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Phenolic Class Analysis in Honey: Comparison of Classical and Single UV Spectrum Methodologies

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Abstract: The analytical results from a study of 16 honey samples (extra white to dark honey color range) of phenolic compounds obtained using the single UV spectrum methodology and classical spectrophotometric methods (Folin–Ciocalteu and $AlCl_3$ methods) are presented. The first method quantified all classes of phenolic compounds in honey's SPE-C18 extract: the total hydroxybenzoic acid content (concentrations between 0.37 ± 0.05 and 4.46 ± 0.37 mg of gallic acid/g of honey), total hydroxycinnamic acid content (0.13 ± 0.03 and 2.76 ± 0.13 mg of ferulic acid/g of honey), and total flavonoid content (0.15 ± 0.03 and 1.63 ± 0.17 mg of quercetin/g of honey). The total phenolic contents were, on average, 1.86 ± 0.72 and 1.78 ± 0.79 times higher than the results obtained for raw honey and the SPE-C18 extract, respectively, using the classical Folin–Ciocalteu method. The total flavonoid contents, on average, were 6.02 ± 3.14 times larger and 0.66 ± 0.33 times smaller than the results obtained using the classical $AlCl_3$ method for raw honey and SPE-C18 extract, respectively.

Keywords: raw honey; honey SPE-C18 extracts; phenolic compounds groups; single UV spectrum methodology; Folin–Ciocalteu method; $AlCl_3$ method

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1. Introduction

Honey is a natural product produced by *Apis mellifera*, which contains a highly concentrated solution of sugars, mostly fructose and glucose. It also presents lower amounts of phenolic acids, flavonoids, minerals, proteins, vitamins, organic acids, enzymes, and other phytochemicals [1–3]. The overall quantity of these compounds varies according to the floral and geographical origin of honey, influencing its color and flavor [2,4,5].

The phenolic compounds of honey are classified into two main families: phenolic acids, including their derivatives, and flavonoids [6]. Phenolic acids are divided into two subclasses: benzoic acids and cinnamic acids. Flavonoids are divided into three classes with similar structures: flavonols, flavones, and flavanones [5]. An overview of the large variety of phenolic compounds present in monofloral honeys can be found in the work performed by Lawag et al. [7]. These compounds are not only valuable for detecting honey adulteration [8] but also for authenticating honey varieties, as demonstrated in studies using biochemical markers like total phenolic and flavonoid content to differentiate between honeys from various botanical origins, including blossom and honeydew honeys [9]. Their presence is intricately linked to the bioactive properties of honey, particularly its antioxidant capacity, which enhances the potential medicinal applications of various honey types [10,11]. In addition to contributing to antioxidant activity, these phenolic compounds, alongside ascorbic acid, catalase, peroxidase, carotenoids, and Maillard reaction products, play a pivotal role in honey's overall bioactivity [1,3,12].

These compounds are also responsible for the biological properties of honey, such as its antibacterial, anticarcinogenic, anti-inflammatory, antiatherogenic, anti-allergic, antithrombotic, immune-modulating, and analgesic activities, among others [3,5,13].

So, to infer honey's properties, analyzing honey's phenolic compounds is a critical step [14] due to the high amount of sugar, which usually demands the pre-concentration of these analytes before analysis [3,6,15,16]. Three steps are frequently necessary to determine individual phenolic compounds: extraction from the sample, analytical separation, and quantification [3,17]. The most commonly used methodology to concentrate phenolic compounds from honey is liquid–liquid extraction (LLE) with organic solvents and solid-phase extraction (SPE) [3,15,16,18]. This first methodology has disadvantages, including being expensive and time-consuming and using large amounts of solvents [18].

There are several analytical separation methods used to analyze the phenolic compounds in honey: thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE), among others [3,6,17]. These four methodologies can be used to identify the phenolic compound composition of samples, and the last three methods can be used to quantify them.

Also, spectrophotometric methods, usually colorimetric, are applied for the determination of honey's phenolic compounds: the total phenolic content (TPC) and total flavonoid content (TFC). The most common method for the determination of TPC is the Folin–Ciocalteu procedure, and the TFC is generally determined based on the formation of aluminum–flavonoid complexes ($AlCl_3$ method).

The Folin–Ciocalteu method is not very selective [14], since it determines the total reducing capacity not only of phenolic compounds but also any reducing agent and the color reaction that can take place with any oxidizable phenolic hydroxy group such as aromatic amines, ascorbic acid, Cu(I), and Fe(II) [4,19]. Due to this, several authors [20,21] explain that it is not appropriate to measure the total phenol content until interfering agents, such as sugars, are removed. In the case of honey samples, the extraction of phenolic compounds from honey with SPE-C18 should be considered [22,23]. The colorimetric $AlCl_3$ method, based on the formation of a composite between the aluminum ion Al(III) and the carbonyl and hydroxyl groups of flavonoids [24], also suffers from interferences, such as the method referred to by Raval et al. [25], which showed that non-flavonoid compounds bearing the o-diphenol group represent a serious interference.

However, another methodology can also be applied that uses a single UV spectrum analysis to simultaneously determine the total phenolic content, total flavonoid content, and hydroxycinnamic acid and hydroxybenzoic acid content in a single run. Several works inspired this study, such as those by Mazza et al. [26] (wine analysis), Obied et al. [27] (olive mill waste analysis), and Paula et al. [28] (propolis analysis). These works are relevant since the analysis of hydroxybenzoic acid and hydroxycinnamic acid classes is not often performed in studies of phenolic compounds, whose information may be important to explain the biological properties of the extracts of phenolic compounds. For instance, Spilioti et al. [29] showed that Greek honeys, rich in phenolic acids, exhibit significant antioxidant, anticancer, and antiatherogenic activities, which may be attributed, at least in part, to their phenolic acid content. Combarros-Fuertes et al. [30] reported that honey might become an alternative in combating multi-drug-resistant bacteria due, particularly, to its low pH values and the presence of hydrogen peroxide; phenolic acids; and flavonoids, methylglyoxal, and Bee Defensin-1 Peptide. In Portuguese honey extracted with Amberlite XAD-2, Estevinho et al. [5] found that the antioxidant and antimicrobial activities resulted mainly from the presence of phenolic compounds, particularly *p*-hydroxibenzoic acid, cinnamic acid, naringenin, pinocembrin, and chrysin.

The significant interest in the phenolic acids' properties [31] is associated with their preventive effect against a variety of chronic diseases, such as cancer [32] and cardiovascular diseases [33]. However, these properties are not evaluated considering the total levels of hydroxybenzoic and hydroxycinnamic acids present (contents rarely established) but are generally based on the profile of phenolic acids obtained by HPLC [5,29,30,34].

Considering this, the objective of this study was to compare results from the single UV spectrum methodology, which quantifies all classes of phenolic compounds (hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids) and TPC in honey's SPE-C18 extract, with two classical methods for analyzing total phenolic compounds (Folin–Ciocalteu method) and total flavonoids ($AlCl_3$ method) in raw honey and honey's SPE-C18 extract.

2. Materials and Methods

All reagents were of analytical quality and used as purchased. The following compounds were used as standards: gallic acid (1-Hidrate) from Panreac (99%, Barcelona, Spain), ferulic acid from Fluka (Buchs, Switzerland), and quercetin from Sigma-Aldrich (>95%, Hoeilaart, Belgium). The solvents used were ethanol acquired from Panreac (HPLC quality, Barcelona, Spain, 99.9%) and methanol from Fisher Chemical (HPLC quality, Leics, UK, >99.9%).

Other reagents were concentrated hydrochloric acid (HCl) from Carlo Erba (Val de Reuil, France, 37% and d = 1,18), sodium carbonate (Na_2CO_3) from Merck (Darmstadt, Germany), Folin–Ciocalteu reagent from Panreac (Barcelona, Spain, d = 1.234), aluminum chloride ($AlCl_3$) from Merck (Darmstadt, Germany), (85%, Barcelona, Spain), sodium nitrite ($NaNO_2$) from Himedia (Mumbai, India), and sodium hydroxide (NaOH) from José Manuel Gomes dos Santos (Odivelas, Portugal). Type II deionized water was used in all analytical work.

For the extraction of phenolic compounds, solid-phase extraction (SPE) was applied using SPE C18 ec Chromabond from Macherey-Nagel (polypropylene column with polyethylene filter elements with silica gel-based octadecyl phase, endcapped, 45 µm particle size, 3 mL column volume, and 200 mg filling quantity, Hoerdt, France).

All analyses were performed in triplicate, and all aqueous solutions were prepared using type II deionized water.

2.1. Samples of Honey

In this work, 16 commercial honeys were purchased from the local market. The samples were stored in the dark at room temperature until analysis. Table 1 presents information on the labels regarding the geographical origin and floral predominance (defined by pollinic analysis).

Table 1. Honeys' origin and floral predominance.

Honey Samples	Geographic Origin in Portugal	Floral Predominance
H1	Vila Velha de Rodão	Monofloral Rosemary
H2	Serra da Estrela	Monofloral Orange
H3	Leiria	Monofloral Echium
H4	Mirandela	Multifloral
H5	Mirandela	Monofloral Lavandula
H6	Palmela	Monofloral Rosemary
H7	Macedo de Cavaleiros	Multifloral
H8	Porto	Multifloral Wild Flowers
H9	Porto	Monofloral Eucalyptus
H10	Arcos de Valdevez	Multifloral, predominance of Chestnut and Heather
H11	Boticas	Monofloral Heather
H12	Boticas	Monofloral Heather
H13	Serra da Estrela	Monofloral Orange
H14	Bragança	Multifloral, predominance of Rosemary
H15	Bragança	Multifloral, predominance of Heather
H16	Bragança	Multifloral, predominance of Heather

2.2. Determination of Color

Honeys' color was determined according to the spectrophotometric method described by Montenegro et al. [35]. In a 50 mL Falcon tube, 5 g of the honey samples was weighed and dissolved in 10 mL of water. The prepared solutions were placed in plastic cuvettes and allowed to stand for 10 to 15 min. The absorbance on the UV-Vis spectrophotometer (UV-3100PC spectrophotometer, VWR International LLC, Avantor, Radnor, PA, USA) was read at 635 nm using water as blank solution. To determine the honey's color on the mm Pfund scale, the absorbance value was converted using the following equation (White, 1984) [36]:

$$\text{mm Pfund} = -38.70 + 371.39 \times \text{Absorbance} \tag{1}$$

The color classification was conducted using the scale defined by the U.S. Department of Agriculture (USDA) [37], which categorizes honey into seven color groups based on their Pfund value range: water white color for values from 0 to 8 mm Pfund, extra white color for 8 to 16.5 mm Pfund, white color for 16.5 to 34 mm Pfund, extra light amber color for 34 to 50 mm Pfund, light amber color for 50 to 85 mm Pfund, amber color for 85 to 114 mm Pfund, and dark color for values more than 114 mm Pfund.

2.3. Determination of Total Phenolic Compound Content

The total phenolic compound content (TPC) of honey was determined using the Folin–Ciocalteu spectrophotometric method (FC method) modified by Singleton et al. [21]. Preparation involved dissolving 10 g of honey in acidified water at pH 2, making up a volume of 25 mL, except for the dark-colored honeys, where 1 g of honey was dissolved in 20 mL. For the analysis, 0.5 mL of the honey solution was pipetted and mixed with 2.5 mL of Folin–Ciocalteu (10%, v/v) and 2.0 mL of sodium carbonate solution (Na_2CO_3, 75 g/L). The reaction was left in the dark for 2 h, after which absorbance was read at 750 nm (UV-3100PC spectrophotometer, VWR). To perform the calculations of TPC_{FC} levels, a calibration curve with the gallic acid (standard) was used, and the final results were expressed as mg of gallic acid per g of honey.

2.4. Determination of Total Flavonoid Compound Content

The total flavonoid content (TFC) was determined spectrophotometrically using aluminum chloride method ($AlCl_3$ method), as described by Zhishen et al. [24]. A total of 10 g of honey was weighed and diluted into 25 mL of acidified water at pH 2. For the analysis, 1 mL of the honey solution was pipetted and mixed with 4 mL of water. Then, 0.3 mL of 5% (w/v) sodium nitrite ($NaNO_2$) was added. After 5 min, 0.3 mL of 10% (w/v) aluminum chloride ($AlCl_3$) was added, and after 6 min, 2 mL of 1 M sodium hydroxide (NaOH) was added. The final volume was adjusted to 10 mL by adding 2.4 mL of water. The reaction was left in the dark for 1 h, after which absorbance was measured at 510 nm (UV-3100PC spectrophotometer, VWR). A calibration curve with quercetin (standard) was used to calculate TFC_{AlCl3} levels, with results expressed as mg of quercetin per g of honey. It should be noted that some authors suggest that catechin is the most suitable standard for the analysis of total flavonoids [38,39]. Another important aspect to consider, as mentioned by Sancho et al. [40], is the composition of the blank and the need for color correction in the analysis of total flavonoid content in honey.

2.5. Calibration and Analysis of Phenolic Classes

For the determination of different phenolic classes using a single spectrum, the method described by Mazza et al. [26] and by Obied et al. [27] for spectral analysis was applied.

Calibration solutions. The hydroxybenzoic acids, hydroxycinnamic acids, and flavonoid groups were represented by three standard compounds: gallic acid; ferulic acid, and quercetin, respectively. Precisely 0.02 g of each compound was weighed and dissolved in 80% ethanol (20% water) in 20 mL volumetric flasks, except for gallic acid, which was dissolved in deionized water. For the multivariate calibration, 25 standard solutions were prepared to obtain the UV-Vis spectra in the wavelength range from 190 to 600 nm, using 2% aqueous HCl to adjust the final volume. A fractional factorial experimental design was used to ensure that the concentrations of the three compounds were orthogonal (not correlated). This approach also allowed for the determination of another quantitative parameter: the total phenolic compound content. The concentrations of the 25 standard solutions (see Table S1, Supplementary Data) varied between 9.1 and 73.1 mg/L of gallic acid, 10.6 and 84.8 mg/L of ferulic acid, 10.2 and 61.2 mg/L of quercetin, and 40.5 and 197.9 mg/L of TPC. The TPC was determined as mg of mix phenolic compounds (gallic acid, ferulic acid, and quercetin) per liter.

Repeatability and recovery assays. To evaluate method precision, repeatability assays (5 repetitions) were conducted using a mixed standard solution prepared by dissolving 0.1 g of each standard compound in the same 20 mL volumetric flask using 2% aqueous HCl to adjust the final volume. Recovery assays were used to assess the accuracy of sample analysis, aiming to verify whether the honey extract matrix allows for an additive effect when phenolic compounds are added. These assays were conducted on all honey samples analyzed in this study by mixing the spiked honey extract solutions in a 5:1 ratio. The recovery was calculated as follows:

$$R\% = \frac{[C_T - C_S]}{C_A} \times 100 \tag{2}$$

where C_T is the concentration detected in the spiked sample, C_S is the concentration detected in the sample before the spiking, and C_A is the true added concentration. Evaluating the matrix effect through recovery is essential when linearity, accuracy, and precision are assessed using standard solutions, especially given the high variability in the honey matrix. Satisfactory recoveries provide confidence in the accuracy of the experimental method in honey analysis [41].

UV-Vis spectrum. The procedure for obtaining spectra for all solutions (mixture standard solutions, honey solutions, repeatability assays, and spiked honey solutions) was the same, as described by Obied et al. [27]. Into 10 mL volumetric flasks, 1 mL of a 96% ethanolic solution containing 0.1% HCl and 1 mL of the final extract solution of honey or standard solution were added, and the volume was adjusted with 2% HCl. The UV-Vis spectrum was then obtained by scanning wavelengths between 190 and 600 nm (UV-3100PC spectrophotometer, VWR), with a quartz cuvette, a scan step of 1.0 nm, a recording speed of 35 nm/s, and an absorbance measuring reproducibility of 0.002. The spectral data obtained from the mixed standard solutions were used to obtain the calibration models as defined in the work of Paula et al. [28], which are discussed in the Section 2.7. These models were applied to determine honey's total content of hydroxybenzoic acids ($THBA_{UV}$), hydroxycinnamic acids ($THCA_{UV}$), and flavonoid compounds (TFC_{UV}) groups (biophenols groups), expressed as mg of the standard compound (gallic acid, ferulic acid, and quercetin, respectively) per liter of solution. The total phenolic compound content (TPC_{UV}) was determined as mg of mixed phenolic compounds (gallic acid, ferulic acid, and quercetin) per liter of solution (mg mix PC/L). The results from the mixed standard solution, used as an independent quality control solution (reference solution), allowed the determination of precision through repeatability assays. Accuracy was assessed using the results of the spiked honey solutions.

2.6. Extraction of Phenolic Compounds from Honey

Extraction of phenolic compounds from honey was performed as described by Truchado et al. [22], with some modifications, using the SPE-C18 procedure to reduce possible interference from sugars and other organic compounds. For extraction, a manifold chamber (Varian) and SPE columns (C18ec Chromabond) were used. Honey solutions were prepared by dissolving 10 g of honey in 50 mL of HCl solution at pH 2. The SPE-C18 columns were activated with 10 mL of methanol followed by 10 mL of deionized water. After activation, each honey solution was passed through a SPE column using the entire volume. The column was then washed with 10 mL of deionized water, and the phenolic compounds were extracted with 4 mL of methanol, which underwent a final dilution to obtain the honey's final extract solution. The final volume (10, 20, 25, or 50 mL) of this solution was selected to ensure the UV spectrum was within the limits defined by the standard calibration solutions, introduced in Section 2.5. The honey's final extract solution was used to determine the following: TPC_{FC} and TFC_{AlCl3} according to the methodology described in Sections 2.3 and 2.4; the different bio phenolic classes, as explained in Section 2.5 for honey analysis; and the recovery assays. All results were presented relative to the initial honey mass.

2.7. Statistical Analysis

All statistical analyses were performed with R software (R version 3.3.2, 2016-10-31), a free software environment for statistical computing and graphics.

Multivariate calibration: For the multivariate calibration, a multilevel fractional factorial design ($5^{k-(k-2)}$, where k is the number of factors and 5 is the number of concentration levels) was applied [42]. This experimental design involves mixing three factors and five levels (concentrations), ensuring that the compound concentrations in the mixing solutions are uncorrelated or orthogonal to each other (see Table S1, Supplementary Data). The data matrix was divided into training group (20 solution, 80%) and a test group (5 solutions, 20%) using the k-means clustering algorithm (package prospectr) [43] to verify that the calibration models obtained from the training group data have predictive power over the test group data, which were not used in the model adjustment.

Multiple and simple linear regression. To determine the total content of hydroxybenzoic acids, hydroxycinnamic acids, flavonoids compounds, and the total phenolic compounds from the UV spectrum, the equations presented in a previous work [28] were used. The analysis of TFC_{UV} (results in mg of quercetin per L of solution) was conducted by measuring the solution's absorbance at 375 nm and using the simple linear regression (a_1, coefficient of the linear model):

$$C_{Quercetin} = a_1 \times Abs_{375} \tag{3}$$

Using multiple linear regression (a_1 and a_2 represent coefficients of the multiple linear model), the determination of $THBA_{UV}$ (results in mg of gallic acid per L of solution) followed the following equation:

$$C_{GallicAcid} = a_1 \times Abs_{275} + a_2 \times Abs_{345} \tag{4}$$

For the $THCA_{UV}$ (results in mg of ferulic acid per L of solution), the calibration used the following equation:

$$C_{FerulicAcid} = a_1 \times Abs_{325} + a_2 \times Abs_{380} \tag{5}$$

and the TPC_{UV} (results in mg of mixed phenolic compounds per L of solution) used

$$C_{TPC} = a_1 \times Abs_{220} + a_2 \times Abs_{345} \tag{6}$$

To evaluate the predictive capacity of these models, a simple linear regression model was established between the concentrations predicted by each model and real values for train and test data groups, which resulted from the division of the 25 mixing solutions (expected concentrations are shown in Table S1, Supplementary Data). The results are considered satisfactory if the linear regression parameters are close to the theoretical values [44,45]: 'zero' (0) for root square error (RSE) and intercept; 'one' (1) for slope and the determination coefficient (R^2). Additionally, the 95% confidence interval for the slope and intercept can be used to confirm whether they can be statistically considered as the theoretical values of 'one' and 'zero,' respectively. Model validation was performed by checking randomness and normality of the residuals; cook's distance (values greater than 1 indicate that these are excessively influential in the model); leverage values (values below

0.2 are acceptable, values between 0.2 and 0.5 are risky, and values higher than 0.5 indicate the presence of an influential value or outlier); model's *p*-value (to evaluate the significance of the model obtained using the significance level of 0.05); determination coefficient value (R^2, to assess the amount of variance explained by the model); and relative standard error (RSE, to confirm the magnitude of the model errors) [46].

3. Results and Discussion

3.1. Honey Color

Honey color was determined spectrophotometrically, and the results in mm Pfund scale, along with the corresponding color classification, are included in Table 2.

Table 2. Results of total phenolic and flavonoid content in raw honeys and honey SPE extracts.

	Raw Honey				Honey SPE Extract	
Honey Samples	**Color mm Pfund**	**Color**	**TPC_{FC} (mg GA/g)**	**TFC_{AlCl3} (mg Q/g)**	**TPC_{FC} (mg GA/g)**	**TFC_{AlCl3} (mg Q/g)**
H1	11.44	Extra White	1.19 ± 0.12	0.046 ± 0.002	0.73 ± 0.06	0.37 ± 0.03
H2	27.04	White	1.02 ± 0.10	0.042 ± 0.002	0.88 ± 0.02	0.50 ± 0.03
H3	54.52	Light Amber	1.42 ± 0.06	0.083 ± 0.005	1.34 ± 0.03	0.93 ± 0.07
H4	71.23	Light Amber	1.82 ± 0.10	0.067 ± 0.002	1.62 ± 0.02	0.94 ± 0.07
H5	57.86	Light Amber	1.94 ± 0.07	0.135 ± 0.005	2.12 ± 0.06	1.62 ± 0.10
H6	23.32	White	1.09 ± 0.04	0.041 ± 0.003	0.74 ± 0.03	0.55 ± 0.02
H7	84.60	Light Amber	2.10 ± 0.09	0.109 ± 0.003	2.01 ± 0.08	0.76 ± 0.07
H8	67.15	Light Amber	1.72 ± 0.10	0.127 ± 0.005	2.13 ± 0.02	1.23 ± 0.09
H9	101.31	Amber	2.01 ± 0.15	0.142 ± 0.005	2.12 ± 0.02	0.91 ± 0.05
H10	106.14	Amber	2.37 ± 0.04	0.105 ± 0.004	3.13 ± 0.09	1.20 ± 0.15
H11	153.68	Dark	3.08 ± 0.06	0.092 ± 0.006	3.87 ± 0.37	0.98 ± 0.13
H12	178.56	Dark	3.72 ± 0.07	0.363 ± 0.017	6.13 ± 0.22	1.71 ± 0.22
H13	36.32	Extra Light Amber	1.43 ± 0.10	0.087 ± 0.005	1.64 ± 0.03	0.64 ± 0.07
H14	72.35	Light Amber	2.18 ± 0.04	0.114 ± 0.008	3.34 ± 0.07	1.15 ± 0.04
H15	130.65	Dark	2.66 ± 0.11	0.175 ± 0.017	2.90 ± 0.07	1.20 ± 0.04
H16	156.65	Dark	2.87 ± 0.07	0.298 ± 0.016	2.98 ± 0.12	1.65 ± 0.13

SPE—solid-phase extraction; TPC_{FC}—total phenolic content (mg/g, expressed as mg of gallic acid per g of honey) measured using Folin–Ciocalteu method; TFC_{AlCl3}—total flavonoid content (mg/g, expressed as mg of quercetin per g of honey) measured using AlCl3 method.

The honeys used in this work showed a high variability in color, ranging from extra white to dark, based on the values obtained on the Pfund mm scale (continuous scale), which ranged from 11 to 179 mm Pfund. This ensures variability within samples since physicochemical properties, including color, are influenced by the nectar source and botanical origin [9].

3.2. Folin–Ciocalteu and $AlCl_3$ Methods

The mean analytical results obtained for TPC_{FC} and TFC_{AlCl3} composition in raw honeys and SPE honey extracts are also shown in Table 2. The results of the raw honey analysis were satisfactory since all mean values showed relative standard errors of less than 5%. Overall, the magnitude of the results was in agreement with those reported in other studies, where it has been seen that honey samples exhibit significant variability in TFC (measured using the $AlCl_3$ method), potentially ranging from 0.97 to 289.0 mg/100 g of honey, and in TPC (assessed using the Folin–Ciocalteu method), with values varying from 7.57 to 289.8 mg/100 g of honey [47–50]. Some samples exceed the TPC presented ranges but were considered acceptable, as the results of the Folin–Ciocalteu method depend on honey's complex composition, particularly fluctuations in sugar and water content, as well as the conditions under which the method is applied [51]. The TPC_{FC} higher values obtained from the analysis of the SPE extracts showed a linear relationship with those of the raw honeys, with a slope of 1.71 ± 0.16, an intercept of −1.13 ± 0.34, and a correlation coefficient of 0.94. For flavonoid compounds, the TFC_{AlCl3} results of the SPE extracts were much higher than expected, indicating that the extraction procedure greatly influenced this analysis. In general, the concentrations were 4.7 to 14.1 times higher than those obtained in raw honey. Due to this high variability, the linear relationship showed a low correlation coefficient (R = 0.81), a slope of 3.76 ± 0.72, and an intercept of 0.54 ± 0.11. It is also important to note that the relationship between color and TPC obtained using the Folin–Ciocalteu method presented a linear relationship identical to that reported in the literature, with a correlation coefficient of 0.97, slope of 0.014 ± 0.001, and intercept of 0.83 ± 0.10. However, the linear relationships with the other parameters showed correlation coefficients between 0.72 and 0.87, with the highest for the TPC results obtained in the SPE extracts, which showed high dispersion from the line. Linear trends in raw honey analysis have already been studied in previous works [5]. However, the SPE extraction method should be applied in honey work in the future, as it allows for the identification of a larger group of phenolic compounds and may contribute to explaining the variability of properties among honeys.

3.3. UV-Vis Spectra of Individual and Mixed Standard Solutions

The simultaneous quantification of the three classes of phenolic compounds (hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids) was performed using mixed standard solutions of gallic acid, ferulic acid, and quercetin, which are representative compounds of these classes, respectively.

The spectrum of gallic acid shows absorption peaks at 215 and 275 nm; ferulic acid at 320 and a shoulder at 280 nm; and quercetin at 205, 255, and 370 nm, indicating distinct UV-Vis absorption regions for the three compounds (Figure S1, Supplementary Data). The gallic acid spectrum has a greater overlap with two other spectra, making the estimation/prediction model more complex. Overall, it is possible to extract information from the spectra to quantify the total levels of these phenolic compound classes because each class has a characteristic spectrum [52,53], although with variations in absorption intensity patterns. The UV spectra of the raw honey and the respective SPE extract showed different profiles due to differences in the matrix (see Figure S1, Supplementary Data). The SPE extraction also helps remove the most abundant compounds in honey, the sugars, which can also contribute to the absorption values, mainly fructose, since the contributions of glucose and sucrose are low (see Figure S1, Supplementary Data). The spectra of all mixed standard solutions have, in general, absorbances below 1.4, except for those obtained in the wavelength range of 200–225 nm (see Figure S2, Supplementary Data).

3.4. Multiple and Simple Linear Regression

In this study, the determinations of $THBA_{UV}$, $THCA_{UV}$, and TFC_{UV}, as well as TPC_{UV} (dependent variables), were made by applying the calibration equations defined in Section 2.7 [28]. For the calibration procedure, a set of 25 spectra (with known concentrations of standard compounds) representing the 3 classes of phenolic compounds was used (Table S1, Supplementary Data). The spectral data were divided into a training group (80% of the spectra of the standard mixing solutions) and a test group (20%, corresponding to 5 spectra) using the k-means clustering algorithm. The test group presented concentration levels within the limits defined by the mixed standard solutions of the training group: gallic acid ranged from 9 to 55 mg/L, ferulic acid ranged between 10 and 64 mg/L, quercetin ranged from 10 to 51 mg/L, and total phenolic content ranged between 77 and 123 mg/L.

The overall results are presented in Table 3, showing the coefficients and errors obtained for each regression model, as well as the prediction results for the training and test groups. In general, the coefficients are statistically similar to those reported by Paula et al. [28]. Only the TPC model had different coefficient magnitudes, showing that the models should always be developed through experimental assays. All coefficients of the linear models were highly significant, as indicated by *p*-values below 0.001. The models showed robust fitting, as can be verified by the satisfactory results from the predictive performance with the training and test data. The linear adjustment between the predictive and expected values for each phenolic group, as well as for the TPC parameter, was significant (p-values < 0.001), with RSE values lower than 7.40, R^2 values higher than 0.994 (explaining more than 99.4% of the original variability), and slopes approximately equal to 1 (an intercept equal to 0 statistically since it was not significant at the level of 5%). The quantification of TPC showed errors (RSE) greater than those of the quantification models of the individual phenolic compounds. Each model was significant ($p < 0.001$), indicating a good fit to the analytical data, as well as the independent variables selected for each model ($p < 0.001$), considering the ANOVA results. Moreover, all adjusted models presented normality and randomness in the residues, as well as low Cook distances (values lower than 0.5) and leverage values (values lower than 0.6), indicating that there was no evidence of extreme values or outliers.

Table 3. Single or multiple linear models for each analytical parameter and the fitting quality results using the train and test data.

Compound	**Phenolic Group**	**Model Equation**	**R^2**	**RSE**	***p*-Value**
Gallic acid	Hydroxybenzoic acids	$C = 180\ (\pm 4,\ p\text{-value} < 0.001) \times Abs_{275} - 141\ (\pm 6,\ p\text{-value} < 0.001) \times Abs_{345}$	0.9961	2.89	<0.001
Ferulic acid	Hydroxycinnamic acids	$C = 130\ (\pm 2,\ p\text{-value} < 0.001) \times Abs_{325} - 81\ (\pm 6,\ p\text{-value} < 0.001) \times Abs_{380}$	0.9975	2.89	<0.001
Quercetin	Flavonoids	$C = 178\ (\pm 3,\ p\text{-value} < 0.001) \times Abs_{375}$	0.9951	3.05	<0.001
TPC		$C = 66\ (\pm 4,\ p\text{-value} < 0.001) \times Abs_{220} + 168\ (\pm 10,\ p\text{-value} < 0.001) \times Abs_{345}$	0.9981	5.97	<0.001

Estimation with train data:

Compound	R^2	RSE	*p*-value	Slope
Gallic acid	0.9961	2.81	<0.001	1.00 (±0.01, *p*-value < 0.001)
Ferulic acid	0.9975	2.81	<0.001	1.00 (±0.01, *p*-value < 0.001)
Quercetin	0.9945	3.04	<0.001	1.00 (±0.02, *p*-value < 0.001)
TPC	0.9981	5.80	<0.001	1.00 (±0.01, *p*-value < 0.001)

Prediction with test data:

Compound	R^2	RSE	*p*-value	Slope
Gallic acid	0.9976	2.66	<0.001	0.98 (±0.02, *p*-value < 0.001)
Ferulic acid	0.9983	1.80	<0.001	1.05 (±0.02, *p*-value < 0.001)
Quercetin	0.9933	2.84	<0.001	1.03 (±0.04, *p*-value < 0.001)
TPC	0.9965	7.04	<0.001	1.00 (±0.03, *p*-value < 0.001)

C—concentration; Abs—absorbance; TPC—total phenolic content; R^2—determination coefficient; RSE—residual standard error.

Since the test data were not used in the model's calibration, it served to verify the predictive capability of the obtained calibration models and confirm their adequacy to posterior application in the honey sample analysis. The models gave good prediction results because the slopes were close to the unit (generally varying between 0.98 and 1.05), the determination coefficients were greater than 0.993, and the RSE values were satisfactory (less than 2.9). Figure 1 shows the satisfactory linear relations obtained in the representations between the predictive and expected values for each phenolic group and the TPC parameter for both the training and test data groups, representing the orthogonal mixture standard solutions.

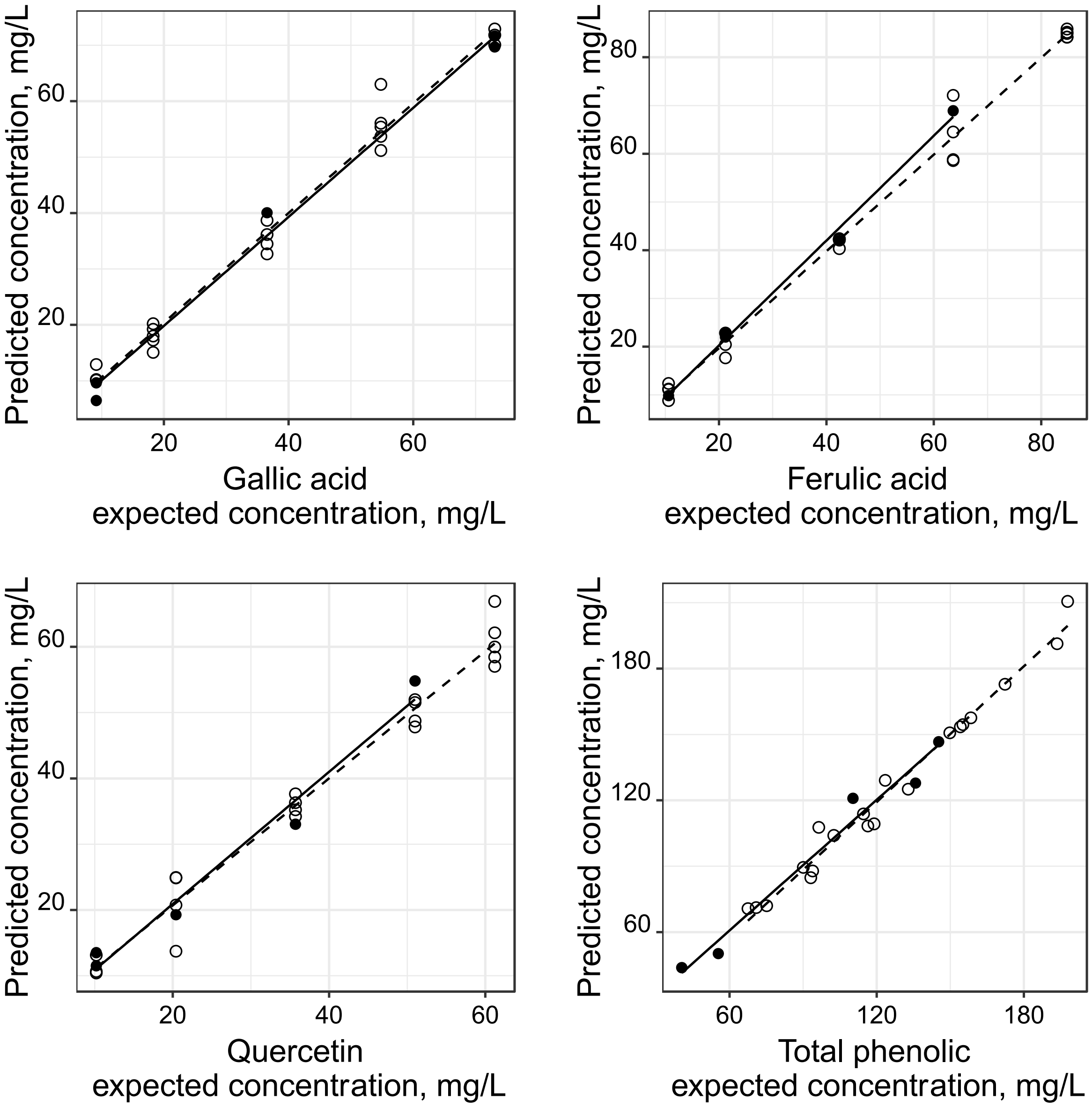

Figure 1. Linear fittings between the predictive and expected values for each phenolic group, as well as for the TPC parameter for the training (open dot) and testing (closed dot) data groups.

3.5. Honey Extract Analysis

The spectra from honey extracts are within the limits defined by the spectra of the standard mixing solutions. In the honeys analyzed, the results generally showed percentage deviations from the standard deviations below 5% and with marked variability: hydroxybenzoic acid levels ranged between 0.37 ± 0.05 and 4.46 ± 0.37 mg of gallic acid per g of honey, hydroxycinnamic acid levels ranged between 0.13 ± 0.03 and 2.76 ± 0.13 mg of ferulic acid per g of honey, flavonoid levels varied between 0.15 ± 0.03 and 1.63 ± 0.17 mg of quercetin per g of honey, and total phenolic content levels ranged between 0.70 ± 0.08 to 7.26 ± 0.13 mg of mix phenolic compounds per g of honey.

The results showed that the hydroxybenzoic acid compounds generally predominated in the honey samples, with concentration levels, on average, 1.9 ± 0.6 times higher than the levels of hydroxycinnamic acid compounds and 3.4 ± 1.1 times higher than flavonoid levels.

The linear relation between the values of TPC_{UV} and the calculated TPC, as the sum of the $THBA_{UV}$, $THCA_{UV}$, and TFC_{UV} values, showed highly correlated results ($R = 0.989$), with a slope close to 1 (1.01 ± 0.04) and an intercept close to zero (0.08 ± 0.17).

This indicates that the UV spectrum methodology showed coherence and robustness in the results, considering the high variability presented by the studied honey samples. To visualize this variability, Figure 2 shows the levels of the four analyzed parameters as a function of the honey's color (in ascending order of mm Pfund values). As can be seen, the color of honey influences the phenolic compound composition, with darker honeys showing higher levels of phenolic compounds. However, the expected trend obtained in raw honey analysis [5] was not so obvious in the results obtained for the SPE extracts. The linear relationship between the parameters determined by the UV spectrum methodology and the color values in the Pfund scale was between 0.53 (THCA parameter) and 0.82 (THBA parameter). Overall, these results show the variability of experimental data obtained using different analytical methods, which may be relevant for research studies as they analyze phenolic compounds in distinct ways.

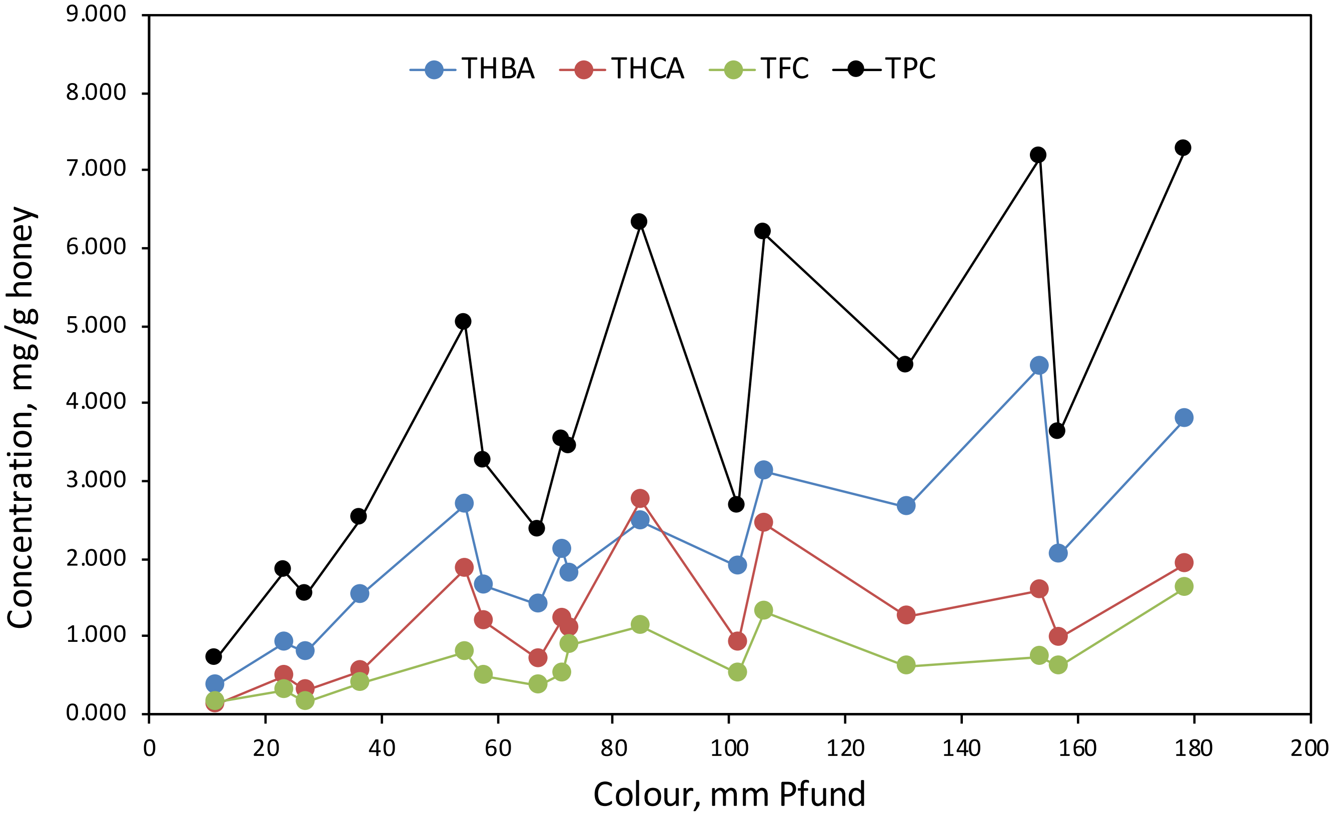

Figure 2. Levels of the four analyzed parameters with the single UV spectrum methodology as function of the honey's color: THBA—total hydroxybenzoic acids (mg of gallic acid per g of honey); THCA—total hydroxycinnamic acids (mg of ferulic acid per g of honey); TFC—total flavonoid content (mg of quercetin per g of honey); TPC—total phenolic content (mg of mix standards GA, FA, and Q per g of honey).

In the SPE extracts, the TPC_{UV} values were usually higher than those from TPC_{FC}, on average 1.78 ± 0.79 times, while the TFC_{UV} results were on average 0.66 ± 0.33 lower than TFC_{AlCl3}.

Comparing the TPC_{UV} values of SPE extracts with the TPC_{FC} of raw honey, the TPC_{UV} values were, on average, 1.86 ± 0.72 times higher, and for TFC_{UV} results, on average 6.02 ± 3.14 times larger than TFC_{AlCl3} of raw honey. These results suggest that interferences present in the honey matrix (for example, sugars) influence the analysis of phenolic compounds, leading to determinations of concentrations by default, mainly of flavonoids. Regarding phenolic acids, there is not enough information to draw conclusions.

All the results presented are related to the SPE-C18 extraction of a honey sample dissolved in acidified water to ensure that both flavonoid aglycones and phenolic acids were extracted in their molecular forms [54]. SPE-C18 extraction is important for removing interfering compounds, allowing the attainment of more reliable results in honey analysis [14,55]. Different extraction materials can be used, such as Amberlite XAD-2 and Amberlite XAD-4, reported to allow acceptable flavonoid recoveries and effective elimination of sugars, acids, pigments, and other interfering compound reversed-phase cartridges [14,54]. However, there are disadvantages, such as a low affinity for some phenolic compounds and polar glycoside flavonoids and the need for high amounts of honey samples and organic solvents [14,56]. Michalkiewicz et al. [56] presented work on phenolic acid and flavonoid extraction from a honey matrix using four solid sorbents (Bond Elut octadecyl C18, Oasis HLB, Strata-X, and Amberlite XAD-2). The best HPLC results for nine phenolic compounds (gallic, p-HBA, p-coumaric, vanillic, caffeic, syringic acid, rutin, quercetin, and kaempferol) were obtained with the microcolumn Oasis HLB microcolumn, while C18 silica presented less appropriate recovery for most of the compounds tested, except for quercetin, where it achieved the highest recovery (>90%). An et al. [57] performed an HPLC comparative study of the extraction efficiency of nine phenolic acids and flavonoids (benzoic acid, dihydroxybenzoic acid, gallic acid, trans-cinnamic acid, vanillic acid, naringenin, naringin, quercetin, and rutin)) using two SPE methods: one using Amberlite XAD-2 resin and another with a C18 cartridge. The C18 cartridges provided the best recovery values (a mean recovery of 74.2%) compared to Amberlite XAD-2 resin (43.7%), although gallic acid was not extracted using C18 cartridges, a result consistent with the results in a study by Michalkiewicz et al. [56]. Overall, obtaining different honey phenolic profiles considering the solid sorbent used is expected, which shows the importance of conducting further studies on sorbents (for instance, a mixture of several sorbents) to develop an extraction method that provides a more representative phenolic profile of the honey matrix.

3.6. Repeatability and Recovery Assays

To evaluate repeatability, five analyses were performed on a quality control solution of a standard mix with concentrations of 43.4 mg/L of gallic acid, 50.5 mg/L of ferulic acid, 50.0 mg/L of quercetin, and 143.9 mg/L of TPC. The predicted concentrations in the five assays were 41.3 ± 0.2 mg/L of gallic acid, 49.4 ± 0.5 mg/L of ferulic acid, 47.8 ± 0.2 mg/L of quercetin, and 138.2 ± 0.7 mg/L of TPC. These results presented percentage relative standard deviation (RSD%) and percentage relative error (RE%) values lower or equal to 1.0% and 4.7%, respectively, indicating that the calibration models have satisfactory precision and accuracy in the analysis of standard mixing solutions, as expected considering the results obtained from the test group.

To evaluate if there were interferences within the honey extract matrix, recovery assays were carried out using the quality control solution standard mix. The standard addition to honey samples increased the concentrations of 8.7 mg/L of gallic acid, 10.1 mg/L of ferulic acid, 10.0 mg/L of quercetin, and 28.8 mg/L of TPC. The recovery assay results obtained from all the honey samples varied ranged from 90.8 to 121.0% for gallic acid spiking (a mean value of 102 ± 9%), 83.4 to 101.9% for ferulic acid (a mean value of 94 ± 7%), 85.0 to 111.2% for quercetin (a mean value of 100 ± 7%), and 85.4 to 104.4% for mixed phenolic compounds (TPC parameter; a mean value of 96 ± 6%). These results presented RSD% values lower than 8.5%, indicating satisfactory precision considering the high matrix variability in honey samples, which also allows us to infer that the calibration models for honey analysis were robust.

Considering the high variability of honey extracts, the recovery test results showed that the final honey extract solutions have additive effects. This fact can be visualized in Figure 3, where the representation of the concentrations of the honey final extract solutions with and without spiking with the standards mix quality control solution showed a linear relationship for all groups analyzed (UV spectrum analysis). Therefore, it can be considered that in these analyses, there are no hidden interference problems.

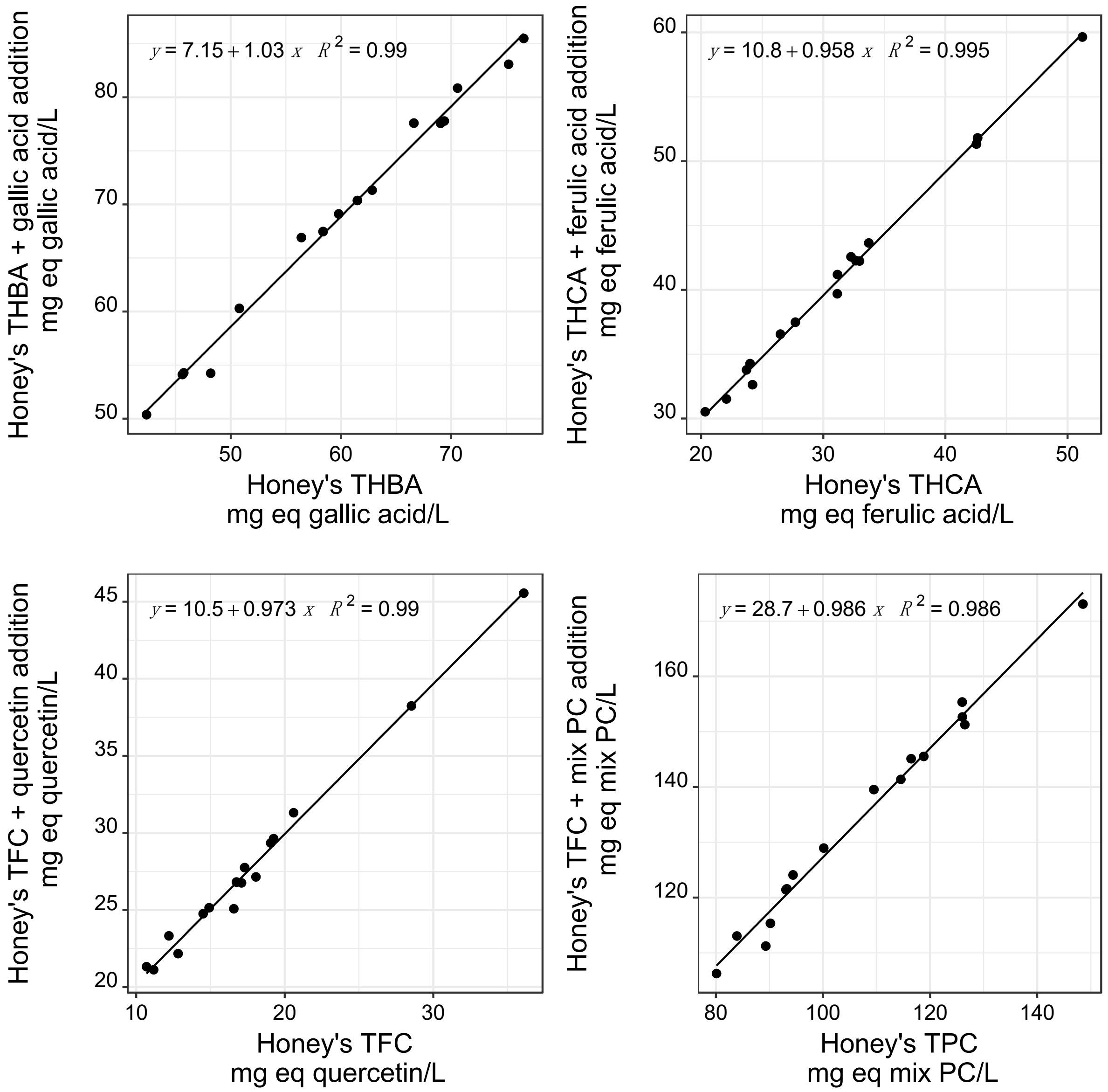

Figure 3. Linear relation between the concentrations of the honey's final extract solutions with and without spiking the standard mix quality control solution.

4. Conclusions

This work used 16 honeys with different colors (extra white to dark), ensuring the variability to evaluate their phenolic compound composition. The color of honey influences its phenolic compound composition, with darker honey samples containing higher levels of phenolic compounds. The single UV spectrum method, characterized by its simplicity, speed, precision, and accuracy, when applied to SPE-C18 honey extracts, allowed the determination of four analytical parameters: the total hydroxybenzoic acid content, total hydroxycinnamic acid content, total flavonoid content, and total phenolic content

The results obtained using this method showed higher levels of total hydroxybenzoic acid content, followed by total hydroxycinnamic acid content, and lower levels of total flavonoid content. Also, the total phenolic contents were higher than the results obtained using the Folin– Ciocâlteu method for both raw honey and the SPE-C18 extract. However, the total flavonoid content, measured using the single UV spectrum method, was higher than in raw honey but lower than in the SPE-C18 extract when using the $AlCl_3$ method.

Overall, the results provide evidence that the Folin–Ciocalteu and $AlCl_3$ methods analyze the phenolic compounds differently from the single UV spectrum method proposed in this work. The characteristics of this method suggest that it may serve as a first approach for the chemical characterization of phenolic compounds present in honey samples. In addition to determining the flavonoids and phenolic total contents, it also allows quantification of total hydroxycinnamic and hydroxybenzoic compounds, increasing the information possible to relate to a given biological property.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12102297/s1, Figure S1: Spectrum of an amber honey (sample H10, 4 g/20 mL); amber honey final extract solution (H10), fructose (4 g/20 mL); and phenolic compounds, gallic acid, ferulic acid, and quercetin (concentrations of 0.02 g/20 mL). Figure S2: Spectra of the following: A—all mixed standard solutions; B—honey ethanolic extract solutions with limits defining the range represented by the spectra of the standard mixing solutions. Table S1: A total of 25 solutions prepared with a mixture of gallic acid, ferulic acid, and quercetin according to the fractional factorial experimental design.

Author Contributions: Investigation, V.B.P. and N.L.S.; Formal Analysis and Writing—Original Draft, V.B.P., M.L.S.-D. and P.C.-F.; Conceptualization, Methodology and Supervision, L.G.D.; Project Administration, Funding Acquisition and Writing—Review and Editing, L.M.E. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Nomenclature

$AlCl_3$	Aluminum chloride.
CE	Capillary electrophoresis.
FC	Folin–Ciocalteu.
GC	Gas chromatography.
HCl	Hydrochloric acid.
HPLC	High-performance liquid chromatography.
LLE	Liquid–liquid extraction.
Na_2CO_3	Sodium carbonate.
$NaNO_2$	Sodium nitrite.
NaOH	Sodium hydroxide.
PC	Phenolic compounds.
RSE	Root square error.
SPE	Solid-phase extraction.
TFC	Total flavonoid content.
TFC_{AlCl3}	Total flavonoid content measured using the aluminum chloride method.
TFC_{UV}	Total flavonoid compounds measured using the UV spectrum method.
$THBA_{UV}$	Total hydroxybenzoic acids measured using the UV spectrum method.
$THCA_{UV}$	Total hydroxycinnamic acids measured using the UV spectrum method.
TLC	Thin-layer chromatography.
TPC	Total phenolic content.
TPC_{AlCl3}	Total phenolic content measured using the aluminum chloride method.
TPC_{FC}	Total phenolic content measured using the Folin–Ciocalteu method.
TPC_{UV}	Total phenolic content measured using the UV spectrum method.

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