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Adulteration of anthocyanin- and betalain-based coloring foodstuffs with the textile dye 'Reactive Red 195' and its detection by spectrophotometric, chromatic and HPLC-PDA-MS/MS analyses

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ABSTRACT

A wide range of natural food colorants and coloring foodstuffs is available for the food industry to meet current costumer trends. Most natural pigments are more sensitive towards heat, light, and pH changes compared to their synthetic counterparts. Additionally, high dosages are often required to attain desired color hues and intensities. In this research article, we report on the broad and worldwide incidence of a fraudulent practice to overcome these disadvantages by adding a non-approved azo-dye preparation originating from the textile dye Reactive Red 195 to natural pigment extracts. Since the respective products and their derivatives have been widely distributed, we present a rapid method allowing the differentiation of the fraudulent azo-dye from *Hibiscus sabdariffa* (roselle) flower and *Beta vulgaris* (red beet) root extracts, the two coloring foodstuffs that are most frequently adulterated. Furthermore, detailed HPLC-PDA-MS/MS data is presented for the unambiguous identification of Reactive Red 195 and its derivatives.

1. Introduction

During the past two years, red beet- and roselle flower-based coloring foodstuffs appeared on worldwide markets, instantly gaining an enormous interest of the food industry due to their apparently exceptional processing and storage stability. These preparations or extracts solely containing natural plant pigments would be highly desired by the food industry producing savory meat and neutral dairy products. Most of the currently available, highly stable colorants permitted for these foods are either synthetic or animal origin (e.g. carmine). However, both synthetic dyes and carmine have clear disadvantages limiting their application. For instance, several synthetic food dyes such as carmoisine, allura red, tartrazine, and ponceau 4R have been related to an increased hyperactivity in 3-year-old and 8/9-year-old children (McCann et al., 2007), thus requiring a warning. Deriving from the insect *Dactylopius coccus* Costa, carmine was previously related with allergenic and toxicological risks, furthermore being nauseating to many customers. In addition, the presence of aluminium in carmine lakes has been frequently criticized, often hampering its use for coloring foods (Müller-Maatsch & Gras, 2016). In contrast, plant-based natural colorant preparations often comprise potential health benefits beyond their tinctorial strength, such as relevant levels of health-promoting constituents like pro-vitamin A and phenolic antioxidants (Delgado-Vargas, Jiménez, & Paredes-López, 2000; Stintzing & Carle, 2004). Since consumers are widely aware of these facts, they increasingly reject artificial and carmine-based colorants. Hence, the production of food, colored with expensive and less effective natural colorants, puts pressure on the market. Consequently, committing fraud is driven by considerable incentives (Carocho, Morales, & Ferreira, 2015).

The above mentioned specific coloring foodstuffs, labelled as red beet and roselle flower extracts, as well as further plant-based colorant preparations appeared on several markets within Europe and worldwide. Although doubts about the authenticity of such extracts have been raised, and adulterations with a synthetic food colorant have been suspected by several food companies and state agencies, the fraudulent pigment remained unknown. In the

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present communication, we elucidate the identity of the pigment in question. Besides presenting a newly developed HPLC-PDA-MS/MS method for its unambiguous detection, we present a methodology to distinguish the anthocyanins from roselle and the betalains from red beet from this fraudulent textile colorant by simple spectrophotometric, chromatic, thermal, and adsorptive methods.

2. Materials and methods

2.1. Reagents and materials

Methanol and formic acid were purchased from VWR International (Leuven, Belgium) and Merck (Darmstadt, Germany), respectively. Polyamide (particle size 0.05–0.16 mm, CAS 63428-84-2) was purchased from Carl Roth (Karlsruhe, Germany). Ultrapure water was used throughout and all chemicals were of analytical purity. The textile dye Reactive Red 195 was provided by Yorkshire Farben (Krefeld, Germany). Dried roselle flowers (*Hibiscus sabdariffa* L.) were provided by Martin Bauer (Vestenbergsgreuth, Germany) and red beet extract (*Beta vulgaris* L. ssp. *vulgaris* var. *conditiva* Alef.) by Colin Ingrédients (Mittelhausen, France). Three adulterated preparations from Turkey (Sample 1), France (Sample 2), and Germany (Sample 3) were included in this study, being obtained from undisclosed providers.

2.2. Sample preparation and chromatic analyses

Red beet extract, roselle flowers, and Reactive Red 195 were dissolved in citric acid sodium phosphate buffer (McIlvaine buffer) at seven different pH values (pH 2, 3, 4, 5, 6, 7, and 8) and filtered using cellulose filter paper (Whatman grade 2) prior to further analyses (Herbach, Maier, Stintzing, & Carle, 2007; Malien-Aubert, Dangles, & Amiot, 2001). The solutions were diluted with the respective buffer to adjust an absorbance of 1.0 ± 0.1 AU at the wavelength of the absorption maximum, further named standard solutions. CIE-L*a*b* values of the standard solutions were recorded in transmission by an UltraScan Vis spectrophotometer (Standard illumination D65/10°, HunterLab, Murnau, Germany) in 1 cm path length disposable cuvettes. All treatments and measurements were carried out in duplicate.

2.3. Adsorption on polyamide

The adsorption experiment for the separation of synthetic and natural pigments followed the instructions of Lehmann and Hahn (1968) with slight modifications. Aliquots of 10 mL of the respective standard solutions of red beet, roselle flowers, and Reactive Red 195 (all at pH 4) were combined with 1 g polyamide. After adsorption of the pigments, the adsorbent was transferred into glass columns and washed with 50 mL water, 50 mL acetone, and 50 mL methanol containing 10% ammonia in the respective order. All eluates were collected and visually inspected. Adsorption experiments were carried out in duplicate.

2.4. Thermal degradation

Standard solutions diluted in McIlvaine buffer (pH 3) were subjected to a heat treatment at 80 °C in sealed glass tubes (Pyrex), as previously described by Dyrby, Westergaard, and Stapelfeldt (2001). Samples were taken at 30 and 60 min and subsequently cooled to room temperature (25 °C). UV/Vis absorption spectra from 200 to 700 nm were recorded prior to and after heating using a Perkin Elmer UV/Vis spectrophotometer Lambda 35 (Überlingen, Germany) and 1 cm path length disposable cuvettes. All treatments and measurements were carried out in duplicate.

2.5. HPLC-PDA-MS/MS analyses of individual compounds

The developed HPLC-PDA-MS/MS method was based on a previously reported HPLC method for the separation of anthocyanins and betalains (Stintzing, Trichterborn, & Carle, 2006) with several modifications. Prior to the separation, the aqueous extracts were membrane-filtered (cellulose, 0.45 μm, Macherey-Nagel, Düren, Germany). Separation of individual compounds in the aqueous extracts was performed on an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany), using aqueous formic acid (1%, v/v) and formic acid in methanol (1%, v/v) as eluents A and B, respectively. Pigments were separated at 40 °C on a Phenomenex (Torrance, CA, USA) Synergi Hydro-RP 80A C_{18} column (150 × 3.0 mm i.d., 4 μm particle size, 80 Å pore size) combined with a guard column (4.0 × 2.0 mm i.d.) of the same material. The gradient program was as follows: 0% B to 40% B (20 min), 40% B to 100% B (5 min), 100% B isocratic (5 min), 100% B to 0% B (1 min), 0% B isocratic (4 min). Total run time was 35 min at a flow rate of 0.6 mL/min. Pigments were monitored at 520 nm, additionally recording UV/Vis spectra from 200 to 600 nm. For mass spectrometric analyses, the HPLC system described above was coupled on-line to a Bruker (Bremen, Germany) model Esquire 3000 + ion trap mass spectrometer applying an electrospray ionization (ESI) interface. Positive ion mass spectra of the column eluate were recorded in the range of m/z 50-2000 at a scan speed of 13,000 m/z s^{-1}. Nitrogen was used both as drying gas at a flow rate of 9 L/min and as nebulizing gas at a pressure of 40 psi.

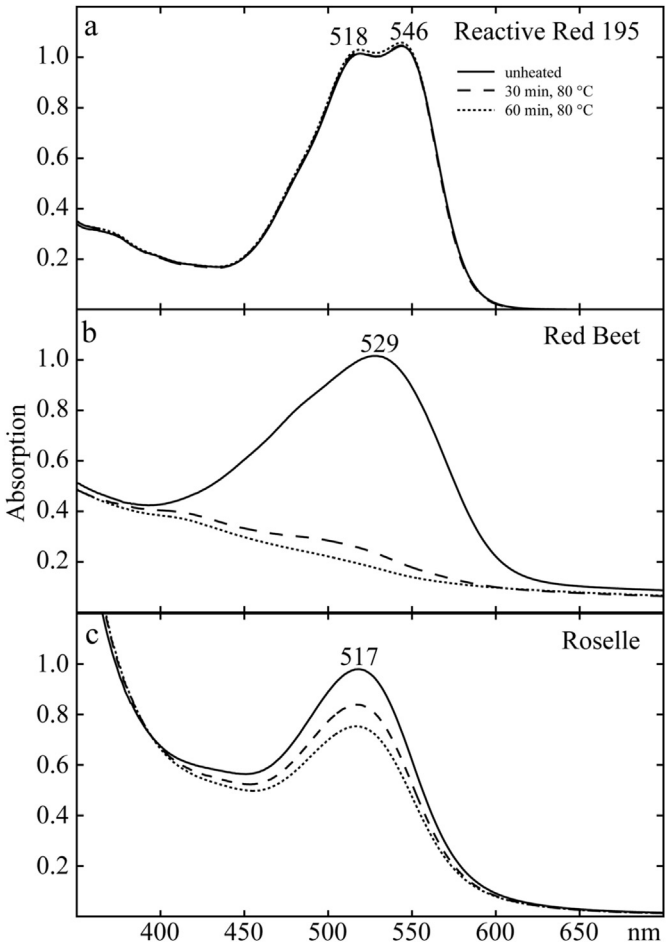

Fig. 1. Visible absorption spectra of aqueous solutions (pH 3) of Reactive Red 195 (a), red beet extract (b), and roselle extract (c) monitored before heating (solid line) as well as after 30 min (dashed line), and 60 min (dotted line) of heating at 80 °C.

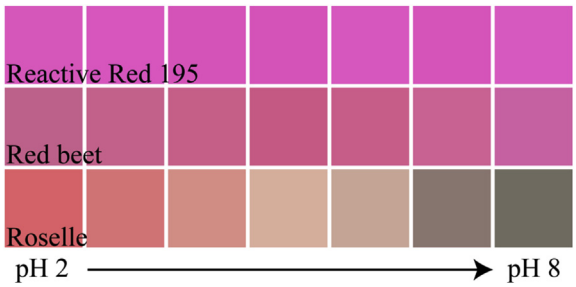

Fig. 2. Hues of aqueous solutions of Reactive Red 195, red beet extract, and roselle extract at different pH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The nebulizer temperature was set at 365 °C. For collision induced dissociation (CID) experiments, helium was used at a pressure of 4.9×10^{-6} mbar and a fragmentation amplitude of 1.00 V was applied.

2.6. Statistical analyses

Mean values were analyzed for significant differences by analysis of variance (ANOVA) and the least significant difference (LSD) post-hoc test ($p < 0.05$) using SPSS software version 22.0 (IBM, Armonk, NY, USA).

3. Results and discussion

3.1. Spectral and chromatic observations during pH changes

Upon dissolution in acidic media (pH 3), Reactive Red 195, the roselle flower extract, and the red beet extract exhibited similar absorption spectra with maxima at ca. 520 nm. However, as displayed in Fig. 1 a (solid line), the textile dye Reactive Red 195 revealed two characteristic absorption bands at 518 nm and 546 nm (at pH 3), being similar to the UV/Vis spectra of the textile dye previously reported by Peralta-Zamora (1998). In contrary, the above mentioned red beet (Fig. 1b, solid line) and roselle (Fig. 1c, solid line) extracts solely showed one absorption maximum at 529 nm and at 517 nm at pH 3, respectively. Despite these slight spectral differences, the roselle, and particularly, the red beet extract solutions evoked a color similar to the Reactive Red 195 solution in acidic media (pH 2–4, Fig. 2). As listed in Table 1, Reactive Red 195 reached hue angles (h°) of ca. 346 (cool red), while the red beet and roselle standard solutions exerted h° of 1 (mid red) and 23 (warm red to orange), respectively.

These rather slight differences became more obvious when increasing the pH from 2 to 8 (Fig. 2). For instance, the hue of the roselle standard solutions expectedly changed from red (h° 23.8 ± 0.2) to bluish (h° 88.1 ± 1.6, Table 1). These changes have been previously related to the deprotonation of flavylium cations (red in acidic media) and the formation of quinoidal bases (blue in alkaline media) (Pina, Melo, Laia, Parola, & Lima, 2012). Red beet extract, consisting majorly of betalains, widely maintained its respective hue values of 0.3 ± 0.1 to 349.5 ± 0.4 over the pH range from 2 to 8, being supported by earlier literature reports on the maintained color of betalain solutions at pH values between 3.5 and 7 (Delgado-Vargas et al., 2000; Kugler, Graneis, Stintzing, & Carle, 2007). Similarly to the diluted red beet extract, the textile dye solution maintained its hue throughout the pH shift (Table 1, Fig. 2). Therefore, fraud may be easily detected by simply changing pH from acidic to alkaline, when anthocyanins are part of the preparation. However, adulterations of red beet extracts cannot be detected by simple pH shifts.

3.2. Adsorption to polyamide resin

While an adsorption of the betalains from red beet extract and anthocyanins from roselle flowers was absent, they were largely removed from the polyamide resin by washing with water. In contrast, the azo-dye Reactive Red 195 strongly adsorbed to the positively-charged polyamide due to its five negatively-charged sulfonyl groups (Fig. 3a), remaining attached to the adsorbent during rinsing with water. Alkaline methanol allowed deprotonation of the polyamide backbone, thus annulling the attractive force between the polyamide and the azo-dye. Consequently, applying alkaline methanol as the eluent, the azo dye was completely rinsed of the column. In brief, this adsorption method allowed the separation of two natural pigment classes from synthetic ones with strongly negatively charged functional groups, e.g., sulfonyl groups, and may be used as a first indicator of adulterated samples.

Table 1
CIE-L*a*b color values of aqueous solutions of roselle flower extract, red beet extract, and Reactive Red 195 at different pH after adjusting to an absorbance of 1 (~520 nm).

pH		L*	a*	b*	C*	H°
2	Reactive Red 195	68.7 ± 0.3 ^{A}	67.6 ± 0.7 ^{A}	−16.1 ± 0.0 ^{F}	69.5 ± 0.7^{A}	346.6 ± 0.1 ^{E}
3		68.6 ± 0.2 ^{A}	67.5 ± 0.5 ^{A}	−16.5 ± 0.0 ^{E}	69.5 ± 0.4 ^{A}	346.2 ± 0.1 ^{D,E}
4		68.0 ± 0.6 ^{A}	67.7 ± 1.3 ^{A}	−16.9 ± 0.1 ^{D}	69.8 ± 1.2 ^{A}	345.9 ± 0.3 ^{C,D,E}
5		68.2 ± 0.6 ^{A}	67.7 ± 1.2 ^{A}	−17.3 ± 0.0 ^{C}	69.9 ± 1.2 ^{A}	345.7 ± 0.3 ^{B,C,D}
6		68.5 ± 0.5 ^{A}	67.0 ± 0.6 ^{A}	−17.4 ± 0.1^{B,C}	69.2 ± 0.6 ^{A}	345.4 ± 0.2 ^{A,B,C}
7		68.5 ± 0.1 ^{A}	66.8 ± 0.0 ^{A}	−17.7 ± 0.0 ^{A,B}	69.2 ± 0.0 ^{A}	345.2 ± 0.0 ^{A,B}
8		68.7 ± 0.6 ^{A}	66.2 ± 1.1 ^{A}	−18.0 ± 0.1 ^{A}	68.6 ± 1.1 ^{A}	344.8 ± 0.1 ^{A}
2	*Beta vulgaris*	63.2 ± 0.3 ^{A}	47.4 ± 0.7 ^{A,B}	0.2 ± 0.1 ^{C}	47.4 ± 0.7 ^{A}	0.3 ± 0.1 ^{A}
3		64.3 ± 0.4 ^{A}	49.1 ± 0.4 ^{A,B,C}	1.1 ± 0.1 ^{C,D}	49.1 ± 0.4^{A}	1.3 ± 0.1 ^{A,B}
4		64.5 ± 1.0^{A}	51.0 ± 1.2 ^{B,C}	1.8 ± 0.4 ^{D,E}	51.1 ± 1.3 ^{A}	2.0 ± 0.4 ^{B}
5		63.3 ± 1.5 ^{A}	53.2 ± 1.7 ^{C}	2.6 ± 0.8 ^{E}	53.3 ± 1.7 ^{A}	2.8 ± 0.7 ^{B}
6		63.9 ± 0.0 ^{A}	53.1 ± 0.2 ^{C}	1.2 ± 0.1 ^{C,D}	53.1 ± 0.2 ^{A}	1.3 ± 0.1 ^{A,B}
7		65.2 ± 0.8 ^{A}	51.3 ± 1.0 ^{B,C}	−1.9 ± 0.3 ^{B}	51.3 ± 1.0 ^{A}	357.8 ± 0.3 ^{C}
8		64.4 ± 1.4 ^{A}	46.4 ± 1.5 ^{A}	−8.6 ± 0.1 ^{A}	47.2 ± 1.5 ^{A}	349.5 ± 0.4 ^{D}
2	*Hibiscus*	66.1 ± 0.1 ^{C}	53.4 ± 0.1 ^{F}	23.5 ± 0.2 ^{E}	58.3 ± 0.2 ^{G}	23.8 ± 0.2 ^{A}
3		69.3 ± 0.0 ^{C,D}	45.6 ± 0.1 ^{E}	19.7 ± 0.0 ^{D}	49.7 ± 0.1 ^{F}	23.3 ± 0.0 ^{A}
4		73.5 ± 1.4 ^{D,E}	31.5 ± 1.5 ^{D}	17.9 ± 1.3 ^{D}	36.2 ± 2.0 ^{E}	29.6 ± 0.6 ^{A}
5		79.6 ± 0.4^{F}	15.6 ± 0.8 ^{C}	15.0 ± 0.0 ^{C}	21.6 ± 0.6 ^{D}	43.9 ± 1.3 ^{B}
6		76.3 ± 0.8 ^{E,F}	13.0 ± 1.0 ^{C}	11.8 ± 1.2 ^{B}	17.6 ± 0.1 ^{C}	42.2 ± 5.1 ^{B}
7		58.9 ± 2.4 ^{B}	8.2 ± 0.7 ^{B}	7.5 ± 0.2 ^{A}	11.1 ± 0.7 ^{B}	42.4 ± 1.6 ^{B}
8		53.1 ± 0.9 ^{A}	0.2 ± 0.2 ^{A}	5.7 ± 0.2 ^{A}	5.7 ± 0.2 ^{A}	88.1 ± 1.6 ^{C}

Different characters indicate significant differences of mean color values of an individual colorant within a column, i.e., at different pH values ($p < 0.05$).

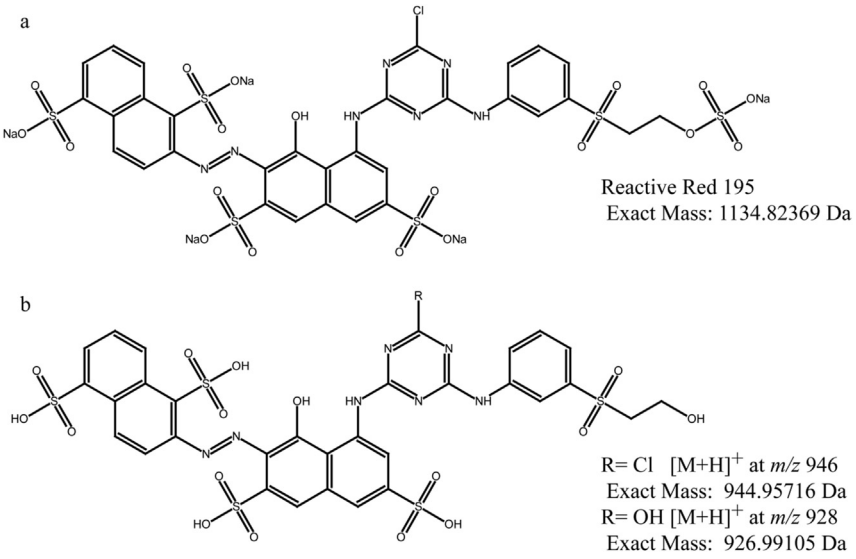

Fig. 3. Structure of Reactive Red 195 (a) and its derivatives (b) as obtained after dissolution in aqueous media.

3.3. Thermal degradation

Since most natural pigments, e.g. betalains and non-acylated anthocyanins, are known to be rather labile towards thermal treatment in contrast to synthetic azo-dyes (Delgado-Vargas et al., 2000; Stintzing & Carle, 2004), thermal degradation experiments may provide further indications of coloring foodstuffs adulterated by the addition of synthetic dyes. As displayed in Fig. 1 b, red beet extracts (pH 3) showed a decrease of absorbance of 73% and 83% at the absorption maxima (529 nm) after 30 min and 60 min at 80 °C, respectively. Although the absorbance of the roselle flower extract (Fig. 1c) was slightly more stable, a degradation of 15% and 23% was observed after 30 and 60 min at 80 °C, respectively. In clear contrast, the textile dye showed identical UV/Vis absorption spectra in Fig. 1 a throughout the thermal treatment, behaving notably different than the colorants of plant origin.

In brief, an exceptionally high thermal stability in combination with the above mentioned adsorption behavior on polyamide resins as well as the presence of two UV/Vis absorption bands should give rise to doubts about the origin of an allegedly natural pigment preparation, prompting more detailed analyses by HPLC-PDA-MS/MS as described below.

3.4. Pigment characterization by HPLC-PDA-MS/MS

All major pigments being characteristic of red beet and roselle extracts as well as the Reactive Red 195 solution were separated by HPLC as shown in the respective chromatograms (Fig. 4). In the mentioned authentic extracts of natural origin, each two main tinctorial constituents, i.e. betanin (compound **1**, Table 2) and isobetanin (compound **2**) in red beet extract as well as delphinidin-3-sambubioside (compound **4**) and cyanidin-3-sambubioside (compound **5**) in the roselle flower extract, were identified by comparing their UV/Vis absorption and mass spectra to those previously reported (Giusti, Rodríguez-Saona, Griffin, & Wrolstad, 1999; Stintzing et al., 2006). As shown in Fig. 4, the aqueous Reactive Red 195 preparation exhibited two major coloring principles (compounds **3** and **6**). Both displayed the above described characteristic UV/Vis absorption maxima (Table 2). In positive ionization mode, compound **3** and compound **6** revealed molecular ions $[M+H]^+$ at m/z 946 and m/z 928. The recorded mass spectra as well as CID fragmentation spectra of the parent ions are displayed in Fig. 5. Presumably, compound **3** represents the initially present main component of aqueous Reactive Red 195 (1,5-

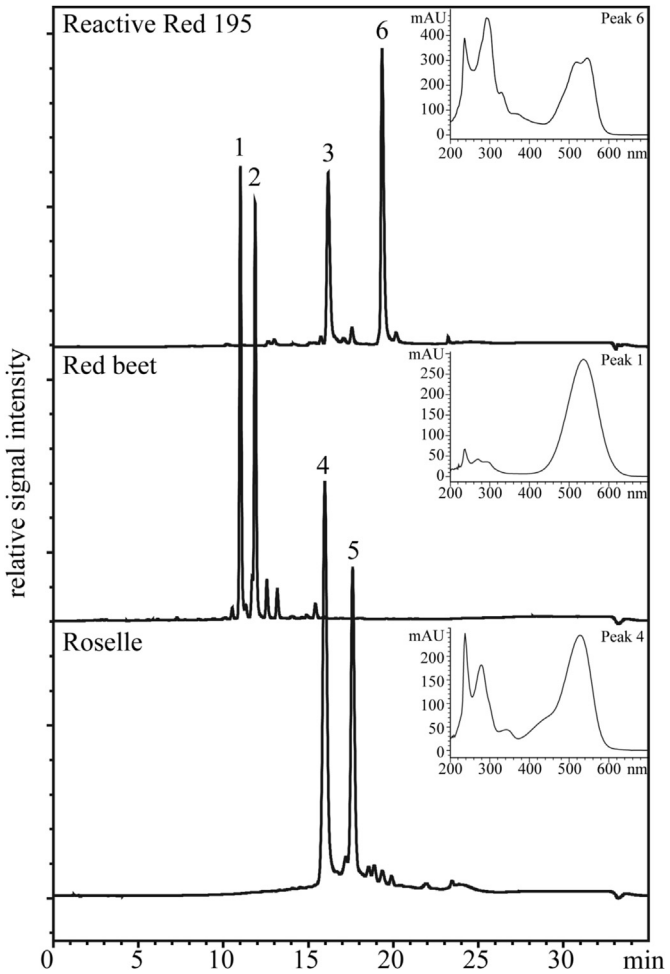

Fig. 4. HPLC separation of Reactive Red 195′, red beet extract, and roselle extract monitored at 520 nm. Peak assignment in Table 2.

Table 2
UV/Vis and mass spectral characteristics of pigments from roselle flower extract, red beet extract, and an aqueous solution of Reactive Red 195.

	Origin	Identity	Retention time [min]	UV/Vis abs max [nm]	$[M+H]^+$ m/z	HPLC-ESI(+)-MS/MS experiment m/z (intensity, % base peak)
1	*Beta vulgaris*	Betanin	10.97	268sh, 536	551	[551]: 389 (100), 390 (20)
2	*Beta vulgaris*	Isobetanin	11.83	268sh, 534	551	[551]: 389 (100), 390 (13)
3	Reactive Red 195	Pigment 1	15.93	292, 328, 518, 546	946	[946]: 928 (100), 866 (45)
4	*Hibiscus*	Delphinidin-3-sambubioside	16.12	278, 340, 526	597	[597]: 303 (100), 304 (16)
5	*Hibiscus*	Cyanidin-3-sambubioside	17.57	280, 520	581	[581]: 287 (100), 190 (3)
6	Reactive Red 195	Pigment 2	19.30	296, 328, 518, 546	928	[928]: 910 (100), 892 (54)

naphthalenedisulfonic acid, 2-[2-[8-[[4-chloro-6-[[3-[[2-(sulfooxy) ethyl]sulfonyl]phenyl]amino]-1,3,5-triazin-2-yl]amino]-1-hydroxy-3,6-disulfo-2-naphthalenyl]azo]-, sodium salt (1:5), Zhang, Yuan, and Liu (2015)) after spontaneous hydrolysis of the terminal sulfonyl group (Fig. 3). Dissolving this compound in an aqueous acidic media might have additionally resulted in the electrophilic substitution of the chlorine at the 1,3,5-triazine ring by an OH group, yielding compound **6** (Fig. 3). In brief, comparing the HPLC retention time, UV/Vis absorption and mass spectra of roselle anthocyanins and red beet betalains to those of Reactive Red 195 and its putative derivatives clearly allowed their unambiguous differentiation.

3.5. Examples of adulterations

The supplier of sample 1, which originated from Turkey, claimed that the preparation solely contained extracts from roselle flower and red beet. By analogy, similarly labelled samples were collected in France (sample 2) and Germany (sample 3). Although the color of aqueous solutions of these samples was matching the color of roselle and red beet blends, they revealed uncharacteristic UV/Vis absorption spectra, an unusual polyamide adsorption behavior, and an uncommonly high thermal stability. Thus, they were subjected to HPLC-PDA-MS/MS analyses. As shown in the chromatograms in Fig. 6, the tinctorial constituents of these samples were unambiguously different to those that had been labelled. The UV/Vis absorption, retention time, and mass spectra of these constituents clearly revealed the identity of these compounds to be the same as in the previously mentioned Reactive Red 195 solution. Minor components of red beet extract were identified in the samples 2 and 3, while sample 1 was virtually devoid of natural pigments. As a consequence of our analyses, the respective providers from food industry refrained from incorporating the adulterated samples into real food products.

4. Conclusions

Authentication of food ingredients is required for their differentiation from food additives, particularly, under the strict regulations of the Codex Alimentarius and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Approved artificial food colors may be easily analyzed by official methods, but the detection

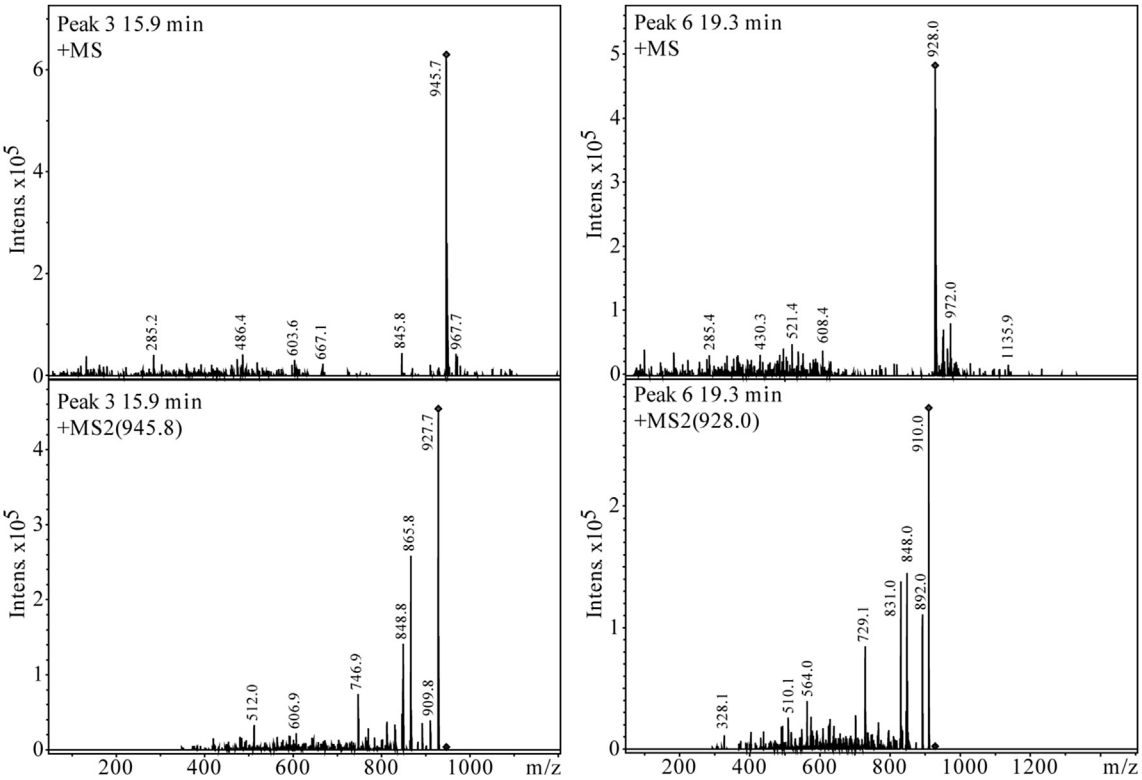

Fig. 5. Positive ionization mass spectra of the main tinctorial constituents of aqueous solutions of Reactive Red 195 (upper panels) and the CID fragmentation pattern of the respective parent ions (lower panels). Peak assignment in Table 2.

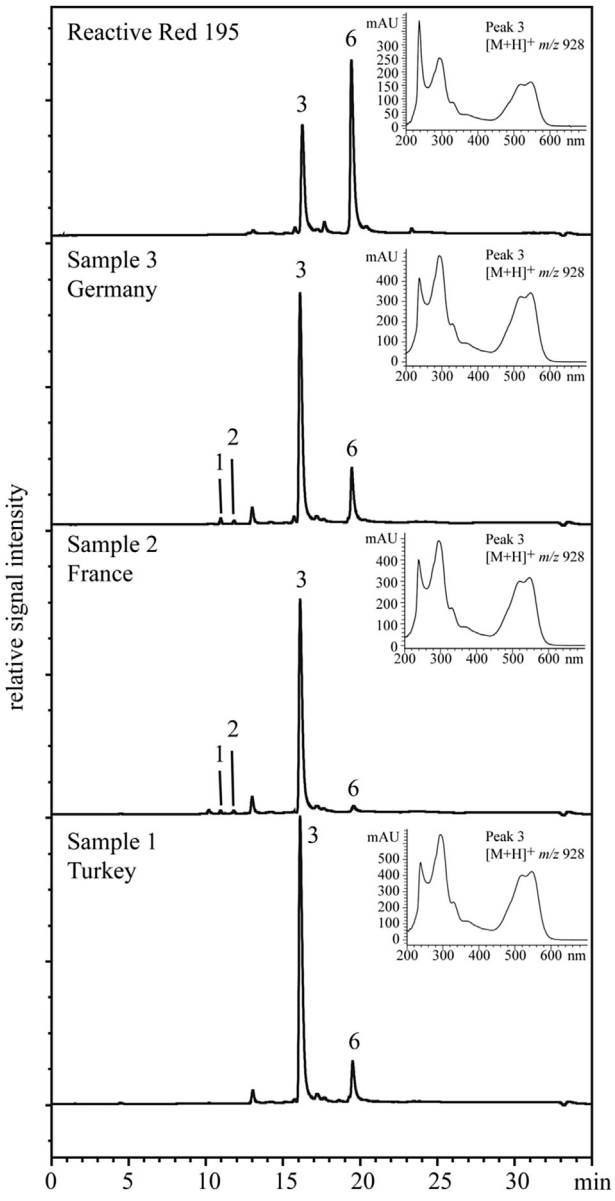

Fig. 6. HPLC separation of aqueous solutions of Reactive Red 195 as well as adulterated samples from business-to-business markets obtained from Germany, France, and Turkey monitored at 520 nm. Peak assignment in Table 2.

and identification of unknown synthetic dyes in food is challenging. Differentiation of synthetic colorants from coloring foodstuffs and the detection of adulterations are of utmost importance to consumer health, since several synthetic dyes have been associated with adverse effects on children (McCann et al., 2007). The consequences of acute and chronic oral intake of the identified textile dye Reactive Red 195 to human health are widely unknown. Therefore, our chromatic, spectrophotometric, adsorptive, and thermal investigations of suspicious water-soluble coloring foodstuff should be helpful to raise preliminary concerns about the authenticity of doubtful natural colorant preparations. In order to prevent further attempts of fraud, the pigment identity of suspicious preparations should be subsequently ascertained, e.g., by the HPLC-PDA-MS/MS method presented.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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