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Antioxidant Activity, Total Phenolic and Flavonoid Contents in Floral Saffron Bio-Residues

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Abstract: Saffron spices are composed of dehydrated stigmas of *Crocus sativus* L. A large amount of bio-residues (stamens, tepals, and stigmas) is generated during the production of this spice (~92 g of bio-residues per 100 g of flowers). These bio-residues are usually discarded losing the chance to obtain interesting bioactive compounds from them. In this work, we use the saffron bio-residues as possible source of natural bioactive compounds. Different extraction conditions were applied obtaining hydrophilic and lipophilic components. The antioxidant activity of the bioactive compounds in the different conditions were analysed using the ABTS method developed by our team. Furthermore, the total content of phenolic compounds and flavonoids present in the bio-residues were estimated. We demonstrated that bio-residues contain a high amount of both phenolic and flavonoid compounds with a strong antioxidant potential effect. Moreover, we compared the antioxidant activities of saffron bio-residues (obtained from stamens, tepals, and stigmas). The extraction was made at two different pH (4.5 and 7.5) and measured (antioxidant activity, flavonoid and phenol content) at pH 7.5. Another extraction done at pH 7.5 measured antioxidant activity, flavonoid and phenol content at time 0 (t = 0 h) and after 24 h (t = 24 h) at pH 4.5 and 7.5. We point out that bioresidues contain antioxidant activity (up to 8.42 µmoles Trolox/100 g DW), total phenols (up to 111.91 µmoles EG/100 g DW) and total flavonoids (up to 109.25 µmoles QE/100 g DW) as bioactive compounds. The applicability of bio-residues as additives is promising, both in the pharmaceutical and in the food industry as nutraceuticals and phytogenics. The variability in pH and the colour originating from it must be taken into account.

Keywords: saffron; antioxidant activity; phenolic compounds; flavonoids; bio-residues; by-products; waste reuse; waste reduction; nutraceutical; bifunctional compounds; food additive; environmental safety

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1. Introduction

Saffron (*Crocus sativus* L.) is a perennial herb that belongs to the Iridaceae family and is composed of stamens, tepals, and stigmas. However, tepals and stamen are simply discarded while just flower stigmas are utilized to make saffron [1]. It is cultivated in Iran, Morocco, Greece, Azerbaijan, Pakistan, Italy, and Spain, which are the largest producers of saffron [2]. One of the oldest, most precious, and most expensive spices is saffron. Its production is of tremendous significance because 1 kg of saffron costs around $10,000 and requires roughly 60,000 flowers to produce it [3]; in fact, it can cost $40–50 per gram [4].

The current system of production is not sustainable because it generates a huge amount of bio-residue; in total, the production of 1 kg of saffron produces 30 kg of tepals as bio-residues [5], this means that approximately 92 g of bio-residues are generated for every 100 g of flowers [6]. Thus, estimating a global production of saffron of about 900 tons, the amount of bio-residue would be about 10,350 tons. Saffron is a very low yielding crop and there are many barriers to its widespread use in the industry [7].

The essential oil of the tepals contains kaempferol, quercetin, rutin, hesperidin and luteolin, tannins and anthocyanins [8]. The bio-residues could contain, similarly to the spice, substances such as vitamin A, vitamin C and folic acid, as well as proteins, lipids and carbohydrates, iron, calcium, magnesium, copper, manganese, potassium, phosphorus, zinc and sodium. It may even possess other secondary metabolites such as monoterpenoids, flavonoids and carotenoids [9,10]. Dried saffron tepals are a natural source of antioxidant chemicals. The method of extraction has been shown to affect the phytochemical content, antioxidant potential and total phenolic content [11]. Saffron bio-residue, which has a lower cost compared with the spice, has been shown to have antioxidant properties and to improve many diseases, just as the spice does [4,12].

The ABTS method, used to determinate antioxidant activity, has many benefits compared to other methods such as the avoidance of unintended side effects (unwanted side-reactions, high temperatures are not needed to generate ABTS radicals, and antioxidant activity can be studied over a wide range of pH values), the ability to study antioxidant activity across a wide pH range, and the fact that ABTS radicals can be produced at low temperatures. Moreover, this method also eliminates interference from endogenous peroxidase activity in samples, resulting in a more precise and rigorous assessment of the antioxidant activity in plant and other extracts. Moreover, it can quantify hydrophilic (in buffered media) and lipophilic antioxidants nature (in organic media) [13].

Nowadays the processing of food bio-residues and wastes to create high-value products for industrial use has piqued both scientific and commercial interest [14,15]. Therefore, it is necessary to provide and organize the existing information on these issues from a broad food perspective and consider the use of saffron bio-residues as an alternative with high economic, industrial, and environmental value. In this context, a study on saffron bio-residues as a potential source of organic bioactive chemicals was carried out. Using our ABTS method, various extraction and measurement conditions were established to detect the antioxidant activity capacity from the extracts, and hydrophilic and lipophilic antioxidant activity were obtained. In addition, the total concentration of phenolic and flavonoid components of the bio-residues was evaluated.

2. Materials and Methods

2.1. Chemicals

Ethyl acetate, methanol and ethanol solvents and aluminium chloride were purchased from Scharlau S.L. (Barcelona, Spain). Sodium citrate, sodium phosphate, sodium carbonate, gallic acid, quercetin, the Folin-Ciocalteu reagent, 2′2-azino-bis-(3-ethylbenzothiazole-6-sulfonic acid) (ABTS) in crystallized form of diammonium salt, L-ascorbic acid, H_2O_2 and peroxidase enzyme (HRP Type VI) were supplied by Sigma Chem Co (Madrid, Spain). The concentrations of ABTS, H_2O_2 and HRP were verified by measuring their absorbance and using molar extinction coefficients: ε_{340} = 36,000 M^{-1} cm^{-1} for the ABTS [16], ε_{303} = 100,000 M^{-1} cm^{-1} for the enzyme HRP [17], ε_{240} = 43.6 M^{-1} cm^{-1} for H_2O_2 [18].

A Perkin-Elmer Lambda-2S UV-Visible two-beam spectrophotometer (Überlingen, Germany) connected to a computer was used for these tests. Spectroscopic studies between 190 and 900 nm were performed on each of the samples obtained.

2.2. Plant Material

The floral bio-residues (tepals, styles and stamens discarded after the stigmas were taken for saffron) were subjected to dehydration and grinding, giving a purplish brown and grainy appearance. During the 2022 harvest season, whole saffron flowers were

obtained from a producer in Tomelloso (Toledo, Castilla-La Mancha, Spain) and cultivated in accordance with the requirements established by the Protected Designation of Origin "Azafrán de La Mancha" according to DOCM [19]. The flowers were made up of all the parts of saffron flowers (tepals, stamens, and styles), except the stigmas, which were manually separated from the rest of the flower using traditional DOCM procedures [19].

2.3. Extraction Process

Saffron sample (0.1 g) was weighed in 50 mL PTFE tube to which 5 mL of ethyl acetate were added. Subsequently, either 10 mL of 50 mM sodium phosphate buffer (pH 7.5) or 50 mM citrate buffer (pH 4.5) was added in order to extract the biologically active compounds from the saffron bio-residues. After stirring at 25 °C for 15 min, the solutions were centrifuged at 4000× *g* for 10 min in a Sorvall RC-5B Plus (Waltham, MA, USA) to improve phase separation. The fully decolorized solid was discarded. The aqueous and organic phases were collected for the measurement of antioxidant activity, polyphenol, and flavonoid content. The extracts were stored at 4 °C in the dark to preserve the properties of bioactive compounds. One extraction was performed at pH 7.5 and pH 4.5 and antioxidant activity was measured at pH 7.5. In addition, another extraction was carried out at pH 7.5 and antioxidant activity was measured at pH 4.5 and pH 7.5 at time 0 (t = 0 h). After 24 h (t = 24 h), a measurement of the pH decrease of this last extraction was made. The outcome is given in µmoles of Trolox equivalents per 100 g dry weight (µmoles Trolox/100 g DW).

2.4. Determination of Antioxidant Activity

Antioxidant activity was determined using the ABTS/H_2O_2/HRP reaction system decolorization method [20]. Antioxidant activity was subdivided into hydrophilic and lipophilic antioxidant activities. They were calculated from Trolox (6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid) and measured on a dose-response curve, which relates the difference in absorbance at 730 nm to the amounts of Trolox. The results are given in µmoles of Trolox equivalents per 100 g dry weight (µmoles Trolox/100 g DW).

2.4.1. Hydrophilic Antioxidant Activity

For the measurement of hydrophilic antioxidant activity (HAA), an ABTS radical solution (suitable for hydrophilic antioxidants) was prepared containing 2 mM ABTS, 30 µM H_2O_2 and 20 nM HRP in 50 mM phosphate buffer (pH 7.5) in a final volume of 25 mL. 1 mL of this dilution was taken and monitored at 730 nm until a stable absorbance was reached (1 min). After that, 20 µL of the aqueous phase of the sample extract were added to the reaction medium and after 5 min a decrease in absorbance proportional to the removed $ABTS^+$ was determined [21].

2.4.2. Lipophilic Antioxidant Activity

This was based on the method of Cano et al., in which the measurement of lipophilic antioxidant activity (LAA), an adaptation of the HAA method. An ABTS radical solution (suitable for lipophilic antioxidants) was prepared containing 1 mM ABTS, 60 µM H_2O_2 and 200 nM HRP in 25 mL of ethanol with 15 µL of 10% H_3PO_4. 1 mL of this dilution was taken and monitored at 730 nm until a stable absorbance was reached (1 min). Following that, 20 µL of the lipophilic phase of the sample extract were added to the reaction medium and after 5 min a decrease in absorbance proportional to the removed $ABTS^+$ was determined [21]. The decrease in absorbance was determined by the difference between absorbance at 730 nm initially and 5 min after to the sample addition. Lipophilic antioxidant activity (LAA) was calculated as Trolox equivalent, measured on a dose-response curve, which relates the difference in absorbance at 730 nm to the amounts of Trolox. The result is expressed in µmoles of Trolox equivalents per 100 g dry weight (µmoles Trolox/100 g DW).

2.5. Determination of Total Phenolic Content

The polyphenols contained in plant extracts are usually assessed by the Folin-Ciocalteu assay, the method developed by Singleton and Rossi [22]. In the case of hydrophilic extracts, the following protocol was carried out: to 100 µL of each hydrophilic extract at the different pHs were added 900 µL of distilled H_2O, 50 µL of 1 M sodium carbonate, 50 µL of Folin-Ciocalteu's reagent, and finally all was diluted with 2 mL of distilled H_2O. In contrast, the extracts in the lipophilic phase were brought to dryness in a Heildolph Laborota 4001 rotary evaporator (Schwabach, Germany) with a 40 °C bath and a Büchi V-700 (Barcelona, Spain) vacuum pump (240 mbar) connected to a VWRRC-10 (Barcelona, Spain) digital chiller to evaporate the solvent. The dried residue was resuspended with 600 µL of 70% methanol. Lastly, 200 µL of the resuspended extracts were taken with a pipette and 800 µL of distilled water were added, followed by the addition of the other reagents used to determine the amount of phenol in the same way and in the same quantities. All reaction mixtures were kept in a water bath at 30 °C for 15 min and then it was measured at 755 nm [23]. The total phenol concentration was expressed as µmoles of gallic acid equivalents (GE) per gram dry weight (µmoles GE/100 g DW).

2.6. Determination of Total Flavonoid Content

The aluminium chloride colorimetric method, modified by Woisky and Salatino [24], was used to determine the flavonoid content. On the other hand, extracts brought to dryness were used to measure lipophilic extracts. To 500 µL of the extract, 1.5 mL of 96% ethanol was added. The other reagents were added in the same way and amount as for the hydrophilic extracts until a final volume of 5 mL was reached. The mixtures were kept in the dark for 30 min and then the optical density was measured at 415 nm [25]. Quercetin (QE), a flavanol found as an O-glycoside in high concentrations in both fruits and vegetables, was used as a standard. A calibration curve was obtained. The total flavonoids were expressed as µmoles of quercetin equivalents per gram dry weight (µmoles QE/g DW). To express the results, all replicates were averaged. The standard error and relative error were calculated.

2.7. Statistical Analysis

The result was analysed for determine statistical significance using an analysis of variance using SPSS20.0 analysis software (IBM, Chicago, IL, USA). The data are represented as means ± SE.

3. Results

The extracts were obtained from the plant material at the two pH conditions tested presented colour differences: the pH 4.5 buffer induced a brown-orange colour, while a dark-green colour was observed when the pH 7.5 buffer was used (Figure 1A). On the other hand, the dark-green colour of pH 7.5 buffer extraction samples at time 0 (t = 0 h) evolved to a light-green colour after 24 h (t = 24 h) of cold storage (Figure 1B), while pH dropped to 5.5. The colour changes were striking, so absorption spectra of the different hydrophilic extracts and the lipophilic one was performed. The colour changes open the way to discover if the colour it is dependent of the values of antioxidant capacity, total phenolics or total flavonoids in the bio-residues.

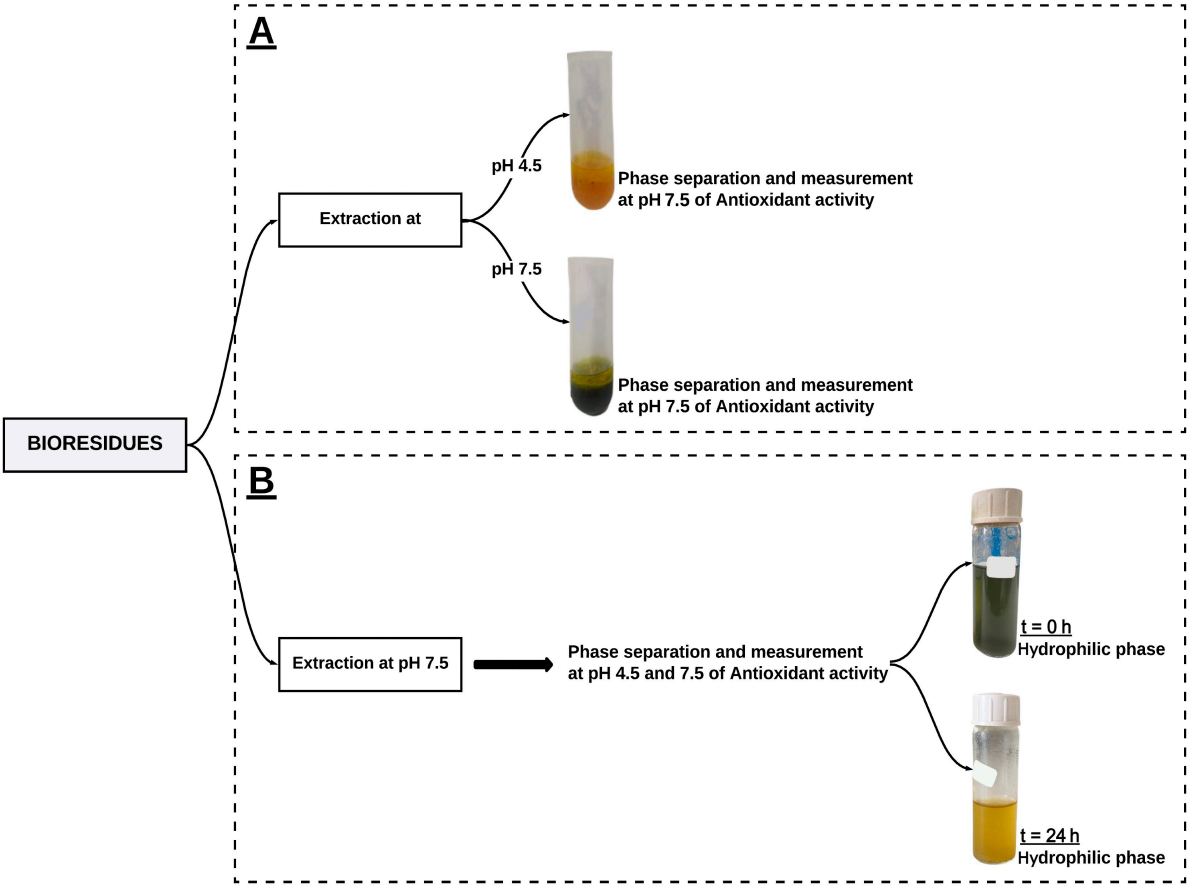


Figure 1. Protocol diagram of extractions and measurements at different pHs. Panel (**A**): Two extractions were made at pH 4.5 (brown–orange colour) and pH 7.5 (dark-green colour) with measurement at pH 7.5. Panel (**B**): Extraction at pH 7.5 (dark-green colour) with measurements at pH 7.5 and pH 4.5. Also, the measurement of the same sample showed a color difference between time 0 (t = 0 h) extraction at pH 7.5 showing a dark-green coloration and after 24 h (t = 24 h) pH decreased to pH 5.5 generating a light-green colour.

Figure 2 show the spectra of hydrophilic fraction extracted at both pH (pH 7.5 and 4.5) (Figure 2A,2B) and by the lipophilic fraction (Figure 2C). The spectra of hydrophilic fraction at both pHs showed no differences except for the region of 600 nm (Figure 2B). The lipophilic extract spectrum showed a profile clearly associated with carotenoids (Figure 2C), whose chemical structure provides absorption maxima around 450 nm [23]. As we observed, none of the compounds present in the extracts were absorbed at 730 nm, avoiding a possible interaction with the ABTS test.

Table 1 show the hydrophilic antioxidant activity (HAA), lipophilic antioxidant activity (LAA), and the sum of both corresponding to total antioxidant activity (TAA). The fractions at pH 7.5 show a higher TAA since we obtain 8.42 (μmoles Trolox/100 g DW), almost double than in the fractions obtained at pH 4.5, observing that this difference is mainly due to hydrophilic antioxidant activity.

Table 2 shows the total phenolic content applying Folin-Ciocalteu method at the different samples. The highest value for the hydrophilic fractions is found when buffer at pH 7.5 was used. Additionally, the highest value for the lipophilic fraction was found when using buffer extraction at pH 7.5. The total phenol contents of the hydrophilic phase were lower in the extraction at pH 4.5 than those obtained in the extraction at pH 7.5. In the hydrophilic phase, very similar values were seen at pH 4.5 and pH 7.5. However, in the lipophilic phases, a higher flavonoid content was observed in the lipophilic extracts at pH 7.5.

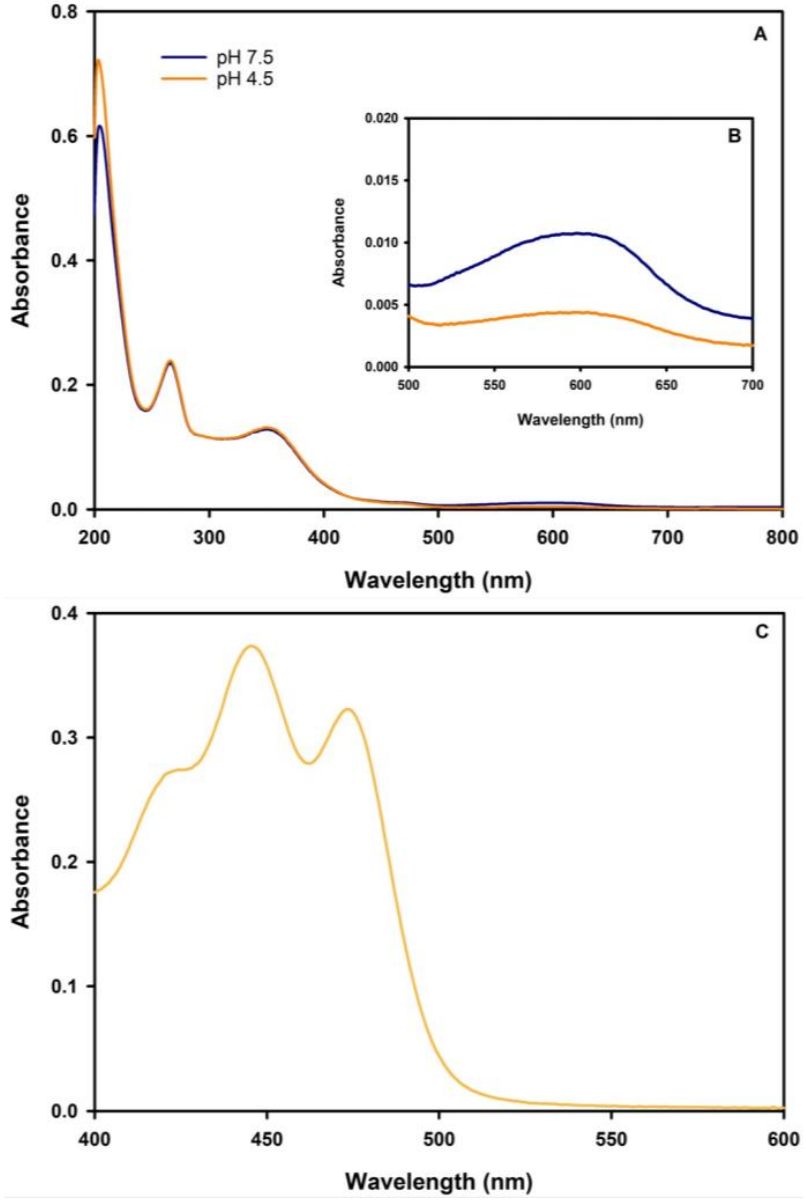


Figure 2. Spectra of the hydrophilic and lipophilic fractions obtained from saffron bio-residue samples. (**A**) Representative spectrum of a hydrophilic fraction at both pHs. (**B**) A close-up of the spectra differences at 500–700 nm. (**C**) Representative spectrum of a lipophilic fraction.

Table 1. Antioxidant activities of different fractions performed at pH 4.5 and pH 7.5.

Extraction	**Antioxidant Activities (μmoles Trolox/100 g DW)**		
Fractions	HAA [2]	LAA	TAA
pH 4.5	4.63 ± 0.11	0.38 ± 0.07	5.02 ± 0.04
pH 7.5	7.92 ± 0.11	0.51 ± 0.10	8.42 ± 0.21
Sig. [1]	***	NS	***

[1] Statistically significant differences at $p < 0.001$ ***; NS, not significative. [2] (HAA) hydrophilic antioxidant activity, (LAA) lipophilic antioxidant activity, (TAA) total antioxidant activity.

Table 2. Total phenolic and flavonoid contents in different fractions.

Extraction	Total Phenolic Content (μmoles EG/100 g DW)		Total Flavonoid Content (μmoles QE/100 g DW)	
Fractions	Hydrophilic	Lipophilic	Hydrophilic	Lipophilic
pH 4.5	88.89 ± 2.42	8.57 ± 0.06	99.33 ± 0.27	4.23 ± 0.14
pH 7.5	95.29 ± 2.16	16.62 ± 0.29	91.91 ± 2.40	17.34 ± 1.28
Sig. [1]	NS	***	*	***

[1] Statistically significant differences at $p < 0.001$ ***; at $p < 0.05$ *; NS, not significative.

Table 3 shows the effect of time (at time 0 (t = 0 h) and after 24 h (t = 24 h)) on the antioxidant activities, determined at both pH on the day of extraction at pH 7.5 (t = 0 h). The following day is when a colour change was observed (t = 24 h). We can see the extraction was carried out at pH 4.5 and no differences were observed between the measurements at pH 7.5 at time 0 and after 24 h. However, a higher antioxidant activity was showed in the measurement at pH 7.5, when, after 24 h, the pH of the sample was measured and had dropped to pH 5.5 compared to the initial pH of 7.5.

Table 3. Results of antioxidant activity on the day of extraction at pH 7 (t = 0 h) and the following day (t = 24 h) measured at pH 4.5 and pH 7.5.

Extraction	Hydrophilic Antioxidant Activity (μmoles Trolox/100 mg DW)	
	pH 7.5	pH 4.5
t = 0 h	6.87 ± 0.42	3.49 ± 0.22
t = 24 h	7.04 ± 0.53	3.48 ± 0.29
Sig. [1]	NS	NS

[1] Statistically significant differences: NS, not significative.

Table 4 shows the effect of time (at time 0 (t = 0 h) and after 24 h (t = 24 h)) on the phenols and flavonoids, which was determined at the day of extraction at pH 7.5 (t = 0 h) and the following day (t = 24 h). No differences were observed between the measurements at time 0 h and after 24 h.

Table 4. Total phenolic and flavonoid contents in different fractions. Results of phenols and flavonoids at time 0 (t = 0 h) and after 24 h (t = 24 h) measured at pH 4.5 and pH 7.5.

Time	Total Phenol Content (μmoles EG/100 g DW)	Total Flavonoid Content (μmoles QE/100 g DW)
t = 0 h	88.6 ± 6.81	93.16 ± 17.5
t = 24 h	74.1 ± 8.9	75.92 ± 18.8
Sig. [1]	NS	NS

[1] Statistically significant differences: NS, not significative.

4. Discussion

Saffron bio-residues and flavonoids have the potential to be used as natural antioxidants. Most saffron bio-residues are tepals. It has been shown that saffron tepals can be dried while maintaining their quality. The green extraction techniques can be used to produce significant quantities of valuable antioxidant phytochemicals, which is necessary for a sustainable biorefining process [11]. The ABTS/TAC method has proven to be a fast and reliable technique regarding the antioxidant properties of foods and their bio-residues [26]. The diversity of methods used to measure the antioxidant capacity of various bio-residues

contributes to the heterogeneity of the results with their corresponding limitations and controversies [27–30].

According to our results, in Table 1 and Table 2, pH 7.5 buffer extraction is ideal for the assessment of hydrophilic antioxidant activity because it ensures that no hydrophilic compounds are retained in the organic phase [31].

One of the compounds contained in saffron are anthocyanins, molecules with a high instability due to the pH of the medium. Therefore, the colour change in the extracts may be due to changes in the structure of the anthocyanins caused by differences in pH. Compared to our data, pH changes were observed the day after extraction. Prenzler et al., (2021) have confirmed that pale yellow-red structural forms are present at pH 4.5, which explains the colour of the extracts. Furthermore, it could also explain the change from one day (t = 0 h) to the next one (t = 24 h). Factors such as pH, temperature, light and oxygen largely determine their stability [32]. Cerdá-Bernad et al., (2022) showed that the amount of bioactive chemicals with coloring properties in the various portions of the plant is correlated to the colour parameters diversity within the flowers and stigmas of *Crocus sativus* [33]. Therefore, the influence of pH must be considered both in the extraction protocols and in the measurement of the antioxidant capacities of the biological residues of saffron.

Total phenolic content obtained in our study from saffron bio-residues is similar to the obtained before by Serrano-Díaz et al., (2012, 2013) using the ABTS method [6,34]. However, different results reported in the literature may be due to the different extraction protocols performed and even to the origin of the raw plant material. Abe et al., (2014) have shown that there are different compositions of *C. sativus* depending on its geographical origin [35]. As we can see, our results are a little lower than the rest; this could also be since we have calculated the content in phenolic compounds separately, i.e., in a double fraction (hydrophilic and lipophilic) and not in a single fraction as they did in the other trials [34].

We also carried out a comparison between the total phenolic content of our bio-residues and other plant organs of *C. sativus*, according to data from Azghandi-Fardaghi et al. (2021) [36]. The lowest values were found in the corms, styles, and stamens, as these are organs with the few bioactive compounds [6]. However, the highest values can be found in the stigmas, which is the part used to produce the spice but has many bioactive compounds, including phenolic ones. In the petals we find a high value of phenolic compounds. They hypothesized that this could be due to the high concentration of kaempferol in the petals [37]. The total phenolic content values in our bio-residues are close to the values of the petals, which is why they are of great interest. The total phenol content results are similar to Azghandi-Fardaghi et al., (2021), which use bio-residues of saffron [36].

Stelluti et al., (2021) compared dried saffron tepals with the spice (stigmas) and found that tepals have fewer total phenolics and anthocyanins but more antioxidant activity, as assessed by the FRAP, ABTS and DPPH methods [11]. The fact that the solvents (water or methanol) employed in the extraction were different from those used in our investigation may account for the variations in the results when compared to this work.

Cerdá-Bernad et al., (2022) [33] concluded that Spanish saffron had a significantly higher concentration of total polyphenols. Saffron stigmas from Spain, Iran and Greece had high amounts of flavonoids, in the range of 15–18 mg GAE/g DW, which is a measure of total flavonoid content. However, compared to stigmas, saffron flowers contained lower total flavonoids (4–5 mg QE/g DW) [33]. We compared our results of total flavonoid content with those obtained by Serrano-Díaz et al., (2012) [6]. Both works have determined total flavonoid content in the saffron bio-residues of similar composition.

In the hydrophilic phase, we obtained the highest total flavonoid content at pH 4.5 (99.33 $\pm$ 0.27µmoles QE/100 g DW), the greatest in antioxidant capacity at pH 7.5 (7.92 $\pm$ 0.11µmoles Trolox/100 g DW) and highest phenolic content at pH 7.5 (95.29 $\pm$ 2.16 µmoles EG/100 g DW). The lowest values in total flavonoid content and

phenol content were obtained after 24 h in pH 4.5 (t = 24 h) in the hydrophilic phase; however, the antioxidant capacity was the lowest in the lipophilic phase at pH 4.5.

The lowest flavonoid content values are found in the stamens and styles. However, the highest values are found in the tepals, approximately 43 mg QE/g DW, exceeding the flavonoid content of the bio-residues. According to some authors, a large variety of glycosylated flavanol derivatives esterified with phenylpropanoic acids, as well as kaempferol, quercetin, myricetin and naringenin, which are found in tepals [29]. The tepals contain a large amount and variety of flavonoids, as demonstrated Zeka et al., (2015), who determined a total flavonoid content in saffron tepals of about 130 mg rutin equivalents/g DW [37].

As we can see, our bio-residues flavonoid content values are similar to those obtained by Serrano-Díaz et al., (2021), although a different extraction method has been used. The values of the bio-residues are around 31–33 mg QE/g DW, being at the highest value when extracted at pH 7.5. This could be due to the composition of the bio-residues which, as mentioned above, were composed of tepals, styles and stamens discarded after taking stigmas for saffron. Therefore, the bio-residues probably owe their high flavonoid content to the tepals [6].

5. Conclusions

We showed that saffron bio-residues had a significant potential for antioxidant activity, as well as a high content of phenolic and flavonoid compounds, compared to other plant organs. The hydrophilic phase has been shown to be the one with the highest antioxidant activity capacity at pH 7.5 in both extraction and measurement.

Additionally, we found a striking colour difference (brown-orange, dark-green and light-green) depending of the pH that the extraction was carried out. Further work is needed to seek the ethology of the colour modification. We should consider these colour changes when saffron bio-residues are added to other products in order to obtain the undesired hues in the product. However, we reported that color differences do not statistically significant change the antioxidant activity of the compounds.

Future studies are needed to demonstrate the potential utilization of saffron bio-residues in food, animal feed, cosmetic and pharmaceutical industries, among others. Furthermore, saffron bio-residue use could lead to reduced economic and industrial costs and be a better option for environmental value.

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