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Co-pigmentation of strawberry anthocyanins with phenolic compounds from rooibos

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ABSTRACT

Anthocyanin-rich strawberry model solutions were co-pigmented with rooibos phenolics to enhance color and heat stability. The addition of green and fermented rooibos extracts at pigment-to-co-pigment molar ratios of 1:10, 1:50, and 1:100 pelargonidin-3-glucoside equivalents: orientin equivalents induced hyper- and bathochromic shifts at room temperature and during thermal processing at 80 °C for an hour. Co-pigmentation effects on hyperchromic shift were up to 96%, and bathochromic shift reached 19 nm when adding flavonoid-rich fractions of green rooibos phenolics. Following the co-pigmentation tests with rooibos extracts, selected pure phenolic co-pigments were tested for their monomeric contribution to the observed co-pigmentation effects. Orientin was identified as a potent co-pigment for pelargonidin-3-glucoside, showing stronger co-pigmentation effects than that of its aglycon luteolin. Additionally, orientin had the most pronounced bathochromic shift in heat-treated solutions. Rooibos extracts, particularly flavonoid-rich fractions composed of luteolin, apigenin, and quercetin glycosides, are suggested as color enhancers and stabilizers for strawberry products.

1. Introduction

Food products based on strawberries (*Fragaria* × *ananassa* Duch.) suffer from color instability during their processing and subsequent storage, thus, eventually leading to the loss of their quality and marketability. The main reason for their color loss is the degradation of the colored pigments anthocyanins. Anthocyanins are inherently susceptible to light, temperature, oxygen, or acids and bases present in the surrounding matrix (Markakis & Jurd, 1974). One way of stabilizing anthocyanins is intermolecular co-pigmentation, in which a noncovalent association of the aromatic chromophore of the anthocyanin and a co-pigment molecule occurs (Müller-Maatsch, Bechtold, Schweiggert, & Carle, 2016). Thereby, the red-colored flavylium cation of the anthocyanin is protected from nucleophilic attacks of water present in the surrounding matrix and becomes the prevalent anthocyanin form in place of its hydrated and colorless forms. As a result, increased color intensities, as described by hyperchromic shifts at λ_{max}, and enhanced color stabilities are often observed. Interaction of anthocyanins and co-pigments may also lead to bathochromic shifts, i.e., the shift of λ_{max} to higher wavelengths, thus affecting the color hue (Trouillas et al., 2016).

Besides being prevalent among many colorful flowers, fruits, and vegetables, intermolecular co-pigmentation is also of interest for the food industry, where co-pigment sources are intentionally added to those food products suffering from poor color stability. Color and stability of strawberry anthocyanins were enhanced to a limited extent by the addition of phenolic-rich plant extracts from mango peel, quince, rhubarb, chokeberry, and rose petals (Mollov, Mihalev, Shikov, Yoncheva, & Karagyozov, 2007; Müller-Maatsch et al., 2016; Shikov, Kammerer, Mihalev, Mollov, & Carle, 2008; Wojdyło, Oszmiański, & Bober, 2008). Several individual phenolic co-pigments such as gallic, ferulic, caffeic, rosmarinic and chlorogenic acids, and catechin were also potent co-pigments for strawberry anthocyanins (Davies & Mazza, 1993; Eiro & Heinonen, 2002; Zou et al., 2018). Nevertheless, there is still a need for novel and readily available co-pigment sources providing improved color and heat stability of strawberry anthocyanins.

Rooibos (*Aspalathus linearis* (Burm.f.) R. Dahlgren), a Leguminosae originating from South Africa, has been long used in herbal tea preparations and traditional medicine (Joubert & de Beer, 2011). Since the

Abbreviations: AU, absorption unit; DAD, diode array detector; ESI, electrospray ionisation; HPLC, high-performance liquid chromatography; MS^n, multiple-stage mass spectrometry; OE, orientin eqivalents; PE, pelargonidin-3-glucoside equivalents; RT, room temperature; SPE, solid-phase extraction; $\Delta\lambda_{max}$, bathochromic shifts of the absorbance maximum.

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1900s, its dried and fermented twigs and leaves have been exported to Europe, the USA, and Japan and marketed as a caffeine-free hot beverage alternative to coffee and tea of *Camellia sinensis* (L.) Kuntze. Apart from being used in beverage preparations, value-added rooibos plant extracts have been utilized as a functional food ingredient considering their composition rich in bioactive phenolic compounds (Joubert & de Beer, 2011). Rooibos is a unique source of aspalathin, a dihydrochalcone glucoside, concomitant with various flavone *C*-glucosides such as orientin and vitexin, and flavonol *O*-glycosides including isoquercitrin and rutin (Stander, van Wyk, Taylor, & Long, 2017). Several of these phenolic compounds are antioxidant markers, contributing to the health benefit of tea infusions (Orzel et al., 2014). Attributing to the co-pigmentation effects of these phenolic constituents, the addition of ethyl acetate fraction of rooibos tea has successfully enhanced the color of cyanidin-based açai anthocyanins during 30-days-of-storage at 30 °C (Pacheco-Palencia & Talcott, 2010). Thus, rooibos has great potential use in food systems as a co-pigment source.

Rooibos has not yet been explored as a color enhancer for pelargonidin-based strawberry products, and their contribution to anthocyanin stability during thermal food processing remains to be explored. Besides, there is still a lack of information on the co-pigmentation potential of individual rooibos phenolics, specifically dihydrochalcones such as aspalathin and phloretin. So far, flavone *C*-glycosides got only limited attention as co-pigments. They were previously added to blueberry juice as color and anthocyanin stabilizer (Pan et al., 2014) and reported to be naturally present as the stable complex with an anthocyanin, delphinidin-3-coumarylrutinoside-glucoside, in Dutch Iris Prof Blaauw flower (*Iris* × *hollandica* Hort. Exx. Todd.) (Asen, Stewart, Noris, & Massie, 1970). Therefore, in the present study, phenolic-rich extracts from green and fermented rooibos were examined as co-pigments to modulate the spectral properties of anthocyanin-rich strawberry model solutions. Co-pigmentation effects were tested for different concentrations and molar ratios of strawberry pigments, i.e., anthocyanins and co-pigments from green and fermented rooibos. Upon observing promising results, the green rooibos extract was fractionated by preparative HPLC and subjected to co-pigmentation experiments to identify the most potent co-pigment fraction. In addition, the thermal stabilities of strawberry model solutions with added green and fermented rooibos extracts and isolated co-pigment fractions were investigated after heating at 80 °C for an hour. This way, the extent of the co-pigment – pigment interactions was evaluated under prolonged heating conditions as a stress test to predict its application in industrial processes such as the production of strawberry jam, preserved juices, or others. To provide insights into the structural prerequisites for the interaction of phenolic co-pigments with strawberry anthocyanins, two rooibos phenolics, luteolin and orientin, and two of previously suggested potent co-pigments, gallic acid and penta-galloyl-glucose (Müller-Maatsch et al., 2016), were tested in their pure form as co-pigments for pelargonidin-3-glucoside, the predominating strawberry anthocyanin. In addition to the co-pigmentation studies, HPLC-DAD-ESI-MS^n profiles of anthocyanin and phenolic compounds were provided for strawberry and rooibos, respectively.

2. Materials and methods

2.1. Reagents

Citric acid, disodium hydrogen phosphate dihydrate, and methanol were from VWR International (Leuven, Belgium), acetone and formic acid from Merck (Darmstadt, Germany), gallic acid (≥98% purity), and penta-galloyl-glucose (≥96% purity) from Sigma Aldrich (Steinheim, Germany), luteolin (≥99% purity) and orientin (≥99% purity) from Extrasynthèse (Genay, France), fermented and green rooibos powder extracts, and aspalathin (≥98% purity) from Phytolab (Vestenbergsgreuth, Germany). Analytical grade chemicals from Sigma Aldrich (Steinheim, Germany) and ultrapure water prepared using a Sartorius 611 Ultrapure Water System (Sartorius, Göttingen, Germany) were used throughout the study.

2.2. Preparation of strawberry model solution

Strawberry macerate was produced from individually quick-frozen strawberries (Mainfrucht, Gochsheim, Germany) after pressed using cheesecloth (Wahler, Stuttgart, Germany) and subjected to an enzymatic maceration using 0.01% Fructozym Color (Erlöb, Geisenheim, Germany) for 1 h at 35 °C (Buchweitz, Speth, Kammerer, & Carle, 2013). Solid-phase extraction (SPE) of the strawberry macerate was performed using a column (500 mm × 30 mm i.d.) filled with the macro-porous adsorber resin Lewatit® VP OC 1064MD PH (Lanxess, Langenfeld, Germany) as described in detail elsewhere (Gras, Bogner, Carle, & Schweiggert, 2016). The obtained anthocyanin-rich strawberry residue was dissolved in citric acid-sodium phosphate buffer (pH 3.5) to produce an "initial strawberry model solution" and stored at −80 °C until further use. The initial strawberry model solution was thawed for 12 h at 7 °C prior to use.

2.3. Preparative separation of phenolic compounds from green rooibos

Green rooibos powder extract was dissolved in citric acid-sodium phosphate buffer (pH 3.5) and fractionated using a Bischoff HPLC system (Leonberg, Germany) equipped with a preparative Phenomenex Aqua C_{18} reversed-phase column (250 × 21.2 mm i.d., 5 µm particle size, 125 Å pore size, Torrance, CA, USA). For chromatographic separation, water (A) and methanol (B), both acidified with 5% (v/v) formic acid, were used as eluents under the following gradient conditions: 30% B to 40% (20 min), 40% B isocratic (15 min), 40% B to 50% B (10 min), 50% B isocratic (10 min), 50% B to 70% B (10 min), 70% B isocratic (5 min), 70% B to 100% B (5 min), 100% B isocratic (5 min), and re-equilibration phase at 30% B (5 min). The flow rate was 6 mL/min. Four fractions were collected successively between 10 and 23 min (Fraction 1), 23–26 min (Fraction 2), 26–47 min (Fraction 3), and 47–80 min (Fraction 4). After drying *in vacuo* at 35 °C, all fractions were stored at −30 °C. Before use, each fraction was dissolved in citric acid-sodium phosphate buffer (pH 3.5). Their pH was adjusted to 3.5 using 0.1 M citric acid solution and 0.2 M disodium phosphate solution when necessary.

2.4. Spectrophotometric determination of total monomeric anthocyanin and phenolic contents

The total monomeric anthocyanin content and browning index of the initial strawberry model solution were determined by the pH differential method according to Giusti and Wrolstad (2003). A Perkin Elmer UV/Vis Spectrophotometer Lambda 35 (Überlingen, Germany) was used as described elsewhere (Müller-Maatsch et al., 2016). Anthocyanin content was expressed as pelargonidin-3-glucoside equivalents (PE) after using a molar extinction coefficient of 15,600 L cm^{-1} mol^{-1}.

The total phenolic content of the rooibos extracts and derived fractions thereof was determined using the Folin-Ciocalteu assay, according to Ainsworth and Gillespie (2007). An orientin calibration curve was used, and phenolic contents were expressed as orientin equivalents (OE).

2.5. HPLC-DAD-ESI-MS^n analyses of strawberry anthocyanins and phenolic compounds from rooibos

An Agilent 1100 series HPLC (Agilent, Waldbronn, Germany) attached with Kinetex C_{18} column (250 × 4.6 mm i.d., 5 µm particle size, 100 Å pore size, Phenomenex, Torrance, CA, USA). A guard column (4.0 × 2.0 mm i.d., Phenomenex, Torrance, CA, USA) of the same material were used. The afore prepared initial strawberry model solution (Section 2.2) and green and fermented rooibos powder extracts dissolved in citric acid-sodium phosphate buffer (pH 3.5) were filtered through 0.45 µm cellulose membranes (Macherey-Nagel, Düren, Germany) into amber

Table 1
HPLC retention times, UV/Vis Spectra and MS data of aqueous green and fermented rooibos extracts.

No.	Ret. time (min)	UV/Vis abs. max (nm)	$[M-H]^-$ (m/z)	HPLC-ESI(-)-MS^n (m/z)	Purposed identity	Present [b]
1	6.5	285, 300	357	[357]: 313, 221, 194, 151	Hydroxy caffeic acid hexoside	G
2	8.2	285, 300sh[c]	341	[341]: 179, 161, 135, 107	Caffeoyl hexoside	G
3	9.9	297sh, 338	355	[355]: 235, 192, 163, 119 [355 → 192]: 163	Feruloyl hexoside	G
4	10.5	282	577	[577]: 559, 533, 451, 425, 407, 299, 287, 187 [577 → 425]: 407, 339 [577 → 425 → 407]: 389, 285, 255	Procyanidin dimer	G/F
5	10.9	295, 320	357	[357]: 313, 195, 151, 123	Hydroxy caffeic acid hexoside	G/F
6	12.9	280, 303sh	339	[339]: 321, 249, 219 [339 → 219]: 191	Esculin (Esculetin glucoside)	F
7	14.4	285sh, 310	337	[337]: 191, 163, 119 [337 → 163]: 119	Coumaroylquinic acid	G/F
8	14.8	302	341	[341]: 179, 135, 107	Caffeoyl hexoside	G/F
9	16.8	283	325	[325]: 163, 119	Phenylpropenoic acid glucoside (PPAG)	G
10	17.3	287, 324	353	[353]: 191, 173, 135	Caffeoylquinic acid	G/F
11	18.3	281, 306sh	501	[501]: 457, 395, 357, 313, 195, 151 [501 → 457]: 355, 313, 295, 151	Hydroxy caffeic acid hexoside derivative	G/F
12	19.4	286, 320	353	[353]: 191, 179 [353 → 191]: 125	Chlorogenic acid (3-*O*-Caffeoylquinic acid)	G/F
13	21.8	288	449	[449]: 359, 329 [449 → 329]: 193	Eriodictyol-hexoside	F
14	22.0	272	597	[597]: 567, 549, 509, 477, 441, 389, 357 [597 → 477]: 458, 441, 375, 357 [597 → 477 → 357]: 339, 235, 193	Hydroxy caffeic acid hexoside derivative	G/F
15	23.1	290, 310	337	[337]: 173, 163 [337 → 173]: 137, 109, 93, 83	Coumaroylquinic acid	G/F
16	23.9	288	449	[449]: 329, 193 [449 → 329]: 193	Eriodictyol-hexoside	F
17	24.2	288	449	[449]: 413, 329, 193 [449 → 329]: 193	Eriodictyol-hexoside	F
18	24.8	288	449	[449]: 329, 193 [449 → 329]: 193, 151, 135	Eriodictyol-hexoside	F
19	25.6	275, 341	609	[609]: 591, 556, 519, 501, 489, 471, 453, 429, 399, 369 [609 → 489]: 399, 369, 341, 323 [609 → 489 → 341]: 341, 323, 297, 233, 191, 132	Luteolin dihexoside	G/F
20	29.8	274, 334	593	[593]: 575, 545, 503, 485, 473, 437, 383, 353, 325 [593 → 473]: 455, 383, 353, 325, 297, 243 [593 → 473 → 353]: 235, 297	Apigenin dihexoside	G/F
21	30.3	280	325	[325]: 161, 119	Phenylpropenoic acid glucoside (PPAG)	G/F
22	32.9	288, 338sh	451	[451]: 361, 331, 313, 209, 167 [451 → 331]: 313, 209, 167, 125 [451 → 331 → 209]: 167, 123	Aspalathin (Dihydroxychalcone glucoside) [a]	G
23	35.1	286, 320	563	[563]: 545, 503, 473, 443, 407, 383, 353, 325, 297 [563 → 473]: 353, 325, 297	Apigenin hexoside-pentoside	F
24	36.2	267, 350	447	[447]: 399, 367, 357, 327, 297 [447 → 327]: 299, 285, 259, 119	Orientin (Luteolin 8-*C*-glucoside) [a]	G/F
25	37	286, 335	613	[613]: 595, 523, 493, 475, 457, 403, 373 [613 → 493]: 404, 373 [613 → 493 → 373]: 355, 337, 293, 251, 233, 209, 167, 123	Hydroxyphloretin dihexoside	G/F
26	39.7	270, 348	447	[447]: 429, 411, 399, 357, 327, 285 [447 → 327]: 339, 297, 285, 247	Isoorientin (Luteolin 6-*C*-glucoside) [a]	G/F
27	40.5	270, 337	431	[431]: 341, 311, 283, 183 [431 → 311]: 283, 239, 191, 135 [431 → 311 → 283]: 255, 239, 221, 163, 117	Vitexin/Isovitexin (Apigenin *C*-glucoside)	G/F
28	43.7	286, 336sh	435	[435]: 418, 345, 315, 273, 169 [435 → 315]: 209, 167	Nothofagin (Phloretin 3′-*C*-glucoside)	G/F
29	47.7	266, 350	463	[463]: 403, 343, 301, 271, 243, 211, 179, 151 [463 → 301]: 271, 255, 179, 151, 107	Hyperoside (Quercetin 3-*O*-galactoside) [a]	G/F
30	48.5	265, 350	609	[609]: 463, 355, 301, 273, 255, 217 [609 → 301]: 271, 255, 193, 179, 107	Bioquercetin (Quercetin 3-*O*-robinobioside)	G/F
31	49.5	265, 288, 350	463	[463]: 325, 301, 273, 253, 227, 179, 151 [463 → 301]: 271, 255, 193, 179, 151	Isoquercetin (Quercetin 3-*O*-glucoside) [a]	G/F
32	50.1	266, 287, 352	609	[609]: 591, 343, 301, 271, 255, 233, 179 [609 → 301]: 255, 179, 151	Rutin (Quercetin 3-*O*-rutionoside) [a]	G/F

sh: shoulder;.
[a] Identities were confirmed by authentic standard compounds.
[b] G: green rooibos; F: fermented rooibos.
[c] sh: shoulder

HPLC vials before analyses. Water (A) and methanol (B), both acidified with 5% (v/v) formic acid, were used as eluents. Strawberry anthocyanins were separated following the previously published method of Holzwarth, Korhummel, Carle, and Kammerer (2012). Phenolic compounds from rooibos were separated under the following solvent gradient conditions: 9% B to 11% B (4 min), 11% B to 17% B (11 min), 17% B to 20% B (7 min), 20% B isocratic (3 min), 20% B to 25% B (7 min), 25% B isocratic (2 min), 25% B to 28% B (8 min), 28% B isocratic (6 min), 28% B to 50% B (14 min), 50% B to 100% B (2 min), 100% B isocratic (2 min), 100% B to 9% B (2 min), 9% B isocratic (4 min). The total run time was 76 min at a 0.5 mL/min flow rate. UV/Vis spectra were recorded in the range of 200–700 nm. For multi-stage mass spectrometric analyses, a Bruker Daltonics Esquire 3000 + ion trap mass spectrometer fitted with an electrospray ionization (ESI) interface (Bremen, Germany) was connected to the above mentioned HPLC and operated under previously described conditions (Müller-Maatsch et al., 2016).

Compound identities were established by comparing their HPLC-DAD-ESI-MS^n data with authentic reference standards (Table 1) and literature.

2.6. Production of model solutions and co-pigmentation experiments using rooibos extracts

Three different concentrations of initial strawberry model solution (0.2, 0.3, and 0.4 mM PE), giving spectrophotometrically measurable absorption values (<2 AU), were prepared in citric acid sodium phosphate buffer at pH 3.5. Additionally, co-pigment solutions of the green and fermented rooibos extracts, and four fractions derived from green rooibos (Section 2.3), were prepared using the same buffer mentioned above. Model solutions were produced by mixing equal volumes of diluted strawberry and co-pigment solutions, giving the molar ratios of 1:10 (PE:OE) at 0.1, 0.15 and 0.2 mM PE, respectively. Besides, strawberry model solutions at a fixed concentration of 0.2 mM PE were combined with the co-pigment solutions, resulting in varying molar ratios at 1:10, 1:50, and 1:100 (PE:OE). The resulting model solutions were equilibrated at room temperature (RT, 21 °C) in sealed tubes (Eppendorf, Wesseling-Berzdorf, Germany) for an hour, where after their absorption spectra were measured using a microplate spectrophotometer (Biotek, Power Wave XS, Bad Friedrichshall, Germany), as described in detail by Müller-Maatsch et al. (2016). For thermal treatment, separate aliquots of samples were heated at 80 °C for an hour in sealed tubes (Eppendorf, Wesseling-Berzdorf, Germany) and cooled to room temperature before absorbance measurements. Absorption spectra were collected between 450 and 700 nm to determine the absorption maximum (λ_{max}) of the model solutions with or without co-pigments. All absorbances were collected at their λ_{max}. The magnitudes of co-pigmentation effect on hyperchromic shifts were calculated $((A_{Ant+CP}\text{-}A_{CP})\text{-}A_{ANT})/A_{ANT}$, where A_{ANT+CP} represents the absorbance of the model solutions with co-pigment at λ_{max}, A_{ANT} that of the model solution without added co-pigment, and A_{CP} that of the co-pigment in the aforementioned buffer. Bathochromic shifts ($\Delta\lambda_{max}$) were the absolute value of the wavelength difference between the absorption maximum of the model solutions with co-pigment and the absorption maximum of the model solutions without added co-pigment (Müller-Maatsch et al., 2016). All mixtures of pigments and co-pigments were prepared three times and measured in triplicate.

2.7. Production of model solutions and co-pigmentation experiments by pure phenolic co-pigments

Pure gallic acid, penta-galloyl-glucose, orientin, and luteolin were tested as co-pigments for pelargonidin-3-glucoside, the predominant strawberry anthocyanin. Equal volumes of pelargonidin-3-glucoside solutions (0.2 mM) and co-pigment solutions, both prepared in citric acid sodium phosphate buffer at pH 3.5, were mixed, resulting in the final pigment-to-co-pigment molar ratios of 1:1 and 1:10. Spectral changes were measured after an hour of equilibration at room temperature, during subsequent thermal treatments applied in the cycles of 15 min up to a total of an hour, and after final cooling to room temperature. An integrated water bath and a temperature control unit (Lauda Ecoline RE106, Lauda Dr. R. Wobser, Lauda-Königshofen, Germany) were used for heating experiments. For data acquisition and during heating, 2 mL of samples were placed in capped precision cells (quartz SUPRASILR 100-QS, 10 mm layer depth, Hellma Analytics, Müllheim, Germany). Their absorption spectra were measured using a UV/Vis spectrophotometer (Varian Cary 50 Conc, Agilent Technologies, Waldbronn, Germany). Data recorded using the Scan Application Software (Varian, Cary WinUV, Version 02.00, Agilent), collecting data points in a fast mode numbering 75 s^{-1}. Before each absorbance measurement, samples were cooled in an ice bath.

2.8. Statistical analysis

Statistical analyses were performed using the general linear model (GLM) procedure of SPSS 20 (IBM, Armonk, NY, USA). Univariate GLM, including analysis of variance (ANOVA) procedures, were used for the co-pigmentation data of different phenolic fractions. Significant differences between means were evaluated by Tukey's posthoc test at a level of $p < 0.05$.

3. Results and discussion

3.1. Identification and quantitation of anthocyanins in the strawberry model solution

The monomeric anthocyanin content of the initial strawberry model solution was 0.6 mM PE. As determined by HPLC-DAD-ESI(+)-MS, the predominant strawberry pigment was pelargonidin-3-glucoside ($[M]^+$ *m/z* 433; Fig. 1), accompanied by other minor anthocyanins, i.e. pelargonidin-3-malonyl-glucoside ($[M]^+$ *m/z* 519), cyanidin-3-glucoside ($[M]^+$ *m/z* 449), pelargonidin-3-rutinoside ($[M]^+$ *m/z* 579), and cyanidin-3-malonyl-glucoside ($[M]^+$ *m/z* 535). The browning index, describing the rate of anthocyanin degradation in the initial strawberry model solution, was 11.3%. These results were in agreement with those of previous reports (Holzwarth et al., 2012; Müller-Maatsch et al., 2016).

3.2. Identification and fractionation of phenolic compounds from green and fermented rooibos

As revealed by HPLC-DAD-ESI(–)-MS^n analysis (Fig. 2), green and fermented rooibos extracts contained a total of 25 phenolic compounds, including several phenolic acid derivatives and flavonoids (Table 1). Phenolic acid derivatives were caffeoyl- (**2**, **8**), hydroxy caffeoyl- (**1**, **5**, **11**, **14**), and feruloyl hexosides (**3**), caffeoyl- (**10**, **12**) and coumaryl quinic acids (**7**, **15**), and phenylpropenoic acid glucosides (**9**, **21**). Flavonoids included luteolin (**19**, **24**, **26**), eriodictyol (**13**, **16**–**18**), apigenin (**20**, **23**, **27**), quercetin glycosides (**29**–**32**), and dihydrochalcones aspalathin (**22**), hydroxyphloretin dihexoside (**25**), and nothofagin (**28**). In addition, a coumarin glucoside, esculin (**6**), and a procyanidin dimer (**4**) were tentatively assigned (Iswaldi et al., 2011; Stander et al., 2017). The poorly defined hump at 55–70 min of the chromatogram was attributed to high-molecular-weight polymeric phenolic compounds, e.g., tannins (Orzel et al., 2014).

Comparing the phenolic profile of green and fermented rooibos extracts (Table 1 and Fig. S1), several phenolic acids (**1**–**3**, and **9**) and aspalathin (**22**) were lacking in the fermented rooibos extract, possibly due to their oxidation during fermentation (Stander et al., 2017). Nevertheless, two minor compounds (**23,** apigenin hexoside-pentoside, and **6**, esculin) and eriodictyol hexosides (**13**, **16**–**18**) appeared in the chromatogram of fermented rooibos.

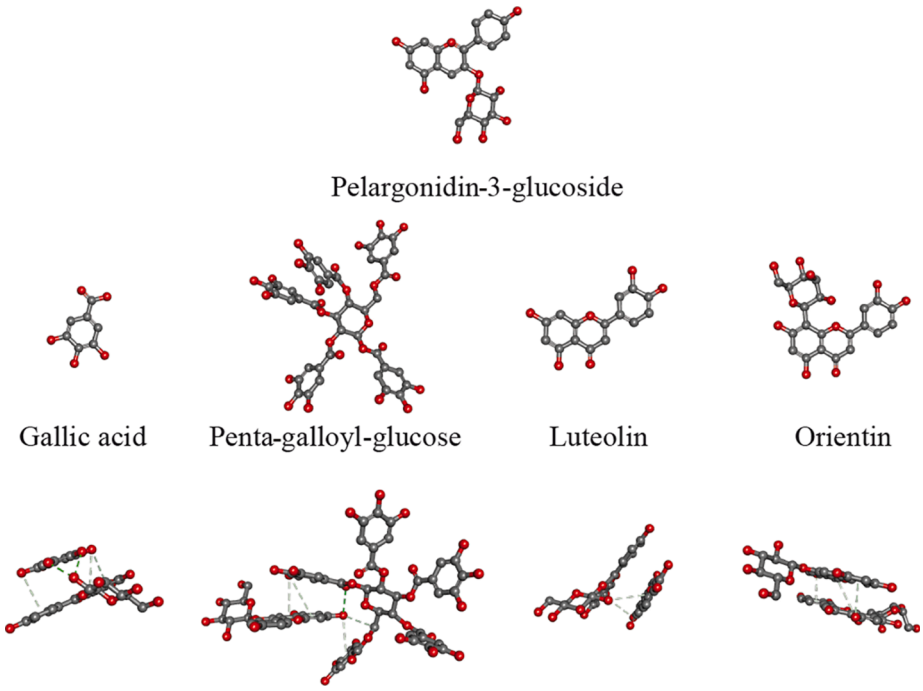

Fig. 1. Three-dimensional structures of the anthocyanin pigment (upper part) and selected phenolic co-pigments (middle part) and their prototypical noncovalent interactions by intermolecular co-pigmentation (lower part). The red color represents hydroxyl groups, while grey colored parts are carbon atoms. Dotted lines show the potential noncovalent interactions between two molecules. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

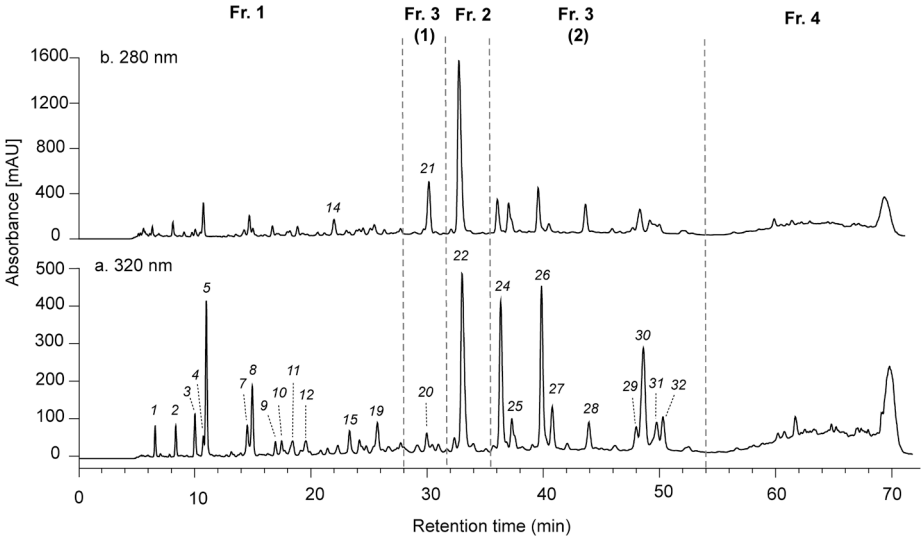

Fig. 2. HPLC-DAD chromatogram of green rooibos extract at 320 (a) and 280 nm (b), and co-pigment fractions (Fr. 1–4) derived thereof. Peak assignments are shown in Table 1. All fractions were collected based on their retentions times in preparative HPLC set-up (Section 2.3). Vertical dotted lines show their tentative separation in analytical HPLC. Of note, in comparison to the preparative HPLC method, Fr. 3 (1) eluted later than Fr. 2 during the analytical HPLC separations and was collected together with Fr. 3 (2) as one complete fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

By preparative HPLC fractionation of green rooibos extract (Section 2.3), four phenolic fractions were produced that mainly contained low-molecular-weight phenolic acid derivatives (Fraction 1), aspalathin (Fraction 2), flavonoids (Fraction 3), or presumably high-molecular-weight polymeric phenolic compounds (Fraction 4).

3.3. Spectral observations on strawberry model solutions with co-pigments from rooibos

3.3.1. Addition of green and fermented rooibos extracts to strawberry model solutions

Strawberry model solutions (0.1 mM PE) were mixed with different concentrations of green or fermented rooibos extracts to obtain molar ratios of 1:10, 1:50, and 1:100 (PE:OE) (Table S1 and Fig. 3). Hyperchromic shifts of green and fermented rooibos extracts were 8.1 and 17% at a low molar ratio (1:10 PE:OE), respectively, and significantly elevated up to 76 and 96% at a high molar ratio of 1:100 (PE:OE). Bathochromic shifts were 6–7 nm at the low ratio (1:10 PE:OE) and boosted to 18–19 nm at the high ratio by both rooibos extracts. Similarly, Müller-Maatsch et al. (2016) previously observed increased hyper- and bathochromic shifts caused by adding an excess of mango peel extract, up to 1:100 pigment-to-co-pigment molar ratio, to red radish and strawberry model solutions rich in acylated or non-acylated pelargonidin derivatives, respectively. In another instance, the color stability of Chinese bayberry anthocyanins was enhanced by adding ferulic, sinapic, and syringic acid co-pigments, providing the most prominent effects at their highest pigment-to-co-pigment molar ratio, that is 1:30 (Zhu et al., 2020). Chlorogenic acid addition to acylated and non-acylated cyanidin-3-glucosides isolated from black carrot similarly showed concentration-dependent hyper- and bathochromic shifts (Gras, Bause, Leptihn, Carle, & Schweiggert, 2018). These effects are attributed to the shift in the hydration equilibrium of anthocyanin in favor of the

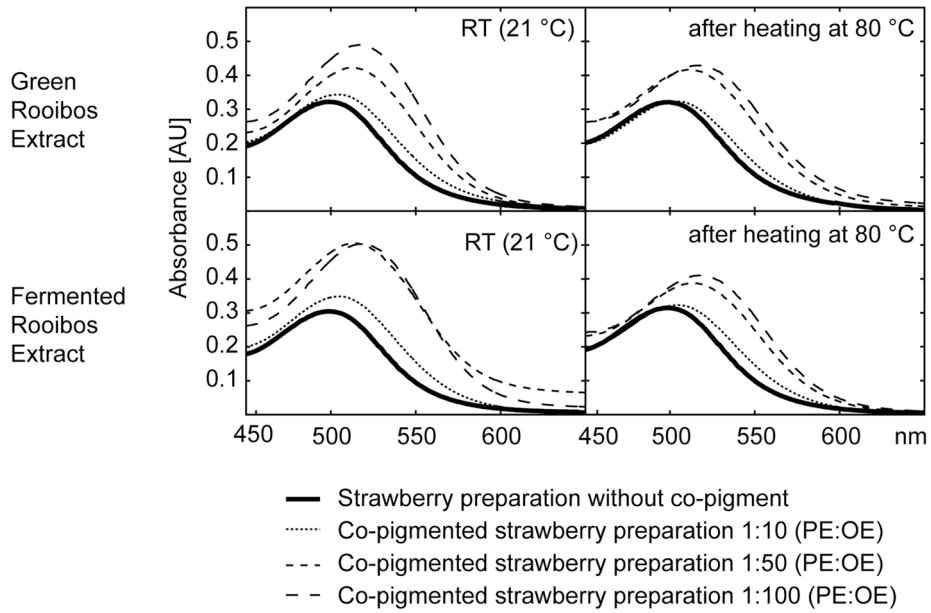

Fig. 3. UV/Vis spectra of strawberry model solutions (0.1 mM PE) with or without added green or fermented rooibos at room temperature (RT, 21 °C) and after heating at 80 °C for an hour. Pigment-to-co-pigment molar ratios ranged from 1:10 to 1:100 (PE:OE). PE: pelargonidin-3-glucoside equivalents. OE: orientin equivalents. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

flavylium cation. In addition, increased proton transfer between pigment and co-pigments due to the increased number of co-pigments and their acid-base couples may contribute (Trouillas et al., 2016).

In addition, green and fermented rooibos extracts were co-pigmented at a constant molar ratio of 1:10 (PE:OE) with strawberry model solutions at final anthocyanin concentrations of 0.1, 0.15, and 0.2 mM PE. By this, hyperchromic shifts ranging from 8.1 to 26% and bathochromic shifts of 6–10 nm were obtained (Table S1). Increasing anthocyanin concentrations from 0.1 to 0.15 mM PE led to an elevated hyperchromic and bathochromic shift by both rooibos extracts, indicating the dependence of their co-pigmentation effect on the anthocyanin concentration at these concentration ranges. A further increase in anthocyanin concentration to 0.2 mM for green rooibos-containing solutions made no difference. Diversely, in the solutions containing fermented rooibos extract, the magnitude of hyperchromic shifts decreased at high anthocyanin levels (0.2 mM PE) while the bathochromic shift was increased.

The above described boosted co-pigmentation effects by hyperchromic and bathochromic shifts by increased anthocyanin concentrations were attributed to the self-association of anthocyanins to provide increased color intensities and hues (Müller-Maatsch et al., 2016; Trouillas et al., 2016). However, after a specific concentration, the increased anthocyanin or co-pigment competitions may lead to inhibited pigment-to-co-pigment associations (Trouillas et al., 2016) and less observable co-pigmentation effects as realized for fermented rooibos extract in the ratios 1:10 PE:OE in the present study. Besides, the differences in chemical composition between fermented and green rooibos extracts (Heinrich, Willenberg, & Glomb, 2012; Krafczyk, Heinrich, Porzel, & Glomb, 2009) might have contributed to the difference in the observed effects for these two extracts. Particularly, tannin-like condensed structures, such as aspalathin dimers, exist in fermented rooibos (Krafczyk et al., 2009). Low-molecular-weight colored compounds, such as dibenzofuran derivatives, are also produced during the fermentation of rooibos by non-enzymatic oxidative degradation of aspalathin (Heinrich et al., 2012). These structures might enhance the formation of co-pigment aggregates and hamper other pigment and co-pigment associations (Bayach, Sancho-García, Di Meo, Weber, & Trouillas, 2013).

These results show that both green and fermented rooibos extracts are the sources of potent co-pigments, successfully interacting with strawberry anthocyanins. Compared to a previous study by Müller-Maatsch et al. (2016), higher co-pigmentation effects were observed by adding rooibos extracts in our study than those obtained by mango peel extracts.

3.3.2. Addition of the co-pigment fractions isolated from green rooibos extract to strawberry model solutions

The green rooibos extract was fractionated, and derived fractions were subjected to co-pigmentation studies. Green rooibos was selected over the fermented one due to its less complex composition devoid of the aforementioned oxidized fermentation products interfering with co-pigmentation effects (Section 3.3.1) while being equally effective. Strawberry model solutions of different concentrations (0.1, 0.15, and 0.2 mM) were co-pigmented with the four fractions isolated from green rooibos extract at the final molar ratio of 1:10 (PE:OE). By this, the flavonoid-rich fraction 3 produced the highest hyperchromic shifts at all concentrations (Table S2). The other fractions were equally effective in increasing hyperchromic shifts at low and mid-concentrations. Only at the highest anthocyanin concentration (0.2 mM PE), a higher hyperchromic shift was attained by fraction 4, presumably containing polymeric compounds, followed by fraction 1, composed of phenolic acid derivates, and fraction 2 with aspalathin. Bathochromic shifts by fractions 3 and 4 were higher than fractions 1 and 2 and only slightly affected by pigment concentrations, resulting in 1–2 nm shifts (Table S2).

The effect of the increasing molar ratios from 1:10 to 1:50 and 1:100 (PE:OE) on spectral properties of the strawberry model solution was only tested for selected fractions, i.e., fraction 1 and 3 (Table S2), due to solubility restrictions of the containing compounds in fraction 4 and limited availability of fraction 2. By these means, fraction 3 provided the highest hyperchromic shifts (94%) among all tested conditions at the molar ratio of 1:100 (PE:OE), even exceeding that of the green rooibos extract (Table S1). Significant but lower hyperchromic (56%) and bathochromic shifts (9 nm) were observed for fraction 1 than fraction 3 (23 nm bathochromic shift).

The aforementioned substantial co-pigmentation effect of fraction 3 that predominantly contained *C*-glycosyl flavones and *O*-glycosyl flavonols were in agreement with those of previous findings (Davies &

Mazza, 1993; Pacheco-Palencia & Talcott, 2010; Pan et al., 2014; Shikov et al., 2008). Trouillas et al. (2016) and Cortez, Luna-Vital, Margulis, and Gonzalez de Mejia (2017) stated that flavonols and flavones are efficient co-pigments, providing the most significant hyper- and bathochromic shifts by interacting with anthocyanins via π-π stacking and extending the π-conjugation through their tricyclic structure. Fraction 1 was rich in hydroxycinnamic acid glycosides, which are also reported as potent co-pigments (Davies & Mazza, 1993; Eiro & Heinonen, 2002; Galland, Mora, Abert-Vian, Rakotomanomana, & Dangles, 2007). However, fraction 1 provided limited hyper- and bathochromic shifts compared to the flavonoids-rich fraction in our study. This observation could also be influenced by the applied Folin-Ciocalteu quantification approach, which is well-correlated with phenolic compositions. However, it may lead to over- or under-estimation of OEs and the observed co-pigmentation effects due to differences in molar extinction coefficients of specific compounds present in rooibos extracts. Noteworthy, additive or synergistic co-pigmentation effects and even the formation of ternary co-pigmentation complexes boosting the observed co-pigmentation effects might already present, e.g., in fraction 3 (Trouillas et al., 2016).

The significant co-pigmentation effect of fraction 4 at high pigment concentration (Table S2) might be attributed to its genuine composition, presumably containing polymeric compounds. Some polymeric phenolic structures, e.g., gallotannins in mango peel, were previously shown to possess great potential to stabilize strawberry anthocyanins (Müller-Maatsch et al., 2016). In this example, Müller-Maatsch et al. (2016) proposed that co-pigment-to-pigment interactions increase by the number of attached galloyl units up to pentamer, i.e., penta-galloyl-glucose, in a gallotannin structure. Gallotannins with higher attached units were possibly sterically hindered, and thus, less interaction occurred with the anthocyanin pigment. Furthermore, condensed tannins, i.e., procyanidin oligomers, were shown to be poor co-pigments for oenin solutions due to the lack of conjugation in the heterocyclic core ring of catechin subunits (Malien-Aubert, Dangles, & Amiot, 2001). Despite the evidence provided by Marais, Marais, Steenkamp, Malan, and Ferreira (1998) on the presence of proanthocyanins in rooibos as determined spectrophotometrically by phloroglucinol method, detailed investigations of the exact tannin composition of fraction 4 from green rooibos extract will provide a better understanding of the observed co-pigmentation effects before their potential application as a color enhancer.

Finally, the low impact observed for the aspalathin-rich fraction 2 might be attributed to the lack of double bonds between its A- and B-rings, limiting the extent of π-conjugation. This study is the first time reporting the co-pigmentation capacity of a chalcone derivative.

3.4. Thermal stability of strawberry model solutions with green and fermented rooibos extracts and co-pigment fractions

3.4.1. Addition of green and fermented rooibos extracts to strawberry model solutions

Strawberry model solutions (0.1 mM PE) were co-pigmented with green and fermented rooibos extracts at differing molar ratios (1:10, 1:50, and 1:100 PE:OE) and heated at 80 °C for a total of an hour. By the addition of green rooibos extracts at intermediate (1:50 PE:OE) and high molar ratios (1:100 PE:OE), hyperchromic shifts were largely preserved after heating by 38 ± 4 and 55 ± 16%, respectively, with slight decreases compared to respective model solutions at room temperature (41 and 76%, respectively, Table S1, Fig. 3). The thermal effect was substantial at a low molar ratio (1:10 PE:OE) of green rooibos extract added model solutions, inducing significantly lower hyperchromic shifts (1.9 ± 1.2%) compared to that of non-heated model solutions (8.1%, Table S1). Hydroxycinnamic acid addition to Chinese bayberry anthocyanins (Zhu et al., 2020) and hydroxybenzoic acid and flavonoid additions to blackberry wine residue anthocyanins similarly enhanced their heat stability up to 90 °C (Fan et al., 2019). Enhanced thermostabilities of co-pigmented anthocyanins are attributed to the increased activation energies (E_a) of co-pigment-anthocyanin complexes that require high energy for degradation reactions (Fan et al., 2019).

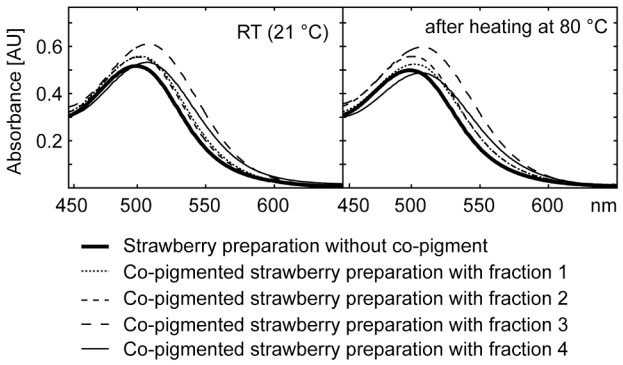

Fig. 4. UV/Vis spectra of strawberry model solutions (0.15 mM PE) with or without added co-pigment fractions from green rooibos at room temperature (RT, 21 °C) and after heating at 80 °C for an hour. Pigment to co-pigment molar ratios were 1:10 (PE:OE). PE: pelargonidin-3-glucoside equivalents. OE: orientin equivalents. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Hyperchromic shifts observed by adding different concentrations of the fermented rooibos extract were lowered after thermal treatment, decreasing to 7.0 ± 2.1% at 1:10 PE:OE, 34.8 ± 4.7% at 1:50 PE:OE, and 52.5 ± 8.0% at 1:100 PE:OE. Higher magnitudes of decrease in hyperchromic shifts in model solutions mixed with fermented rooibos extract than green extract might be attributed to its aforementioned chemical composition containing fermentation-related condensed and oxidized structures (Section 3.3.1). The self-aggregation of these structures from fermented rooibos extracts might be enhanced upon heat treatment, as previously observed by Millet, Poupard, Guilois-Dubois, Zanchi, and Guyot (2019) and Zanchi et al. (2009) in the case of oxidized tannins.

3.4.2. Addition of co-pigment fractions isolated from green rooibos to strawberry model solutions

The thermal stabilities of the strawberry model solutions co-pigmented with individual fractions from green rooibos were investigated (Fig. 4). For this, pigment concentration of 0.15 mM, producing high hyperchromic shifts at room temperature (Table S1), and the molar ratio of 1:10 (PE:OE), in which significant thermal effects were observed (Fig. 3), were tested.

Despite differing susceptibilities of hyperchromic shifts, bathochromic shifts were preserved after thermal treatment for all model solutions with added rooibos fractions (Figs. 3 and 4, Table S2). Among all heat-treated fractions, fraction 3 (17.8 ± 0.8%), fraction 2 (6.4 ± 0.9%), and fraction 1 (16.7 ± 0.9%) successfully preserved their hyperchromic shifts after heat treatment compared to those of non-heated solutions (Table S2). The hyperchromic shift of fraction 4 was significantly reduced by heating from 7.5% to 1.2 ± 2.4%. Thus, the addition of co-pigments, particularly ones deriving from fraction 3 of green rooibos to the strawberry model solutions, seems to slow the thermal degradation of anthocyanins. In agreement, thermostability of co-pigmentation effects was superior at 90 °C for anthocyanins of blackberry wine residue co-pigmented with flavonoids than phenolic acids, which also provided considerable co-pigmentation effect at low temperature heat treatment at 50 °C (Fan et al., 2019).

3.5. Limitations of the investigation into co-pigmentation effects in strawberry model solutions and their application in food systems

The strawberry model solution used in the current study, with 0.6 mM PE anthocyanin content, may contain trace amounts of non-anthocyanin phenolic compounds. A total of 8 non-anthocyanin

phenolic compounds including hydroxybenzoic, hydroxycinnamic, *p*-coumaroyl-hexosides, quercetin-3-glucuronide, and kaempferol-3-glucoside were present in similarly prepared strawberry anthocyanin solution (~1300 mM) and constituted 28–46% of peak area at 280 nm (Buchweitz et al., 2013). These phenolic constituents are expected to only make minor contributions to the observed co-pigmentation effects considering co-pigments added to strawberry model solutions at high and excess molar ratios (1:10, 1:50, and 1:100 PE: OE) in comparison to their aforementioned genuine ratios (~1:0.5 to 1:1 anthocyanin-to-non-anthocyanin phenolic compounds based on peak areas at 280 nm). Nevertheless, as strawberries reportedly contain non-anthocyanins phenolic compounds (Aaby, Mazur, Nes, & Skrede, 2012), the contribution of these to expected co-pigmentation effect should be accounted for during the application of current findings into food systems.

Another limitation of current study is that applied pigment-to-co-pigment molar ratios (1:10, 1:50 and 1:100 PE: OE) exceed those found in nature by far. It has been reported to reach the highest ratio of up to 1:18 flavone: anthocyanin in blue flower *Ceanothus papillosus* (Torr. & A. Gray) petals (Andersen & Jordheim, 2010). Although being achievable in our study in strawberry model solutions, reaching these excess co-pigment concentrations in complex food systems may remain a technological challenge. Phenolic compounds could affect textural and sensorial properties of food products by interacting with other food components or producing undesirable astringent flavor, if not their solubility being an issue. Therefore, the effects of rooibos extract on product quality, e.g., their textural and sensorial properties and storage stabilities of the color of rooibos added strawberry products should be further evaluated in food systems.

3.6. Spectral observations on pelargonidin-3-glucoside co-pigmented with pure phenolic compounds and their thermal stability

Comparable insights into the structural prerequisites for the intermolecular co-pigmentation of selected phenolic co-pigments and pelargonidin-3-glucoside (0.1 mM) were investigated at molar ratios of 1:1 and 1:10 (PE:OE) due to solubility restrictions. Luteolin and orientin were selected as representative phenolic co-pigments of green rooibos fraction 3, the most potent co-pigment fraction (Section 3.3.2 and 3.4.2). Also, penta-galloyl-glucose and its monomeric counterpart gallic acid were included for comparative purposes since penta-galloyl-glucose-rich mango peel extract was previously suggested to be a potent co-pigment for strawberry anthocyanins (Müller-Maatsch et al., 2016). In addition to the effects of individual co-pigments on absorbance maxima (λ_{max}) and absorbance magnitudes of pelargonidin-3-glucoside at room temperature, the co-pigmentation effects were monitored during thermal treatment at 80 °C for an hour, and after subsequent cooling to room temperatures (Fig. 5).

3.6.1. The effect of pure phenolic compounds on spectral properties of pelargonidin-3-glucoside

Comparing the co-pigmentation effects at room temperature at the molar ratio of 1:10, penta-galloyl-glucose led to the highest hyperchromic (79 ± 1.9%) and bathochromic shift (11 nm) among all tested phenolic co-pigments, while its monomeric counterpart, gallic acid, had the slightest effect (hyperchromic shift of 5.8 ± 0.4%; $\Delta\lambda_{max} = 0$). The hyperchromic effect induced by orientin was 47 ± 12 %, higher than that of luteolin (18 ± 12%), the aglycon of orientin. Bathochromic shifts were non-existent for the co-pigmentation of luteolin and pelargonidin-3-glucoside (Fig. 5). A hypsochromic shift at λ_{max} (4 nm) was observed by adding orientin as co-pigment. At the molar ratio of 1:1, no bathochromic shifts and slight co-pigmentation effects were observed for luteolin (10 ± 1.4%), orientin (8.0 ± 0.4%), and penta-galloyl-glucose (11 ± 0.7%) added solutions, being similar among each other. Gallic acid was an ineffective co-pigment at this low molar ratio.

As observed in this study, penta-galloyl-glucose had already been suggested as an effective co-pigment (Müller-Maatsch et al., 2016). It potentially allows the formation of numerous molecular contacts with the pigment and forms stable complexes by accommodating the pigment between galloyl residues (Trouillas et al., 2016). Comparably, other phenolic compounds contain limited interaction sites for pigment molecules (Fig. 1). Thus, higher concentrations of flavonoids, i.e., orientin or luteolin, might be required to shift the co-pigmentation equilibrium and observe similar co-pigmentation effects compared to penta-galloyl-glucose co-pigment (Trouillas et al., 2016). The glycosylation of luteolin, resulting in orientin, had a positive impact on the co-pigmentation effect, despite its structure hindering pigment approaches from their glycosyl site and π-π interactions (Galland et al., 2007). However, hydrogen bond interactions between pigment and hydroxyl-rich sugar moiety might additively contribute to the observed co-pigmentation effect of orientin (Li, Li, Wang, & Jiang, 2016). Hypsochromic shifts observed by orientin addition need further investigations into pigment-to-co-pigment interactions and their stability in respective solutions. Despite being rare, a similar hypsochromic shift, i.e., anti-co-pigmentation effect, was previously reported in quercetin and caffeic

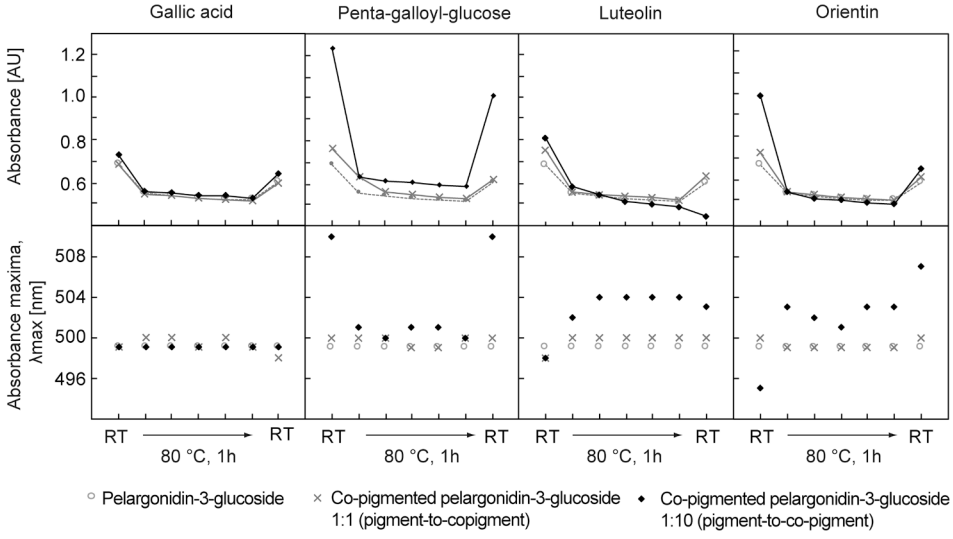

Fig. 5. Effects of the addition of individual phenolic co-pigments on the absorbance and absorbance maxima (λ_{max}) of pelargonidin-3-glucoside at room temperature (RT, 21 °C), during and after thermal treatment. Luteolin and orientin are rooibos flavonoids, while gallic acid and penta-galloyl-glucose found in mango peel were included for comparative purposes.

acid added grape pulp extracts (Rustioni, Bedgood, Failla, Prenzler, & Robards, 2012).

3.6.2. The effect of pure phenolic compounds on the thermal stability of pelargonidin-3-glucoside

During thermal treatment at 80 °C for an hour, hyperchromic effect of gallic acid, orientin, and luteolin vastly diminished (Fig. 5). Penta-galloyl-glucose at a molar ratio of 1:10 was the only co-pigment exerting hyperchromic shifts (13–14%) with 1–2 nm bathochromic shifts throughout the thermal treatment.

After cooling to room temperature at the end of the thermal treatment, hyperchromic effects of gallic acid (5.9 ± 0.9% at 1: 10 M ratio), penta-galloyl-glucoside (67 ± 12% and 8.9 ± 0.6, at 1:10 and 1:1 M ratios, respectively) and orientin (10 ± 1.8% and 4.7 ± 0.6, at 1:10 and 1:1 M ratios, respectively) recovered, although not totally to pre-heating values. These recoveries are possibly due to the exothermic character of the co-pigmentation process (Trouillas et al., 2016). By exception, the hyperchromic effect of luteolin at 1:10 M ratio further decreased even after subsequent cooling, which may indicate its thermal degradation at a higher rate than its glycosylated counterpart, i.e., orientin (Chaaban et al., 2017) (Fig. 5).

The bathochromic shift provided by adding penta-galloyl-glucose was recovered to its initial value (11 nm) after cooling to room temperature. Significant bathochromic shifts were induced by luteolin (up to 5 nm) and orientin (up to 12 nm) during and after thermal treatment (Fig. 5) despite their aforementioned insignificant or adverse effects at room temperature, respectively (Section 3.5.1). These significant bathochromic shifts might be promising for their future applications. However, the underlying mechanisms for them remain unknown to us. These results show that penta-galloyl-glucoside, gallic acid, and orientin are promising co-pigments for pelargonidin-3-glucoside, protecting its degradation upon thermal treatment.

4. Conclusion

Rooibos is already commercially available as tea and tea extracts, thus considered as food being accessible to food producers worldwide. In this study, green and fermented rooibos extracts exerted thermally stable hyper- and bathochromic shifts in strawberry model solutions. Upon fractionation of green rooibos phenolics, all co-pigment fractions induced hyper- and bathochromic shifts, while the most pronounced effect was induced by a flavonoid-rich fraction 3. Investigations of pure pigment and co-pigments revealed that orientin, a selected rooibos phenolic, successfully stabilized the color intensity of strawberry model solutions even after thermal processing. Therefore, rooibos may be used as a food additive to enhance the color of strawberry products and stabilize the pigments throughout food processing. As a future perspective in the co-pigmentation research, investigations into the interaction of anthocyanins with fermentation-related oxidized structures from fermented rooibos may provide additional insights for the application of these co-pigment sources. In addition, heat-induced hyper- and bathochromic shifts by green rooibos fraction 1, rich in hydroxycinnamic acid glycosides, and pure phenolic compounds, i.e., luteolin and orientin, respectively, are to be explored.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2022.100097.

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