, and sonication = S).

**Figure 5.** Effects of extraction solvent and extraction method on the DPPH antioxidant activity for bee pollen samples. DPPH values were expressed as $\mu$mol Trolox equivalent per gram pollen, B: extraction solvent (70% ethanol:30% = E70:30, ethanol = EtOH, water = H2O, and methanol = MtOH) and C: extraction process (agitation = A, maceration = M, reflux = R, and sonication = S).
Figure 6. Interaction effects of extraction solvent and extraction method on the FRAP antioxidant activity for bee pollen samples. FRAP values were expressed as μmol Fe^{+2} equivalent per gram pollen, B: extraction solvent (70% ethanol:30% = E70:30, ethanol = EtOH, water = H₂O, and methanol = MtOH) and C: extraction process (agitation = A, maceration = M, reflux = R, and sonication = S).

3.2. Correlation of TPC, DPPH and FRAP Antioxidant Activity

Bee pollen has been reported to contain many types of polyphenols [99] which are strongly correlated to the antioxidant activity of bee pollen [9]. In this study, a strong positive correlation was observed between the TPC and DPPH data, TPC and FRAP data as well as the DPPH and FRAP data, with correlation values of $\rho = 0.6925$ ($p < 0.001$), $\rho = 0.7295$ ($p < 0.001$) and $\rho = 0.6520$ ($p < 0.001$), respectively. Thus, the presence of polyphenols, captured in the pollen’s TPC, appears to positively influence its antioxidant capacity expressed in the DPPH and FRAP assays. However, it needs to be acknowledged that there are other pollen constituents, such as carotenoids, that could also influence the antioxidant capacity [82,106].

3.3. Choosing Optimum Conditions

The optimisation of the extraction process to maximise the three dependent variables was determined by employing a new variable desirability, which represents all responses simultaneously. Desirability is an objective function that is determined by ranked responses and its value ranges from zero to one, with one being most desirable. When there are several responses, the individual goals are combined to generate one desirability function, which was automated by Design Expert software 12 (StatEase, Inc. Minneapolis, MN, USA). The numerical optimisation based on the goal of the study finds a point that maximises the desirability function. The criteria adopted to determine the desirability function for this study are to maximise all responses. Figures 7–9 provide predicted TPC, DPPH and FRAP values obtained for the three optimum conditions identified for the extraction of bee pollen samples.
Figure 7. Proposed optimised extraction condition 1: crude/non-pulverised sample extracted using 70% ethanol:30% water (E70:30) and agitation (A), corresponding to the optimal conditions see in Figure 4 for TPC data, Figure 5 for DPPH data, and Figure 6 for FRAP data (desirability = 0.925).

| Treatments | A: Pulverisation = - | B: Solvent = E70:30 | C: Extraction = A |
|------------|---------------------|----------------------|------------------|
| TPC        | 3.12754             | 22.4683              | 20.9002          |
| FRAP       | 41.3814             | 406.628              | 329.27           |
| DPPH       | 16.502              | 358.273              | 281.845          |

Figure 8. Proposed optimised extraction condition 2: crude/non-pulverised sample extracted using 70% ethanol:30% water (E70:30) and maceration (M), corresponding to the optimal conditions see in Figure 4 for TPC data, Figure 5 for DPPH data, and Figure 6 for FRAP data (desirability = 0.893).

| Treatments | A: Pulverisation = - | B: Solvent = E70:30 | C: Extraction = M |
|------------|---------------------|----------------------|------------------|
| TPC        | 3.12754             | 22.4683              | 19.699           |
| FRAP       | 41.3814             | 406.628              | 374.139          |
| DPPH       | 16.502              | 358.273              | 274.772          |

Figure 9. Proposed optimised extraction condition 3: crude/non-pulverised sample extracted using 70% ethanol:30% water (E70:30) and reflux (R), corresponding to the optimal conditions see in Figure 4 for TPC data, Figure 5 for DPPH data, and Figure 6 for FRAP data (desirability = 0.883).

| Treatments | A: Pulverisation = - | B: Solvent = E70:30 | C: Extraction = R |
|------------|---------------------|----------------------|------------------|
| TPC        | 3.12754             | 22.4683              | 20.9002          |
| FRAP       | 41.3814             | 406.628              | 329.27           |
| DPPH       | 16.502              | 358.273              | 281.845          |

Based on the model, the crude/non-pulverised pollen sample, extracted with 70% ethanol:30% water (E70:30) by agitation (A) as the extraction process produced the highest desirability (0.92) (Figure 7), followed by the crude/non-pulverised pollen sample, extracted with 70% ethanol:30% water (E70:30) by maceration (M) (desirability = 0.893) (Figure 8), and finally, the non-pulverised pollen sample, extracted with 70% ethanol:30%
water (E70:30) by reflux (R) (desirability = 0.883) (Figure 9). These three conditions can therefore be considered to represent the optimal combination of sample pre-processing treatment, solvent type and extraction process to yield the maximum extraction of constituents from the bee pollen samples for the TPC, DPPH and FRAP assays. The bee pollen extracts prepared using the crude/non-pulverised pollen sample, extracted with 70% ethanol:30% water (E70:30) by agitation (A) as the extraction process were found to have an average total phenolic content of 20.86 mg GAE/g, DPPH antioxidant activity of 320.11 μmol TE/g and 342.28 μmol Fe^{2+} E/g FRAP activities, respectively. These values are very close to the predicted values of 20.83 mg GAE/g, 324.52 μmol TE/g and 351.78 μmol Fe^{2+} E/g, respectively, demonstrating the good fit of the chosen model.

4. Discussion

Commonly, the initial stage in studying the chemical composition and/or bioactivity of natural products, including bee pollen, includes a pre-extraction step, in which the material undergoes drying in order to preserve the biomolecules present in the sample [21]. This is often followed by grinding the dried material using a mortar and pestle, electric blender or various mills to decrease the particle size to enhance surface contact with the extraction solvent [21]. Particles that are too fine will, however, adsorb onto filters and impede filtration [118]. In this study, the effect of the pulverisation of bee pollen samples on its total phenolic content and associated antioxidant activity as captured by DPPH and FRAP assays was analysed. On the basis of these findings, the pulverisation process can be omitted from the extraction protocol.

The selection of solvent is crucial for solvent extraction, with selectivity for the target compounds, the target compound’s solubility as well as cost and safety to be considered. Based on the law of similarity and intermiscibility, solvents with a polarity value near the polarity of the solute are likely to perform better [118]. Water, along with a range of alcoholic and organic solvents such as methanol, ethanol, acetonitrile, acetone, hexane and diethyl ether are commonly utilised in the extraction of bioactive compounds [20]. The current study aimed to analyse the impact of different solvents, including water, methanol (M), ethanol (E) and the combination of ethanol and water at a ratio of 70:30 (v/v) (E70:30) on the total phenolic content as well as DPPH and FRAP antioxidant activity of pollen samples collected from western Australia. The findings of the study demonstrate that the extraction solvent had the strongest influence on the responses of the dependent variables (p <0.05). Among the solvents tested, extracts prepared with 70% ethanol:30% water (v/v) demonstrated the highest activity across all performed assays.

Based on the interactions observed in this study, it appears that TPC values are dependent on solvent type and the extraction process, and it can be concluded that TPC can be maximised when the pollen extraction is carried out either using 70% ethanol:30% water coupled with agitation or reflux. DPPH antioxidant activity is maximised following agitation as an extraction process in any of the investigated solvents, whereas FRAP antioxidant activity is highest when pollen is extracted with maceration coupled with 70% ethanol:30% water. Bee pollen contains 13–55% of carbohydrates [6–8] and it can be assumed that some can be carried over into the extract by polar solvents. Consequently, the prolonged heating of bee pollen at a high temperature might lead to the formation of Maillard products from these carbohydrates during processing [3]. Therefore, an extraction method that does not expose the pollen sample to prolonged high temperatures can be considered favourable. Maceration is an easy process in extracting antioxidant principles; however, it takes 72 h to complete, as compared to agitation, which only requires 6 h. Reflux is a very promising method of extraction, as it only requires 2 h to perform; however, depending on the chosen solvent, it might require high temperature in order to operate. Thus, in this light, agitation can be recommended as the optimal extraction process to maximise antioxidant principles obtained from bee pollen samples.

The term ‘phenolic’ or ‘polyphenol’ is chemically defined as a substance that possesses an aromatic ring bearing one or more hydroxyl substituents, including functional
derivatives such as esters, methyl esters and glycosides. These bioactive compounds are extensively found across the plant kingdom and are closely linked with the sensory and nutritional quality of fresh and processed plant foods, including bee products such as honey and bee pollen [9]. Keskin and Özkök reported various multifloral bee pollen samples from the Czech Republic to have a total phenolic content ranging from 15.2 mg to 22.73 mg GAE/g pollen [38], whereas Mayda, Özkök et al. reported TPC values of 26.69 ± 0.595 and 43.42 ± 0.779 mg GAE/g pollen [58]. TPC for multifloral bee pollen from Morocco was reported as 45.96 ± 0.51 mg GAE/g pollen [63] and samples obtained from Hungary had TPC values ranging from 9.15 ± 0.12 to 13.63 ± 0.11 mg GAE/g pollen [72]. TPC values (mg GAE/g pollen) for the two western Australian monofloral pollen samples investigated as part of this study following the optimised extraction protocol by using non-pulverised samples extracted with 70% ethanol:30% via agitation was 20.86 mg GAE/g. With this, the TPC value of the western Australian pollens are broadly within typical ranges found for a range of bee pollen samples from a wide geographical spread. It needs to be highlighted, though, that comparisons between data generated in different studies need to be treated with caution, as the chosen extraction condition (solvent and extraction method) will impact on the obtained TPC data. Furthermore, the method used in the analysis of TPC in this study utilised a Folin–Ciocalteu method in slightly basic medium (0.75% sodium carbonate) as compared to most researchers that used 7.5% sodium carbonate. This amendment to the common assay protocol was found to be necessary, since reducing sugars present in alcoholic and aqueous pollen extracts can also be reduced by the reagent, and thus, lead to an overestimation of TPC. [119]

The optimised extraction protocol was also used to assess the antioxidant activity of the two western Australian pollen samples investigated in this study. Despite relatively high correlations between FRAP, DPPH and TPC values found in this study, it can be argued that in vitro antioxidant capacity should not be determined by means of a single antioxidant test model because of the diverse types of antioxidant that might be present in the sample as well as the intricacy of the natural product matrix and the variety of free radical reaction mechanisms involved in oxidation. Complementary antioxidant assays might, therefore, produce richer data [78]. Thus, in this study, the antioxidant potential of bee pollen extracts was determined by means of two different radical scavenging assays, namely DPPH and FRAP.

Using the DPPH assay, Rocchetti and Castiglioni reported Magnolia and Lamium bee pollen from Italy to have antioxidant activities of 11.9 and 134.7 µmol TE/g pollen, respectively [78]. Mărghiţaş et al. reported DPPH antioxidant activities ranging from 135 to 2814 µmol TE/g for various monofloral pollens from Romania [28] and Saral et al. found DPPH scavenging activities between 13.87 and 15.04 µg TE/g for multifloral pollen from Turkey [74]. DPPH data generated in this study for the western Australian pollen samples were 320.11 µmol (equivalent to 80.12 mg) TE/g following the optimised extraction protocol using non-pulverised pollen extracted with E70:30 by agitation. These findings are within the range of values reported by others.

Using the FRAP assay, Zuluaga-Dominguez et al. reported 87.2 ± 15.6 µmol Trolox/g for multifloral pollen from Colombia [37], whereas Saral et al. found a FRAP activity ranging from 8.69 to 84.89 µmol Fe²⁺/g for multifloral bee pollen from Turkey [74]. In this study, following the optimised extraction protocol by using non-pulverised pollen extracted with E70:30 by agitation, FRAP antioxidant activity was found to be 342.28 µmol Fe²⁺ g⁻¹, which is higher than the values reported by Saral et al. However, the comparison of FRAP values appears even more difficult, not only because the results are dependent on the chosen extraction conditions but also because the studies use different reference standards (Trolox or Fe²⁺) to express their results.

5. Conclusions

Based on a thorough review of the extant literature, a number of common bee pollen processing steps, solvents and extraction methods were identified, which can all impact on
extraction efficiency and thus result in different TPC, DPPH and FRAP values. The study reports on an in-depth investigation into the optimisation of the most popular extraction conditions for maximum TPC, DPPH and FRAP antioxidant activity using two bee pollen samples from western Australia. The effects of pulverisation, the chosen solvent (70% aqueous ethanol, ethanol, methanol and water) as well as the adopted extraction process (agitation, maceration, reflux and sonication) were determined in order to optimise the extraction parameters. The study’s findings demonstrate that non-pulverised pollen extracted with 70% aqueous ethanol coupled with agitation as the extraction method constitutes the best conditions in order to maximise the extraction of phenolics and antioxidant principles in these bee pollen samples.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/antiox10071113/s1. Table S1: Data of pollen multilevel factor pollen analysis.

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