

Article

Optimization of Green Sample Preparation for the Determination of Hydroxycinnamic Acids in Multi-Floral Honey Using Response Surface Methodology

Konstantina Stavrou, Atalanti Christou  and Vlasios Goulas * 

Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Lemesos 3603, Cyprus; konstantina.stavrou@cut.ac.cy (K.S.); atalanti.christou@cut.ac.cy (A.C.)

* Correspondence: vlasios.goulas@cut.ac.cy; Tel.: +357-2500-2141

Featured Application: The present study provides practical guidelines for the solubilization of hydroxycinnamic acids from honey for their chromatographic determination. The proposed green preparation step can be used for honey hydroxycinnamic fingerprinting for different botanical and/or geographical origins. In addition, it can be also utilized for the evaluation of diverse biological effects of hydroxycinnamic acids present in honey.

Abstract: Hydroxycinnamic acids are an essential phenolic group in honey that are related with its diverse health effects. Furthermore, they can be considered as potential biomarkers for botanical and geographical origin. The extraction of hydroxycinnamic acids from honey requires complicated extraction techniques due to their low contents and matrix particularity. The goal of the present work was to develop a green sample preparation method for the solubilization of hydroxycinnamic acids in honey samples. Thus, a Box–Behnken design has been used to investigate the effect of four factors: (i) percentage of ethanol (60–90%, *v/v*), (ii) temperature (30–60 °C), (iii) solvent-honey ratio (10–30 mL g^{−1}), and (iv) sonication time (10–40 min) on the contents of caffeic, chlorogenic, and ferulic acids. Based on the desirability index, the ultrasound-assisted mixing of honey with 60.0% (*v/v*) ethanol at a solvent-honey ratio of 10 mL g^{−1} for a period of 17.8 min at a temperature of 54.6 °C resulted in the maximum solubilization of hydroxycinnamates. Subsequently, the greenness of developed method was assessed and applied successfully on the analysis of honeys. The present study reports for first time the hydroxycinnamic acid fingerprint of multi-floral honey produced in Cyprus.

Keywords: Box–Behnken design; caffeic acid; chlorogenic acid; ferulic acid; phenolic acids; response surface methodology; recovery; ultrasound-assisted solubilization



Citation: Stavrou, K.; Christou, A.; Goulas, V. Optimization of Green Sample Preparation for the Determination of Hydroxycinnamic Acids in Multi-Floral Honey Using Response Surface Methodology. *Appl. Sci.* **2024**, *14*, 5781. <https://doi.org/10.3390/app14135781>

Academic Editors: Silvia Marzocchi and Maria Caboni

Received: 10 June 2024

Revised: 28 June 2024

Accepted: 29 June 2024

Published: 2 July 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Honey is a traditional sweetener that is produced by honey bees (*Apis mellifera*). It is considered as a nutritive sweetener as it contains a high proportion of sugars. However, it has a lower glycaemic potency than refined sugars due to the presence of relatively high proportion of fructose, non-nutritive components, and moisture ([1,2]. In general, it is mainly composed of a mixture of different sugars (80–85%), water (15–17%), and proteins (0.1–0.4%), and it contains a plethora of micronutrients, namely enzymes, organic acids, vitamins, minerals, and phenolic compounds [3].

Honey is justifiably recognized as a functional food delivering nutritive compounds and treating multifarious ailments. It is noteworthy that several clinical trials correlate the consumption of honey with wound healing [4], antimicrobial [5], anticancer [6], antidiabetic [7], cardio-protective [8], nephro-protective [9], anti-atherosclerotic [10], neuro-protective [11], and anti-aging activities [12]. In addition, various studies support the

potential of honey to treat respiratory diseases, manage mental disorders, control gastrointestinal complications, and promote skin health [13–15]. Researchers also linked the beneficial effects of honey on amelioration of chronic and acute diseases with its special phenolic composition [16].

The term “phenolic compound” describes a substance that possesses an aromatic ring bearing one or more hydroxyl substituents including functional derivatives (esters, methyl esters, and glycosides). Phenolic compounds are widely distributed in the plant kingdom; thus, they are transferred from host plant floral nectars into honey. The phenolic compounds found in honey are mainly flavonoids and phenolic acids, and they can be utilized as biomarkers for the geographical and botanical origin of honey [17]. Hydroxybenzoic and hydroxycinnamic acids are the main sub-groups of phenolic acids found in honey. Hydroxybenzoic acids such as gallic acid, ellagic acid, syringic acid, and protocatechuic acid are more common in honey than hydroxycinnamic acids. Nevertheless, hydroxycinnamic acids exert stronger biological activities because of the unsaturated chain attached to the carboxyl group and the presence of the phenoxy functional group [2]. Caffeic acid, ferulic acid, *trans*-cinnamic acid, and *p*-coumaric acid are widely distributed in mono- and multi-floral honeys. Rosmarinic acid, caftaric acid, chlorogenic acid, and *o*-coumaric acid have also been identified in honeys [18–20]. Furthermore, rosmarinic acid and chlorogenic acid have been proposed as biomarkers for botanical origin in thyme and acacia honey, respectively [21,22].

The extraction of hydroxycinnamic acids from honey is a critical step in their chromatographic analysis, but it is a difficult task due to their chemical complexity and high viscosity of the matrix as well as the low concentrate of these phenolic acids [23]. The extraction of hydroxycinnamic acids is usually performed through liquid-liquid extraction using organic solvents, solid phase extraction with diverse stationary phases, and miniaturized extraction methods such as dispersive liquid-liquid microextraction and solid phase microextraction [23]. Thus, the extraction step is time-consuming, typically requiring 80% of the total analysis time, and has negative impact on greenness of the extraction procedure. Recently, an optimized ultrasound-assisted extraction method was introduced to enhance the solubilization of phenolic substances prior chromatographic analysis. This optimization study was based on total phenolic contents and requires large volumes of solvent for the solubilization [24]. The present work aspires to develop and optimize an effective miniaturized, green sample preparation method for the solubilization of hydroxycinnamic acids from honey using ultrasound irradiation and a hydroethanolic mixture as solvent. In particular, the solubilization temperature, solubilization time, ethanol concentration, and solvent-to-honey ratio were optimized to maximize the solubilization of hydroxycinnamic acids prior chromatographic analysis. Finally, the optimized sample preparation method was applied successfully to determine the hydroxycinnamic acids in commercial multi-floral honey.

2. Materials and Methods

2.1. Chemicals and Reagents

Hydroxycinnamic acids (caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, and rosmarinic acid) were used as standards and were obtained from Sigma Aldrich (Steinheim, Germany). Acetonitrile and water HPLC grade, as well as formic acid, were also purchased from Sigma Aldrich. Regarding sugars, D(-)-fructose and D(+)-glucose anhydrous and sucrose were purchased from Sigma Aldrich, Scharlau Chemie (Barcelona, Spain) and HiMedia Laboratories (Mumbai, India), respectively.

2.2. Honey Samples

An artificial honey was prepared representing the proportions of the three predominant sugars in natural honey samples according to a previous study [25]. More specifically, 4.3 g fructose, 3.6 g glucose, 0.4 g sucrose, and 10 µL for each hydroxycinnamic acid, namely

caffeic acid, chlorogenic acid, and ferulic acid, at a concentration of 10 mg mL^{−1} were mixed thoroughly. Then, 1.67 mL of sterile deionized water was added.

A total of 10 commercial Cypriot multi-floral honey samples were collected from the local supermarkets. The samples were stored in the dark at room temperature until analysis.

2.3. Optimization of Sample Preparation Using Response Surface Methodology (RSM)

The ultrasound-assisted solubilization of hydroxycinnamic acids was optimized with the employment of RSM using an artificial honey solution. An amount of 0.3 g honey solution was mixed thoroughly with different volumes (3, 6, 9 mL) of hydroethanolic mixtures (60, 75, and 90% ethanol in deionized water). The procedures were carried out in an ultrasonic bath (UCI-50, 35 KHz, Raypa-R. Espinar, S.L., Terrassa, Spain) at different temperatures (30, 45, and 60 °C) for varying periods (10, 25, and 40 min). After ultrasonic treatment, the mixture was filtered through a 0.45 µm syringe filter and stored at −20 °C for further analysis. All sample extracts were prepared in triplicate.

The solubilization parameters were optimized using RSM based on Box-Behnken design (BBD). A three-level and four-factor BBD was applied to investigate the effect of ethanol concentration (A), temperature (B), solvent-to-sample ratio (C), and sonication time (D) on solubilization efficiency. The contents of caffeic acid (Y1), chlorogenic acid (Y2), and ferulic acid (Y3) were used as experimental responses for the optimization. Independent variables were selected based on preliminary studies with their coded and actual levels given in Table 1. The complete BBD consisting of 29 combinations including five replications at the central point is given in Table 2, where all measurements were performed in triplicate and in random order to minimize the effects of uncontrolled variables. The mean values of all measured responses were analyzed by multiple regressions and fitted to the quadratic polynomial model described in Equation (1). Analysis of variance (ANOVA) was conducted to assess the significance of linear, quadratic, and interaction regression coefficients (statistically significant at $p < 0.05$) and to determine the validity of the quadratic models. Three-dimensional surface plots were generated based on the developed polynomial models to interpret the relationship between dependent variables and independent responses:

$$Y = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=2}^n \beta_{ij} x_i x_j + \sum_{i=1}^n \beta_{ii} x_i^2 \quad (1)$$

$j > 1$

where β_0 is the constant coefficient, β_i , β_{ii} , and β_{ij} are the regression coefficients for linear, quadratic, and interaction terms, respectively, and x_i and x_j represent the independent variables.

Table 1. Natural and coded levels of independent variables used in three-level, four-factor Box-Behnken design (BBD).

Independent Variables	Symbol	Factor Level		
		Low (−1)	Medium (0)	High (+1)
Ethanol concentration (% <i>v/v</i>)	A	60	75	90
Temperature (°C)	B	30	45	60
Solvent-to-sample ratio (mL g ^{−1})	C	10	20	30
Sonication time (min)	D	10	25	40

Table 2. Box-Behnken design of the independent variables in their actual and coded levels and experimentally obtained data of the investigated responses.

Run	A Ethanol Concentration (%)	B Temperature (°C)	C Solvent-to- Sample Ratio (mL g ^{−1})	D Time (min)	Y1 Caffeic Acid (Peak Area)	Y2 Chlorogenic Acid (Peak Area)	Y3 Ferulic Acid (Peak Area)
1	75 (0)	30 (−1)	20 (0)	40 (+1)	12,722.5	15,773.5	23,352.5
2	75 (0)	45 (0)	20 (0)	25 (0)	17,466.0	18,789.3	28,028.7
3	75 (0)	45 (0)	20 (0)	25 (0)	16,776.3	19,438.7	29,551.6
4	75 (0)	45 (0)	20 (0)	25 (0)	17,572.0	19,909.7	31,064.8
5	75 (0)	30 (−1)	30 (+1)	25 (0)	10,413.7	13,209.7	17,216.8
6	60 (−1)	30 (−1)	20 (0)	25 (0)	20,375.3	21,522.7	30,233.8
7	60 (−1)	45 (0)	10 (−1)	25 (0)	38,171.3	39,178.0	60,649.7
8	75 (0)	60 (+1)	30 (+1)	25 (0)	9574.0	11,310.7	13,541.3
9	60 (−1)	60 (+1)	20 (0)	25 (0)	16,840.3	19,251.0	27,750.7
10	75 (0)	60 (+1)	20 (0)	10 (−1)	15,862.3	19,454.7	28,462.3
11	75 (0)	45 (0)	10 (−1)	10 (−1)	34,675.7	36,232.0	60,867.0
12	90 (+1)	30 (−1)	20 (0)	25 (0)	10,882.3	17,449.3	28,437.7
13	75 (0)	45 (0)	20 (0)	25 (0)	17,864.0	19,771.7	30,452.4
14	75 (0)	60 (+1)	20 (0)	40 (+1)	10,951.0	18,405.3	28,492.7
15	75 (0)	45 (0)	30 (+1)	40 (+1)	6921.33	11,361.0	13,380.7
16	60 (−1)	45 (0)	30 (+1)	25 (0)	10,464.7	13,882.0	18,576.3
17	75 (0)	45 (0)	30 (+1)	10 (−1)	10,129.7	12,203.3	16,713.0
18	75 (0)	60 (+1)	10 (−1)	25 (0)	34,704.0	35,736.9	61,265.7
19	90 (+1)	60 (+1)	20 (0)	25 (0)	14,985.3	19,461.7	27,290.7
20	75 (0)	30 (−1)	10 (−1)	25 (0)	31,777.7	30,114.3	45,641.5
21	90 (+1)	45 (0)	30 (+1)	25 (0)	9452.33	11,552.8	18,012.7
22	90 (+1)	45 (0)	20 (0)	10 (−1)	13,375.7	18,080.3	25,066.3
23	60 (−1)	45 (0)	20 (0)	10 (−1)	18,730.3	21,060.6	28,810.7
24	75 (0)	45 (0)	10 (−1)	40 (+1)	30,953.7	36,263.3	52,651.3
25	90 (+1)	45 (0)	20 (0)	40 (+1)	12,233.0	16,806.6	25,643.7
26	75 (0)	30 (−1)	20 (0)	10 (−1)	6904.0	12,291.7	11,082.4
27	75 (0)	45 (0)	20 (0)	25 (0)	14,266.0	17,522.3	24,905.3
28	90 (+1)	45 (0)	10 (−1)	25 (0)	31,235.7	36,688.7	58,201.7
29	60 (−1)	45 (0)	20 (0)	40 (+1)	17,162.3	19,673.7	27,895.3

2.4. Chromatographic Analysis of Hydroxycinnamic Acids in Honey

The chromatographic analysis of individual hydroxycinnamic acids in artificial honey and honey samples was performed according to Combarros-Fuertes et al., 2019 with slight modifications [26]. Aqueous solution of formic acid 1% *w/w* (A) and acetonitrile (B) were used as mobile phase. The gradient profile was as follows: 0–15 min, 5–20% B; 15–18 min, 20–30% B; 18–20 min, 20–30% B; 20–40 min, 30–70% B; 40–45 min, 70–95% B; 45–46 min, 95–95% B; 46–48 min, 95–5% B and until 55 min, 5% B. All analyses were carried out at room temperature, with an injected volume of 20 µL and a flow rate of 1 mL min^{−1}. The chromatograms were monitored at 280 and 320 nm.

The analysis of samples was performed using a Waters series HPLC (Model “1525”) equipped with a vacuum degasser, quaternary pump, autosampler, thermostated column compartment, photodiode array detector (PDA), and Empower™ 2 Software (Waters Corporation, Milford, Ireland). Chromatographic separations were performed on a reversed-phase Waters® Spherisorb® ODS2 25 cm, 4.6 mm, 5 µm column (Waters Chromatography Division, Milford, MA, USA), thermostated at 25 °C. Peak identification was implemented by comparing both retention times and UV spectra with those of authentic standards. The quantities of hydroxycinnamic acids identified in the extract were determined based on calibration curves constructed using reference standards (caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, and rosmarinic acid).

2.5. Determination of Greenness for Developed Sample Preparation Method

The evaluation of greenness of the optimized method for the solubilization of hydroxycinnamic acids in honey samples was carried out with the use of AGREEprep version 1 software. A compiled version of the open access software can be obtained from most-wiedzy.pl/AGREEprep. This online software assesses the overall greenness performance of the sample preparation stage by quantifying weighted score values across ten impact categories, each corresponding to one of the ten principles of a green analytical process. Score values range from zero (considered a non-environmentally friendly approach) to one (the ideal greenest approach) and are accompanied by a color scale from red to green, respectively [27].

2.6. Statistical Analysis

All experimental assays were performed in triplicate. The results obtained were expressed as mean values $\pm$ standard deviation (SD). Experimental design, model building, and determination of optimum conditions were performed using Design Expert software (trial version 11.0, Stat-Ease Inc., Minneapolis, MN, USA).

3. Results and Discussion

3.1. Model Fitting

In the present study, optimization experiments based on RSM were performed to maximize the solubilization of hydroxycinnamic acids in honey samples using ultrasound irradiation. A BBD was applied to investigate the effects of ethanol concentration (A), temperature (B), solvent-to-sample ratio (C), and sonication time (D) on caffeic acid (Y1), chlorogenic acid (Y2), and ferulic acid (Y3) recoveries. Table 2 presents the experimental data for all measured responses, expressed as chromatographically determined peak areas, obtained under different combinations of sample preparation parameters.

The yields of caffeic acid (Y1), chlorogenic acid (Y2), and ferulic acid (Y3) were correlated in terms of coded values of the process variables by the following second-order polynomial equations obtained using regression analysis.

$$Y1 = +17,079.72 - 2465.00 A + 820.12 B - 12,046.86 C - 727.82 D + 1909.50 AB - 2682.46 BD - 1875.96 B^2 + 5934.19 C^2 - 2547.33 D^2$$

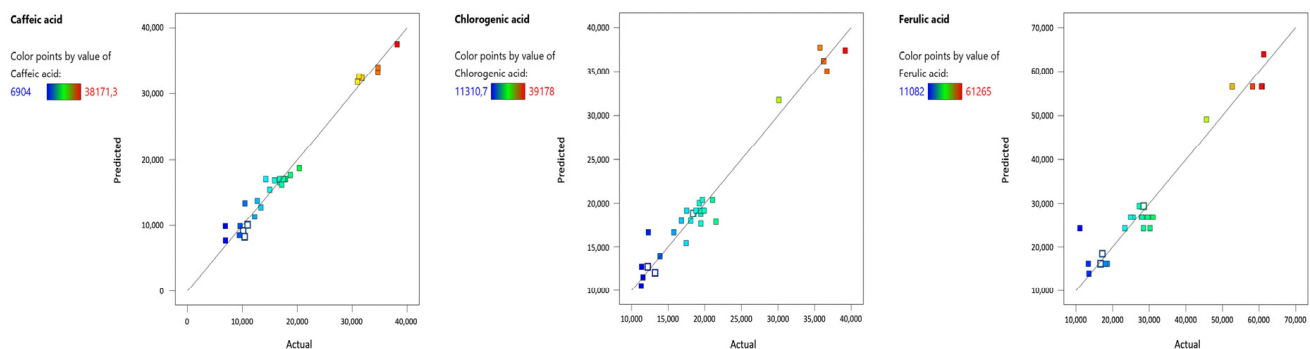
$$Y2 = +19,191.67 - 1210.78 A + 1104.90 B - 11,724.47 C - 1880.17 BC - 1449.74 B^2 + 5269.16 C^2$$

$$Y3 = +28,853.87 + 2569.99 B - 20,152.97 C - 4824.67 BC + 9539.10 C^2$$

The significance and goodness of fit of the quadratic models developed were investigated by analysis of variance (ANOVA) and descriptive statistics, as presented in Table 3. The polynomial models provided a good fit to the data, with the respective coefficients of determination (R^2) being higher than 0.94. At the same time, the reasonable agreement between predicted and adjusted coefficients of determination, with the difference between R^2_{pred} and R^2_{adj} being lower than 0.2, demonstrates the good agreement between the experimental and predicted values. Figure 1 demonstrates the close agreement between the experimental results and those calculated from the model values. Additionally, ANOVA demonstrated significant model F- (>89.46) and p -values (<0.0001) and non-significant lack of fit terms ($p > 0.05$), which validate the models' adequacy to describe the experimental data. As for coefficients of variation (CV), they were in all cases lower than 10%, demonstrating good precision and reproducibility of the models. Desirable signal-to-noise ratios were also obtained for all models developed, as demonstrated by the adequate precision values (>4). In general, the results verify the accuracy of the polynomial models in reflecting the relationship between process variables and measured responses, as well as predicting the optimal extraction conditions.

Table 3. Analysis of variance (ANOVA) and descriptive statistics for the developed quadratic models.

Term	Caffeic Acid		Chlorogenic Acid		Ferulic Acid	
	F-Value	p-Value	F-Value	p-Value	F-Value	p-Value
Model	95.57	<0.0001	108.57	<0.0001	89.46	<0.0001
A	28.10	0.0001	5.97	0.0231	-	-
B	3.11	0.0939	4.97	0.0363	4.99	0.0351
C	671.19	<0.0001	559.43	<0.0001	306.70	<0.0001
D	2.45	0.1340	-	-	-	-
AB	5.62	0.0285	-	-	-	-
AC	-	-	-	-	-	-
AD	-	-	-	-	-	-
BC	-	-	4.80	0.0394	5.86	0.0234
BD	11.09	0.0035	-	-	-	-
CD	-	-	-	-	-	-
A ²	-	-	-	-	-	-
B ²	9.12	0.0070	4.80	0.0394	-	-
C ²	91.30	<0.0001	64.99	<0.0001	40.28	<0.0001
D ²	16.82	0.0006	2.31	0.1509	-	-
Lack of fit	1.26	0.4514	3.56	0.1133	2.95	0.1513
R ²		0.9784		0.9673		0.9371
R ² _{adj}		0.9682		0.9584		0.9267
R ² _{pred}		0.9365		0.9304		0.9064
Adeq Precision		31.540		32.2520		30.1803
C.V. (%)		9.10		8.27		9.94

**Figure 1.** Comparison between experimental data (actual) and calculated from the model values (predicted).

3.2. Effect of Sample Preparation Variables on Hydroxycinnamic Acid Recoveries

According to the ANOVA results, the solvent-to-sample ratio was the most dominant factor, negatively influencing hydroxycinnamic acid responses in its linear form (C) and positively affecting them in its quadratic term (C²). With an increase in solvent-to-sample ratio from 10 to 25 mL g^{−1}, the hydroxycinnamic acids contents gradually decreased. With a further increase in the solvent-to-sample ratio, its positive quadratic effect dominates, and, thus, a slight increase in the responses was observed. The solubilization efficiency probably decreased due to the uneven distribution of the cavitation phenomenon resulting from the over dilution effect [28]. Thus, using lower volumes of solvent may enhance polyphenol solubilization, prevent unnecessary wastage of solvents, and reduce the cost of the process.

Ethanol concentration, in its linear form, demonstrated a weaker but still significant effect on caffeic acid and chlorogenic acid responses. The increase in ethanol concentration resulted in a decrease in chlorogenic acid content. An optimal solvent composition exists at the lower ethanol concentration range. The results may be related to the solvent polarity and the solubility preference of hydroxycinnamic acids. As water content in the aqueous

ethanolic mixture increases, solvent polarity is enhanced, thus improving the solubility of the target analytes [29].

As regards temperature in sample preparation, it significantly affected chlorogenic acid and ferulic acid contents linearly (positive effect) and caffeic and chlorogenic acid contents in a quadratic manner (negative effect). The positive linear effect of temperature can be attributed to its positive influence on the solubilization kinetics [30]. Increased solubility of polyphenols with increasing temperature was also reported in previous studies [31]. On the other side, polyphenol thermal degradation due to elevated temperatures may have resulted in the negative quadratic term of temperature for caffeic acid and chlorogenic acid responses [32].

A negative quadratic effect was observed for sonication time only in the case of caffeic acid. Prolonged sonication times increase the possibility of decomposition and/or oxidation of polyphenols, resulting in lower estimated contents. As a result, sonication time should not be prolonged unnecessarily to prevent degradation of polyphenols [31]. It is worth mentioning here that although temperature did not affect caffeic acid content, its interaction with sonication time had a negative impact on the response. In this case, increasing the temperature along with the sonication time might result in the thermal decomposition of the analyte, suggesting that solubilization at elevated temperatures probably requires a short processing time [33].

Temperature also demonstrated a significant interactive effect with solvent concentration in the case of caffeic acid and with solvent-to-sample ratio for chlorogenic acid and ferulic acid responses. A synergistic effect was observed for the former interaction, indicating that both variables result in greater solubilization of caffeic acid in their upper limits. An opposite effect was observed for the interaction between temperature and solvent-to-sample ratio, indicating an antagonistic effect.

3.3. Optimization of Sample Preparation Conditions and Model Validation

Optimization of the process variables was performed with the aim of simultaneously maximizing the estimated contents of three hydroxycinnamic acids. Numerical optimizations, performed to achieve the maximum desirability index ($D = 0.98$), suggested that the mixing of honey samples with 60.0% *v/v* aqueous ethanol for 17.8 min, at 54.6 °C, and using a solvent-to-sample ratio of 10.0 mL g⁻¹, were the optimum parameters for the sample preparation prior chromatographic analysis. Confirmation experiments were then conducted under these optimum parameters to verify the adequacy of the developed models in predicting the optimum response values. The observed and predicted values, along with the computed absolute errors for the optimum parameters, are presented in Table 4. As observed, the experimental results were close to those predicted from the model values, thus confirming the validity of the quadratic models in predicting the optimum sample preparation parameters.

Table 4. Comparison between experimental values and values predicted by the models for the responses obtained under the optimal sample preparation parameters.

Variable	Predicted Value	Experimental Value	Absolute Error (%)
(Y1) Caffeic acid (Peak Area)	36,650.6	37,002.5 ± 204.1	0.9
(Y2) Chlorogenic acid (Peak Area)	38,710.7	39,711.9 ± 333.6	2.6
(Y3) Ferulic acid (Peak Area)	61,265.2	63,354.3 ± 485.2	3.4
Desirability	0.98		

3.4. Determination of Greenness for the Developed Sample Preparation Method

Figure 2 illustrates the greenness of the proposed method for the solubilization of hydroxycinnamic acids in honey samples, resulting a high overall score (0.74/1.00). The non-toxicity, sustainability, renewability, and reusability of materials used (Criteria 1 and 3) contribute significantly to the green character of the method. High scores were also

obtained for sample size (Criterion 5) due to its considerable reduction as well as the low energy consumption (Criterion 8), as the use of an ultrasonic bath offers the advantage of simultaneous processing of multiple samples consuming low energy. The main penalties of the proposed procedure are related to the ex-situ sample preparation (Criterion 1), a usual practice for the sample preparation in determinations of polyphenols in diverse horticultural products by research laboratories, and the lack of integration/automation of the procedure consisting of three distinct steps (Criterion 7). The use of liquid chromatography for the analysis of hydroxycinnamic acids also influences negatively the greenness of the proposed method (Criterion 9).

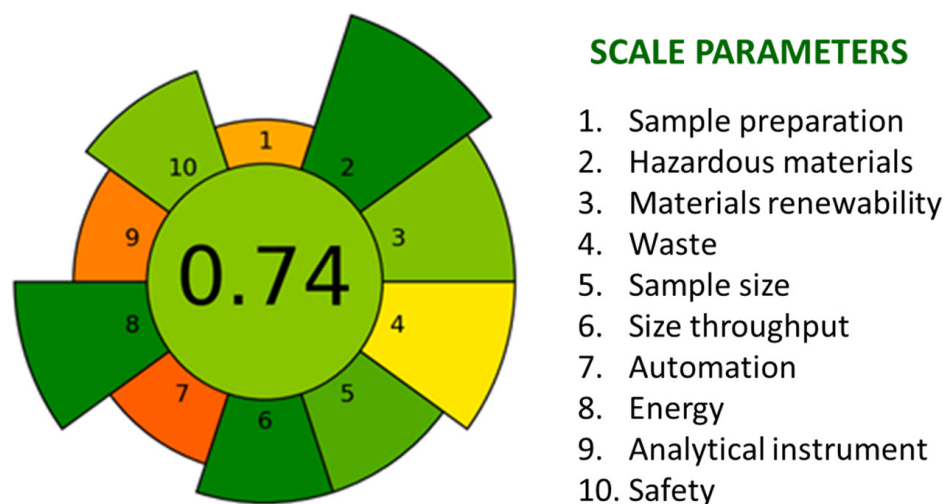


Figure 2. Graphical representation of greenness for the optimum sample preparation method of hydroxycinnamic acids in honey using AGREEprep software. Score values range from zero (considered a non-environmentally friendly approach) to one (the ideal greenest approach) and are accompanied by a color scale from red to green, respectively.

3.5. Application of Optimized Sample Preparation Method on Multi-Floral Honey

The optimized method was applied for the solubilization and determination of hydroxycinnamic acids in commercial multi-floral honey harvested in Cyprus. Multi-floral honey is the most widespread type of honey in Cyprus due to high plant diversity and the geography of the island. This is the first report describing the composition of Cypriot honey in terms of phenolic composition. Table 5 summarizes the amounts of each hydroxycinnamic acid in Cypriot honey collected from local supermarkets. Results showed that the most abundant hydroxycinnamic acid in Cypriot multi-floral honey was the *p*-coumaric acid; its content ranged from 0.35 mg kg⁻¹ to 2.59 mg kg⁻¹. A previous study on Serbian honeys also demonstrated that *p*-coumaric acid was the major phenolic constituent in multi-floral honeys; they contain *p*-coumaric acid at a concentration between 0.40 mg kg⁻¹ and 9.97 mg kg⁻¹ [34]. Recently, Kedzierska-Matysek et al. (2021) also reported that the major hydroxycinnamic acid in Polish multi-floral honeys was *p*-coumaric acid at a concentration of 1.39 mg kg⁻¹ [35]. Caffeic acid was also found in all studied honey samples. Table 5 shows that the concentration of caffeic acid in Cypriot multi-floral honey ranged from 0.46 mg kg⁻¹ to 1.22 mg kg⁻¹. The differences in the contents may be attributed to the diversity in flowering for the production of multi-floral honey [36]. This fluctuation agrees with international literature for multi-floral honey [37–39]; there are multi-floral honeys with no detectable amounts of caffeic acid and honeys with significantly high amounts (up to 20.9 mg kg⁻¹) [40]. Chlorogenic acid, an ester of caffeic acid and quinic acid, was also detected in studied honeys. The median value of chlorogenic acid content in multi-floral honey produced in Cyprus was 0.63 mg kg⁻¹. Our findings are similar to published data [34,37,39]. However, an unusually high amount of chlorogenic acid (22.14 ± 1.32 mg kg⁻¹) was determined in one sample. Ramanauskiene et al. (2012) also

described a similar high concentration of chlorogenic acid in multi-floral forest honey produced in Lithuania [41]. The high chlorogenic acid content is also correlated with honey that is produced from eucalyptus blossoms [42], thus it will be interesting to investigate the botanical origin of the sample. Rosmarinic acid was also quantified in all analyzed samples, but it is found at significantly lower concentrations. The presence of rosmarinic acid has been described in rosemary, mint, and sage honeys [43,44]. Taking into consideration the plethora of native Lamiaceae plants grown in Cyprus, the detection of rosmarinic acid in Cypriot honey is reasonable. Finally, ferulic acid was detected in half of the examined samples at low levels. Its concentration ranged between 0.02 mg kg^{-1} and 0.10 mg kg^{-1} and it was the least abundant studied component in multi-floral honey. A comparable trend was observed in Sicilian honey, though it was not found in multi-floral honeys produced in China and Spain [37,40]. Previous works reported that ferulic acid is distributed in acacia and heather honeys; these plants grow in Cyprus at areas of low altitude [41,45]. Overall, the utilization of the developed ultrasound-assisted method was suitable for the analysis of hydroxycinnamic acids in multi-floral honey produced in Cyprus, providing, for first time, valuable information.

Table 5. Contents of hydroxycinnamic acids in multi-floral honey. The results are expressed as mg hydroxycinnamic acid per kg of honey.

Samples	Caffeic Acid	Chlorogenic Acid	<i>p</i> -Coumaric Acid	Ferulic Acid	Rosmarinic Acid	Total
C1	0.84 ± 0.10	0.46 ± 0.03	0.56 ± 0.03	nd	0.16 ± 0.03	2.02
C2	0.66 ± 0.05	0.57 ± 0.05	0.94 ± 0.05	0.02 ± 0.00	0.19 ± 0.01	2.38
C3	0.83 ± 0.07	0.75 ± 0.06	0.91 ± 0.10	0.04 ± 0.01	0.21 ± 0.01	2.74
C4	0.66 ± 0.05	0.56 ± 0.05	1.00 ± 0.09	0.10 ± 0.02	0.20 ± 0.03	2.52
C5	0.97 ± 0.11	1.04 ± 0.10	1.11 ± 0.11	nd	0.23 ± 0.02	3.35
C6	1.22 ± 0.09	22.14 ± 1.32	1.64 ± 0.08	0.05 ± 0.01	0.45 ± 0.03	25.5
C7	0.46 ± 0.03	nd	0.94 ± 0.07	nd	0.16 ± 0.02	1.56
C8	0.72 ± 0.04	0.69 ± 0.04	2.39 ± 0.18	nd	0.18 ± 0.03	3.98
C9	0.30 ± 0.03	1.74 ± 0.11	2.59 ± 0.20	0.06 ± 0.01	0.30 ± 0.03	4.99
C10	1.04 ± 0.10	nd	0.35 ± 0.01	nd	0.20 ± 0.02	1.59
Range	0.46–1.22	nd–22.14	0.35–2.59	nd–0.10	0.16–0.45	1.56–25.5
Median value	0.78	0.63	0.97	0.01	0.20	2.63

nd: not detected.

4. Conclusions

The present work provides a new, miniaturized, and green sample preparation method for the solubilization of hydroxycinnamic acids in honey prior to chromatographic analysis. The proposed method combined the advantages of ultrasound irradiation and ecofriendly hydroethanolic mixture. More specifically, honey samples were mixed with 60.0% *v/v* aqueous ethanol at a solvent-to-sample ratio of 10.0 mL g^{-1} . The solubilization procedure took place in an ultrasonic bath for a period of 17.8 min at 54.6°C . The proposed method significantly decreases the preparation time, the sample size, and annihilates the volumes of organic solvents. Furthermore, the optimized sample preparation method was applied successfully for the determination of five hydroxycinnamic acids in multi-floral honey, providing, for the first time, the hydroxycinnamic acid fingerprint of honey produced in Cyprus, a Mediterranean island with high biodiversity in flora.

Author Contributions: Conceptualization, V.G.; methodology, A.C. and V.G.; investigation, K.S.; resources, V.G.; writing—original draft preparation, K.S. and V.G.; writing—review and editing, A.C.; project administration, V.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is unavailable due to privacy.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Edwards, C.H.; Rossi, M.; Corpe, C.P.; Butterworth, P.J.; Ellis, P.R. The Role of Sugars and Sweeteners in Food, Diet and Health: Alternatives for the Future. *Trends Food Sci. Technol.* **2016**, *56*, 158–166. [\[CrossRef\]](#)
2. Ayoub, W.S.; Ritu, Zahoor, I.; Dar, A.H.; Farooq, S.; Mir, T.A.; Ganaie, T.A.; Srivastava, S.; Pandey, V.K.; Altaf, A. Exploiting the Polyphenolic Potential of Honey in the Prevention of Chronic Diseases. *Food Chem. Adv.* **2023**, *3*, 100373. [\[CrossRef\]](#)
3. Palma-Morales, M.; Huertas, J.R.; Rodríguez-Pérez, C. A Comprehensive Review of the Effect of Honey on Human Health. *Nutrients* **2023**, *15*, 3056. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Aziz, Z.; Abdul Rasool Hassan, B. The Effects of Honey Compared to Silver Sulfadiazine for the Treatment of Burns: A Systematic Review of Randomized Controlled Trials. *Burns* **2017**, *43*, 50–57. [\[CrossRef\]](#)
5. Nolan, V.C.; Harrison, J.; Wright, J.E.E.; Cox, J.A.G. Clinical Significance of Manuka and Medical-Grade Honey for Antibiotic-Resistant Infections: A Systematic Review. *Antibiotics* **2020**, *9*, 766. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Hizan, N.S.; Hassan, N.H.M.; Haron, J.; Abubakar, M.B.; Mahdi, N.M.N.; Gan, S.H. Tualang Honey Adjunct with Anastrozole Improve Parenchyma Enhancement of Breast Tissue in Breast Cancer Patients: A Randomized Controlled Trial. *Integr. Med. Res.* **2018**, *7*, 322–327. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Akhbari, M.; Jabbari, M.; Ayati, M.H.; Namazi, N. The Effects of Oral Consumption of Honey on Key Metabolic Profiles in Adult Patients with Type 2 Diabetes Mellitus and Nondiabetic Individuals: A Systematic Review of Clinical Trials. *Evid.-Based Complement. Altern. Med.* **2021**, *2021*, 6666832. [\[CrossRef\]](#)
8. Idrus, R.B.H.; Sainik, N.Q.A.V.; Nordin, A.; Bin Saim, A.; Sulaiman, N. Cardioprotective Effects of Honey and Its Constituent: An Evidence-Based Review of Laboratory Studies and Clinical Trials. *Int. J. Environ. Res. Public Health* **2020**, *17*, 3613. [\[CrossRef\]](#)
9. Mazruei Arani, N.; Emam-Djomeh, Z.; Tavakolipour, H.; Sharafati-Chaleshtori, R.; Soleimani, A.; Asemi, Z. The Effects of Probiotic Honey Consumption on Metabolic Status in Patients with Diabetic Nephropathy: A Randomized, Double-Blind, Controlled Trial. *Probiotics Antimicrob. Proteins* **2019**, *11*, 1195–1201. [\[CrossRef\]](#)
10. Rasad, H.; Entezari, M.H.; Ghadiri, E.; Mahaki, B.; Pahlavani, N. The Effect of Honey Consumption Compared with Sucrose on Lipid Profile in Young Healthy Subjects (Randomized Clinical Trial). *Clin. Nutr. ESPEN* **2018**, *26*, 8–12. [\[CrossRef\]](#)
11. Yahaya, R.; Zahary, M.N.; Othman, Z.; Ismail, R.; Nik Him, N.A.S.; Abd Aziz, A.; Dahlan, R.; Jusoh, A.F. Tualang Honey Supplementation as Cognitive Enhancer in Patients with Schizophrenia. *Heliyon* **2020**, *6*, e03948. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Cooper, R.A.; Fehily, A.M.; Pickering, J.E.; Erusalimsky, J.D.; Elwood, P.C. Honey, Health and Longevity. *Curr. Aging Sci.* **2010**, *3*, 239–241. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Shamshuddin, N.S.S.; Mohd Zohdi, R. Gelam Honey Attenuates Ovalbumin-Induced Airway Inflammation in a Mice Model of Allergic Asthma. *J. Tradit. Complement. Med.* **2018**, *8*, 39–45. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Ebrahimi, M.; Ebrahimi, M.; Karimi, M.; Rezaiean, A.; Reza Kazemi, M. Effects of Dietary Honey AndArdehCombination on Chemotherapy-Induced Gastrointestinal and Infectious Complications in Patients with Acute Myeloid Leukemia: A Double-Blind. *Iran. J. Pharm. Res.* **2016**, *15*, 661. [\[PubMed\]](#)
15. McLoone, P.; Oluwadun, A.; Warnock, M.; Fyfe, L. Honey: A Therapeutic Agent for Disorders of the Skin. *Cent. Asian J. Glob. Health* **2016**, *5*, 241. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Hossen, M.S.; Ali, M.Y.; Jahurul, M.H.A.; Abdel-Daim, M.M.; Gan, S.H.; Khalil, M.I. Beneficial Roles of Honey Polyphenols against Some Human Degenerative Diseases: A Review. *Pharmacol. Rep.* **2017**, *69*, 1194–1205. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Dong, R.; Zheng, Y.; Xu, B. Phenolic Profiles and Antioxidant Capacities of Chinese Unifloral Honeys from Different Botanical and Geographical Sources. *Food Bioproc Tech.* **2013**, *6*, 762–770. [\[CrossRef\]](#)
18. Gašić, U.M.; Milojković-Opsenica, D.M.; Tešić, Ž.L. Polyphenols as Possible Markers of Botanical Origin of Honey. *J. AOAC Int.* **2017**, *100*, 852–861. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Kaškonienė, V.; Venskutonis, P.R. Floral Markers in Honey of Various Botanical and Geographic Origins: A Review. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 620–634. [\[CrossRef\]](#)
20. Vazquez, L.; Armada, D.; Celeiro, M.; Dagnac, T.; Llompарт, M. Evaluating the Presence and Contents of Phytochemicals in Honey Samples: Phenolic Compounds as Indicators to Identify Their Botanical Origin. *Foods* **2021**, *10*, 2616. [\[CrossRef\]](#)
21. Andrade, P.; Ferreres, F.; Gilb, M.I.; Tomk+barberhn, F.A. Determination of Phenolic Compounds in Honeys with Different Floral Origin by Capillary Zone Electrophoresis. *Food Chem.* **1997**, *60*, 79–84. [\[CrossRef\]](#)
22. Wang, J.; Xue, X.; Du, X.; Cheng, N.; Chen, L.; Zhao, J.; Zheng, J.; Cao, W. Identification of Acacia Honey Adulteration with Rape Honey Using Liquid Chromatography–Electrochemical Detection and Chemometrics. *Food Anal. Methods* **2014**, *7*, 2003–2012. [\[CrossRef\]](#)
23. Zhang, X.H.; Wang, M.J.; Liu, R.J.; Qing, X.D.; Nie, J.F. Green Sample Preparation Techniques and Their Use in the Extraction and Separation Analysis of Phenolic Compounds in Honey. *Sep. Purif. Rev.* **2023**, *2023*, 1–18. [\[CrossRef\]](#)

24. Pedisić, S.; Čulina, P.; Pavlešić, T.; Vahčić, N.; Elez Garofulić, I.; Zorić, Z.; Dragović-Uzelac, V.; Repajić, M. Efficiency of Microwave and Ultrasound-Assisted Extraction as a Green Tool for Polyphenolic Isolation from Monofloral Honey. *Processes* **2023**, *11*, 3141. [\[CrossRef\]](#)
25. Cooper, R.; Molan, P.; Harding, K. The Sensitivity to Honey of Gram-Positive Cocci of Clinical Significance Isolated from Wounds. *J. Appl. Microbiol.* **2002**, *93*, 857–863. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Combarros-Fuertes, P.; Estevinho, L.M.; Dias, L.G.; Castro, J.M.; Tomás-Barberán, F.A.; Tornadijo, M.E.; Fresno-Baro, J.M. Bioactive Components and Antioxidant and Antibacterial Activities of Different Varieties of Honey: A Screening Prior to Clinical Application. *J. Agric. Food Chem.* **2019**, *67*, 688–698. [\[CrossRef\]](#)
27. Wojnowski, W.; Tobiszewski, M.; Pena-Pereira, F.; Psillakis, E. AGREEprep—Analytical Greenness Metric for Sample Preparation. *TrAC-Trends Anal. Chem.* **2022**, *149*, 116553. [\[CrossRef\]](#)
28. Siddiqui, S.A.; Ali Redha, A.; Salauddin, M.; Harahap, I.A.; Rupasinghe, H.P.V. Factors Affecting the Extraction of (Poly)Phenols from Natural Resources Using Deep Eutectic Solvents Combined with Ultrasound-Assisted Extraction. *Crit. Rev. Anal. Chem.* **2023**, *2023*, 1–22. [\[CrossRef\]](#)
29. Luo, X.; Cui, J.; Zhang, H.; Duan, Y.; Zhang, D.; Cai, M.; Chen, G. Ultrasound Assisted Extraction of Polyphenolic Compounds from Red Sorghum (*Sorghum bicolor* L.) Bran and Their Biological Activities and Polyphenolic Compositions. *Ind. Crops Prod.* **2018**, *112*, 296–304. [\[CrossRef\]](#)
30. Zuorro, A. Optimization of Polyphenol Recovery from Espresso Coffee Residues Using Factorial Design and Response Surface Methodology. *Sep. Purif. Technol.* **2015**, *152*, 64–69. [\[CrossRef\]](#)
31. Belwal, T.; Dhyani, P.; Bhatt, I.D.; Rawal, R.S.; Pande, V. Optimization Extraction Conditions for Improving Phenolic Content and Antioxidant Activity in Berberis Asiatica Fruits Using Response Surface Methodology (RSM). *Food Chem.* **2016**, *207*, 115–124. [\[CrossRef\]](#)
32. Dzah, C.S.; Duan, Y.; Zhang, H.; Wen, C.; Zhang, J.; Chen, G.; Ma, H. The Effects of Ultrasound Assisted Extraction on Yield, Antioxidant, Anticancer and Antimicrobial Activity of Polyphenol Extracts: A Review. *Food Biosci.* **2020**, *35*, 100547. [\[CrossRef\]](#)
33. Christou, A.; Parisi, N.A.; Venianakis, T.; Barbouti, A.; Tzakos, A.G.; Gerothanassis, I.P.; Goulas, V. Ultrasound-Assisted Extraction of Taro Leaf Antioxidants Using Natural Deep Eutectic Solvents: An Eco-Friendly Strategy for the Valorization of Crop Residues. *Antioxidants* **2023**, *12*, 1801. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Gašić, U.; Kečkeš, S.; Dabić, D.; Trifković, J.; Milojković-Opsenica, D.; Natić, M.; Tešić, Z. Phenolic Profile and Antioxidant Activity of Serbian Polyfloral Honey. *Food Chem.* **2014**, *145*, 599–607. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Kędzierska-Matyssek, M.; Strycka, M.; Teter, A.; Skalecki, P.; Domaradzki, P.; Florek, M. Relationships between the Content of Phenolic Compounds and the Antioxidant Activity of Polish Honey Varieties as a Tool for Botanical Discrimination. *Molecules* **2021**, *26*, 1810. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Becerril-sánchez, A.L.; Quintero-salazar, B.; Dublán-garcía, O.; Escalona-buendía, H.B. Phenolic Compounds in Honey and Their Relationship with Antioxidant Activity, Botanical Origin, and Color. *Antioxidants* **2021**, *10*, 1700. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Lo Dico, G.M.; Ulrici, A.; Pulvirenti, A.; Cammilleri, G.; Macaluso, A.; Vella, A.; Giaccone, V.; Lo Cascio, G.; Graci, S.; Scuto, M.; et al. Multivariate Statistical Analysis of the Polyphenols Content for the Discrimination of Honey Produced in Sicily (Southern Italy). *J. Food Compos. Anal.* **2019**, *82*, 103225. [\[CrossRef\]](#)
38. Can, Z.; Yıldız, O.; Sahin, H.; Akyuz Turumtay, E.; Silici, S.; Kolaylı, S. An Investigation of Turkish Honey: Their Physico-Chemical Properties, Antioxidant Capacities and Phenolic Profiles. *Food Chem.* **2015**, *180*, 133–141. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Socha, R.; Juszczak, L.; Pietrzyk, S.; Gałkowska, D.; Fortuna, T.; Witczak, T. Phenolic Profile and Antioxidant Properties of Polish Honey. *Int. J. Food Sci. Technol.* **2011**, *46*, 528–534. [\[CrossRef\]](#)
40. Cheung, Y.; Meenu, M.; Yu, X.; Xu, B. Phenolic Acids and Flavonoids Profiles of Commercial Honey from Different Floral Sources and Geographic Sources. *Int. J. Food Prop.* **2019**, *22*, 290–308. [\[CrossRef\]](#)
41. Ramanauskienė, K.; Stelmakienė, A.; Briedis, V.; Ivanauskas, L.; Jakštas, V. The Quantitative Analysis of Biologically Active Compounds in Lithuanian Honey. *Food Chem.* **2012**, *132*, 1544–1548. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Yao, L.; Jiang, Y.; Singanusong, R.; Datta, N.; Raymont, K. Phenolic Acids and Abscissic Acid in Australian Eucalyptus Honey and Their Potential for Floral Authentication. *Food Chem.* **2004**, *86*, 169–177. [\[CrossRef\]](#)
43. Pavlešić, T.; Poljak, S.; Mišetić Ostojić, D.; Lučin, I.; Reynolds, C.A.; Kalafatovic, D.; Saftić Martinović, L. Mint (*Mentha* spp.) Honey: Analysis of the Phenolic Profile and Antioxidant Activity. *Food Technol. Biotechnol.* **2022**, *60*, 509–519. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Gašić, U.M.; Natić, M.M.; Mišić, D.M.; Lušić, D.V.; Milojković-Opsenica, D.M.; Tešić, Ž.L.; Lušić, D. Chemical Markers for the Authentication of Unifloral *Salvia officinalis* L. Honey. *J. Food Compos. Anal.* **2015**, *44*, 128–138. [\[CrossRef\]](#)
45. Andrade, P.; Ferreres, F.; Teresa Amaral, M. Analysis of Honey Phenolic Acids by HPLC, Its Application to Honey Botanical Characterization. *J. Liq. Chromatogr. Relat. Technol.* **1997**, *20*, 2281–2288. [\[CrossRef\]](#)

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.