



Combining Enzymatic and Subcritical Water Treatments for Green Extraction of Polyphenolic Co-pigments from Saffron Tepals

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Abstract

Purpose Two major developments have taken place in the agri-food industry in recent decades. First, valorization of the plant by-products can contribute significantly for ensuring sustainability, especially applying green technologies for recovery of bio- and/or techno-functional compounds. Second, substitution of synthetic food additives, particularly colorants, by their natural counterparts is of utmost importance to meet consumer demands. The aim of this study was to evaluate a new process for green extraction of polyphenols from *Crocus sativus* tepals, the primary by-product of saffron spice production.

Methods A new strategy for recovery of polyphenols from saffron tepals was studied. An extraction process combining two subcritical water extractions steps with enzymatic treatment incorporated between them was established on a pilot scale. Liquid chromatography–mass spectrometry was used for polyphenols profile characterisation. The co-pigmentation efficiency of the polyphenol extract and its effect on thermal stability of strawberry anthocyanins were evaluated in model solutions.

Results An approximately fourfold increase in the polyphenolic yield was achieved with the new combined extraction method. 16 flavonoids, mainly flavonols, were detected in the saffron tepal extract. To the best of our knowledge, the presence of the flavone apigenin has not been described before in *Crocus sativus*. The addition of saffron tepal polyphenols, acting as co-pigments, significantly reduced the thermal degradation of strawberry anthocyanins.

Conclusion The obtained results demonstrate an innovative approach for the valorisation of saffron tepals through potential application of the by-product polyphenols in fruit processing.

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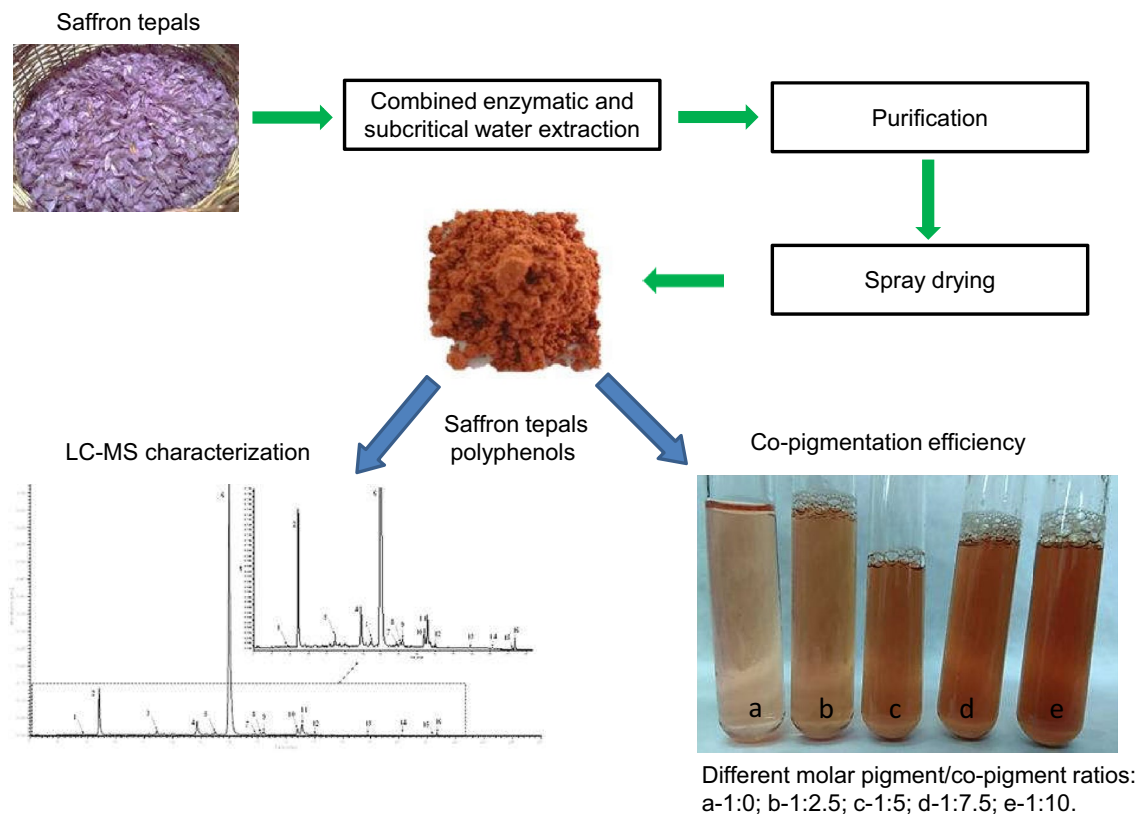
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Graphical Abstract



Keywords *Crocus sativus* · Plant by-product · Eco-friendly extraction · Polyphenols · Co-pigmentation

Statement of Novelty

A new strategy (combining enzymatic treatment and subcritical water extraction) for green extraction of saffron (*Crocus sativus*) tepals polyphenols was developed.

Introduction

Plant by-products originating from the agri-food industry have been demonstrated to contain large amounts of valuable compounds, including polyphenols that could be recovered and used as biologically active substances or as natural food ingredients, replacing synthetic additives [1].

Saffron, *Crocus sativus* L., is systematically cultivated in Greece, Iran, Spain, Morocco and other countries. Its dried stigmas (saffron spice) is used in foods both for the intensive flavouring and colouring properties [2]. Since approximately 150,000 flowers are needed to produce 1 kg of saffron spice [3], representing only 7% of the total flower weight [4], a large amount of by-product (mainly tepals) is generated.

Saffron tepals are rich in biologically active compounds such as apocarotenoids and their glycosides, monoterpeneoids, polyphenols, fatty acids and phytosterols [5]. Saffron tepal polyphenols, particularly flavonol glycosides and anthocyanins, have been demonstrated as potent antioxidants [6, 7].

To overcome the problems encountered when using conventional organic solvent extraction, in particular to reduce the energy consumption and adverse effects on the environment, various green processes have been developed [8]. Among them, subcritical water extraction (SWE), also known as pressurized hot water extraction, is one of the most promising techniques. In this method, water is heated under sufficient pressure to maintain its liquid state at a temperature above the boiling point (100 °C), but without reaching the critical point (374 °C). Under these conditions, water has a lower dielectric constant, thus behaving similarly to less polar organic solvents like methanol and ethanol [9]. SWE has been successfully applied for the recovery of polyphenols from plant by-products such as apple [10] or grape [11, 12] pomace, potato peels [13] and pistachio hulls [14].

Co-pigmentation is a colour-stabilising mechanism in which an anthocyanin (pigment) and another, usually

colourless, molecule (co-pigment) form complexes predominately by π - π stacking interactions in the aqueous environment of the plant vacuoles [15]. Mostly with polyphenols acting as co-pigments, this biological principle is assumed to be responsible for the colour variations that occur within epidermal flower tissues in a pH range where isolated anthocyanins are virtually colourless [16]. While the significance of co-pigmentation for the colour evolution during red wine aging has been thoroughly investigated [17, 18], it has not been frequently considered with respect to anthocyanin stability in fruit processing.

Recently, an extensive cell wall degradation by commercial enzymes was demonstrated to improve the extractability of polyphenolic antioxidants from saffron tepals [19]. Therefore, the present study evaluated the combined effect of subcritical water and enzymatic treatment on the yield of polyphenols from saffron tepals. The polyphenolic composition of the by-product extract was characterised using LC-MS analysis. Finally, the co-pigmentation efficiency of saffron tepal polyphenols was assessed in model solutions of strawberry anthocyanins. The retention of strawberry colour has always been a technological challenge due to the low total content of strawberry anthocyanins [20] and their inherent heat and light sensitivity [21].

Materials and Methods

Chemicals

The following standards were used for LC-MS identification: apigenin (Alfa Aesar, Thermo Fisher Scientific, Kandel, Germany); quercetin (Sigma-Aldrich, Steinheim, Germany); kaempferol (Phytoflan, Heidelberg, Germany). For analytical purposes the following reagents were used: Folin-Ciocalteu's reagent (Merck, Darmstadt, Germany); gallic acid monohydrate (Fluka, Buchs, Switzerland). The adsorbent resin Amberlite® XAD16N was provided by Sigma-Aldrich (Steinheim, Germany). All other reagents and solvents were of analytical or HPLC grade. Deionized water was used throughout.

Plant Materials and Enzyme Preparations

The flowers of *Crocus sativus* L. were collected in the autumn of 2019 in the region of Kozani (Ptelea village), Greece. After removing the stigmas, the saffron tepals (ST) were dried at room temperature (25–27 °C) for 1 week before final hot air drying (60 °C, 1 h). Dried saffron tepals were milled in a laboratory mill (model TSM6A011W/01, Robert Bosch Hausgeräte GmbH, München, Germany) and sieved (700 μ m laboratory sieve). The final saffron tepal

powder was stored in plastic bags at room temperature until usage.

Strawberries (*Fragaria × ananassa* Duch., cultivar 'Clery'; harvest 2020) were from the local market in Plovdiv and were stored frozen until usage.

The following commercial enzyme preparations were used: cellulolytic preparation Celluclast BG (Novozymes A/S, Bagsvaerd, Denmark); hemicellulolytic preparation Xylanase AN (Biovet JSC, Peshtera, Bulgaria).

Extraction of Saffron Tepal Polyphenols

A static mode subcritical water extraction (SWE) was performed using a homebuilt extraction system (constructed by Department of Industrial Heat Engineering, UFT-Plovdiv, Bulgaria) (Fig. 1). Water was heated to the desired temperature in a vessel 1 by an electric heater (InnoSolv LLC, Plovdiv, Bulgaria). A stainless steel extraction vessel (2), with a working volume of 1.7 L, was made by InnoSolv LLC, Plovdiv, Bulgaria. The extract was collected in a cooling vessel (3). To avoid boiling of the water, a back pressure was maintained in the system using carbon dioxide from gas cylinder 4. The heating and extraction vessels were thermally insulated.

Ground saffron tepals (100 g, particle size < 700 μ m) were extracted (160 °C, 0.9–1.0 MPa, 15 min) using a solid-to-solvent ratio of 1:11 (w/v). After separating the extract, the solid residue from the first SWE step (SWE-1) was directly mixed with 60 mL of an enzyme solution (3%, w/w), containing an enzyme preparations mixture (cellulase:xylanase = 1:1) in acidified (HCl, pH 4.0) water. The mixture was incubated for 3.5 h at 50 °C. The

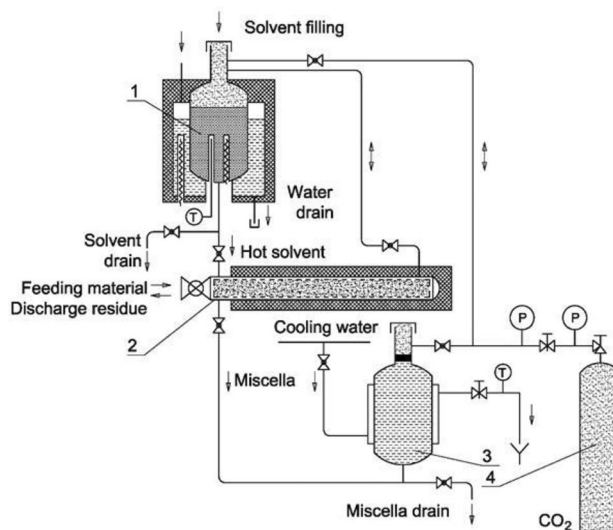


Fig. 1 Schematic diagram of the subcritical water extraction system: 1—water heating vessel; 2—extraction vessel; 3—cooling vessel; 4—CO₂ gas cylinder

enzymatically treated residue was transferred directly in the extraction vessel and was subjected to second SWE step (SWE-2; 1:11 solid-to-solvent, 160 °C, 0.9–1.0 MPa, 15 min). The obtained extracts were clarified through a paper filter.

The same procedure was applied for the control sample, but without enzymatic hydrolysis between the SWE steps.

After concentration under vacuum (40 °C), the combined extract originating from the enzyme-assisted SWE process was purified as described by Shikov et al. [22]. The purified extract was fed into a Büchi B-190 mini spray dryer (BÜCHI Labortechnik AG., Flawil, Switzerland). The operating conditions were as follows: aspirator rate—75% (26 m³/h); drying air temperature—136 °C; pump rate—6.5 mL/min; air-flow 600 L/h; outlet temperature – 62 °C. The dried extract was stored in a glass jar at -20 °C until usage.

Extraction of Strawberry Anthocyanins

Frozen strawberries were thawed and manually squeezed in a beaker. The homogenized purée (approximately 3 kg) was extracted overnight at 4 °C using 3 L of methanol acidified with hydrochloric acid (1%, v/v). The extraction mixture was filtered through a paper filter on a Buchner funnel, and the organic solvent was evaporated under vacuum (40 °C). To remove sugars, salts, and amino acids from the crude extract, the sample was purified as described by Shikov et al. [22]. The organic solvent of the eluate was evaporated under vacuum (40 °C). To separate anthocyanins from colorless phenolics, further purification was performed by extracting the aqueous phase three times with the same volume of ethyl acetate. After evaporation and concentration under vacuum (40 °C), the extract was frozen (-20 °C, overnight) and freeze dried (Cryodos-50, Telstar, Terrassa, Spain) at -46.6 °C under a pressure below 0.191 kPa for 48 h. The lyophilized extract was ground and stored in a glass jar at -20 °C until usage.

LC–MS Analysis

Prior to analysis, 50 mg of saffron extract powder was vortexed with 2 mL 70% methanol and sonicated for 10 min. After centrifugation (10 min, 9600×g, 20 °C), the methanol layer was collected in a 5 mL volumetric flask and the extraction procedure was repeated. The combined methanol layers were filled up with water and filtered through a disposable filter (regenerated cellulose, pore size 0.20 µm, Macherey–Nagel GmbH and Co. KG, Düren, Germany). The filtered sample was tenfold diluted with water.

Analysis was performed according to Dilberger et al. [23] with some modifications. Acquity UHPLC system by Waters (Milford, MA, USA) coupled with an LTQ XL linear ion trap by Thermo Scientific (Waltham, MA,

USA) was used. The Acquity UHPLC consisted of a binary pump, an autosampler (cooled at 20 °C), a column oven set at 40 °C, and a diode array detector scanning from 190 to 700 nm. An Acquity HSS-T3 RP18 column (150 mm×2.1 mm; 1.8 µm particle size) combined with a pre-column (Acquity UPLC HSS T3 VanGuard, 100 Å, 2.1 mm×5 mm, 1.8 µm), both from Waters (Milford, MA, USA), were used for chromatographic separation with water (A) and acetonitrile (B) as eluents, both acidified with formic acid (0.1 + 99.9, v + v). The flow rate was set at 0.5 mL/min with a gradient elution: 0.0–1.5 min: 1.0% B; 1.5–18.0 min: 1.0–25.0%B; 18.0–30.0 min: 25.0–100% B; 30.0–35.0 min: 100% B; 35.0–40.0 min: 100–1% B. The injection volume was 5 µL. A linear ion trap with an electrospray interface operating in negative ion mode was used. The mass spectrometry (MS) settings were as follows: sheath gas flow: 70 arb; auxiliary gas flow: 10 arb; sweep gas flow: 1 arb; capillary temperature: 350 °C. Negative mode: source voltage: 2.50 kV; capillary voltage: -21.00 V; multipole 00 offset: +5.50 V; multipole 0 offset: +6.25 V; multipole 1 offset: +10.00 V; front lens: 6.75 V. Normalized collision energy: MS2 35%, MS3 35%, using helium as collision gas. The system was controlled using Xcalibur Software 2.2 SP1.48 (Thermo Scientific, Waltham, MA, USA).

Spectrophotometric Methods for Chemical Analysis

All measurements were performed on a Helios Omega UV–Vis spectrophotometer equipped with VISIONlite software (all from Thermo Fisher Scientific, Madison, WI, USA), using 1 cm path length cuvettes.

The content of total polyphenols (TPP) was determined by the method of Singleton and Rossi [24] as modified by Dinkova et al. [25]. The results were presented as equivalents of gallic acid (GAE). Prior to analysis, an aliquot (10 mL) of saffron tepal extract was transferred into a 50 mL volumetric flask using 40 mL acidified (0.1% HCl) methanol. After extraction for 24 h at 10 °C, the flask was filled up to the mark with the solvent and filtered through a paper filter. Extractions were performed in triplicate.

The amount of total monomeric anthocyanins of the strawberry extract was determined by the pH-differential method [26]. The result was calculated using a molar extinction coefficient of 15,600 L/(mol cm) and molecular weight of 433.2 g/mol [27–29] and expressed as equivalents of pelargonidin 3-glucoside (PGE).

Co-pigmentation Experiments

The co-pigmentation effect of saffron tepals polyphenols was evaluated at different molar pigment/co-pigment

ratios. Stock solutions of strawberry (prepared as described in “[Extraction of Strawberry Anthocyanins](#)” section) and saffron tepal extracts (prepared as described in “[Extraction of Saffron Tepal Polyphenols](#)” section) were prepared on the basis of total anthocyanins (quantified as PGE) and total polyphenols (quantified as GAE), respectively. The stock solutions were prepared in McIlvaine buffer (0.1 M, pH 3.4) [30]. Model solutions of strawberry anthocyanins (1×10^{-4} M) with and without co-pigment addition were obtained by mixing 5 mL of both stock solutions and were then left for equilibration (30 min at 25 °C). The following molar pigment/co-pigment ratios were used: 1:0; 1:2.5; 1:5; 1:7.5; 1:10. Model solutions were prepared in triplicate.

The thermal stability of the mixtures was evaluated via heating experiments at 85 °C performed in a W3 laboratory water bath (VEB MLW Prüfgeräte-Werk Medingen, Freital, Germany). After 0, 60, 120, 180 and 240 min heating the glass tubes were immediately cooled in an ice bath to stop thermal degradation of anthocyanins. An aliquot of each sample was filtered through a Filtrak 390 paper filter (VEB Spezialpapierfabrik Niederschlag, Niederschlag/Erzgebirge, Germany).

In order to achieve spectrophotometrically measurable absorption values (absorbance < 2 AU), the model solutions were diluted (1:1, v/v) with the McIlvaine buffer. Visible spectra were recorded in a range of 450 to 700 nm. The absorbance at the wavelength of maximum absorption and the wavelength of the absorption maximum were taken down.

The co-pigmentation effect (CE) and spectral shift of the absorption maximum ($\Delta\lambda_{\max}$) were determined as follows Gras et al. [31]:

$$\text{CE (\%)} = ((A - A_0)/A_0) \times 100,$$

where A_0 is the absorbance at the wavelength of maximum absorption (λ_{\max}) of the anthocyanin solution without co-pigments, and A is the absorbance at λ_{\max} of the respective anthocyanin solutions with added co-pigments;

$$\Delta\lambda_{\max} = \lambda_{\max\text{CP}} - \lambda_{\max0},$$

where $\lambda_{\max0}$ is the wavelength of the absorption maximum of the anthocyanin solutions without co-pigments and $\lambda_{\max\text{CP}}$ is the wavelength of the absorption maximum of the corresponding solutions with added co-pigments.

All measurements were performed with a Helios Omega UV–Vis spectrophotometer equipped with ColorCalc Basic software (all from Thermo Fisher Scientific, Madison, WI, USA), using 1 cm path length cuvettes.

Statistical Analysis

The reported in the present study results are the mean values of at least three analytical determinations and the coefficients of variation, expressed as the percentage ratios between the standard deviations and the mean values. The means were compared using one-way ANOVA, performed with Microsoft Excel, and Tukey’s test at a 95% confidence level.

Results and Discussion

Yield of Saffron Tepal Polyphenols

A novel process, enzyme-assisted subcritical water extraction, was established on a pilot-plant scale (Fig. 2). This process combines two subcritical water extraction (SWE) steps with enzymatic treatment after the first SWE (SWE-1). The second SWE (SWE-2) was also used as the enzyme inactivation step.

Ahmadian-Kouchaksaraie et al. [32] determined optimum conditions (159 °C, 54 min) of SWE for recovery of total polyphenols from saffron tepals. However, the efficient extraction of flavonols, which are the main phenolic compounds in saffron tepals [33], has been shown to be faster (15–30 min) at extraction temperatures of 145–165 °C [34, 35]. Therefore, the SWE in the present study was performed at extraction temperature of 160 °C and total extraction time of 30 min (2 × 15 min steps).

The crude extract was purified using adsorber technology [36], which has been successfully applied to the recovery of phenolic compounds from plant by-products such as apple [37] and grape [38] pomace.

The enzymatic treatment resulted in an approximately fourfold increase in the polyphenolic yield (up to 8.37 g GAE/kg saffron tepals for the total SWE) compared with the control sample (without enzymatic treatment) (Fig. 3).

This result confirms the efficiency of the enzymes applied in the cell wall degradation. The structure of the plant cell walls illustrates the mechanism of synergistic effects between xylanase and cellulase. In the cell walls, xylan chains (arabinoxylans) are thought to bind to cellulose microfiber surfaces and bind several cellulose microfibers to form crosslinked structures [39]. This cell wall structure model suggests that the degradation of xylan networks between cellulose microfibers by xylanases can allow cellulases to access and degrade the cellulose. Also, the degradation of cellulose microfibers can help xylanase to access and further degrade xylan chains. This degradation model could explain the unique characteristics of synergy between xylanase and cellulase [40].

Therefore, the mechanism proposed above, in combination with the high temperature and pressure applied during

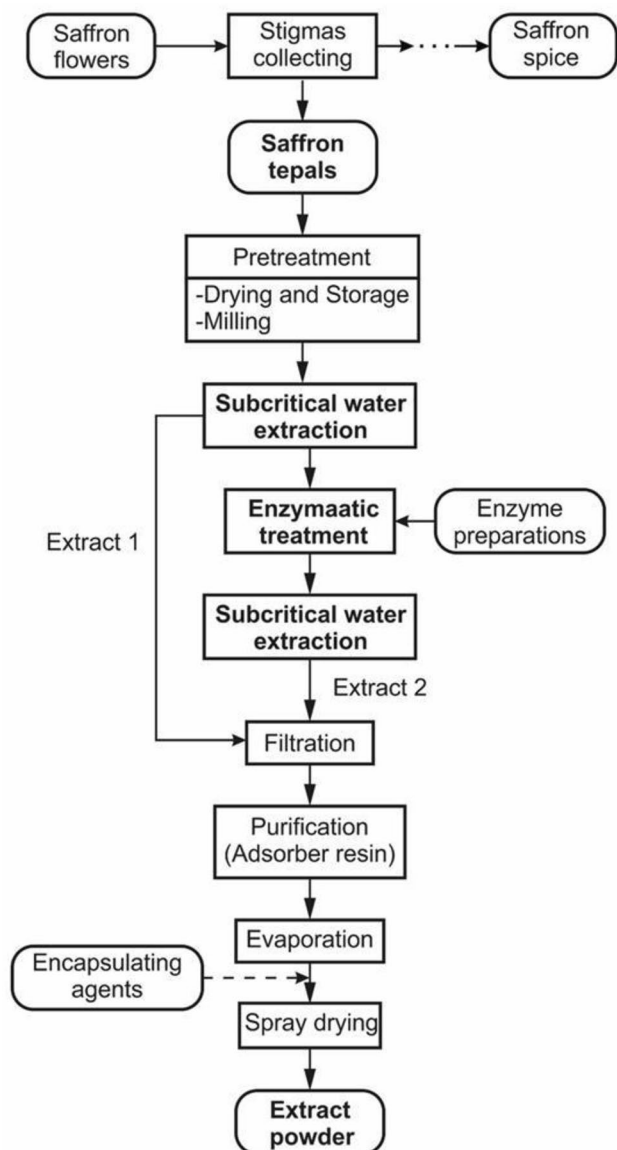


Fig. 2 Flow diagram of the new process for recovery of polyphenols from saffron tepals: — main process step; --- additional process step

the SWE, may be a plausible explanation for the enhanced extractability of polyphenols from saffron tepals.

Characterization of Saffron Tepal Polyphenols

The identification of flavonoids was based on the UV spectra, MSⁿ spectra and chromatographic behaviour, and by comparison with previous studies and to reference compounds where available.

The UHPLC-DAD chromatogram of the saffron tepal extract at 350 nm is shown in Fig. 4. Retention times, UV maxima, and MSⁿ data are shown in Table 1.

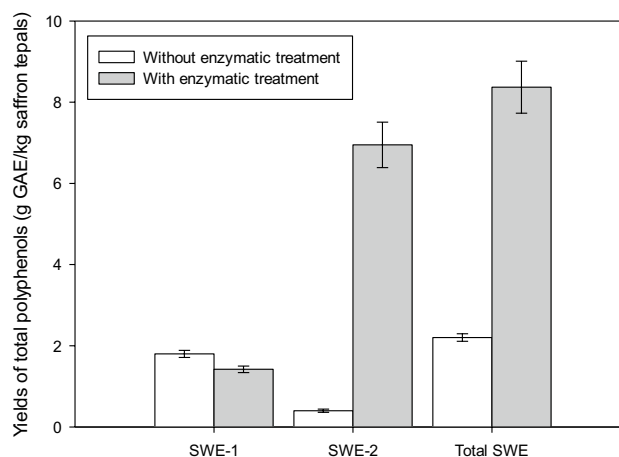


Fig. 3 Yields of total polyphenols (g GAE/kg saffron tepals) depending on the different extraction processes—with or without enzymatic treatment between the two subcritical water extraction (SWE) steps

Compound **1** exhibited a molecular mass $[M-H]^-$ at m/z 787. Diagnostic fragments were recorded at m/z 625 $[M-H-162]^-$, 300 $[M-H-162-325]^-$, 445 $[M-H-162-180]^-$ and 301 $[M-H-162-324]^-$. The fragmentation behavior is in accordance with literature data and corresponds in particular to the loss of glucose in position 7 (m/z 625) and of the terminal glucose of the sophoroside moiety (m/z 445) [41, 42]. Homo- and heterolytic cleavage of the glycosidic bond (m/z 300 and m/z 301) was observed in the MS³ experiment of m/z 625, releasing the aglycone quercetin. Thus, this compound was tentatively characterized as quercetin 3-*O*-sophoroside-7-*O*-glucoside. Analogously, peak **2** (m/z 771), producing in the MS³ experiment the characteristic fragment of the aglycone kaempferol at m/z 285, was attributed to kaempferol 3-*O*-sophoroside-7-*O*-glucoside [41].

The 3,7-diglucosides and 3-sophorosides of flavonols have the same m/z value. Differentiation of both was still possible because of four diagnostic fragments produced by the loss of 324 amu, 180 amu, 162 amu, and 120 amu. In 3,7-diglucosides, a main fragment ion $[M-H-162]^-$ can be observed in the MS² experiment with concurrent absence of the product ion $[M-H-180]^-$ [42]. In both the 3,7-diglucosides and the 3-sophorosides, the loss of 324 amu is specific, but in 3-sophorosides it represents the main fragment. Compound **3** (m/z 609) released the product ions at m/z 447 $[M-H-162]^-$, 489 $[M-H-120]^-$ and 285 $[M-H-324]^-$, indicating the typical fragmentation pattern of the 3,7-di-*O*-glucoside of kaempferol [42]. In contrast, compound **5** (m/z 639) yielded the main fragment ions at m/z 315 $[M-H-324]^-$ and 477 $[M-H-162]^-$. In the extracted ion chromatogram of m/z 639, a second peak was detected referring to the isorhamnetin 3-sophoroside due to typical product ions at m/z 315 $[M-H-324]^-$, 459 $[M-H-180]^-$,

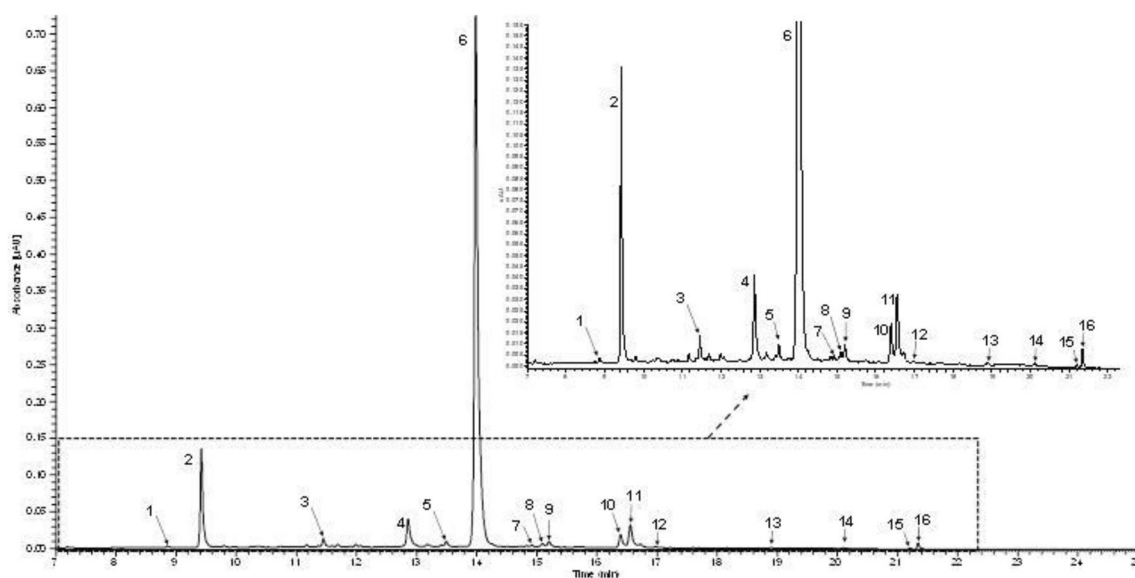


Fig. 4 UHPLC-DAD chromatogram at 350 nm of *Crocus sativus* tepal extract. For peak assignment see Table 1

Table 1 Characteristics of phenolic compounds in saffron tepal extract

Peak	Peak assignment	Retention time (min)	λ_{\max} (nm)	$[M-H]^-$ (m/z)	Fragment ions MS^2 (m/z)	Fragment ions MS^3 (m/z)*
1	Quercetin 3- <i>O</i> -sophoroside-7- <i>O</i> -glucoside	8.86	358	787	625	300/445/301
2	Kaempferol 3- <i>O</i> -sophoroside-7- <i>O</i> -glucoside	9.42	351	771	609	429/285
3	Kaempferol 3,7-di- <i>O</i> -glucoside	11.45	349	609	447 /489/285	284/285
4	Quercetin 3- <i>O</i> -sophoroside	12.86	355	625	300 /301/445	271/255/179/272/151
5	Isorhamnetin 3,7-di- <i>O</i> -glucoside	13.50	349	639	315 /477	300/151/287/272
6	Kaempferol 3- <i>O</i> -sophoroside	13.99	351	609	285 /429	151/241/257/285
7	Kaempferol 3- <i>O</i> -rutinoside	14.92	351	593	285 /429/447	255/256/227/284
8	Quercetin 3- <i>O</i> -glucoside	15.10	353	463	301	179/151
9	Isorhamnetin 3- <i>O</i> -rutinoside	15.20	358	623	315 /477	300/299/287/272
10	Kaempferol 3- <i>O</i> -(6"-acetyl)-glycoside-7- <i>O</i> -glycoside	16.40	351	651	471 /285	429
11	Kaempferol 3- <i>O</i> -glucoside	16.56	351	447	285	257/256/285
12	Isorhamnetin 3- <i>O</i> -glucoside	16.98	352	477	314 /315	285/271/286/300
13	Kaempferol 3- <i>O</i> -(6"-acetyl)-glycoside	18.90	351	489	285	257/256/267/285
14	Quercetin	20.11	364	301	179 /151	151
15	Apigenin	21.20	336	269	225 /149/201	181/169/197/225/183
16	Kaempferol	21.34	370	285	285 /286/151	285/286/164/185/151

*Precursor in bold

519 $[M-H-120]^-$ and 477 $[M-H-162]^-$ (data not shown) [41, 42]. Therefore, compound 5 was assigned to 3,7-di-*O*-glucoside of isorhamnetin, even though the main product ion was produced by the simultaneous loss of both sugar moieties. The fragmentation of compounds 4 and 6 is typical for a 3-*O*-sophoroside, characterized by the loss of 324 amu during the MS^2 experiment, which released the aglycone. The loss of the terminal glucose from the sophorosyl

moiety 180 amu [41, 42] was also observed. Therefore, the 3-*O*-sophoroside derivatives of quercetin (4) and kaempferol (6) were identified. Consistent with Goupy et al. [43], kaempferol 3-*O*-sophoroside was the major phenolic compound in *Crocus sativus* tepals.

After MS^2 fragmentation, peaks 7 (m/z 593) and 9 (m/z 623) exhibited product ions at m/z 285, 447 and m/z 315 and 477, respectively. These diagnostic fragments are indicative

of a rutinosyl moiety, which allowed the identification of compound **7** as kaempferol 3-*O*-rutinoside and compound **9** as isorhamnetin 3-*O*-rutinoside [6, 43].

In accordance with literature data, the 3-glucosides of quercetin (**8**), kaempferol (**11**) and isorhamnetin (**12**) were also characterized by the loss of the glucose moiety $[M-H-162]^-$, by their UV spectra and by their elution order in comparison to the other flavonol glycosides [6, 42, 43].

Two acylated flavonol glycosides were tentatively identified by their fragmentation in MS^2 and MS^3 experiments. For compounds **10** and **13**, product ions at $[M-H-324-42]^-$ and $[M-H-162-42]^-$, corresponding to acetyldiglycosyl and acetylglycosyl units, respectively, were detected. Therefore, these compounds were characterized as kaempferol 3-*O*-(6''-acetyl)-glycoside-7-*O*-glycoside (**10**) and kaempferol 3-*O*-(6''-acetyl)-glycoside (**13**).

Peaks **14** and **16** were attributed to the aglycones quercetin and kaempferol, respectively. Interestingly, compound **15** produced a molecular ion at m/z 269 and fragment ions m/z 225, 149 and 201, which are characteristic of the flavone apigenin [44–46]. The identification of these three compounds was confirmed by comparison with authentic reference compounds. To our knowledge, this is the first report on presence of apigenin in *Crocus sativus* tepals, however in trace amounts. Carryover during chromatography can be ruled out by the analysis of blank samples.

Co-pigmentation Efficiency of Saffron Tepal Polyphenols in Model Solutions of Strawberry Anthocyanins

The interactions between strawberry anthocyanins (pigment) and the saffron tepal polyphenols (co-pigment) were studied spectrophotometrically in model solutions at pH 3.4, which is similar to that of strawberry juice. The kinetic reaction approach was used which allowed studying the influence of saffron tepals polyphenols on the thermal stability of strawberry anthocyanins.

To investigate the co-pigmentation efficiency against strawberry anthocyanins, the saffron tepal extract was purified using a polymeric adsorber resin, which resulted in 6.1% content of total polyphenols (quantified as GAE).

As shown in Fig. 5, in accordance with Petrova et al. [47], the magnitude of the co-pigmentation effect (CE) increased with the co-pigment concentration. For the solutions with the highest molar pigment/co-pigment ratio (1:10), the CE amounted was 116%.

However, in contrast to the addition of rose petal polyphenols [47], no bathochromic shifts ($\Delta\lambda_{max}$) were observed for the saffron tepal polyphenols, implying that the co-pigmentation reaction does not change the colour tonality of the strawberry anthocyanins.

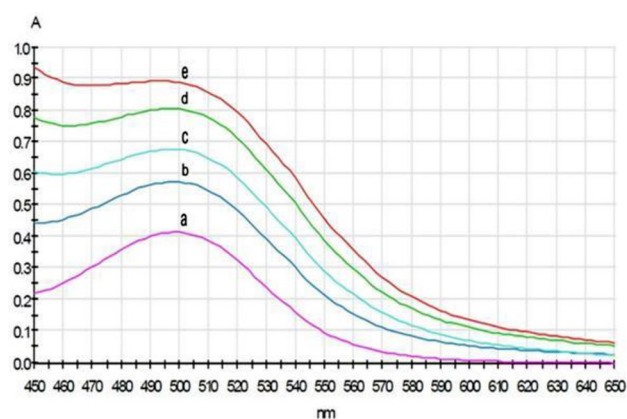


Fig. 5 Visible absorption spectra (450–650 nm) of strawberry anthocyanins in model solutions with added saffron tepal polyphenols. Different molar pigment/co-pigment ratios: (a) 1:0; (b) 1:2.5; (c) 1:5; (d) 1:7.5; (e) 1:10

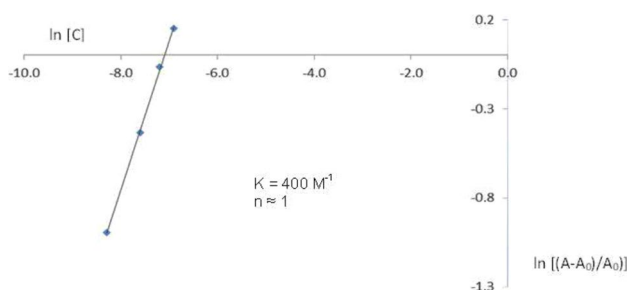


Fig. 6 Plots of co-pigmentation effect ($\ln[(A-A_0)/A_0]$) as a function of the co-pigment concentration ($\ln[C]$), $y = 0.835x + 5.926$ ($R^2 = 0.99$)

Besides spectral variations, some thermodynamic parameters of the co-pigmentation reaction were determined using the following equation:

$$\ln[(A - A_0)/A_0] = \ln[K] + n \times \ln[C],$$

where A is the absorption maximum value for the co-pigmented anthocyanins, A_0 is the absorption maximum value for the anthocyanins alone, C is the molar co-pigment concentration, K is the equilibrium constant of the reaction and n is the stoichiometric ratio of the compounds in the complex [48].

Figure 6 shows co-pigmentation effect ($\ln[(A-A_0)/A_0]$) as a function of the co-pigment concentration ($\ln[C]$) at time 0. The concentrations of saffron tepals polyphenol used to plot Fig. 6 were 2.5×10^{-4} ; 5.0×10^{-4} ; 7.5×10^{-4} and 10.0×10^{-4} M. The plot shows linear relationships with slope close to unity, suggesting a 1:1 pigment/co-pigment complex, which is in agreement with other studies [48, 49]. The calculated value of the equilibrium

Table 2 Absorbance at absorption maximum (A_{max}) and half-life ($T_{1/2}$) values of strawberry anthocyanins (pigment) upon heating at 85 °C in model solutions with added saffron tepal polyphenols (co-pigment)

Heating time (min)	Molar pigment/co-pigment ratio				
	1:0	1:2.5	1:5	1:7.5	1:10
	A_{max} *				
0	0.417 ± 0.021a	0.571 ± 0.029a	0.687 ± 0.034a	0.808 ± 0.040a	0.902 ± 0.045a
60	0.308 ± 0.015b	0.495 ± 0.025b	0.602 ± 0.030b	0.741 ± 0.037a	0.798 ± 0.040b
120	0.281 ± 0.014bc	0.428 ± 0.021cf	0.540 ± 0.027bc	0.642 ± 0.032b	0.774 ± 0.039be
180	0.254 ± 0.013cd	0.394 ± 0.020df	0.525 ± 0.026bc	0.604 ± 0.030b	0.651 ± 0.032cd
240	0.228 ± 0.011d	0.369 ± 0.018ed	0.491 ± 0.025c	0.587 ± 0.029b	0.700 ± 0.035de
	$T_{1/2}$ (min)				
	(R^{**})				
	250.2	348.3	450.1	462.1	513.4
	(0.96)	(0.99)	(0.97)	(0.97)	(0.90)

*Values are presented as means ± standard deviations (n = 3). Different letters within a column indicate significant differences (Tukey's test, $P < 0.05$)

** R —Correlation coefficient

constant, reflecting stability of the complex formed, was ca. 400 M⁻¹.

Further, pigment degradation was assessed by the absorbance measurements [50]. Half-life ($T_{1/2}$) values were determined as described by Sadilova et al. [51] which allowed comparing the times for 50% reduction of the anthocyanins in the model solutions with different co-pigment concentrations. Higher half-life ($T_{1/2}$) means increased anthocyanins stability.

As shown in Table 2, with the increasing of the heating times the absorbance values (A_{max}) were significantly lowered. As indicated by the high values of the correlation coefficient ($R = 0.90–0.99$), anthocyanin degradation ideally followed first-order reaction kinetics (Table 2). These findings are in accordance with previous studies reporting first-order degradation kinetics for strawberry anthocyanins both in model systems [22, 52] and real food matrices [53, 54].

The half-life ($T_{1/2}$) values increased (39–105%) due to the addition of saffron tepal extract, reflecting lower pigment degradation rates in the presence of polyphenolic co-pigments, which is in agreement with other studies [22, 55]. Maximum heat stability was reached at the highest pigment/co-pigment ratio (1:10).

Conclusion

A new process for recovery of saffron tepal polyphenols was developed on pilot-plant scale. Combining 2-step sub-critical water extraction and enzymatic hydrolysis resulted in a notably higher recovery rate compared to the control

(without enzymatic treatment) sample. Together with the application of adsorber technology for the concentration of phenolic compounds, this new process may provide an innovative approach for valorization of the saffron by-product as a source of natural co-pigments.

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Declarations

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References

1. Schieber, A.: Side streams of plant food processing as a source of valuable compounds: selected examples. *Annu. Rev. Food*

- Sci. Technol. **8**, 97–112 (2017). <https://doi.org/10.1146/annur-ev-food-030216-030135>
2. Carmona, M., Sánchez, A.M., Ferreres, F., Zalacain, A., Tomás-Barberán, F., Alonso, G.L.: Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: comparative study of samples from different geographical origins. *Food Chem.* **100**, 445–450 (2007). <https://doi.org/10.1016/j.foodchem.2005.09.065>
 3. Goli, S.A.H., Mokhtari, F., Rahimmalek, M.: Phenolic compounds and antioxidant activity from saffron (*Crocus sativus* L.) petal. *J. Agric. Sci.* **4**, 175–181 (2012)
 4. Serrano-Díaz, J., Sánchez, A.M., Maggi, L., Martínez-Tomé, M., García-Diz, L., Murcia, M.A., Alonso, G.L.: Increasing the applications of *Crocus sativus* flowers as natural antioxidants. *J. Food Sci.* **77**, C1162–C1168 (2012). <https://doi.org/10.1111/j.1750-3841.2012.02926.x>
 5. Mykhailenko, O., Kovalyov, V., Goryacha, O., Ivanauskas, L., Georgiyants, V.: Biologically active compounds and pharmacological activities of species of the genus *Crocus*: a review. *Phytochemistry* **162**, 56–89 (2019). <https://doi.org/10.1016/j.phytochem.2019.02.004>
 6. Tuberoso, C.I.G., Rosa, A., Montoro, P., Fenu, M.A., Pizza, C.: Antioxidant activity, cytotoxic activity and metabolic profiling of juices obtained from saffron (*Crocus sativus* L.) floral by-products. *Food Chem.* **199**, 18–27 (2016). <https://doi.org/10.1016/j.foodchem.2015.11.115>
 7. Termentzi, A., Kokkalou, E.: LC-DAD-MS (ESI+) analysis and antioxidant capacity of *Crocus sativus* petal extracts. *Planta Med.* **74**, 573–581 (2008). <https://doi.org/10.1055/s-2008-1074498>
 8. Chemat, F., Albert-Vian, M., Cravotto, G.: Green extraction of natural products: concept and principles. *Int. J. Mol. Sci.* **13**, 8615–8627 (2012). <https://doi.org/10.3390/ijms13078615>
 9. Teo, C.C., Tan, S.N., Yong, J.W.H., Hew, C.S., Ong, E.S.: Pressurized hot water extraction (PHWE). *J. Chromatogr. A* **1217**, 2484–2494 (2010). <https://doi.org/10.1016/j.chroma.2009.12.050>
 10. Plaza, M., Abrahamsson, V., Turner, C.: Extraction and neof ormation of antioxidant compounds by pressurized hot water extraction from apple byproducts. *J. Agric. Food Chem.* **61**, 5500–5510 (2013). <https://doi.org/10.1021/jf400584f>
 11. Monrad, J.K., Suárez, M., Motilva, M.J., King, J.W., Srinivas, K., Howard, L.R.: Extraction of anthocyanins and flavan-3-ols from red grape pomace continuously by coupling hot water extraction with a modified expeller. *Food Res. Int.* **65**, 77–87 (2014). <https://doi.org/10.1016/j.foodres.2014.04.020>
 12. Vergara-Salinas, J.R., Bulnes, P., Zúñiga, M.C., Pérez-Jiménez, J., Torres, J.L., Mateos-Martín, M.L.: Effect of pressurized hot water extraction on antioxidants from grape pomace before and after enological fermentation. *J. Agric. Food Chem.* **61**, 6929–6936 (2013). <https://doi.org/10.1021/jf4010143>
 13. Singh, P.P., Saldaña, M.D.A.: Subcritical water extraction of phenolic compounds from potato peel. *Food Res. Int.* **44**, 2452–2458 (2011). <https://doi.org/10.1016/j.foodres.2011.02.006>
 14. Erşan, S., Güçlü Üstündağ, Ö., Carle, R., Schweiggert, R.M.: Subcritical water extraction of phenolic and antioxidant constituents from pistachio (*Pistacia vera* L.) hulls. *Food Chem.* **253**, 46–54 (2018). <https://doi.org/10.1016/j.foodchem.2018.01.116>
 15. Brouillard, R., Chassaing, S., Isorez, G., Kueny-Stotz, M., Figueiredo, P.: The visible flavonoids or anthocyanins: from research to applications. In: Santos-Buelga, C., Escribano-Bailon, M.T., Lattanzio, V. (eds.) *Recent Advances in Polyphenol Research*, pp. 1–22. Wiley, New York (2010)
 16. Asen, S., Stewart, R.N., Norris, K.H.: Co-pigmentation of anthocyanins in plant tissues and its effect on color. *Phytochemistry* **11**, 1139–1144 (1972). [https://doi.org/10.1016/S0031-9422\(00\)88467-8](https://doi.org/10.1016/S0031-9422(00)88467-8)
 17. Boulton, R.: The copigmentation of anthocyanins and its role in the color of red wine: a critical review. *Am. J. Enol. Vitic.* **52**, 67–87 (2001)
 18. de Freitas, V.A.P., Fernandes, A., Oliveira, J., Teixeira, N., Mateus, N.: A review of the current knowledge of red wine colour. *Oeno ONE* (2017). <https://doi.org/10.20870/oeno-one.2017.51.1.1604>
 19. Vardakas, A., Shikov, V., Dinkova, R., Mihalev, K.: Optimisation of the enzyme-assisted extraction of polyphenols from saffron (*Crocus sativus* L.) tepals. *Acta Scientiarum Polonorum Technologia Alimentaria* **20**, 359–367 (2021). <https://doi.org/10.17306/J.AFS.2021.0954>
 20. Clifford, M.N.: Anthocyanins—nature, occurrence and dietary burden. *J. Sci. Food Agric.* **80**, 1063–1072 (2000). [https://doi.org/10.1002/\(SICI\)1097-0010\(20000515\)80:7%3c1063::AID-JSFA605%3e3.0.CO;2-Q](https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7%3c1063::AID-JSFA605%3e3.0.CO;2-Q)
 21. Hayashi, K., Ohara, N., Tsukui, A.: Stability of anthocyanins in various vegetables and fruits. *Food Sci. Technol. Int.* **2**, 30–33 (1996). <https://doi.org/10.3136/fsti9596t9798.2.30>
 22. Shikov, V., Kammerer, D.R., Mihalev, K., Mollov, P., Carle, R.: Heat stability of strawberry anthocyanins in model solutions containing natural copigments extracted from rose (*Rosa damascena* Mill.) petals. *J. Agric. Food Chem.* **56**, 8521–8526 (2008). <https://doi.org/10.1021/jf801946g>
 23. Dilberger, B., Passon, M., Asseburg, H., Silaidos, C.V., Schmitt, F., Schmi edl, T., Schieber, A., Eckert, G.P.: Polyphenols and metabolites enhance survival in rodents and nematodes—impact of mitochondria. *Nutrients* **11**, 1886 (2019). <https://doi.org/10.3390/nu11081886>
 24. Singleton, V.L., Rossi, J.A.: Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **16**, 144–158 (1965)
 25. Dinkova, R., Heffels, P., Shikov, V., Weber, F., Schieber, A., Mihalev, K.: Effect of enzyme-assisted extraction on the chilled storage stability of bilberry (*Vaccinium myrtillus* L.) anthocyanins in skin extracts and freshly pressed juices. *Food Res. Int.* **65**, 35–41 (2014). <https://doi.org/10.1016/j.foodres.2014.05.066>
 26. Giusti, M.M., Wrolstad, R.E.: Characterization and measurement of anthocyanins by UV-visible spectroscopy. In: Wrolstad, R.E. (ed.) *Current Protocols in Food Analytical Chemistry*, p. F1.2.1–F1.2.13. Wiley, New York (2001)
 27. Giusti, M.M., Rodriguez-Saona, E.L., Wrolstad, E.R.: Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. *J. Agric. Food Chem.* **47**, 4631–4637 (1999). <https://doi.org/10.1021/jf981271k>
 28. Moyer, R.A., Hummer, K.E., Finn, C.E., Frei, B., Wrolstad, R.E.: Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus*, and *Ribes*. *J. Agric. Food Chem.* **50**, 519–525 (2002). <https://doi.org/10.1021/jf011062r>
 29. Tonutare, T., Moor, U., Szajdak, L.: Strawberry anthocyanin determination by pH differential spectroscopic method—how to get true results? *Acta Scientiarum Polonorum Hortorum Cultus* **13**, 35–47 (2014)
 30. Elving, P.J., Markowitz, J.M., Rosenthal, I.: Preparation of buffer systems of constant ionic strength. *Anal. Chem.* **28**, 1179–1180 (1956). <https://doi.org/10.1021/ac60115a034>
 31. Gras, C.C., Bause, K., Leptihn, S., Carle, R., Schweiggert, R.M.: Effect of chlorogenic acid on spectral properties and stability of acylated and non-acylated cyanidin-3-*O*-glycosides. *Food Chem.* **240**, 940–950 (2018). <https://doi.org/10.1016/j.foodchem.2017.07.137>
 32. Ahmadian-Kouchaksaraie, Z., Niazmand, R., Najafi, M.N.: Optimization of the subcritical water extraction of phenolic antioxidants from *Crocus sativus* petals of saffron industry residues: Box-Behnken design and principal component analysis. *Innov.*

- Food Sci. Emerg. Technol. **36**, 234–244 (2016). <https://doi.org/10.1016/j.ifset.2021.102811>
33. Lakka, A., Grigorakis, S., Karageorgou, I., Batra, G., Kaltsa, O., Bozinou, E., Lalas, S., Makris, D.P.: Saffron processing wastes as a bioresource of high-value added compounds: development of a green extraction process for polyphenol recovery using a natural deep eutectic solvent. *Antioxidants* **8**, 586 (2019). <https://doi.org/10.3390/antiox8120586>
 34. Benito-Román, Ó., Blanco, B., Sanz, M.T., Beltrán, S.: Subcritical water extraction of phenolic compounds from onion skin wastes (*Allium cepa* cv. Horcal): effect of temperature and solvent properties. *Antioxidants* **9**, 1233 (2020). <https://doi.org/10.3390/antiox9121233>
 35. Ko, M.J., Cheigh, C.-I., Cho, S.W., Myong-Soo Chung, M.S.: Subcritical water extraction of flavonol quercetin from onion skin. *J. Food Eng.* **102**, 327–333 (2011). <https://doi.org/10.1016/j.jfoodeng.2010.09.008>
 36. Kammerer, J., Carle, R., Kammerer, D.R.: Adsorption and ion exchange: basic principles and their application in food processing. *J. Agric. Food Chem.* **59**, 22–42 (2011). <https://doi.org/10.1021/jf1032203>
 37. Schieber, A., Hilt, P., Streker, P., Endress, H.-U., Rentschler, C., Carle, R.: A new process for the combined recovery of pectin and phenolic compounds from apple pomace. *Innov. Food Sci. Emerg. Technol.* **4**, 99–107 (2003). [https://doi.org/10.1016/S1466-8564\(02\)00087-5](https://doi.org/10.1016/S1466-8564(02)00087-5)
 38. Kammerer, D., GajdosKljusuric, J., Carle, R., Schieber, A.: Recovery of anthocyanins from grape pomace extracts (*Vitis vinifera* L. cv “Cabernet Mitos”) using a polymeric adsorber resin. *Eur. Food Res. Technol.* **220**, 431–437 (2005). <https://doi.org/10.1007/s00217-004-1078-z>
 39. Carpita, N.: Structure and biogenesis of the cell walls of grasses. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 445–476 (1996)
 40. Murashima, K., Kosugi, A., Doi, H.R.: Synergistic effects of cellulosomal xylanase and cellulases from *Clostridium cellulovorans* on plant cell wall degradation. *J. Bacteriol.* **185**, 1518–1524 (2003). <https://doi.org/10.1128/JB.185.5.1518-1524.2003>
 41. Ferreres, F., Llorach, R., Gil-Izquierdo, A.: Characterization of the interglycosidic linkage in di-, tri-, tetra- and pentaglycosylated flavonoids and differentiation of positional isomers by liquid chromatography/electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **39**, 312–321 (2004). <https://doi.org/10.1002/jms.586>
 42. Vallejo, F., Tomas-Barberan, F.A., Ferreres, F.: Characterisation of flavonols in broccoli (*Brassica oleracea* L. var italica) by liquid chromatography–UV diode array detection–electrospray ionisation mass spectrometry. *J. Chromatogr. A* **1054**, 181–193 (2004). <https://doi.org/10.1016/j.chroma.2004.05.045>
 43. Goupy, P., Vian, M.A., Chemat, F., Caris-Veyrata, C.: Identification and quantification of flavonols, anthocyanins and lutein diesters in tepals of *Crocus sativus* by ultra performance liquid chromatography coupled to diode array and ion trap mass spectrometry detections. *Ind. Crops Prod.* **44**, 496–510 (2013). <https://doi.org/10.1016/j.indcrop.2012.10.004>
 44. Kang, J., Price, W.E., Ashton, J., Tapsell, L.C., Johnson, S.: Identification and characterization of phenolic compounds in hydromethanolic extracts of sorghum wholegrains by LC-ESI-MSn. *Food Chem.* **211**, 215–226 (2016). <https://doi.org/10.1016/j.foodchem.2016.05.052>
 45. Šibul, F., Orčić, D., Berežni, S., Anačkov, G., Mimica-Dukić, N.: HPLC–MS/MS profiling of wild-growing scentless chamomile. *Acta Chromatogr.* **32**, 86–94 (2020). <https://doi.org/10.1556/1326.2019.00546>
 46. Fabre, N., Rustan, I., de Hoffmann, E., Quetin-Leclercq, J.: Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J. Am. Soc. Mass Spectrom.* **12**(6), 707–715 (2001). [https://doi.org/10.1016/S1044-0305\(01\)00226-4](https://doi.org/10.1016/S1044-0305(01)00226-4)
 47. Petrova, I., Mihalev, K., Shikov, V., Gandova, V.: Thermodynamic investigation of the copigmentation process between strawberry anthocyanins and rose petal polyphenols (*Rosa damascena* Mill.) at different temperatures. *E3S Web Conf.* **207**, 01020 (2020). <https://doi.org/10.1051/e3sconf/202020701020>
 48. Oszmiański, J., Bąkowska, A., Piacente, S.: Thermodynamic characteristics of copigmentation reaction of acylated anthocyanin isolated from blue flowers of *Scutellaria baicalensis* Georgi with copigments. *J. Sci. Food Agric.* **84**, 1500–1506 (2004). <https://doi.org/10.1002/jsfa.1815>
 49. Malaj, N., De Simone, B.C., Quartarolo, A.D., Russo, N.: Spectrophotometric study of the copigmentation of malvidin 3-*O*-glucoside with *p*-coumaric, vanillic and syringic acids. *Food Chem.* **141**, 3614–3620 (2013). <https://doi.org/10.1016/j.foodchem.2013.06.017>
 50. Gradinaru, G., Biliaderis, C.G., Kallithraka, S., Kefalas, P., Garcia-Viguera, C.: Thermal stability of *Hibiscus sabdariffa* L. anthocyanins in solution and in solid state: effects of copigmentation and glass transition. *Food Chem.* **83**, 423–436 (2003). [https://doi.org/10.1016/S0308-8146\(03\)00125-0](https://doi.org/10.1016/S0308-8146(03)00125-0)
 51. Sadilova, E., Carle, R., Stintzing, F.C.: Thermal degradation of anthocyanins and its impact on color and in vitro antioxidant capacity. *Mol. Nutr. Food Res.* **51**, 1461–1471 (2007). <https://doi.org/10.1002/mnfr.200700179>
 52. Tanchev, S., Iontcheva, N.: Kinetik des thermischen Abbaues der anthocyane pelargonidin-3-glucosid und cyanidin-3-glucosid. *Nahrung* **19**, 629–633 (1975). <https://doi.org/10.1002/food.19750190803>
 53. Garzón, G.A., Wrolstad, R.E.: Comparison of the stability of pelargonidin-based anthocyanins in strawberry juice and concentrate. *J. Food Sci.* **67**, 1288–1299 (2002). <https://doi.org/10.1111/j.1365-2621.2002.tb10277.x>
 54. Skrede, G., Wrolstad, R.E., Lea, P., Enersen, G.: Color stability of strawberry and blackcurrant syrups. *J. Food Sci.* **57**, 172–177 (1992). <https://doi.org/10.1111/j.1365-2621.1992.tb05449.x>
 55. Brenes, C.H., Del Pozo-Insfran, D., Talcott, S.: Stability of copigmented anthocyanins and ascorbic acid in a grape juice model system. *J. Agric. Food Chem.* **53**, 49–56 (2005). <https://doi.org/10.1021/jf049857w>

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