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Isotope ratio mass spectrometrical analysis of betanin and isobetanin isolates for authenticity evaluation of purple pitaya-based products

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Abstract

Betanin and isobetanin from purple pitaya fruits originating from Israel, commercial red beet samples from Germany and France, as well as from a commercial pigment extract, respectively, were isolated by column chromatography and semi-preparative HPLC. Their $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ values were determined by Elemental Analyser-Isotope Ratio Mass Spectrometry in the Combustion (C) and Pyrolysis (P) modes (EA-C/P-IRMS). While $\delta^{13}C_{V\text{-}PDB}$ values of red beet samples ranged from $-26.9‰$ to $-27.7‰$ indicative of C_3 plants, betanin and isobetanin derived from purple pitaya displayed values of $-17.0‰$ and $-17.7‰$, respectively, reflecting CAM mechanism of CO_2 fixation. As expected, betanin and isobetanin isolated from red beet and purple pitaya juice blends exhibited depletion in the heavy carbon isotope increasing with incrementing red beet content. Therefore, determination of $\delta^{13}C_{V\text{-}PDB}$ is considered a feasible approach for the detection of admixtures from red beet to purple pitaya-based products. In conjunction with $\delta^{13}C_{V\text{-}PDB}$ data, also different $\delta H_{V\text{-}SMOW}$ values of the investigated plant samples proved useful for authenticity evaluation.

Keywords: Betanin; $^{13}C/^{12}C$ ratio; $^{2}H/^{1}H$ ratio; Purple pitaya; Red beet; Colouring foodstuff; EA-C/P-IRMS; Authenticity; Admixture

1. Introduction

In the last century, the use of natural food colourants had mainly been superseded by synthetic dyes, the latter still showing the most important market share (Downham & Collins, 2000). In recent years, there is not only a return of natural alternatives, but also an increased interest in establishing new sources of colouring foodstuffs and improving their performance in food applications (Davies, 2004).

Whereas a broad array of anthocyanin-containing extracts is used for food colouring, hitherto one single betalainic source, the red beet (*Beta vulgaris* L. ssp. *vulgaris*), has been well established on the market (Stintzing, Schieber, & Carle, 2000). Very recently, however, the purple pitaya (genus *Hylocereus*, Cactaceae), was suggested as a promising source for betalains, and the pulp of *H. polyrhizus* is already used in Israel for ice cream colouring (Wybraniec & Mizrahi, 2002). Since the availability of violet-fleshed pitaya is still limited, pitaya-based products are afflicted by considerably high commodity prices. On the other hand, pitayas bear a whiff of novelty and exoticism, thus appealing to consumers.

Due to significantly lower prices of red beet, adulteration of purple pitaya with red beet juice would lead to noticeably lower production costs. Therefore, authentication of the plant materials is challenging. Several systems for the separation of purple pitaya betalains by high-performance liquid chromatography (HPLC) have been proposed

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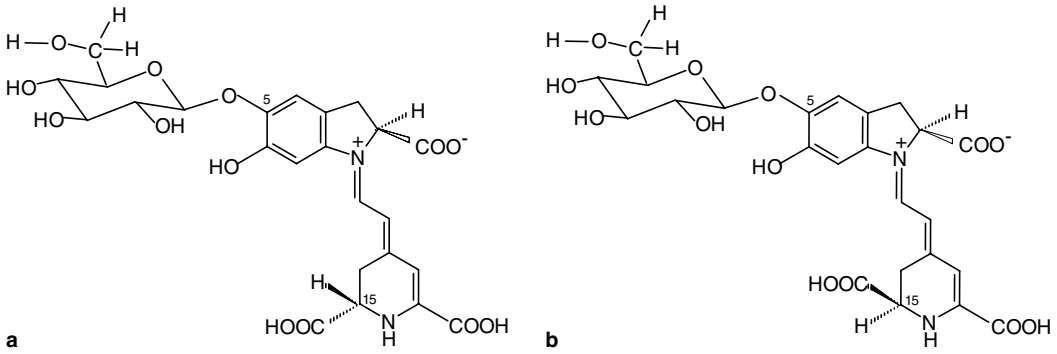

Fig. 1. Structures of betanin (a) and isobetanin (b).

(Herbach, Stintzing, & Carle, 2004; Stintzing, Schieber, & Carle, 2002; Wybraniec et al., 2001). While both purple pitaya and red beet contain betanin (betanidin 5-*O*-β-glucoside) and isobetanin (isobetanidin 5-*O*-β-glucoside, Fig. 1) as major pigment constituents, purple pitaya additionally features acylated constituents, i.e. phyllocactin (betanidin 5-*O*-(6′-*O*-malonyl)-β-glucoside) and hylocerenin (betanidin 5-*O*-(6′-*O*-3″-hydroxy-3″-methyl-glutaryl)-β-glucoside) together with their respective C_{15}-stereoisomers. However, the proportion of acylated and non-acylated betacyanins in purple pitaya is subjected to fluctuations. Depending on the *Hylocereus* species, betanin and isobetanin made up 18–76% and 3–14% of the total betacyanin content, respectively, the remainder being acylated structures (Wybraniec & Mizrahi, 2002). Additionally, the proportions of acylated and non-acylated betacyanins varied during processing due to different pigment stabilities (Herbach, Stintzing, & Carle, submitted for publication). Hence, unequivocal proof of purple pitaya products adulterated with red beet juice by comparison of their respective betacyanin patterns is not possible. Alternatively, betaxanthins only occurring in small amounts or completely lacking in pitaya may be used as authenticity markers. However, by utilisation of early-harvested red beets exhibiting a relatively high betacyanin/betaxanthin ratio (Watson & Gabelman, 1982), red beet juice concentrates poor in betaxanthins are obtained to achieve a more bluish hue. Consequently, the unequivocal detection of red beet admixtures to purple pitaya based on betaxanthin analysis would be difficult.

However, because of different CO_2 fixation mechanisms in plants, distinct carbon isotope ratios were reported. For C_3 plants, $\delta^{13}C_{V\text{-}PDB}$ values are determined by carbon isotope discrimination of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and range between −24‰ and −32‰ (Winkler & Schmidt, 1980). Due to utilisation of phospho*enol*pyruvate (PEP) carboxylase for primary CO_2 fixation, C_4 plants are less depleted in the heavy carbon isotope, resulting in $\delta^{13}C_{V\text{-}PDB}$ values between −10‰ and −16‰ (Winkler & Schmidt, 1980). The ability of Crassulacean Acid Metabolism (CAM) plants to employ both mechanisms for CO_2 fixation leads to intermediate values for $\delta^{13}C_{V\text{-}PDB}$, ranging from −12‰ to 30‰ (Winkler & Schmidt, 1980). Since authentication of isolated compounds has been successful even for plants exerting the same photosynthetic mechanism (Carle, Fleischhauer, Beyer, & Reinhard, 1990), distinction of purple pitaya (Cactaceae) employing the CAM pathway for CO_2 fixation (Raveh, Nerd, & Mizrahi, 1998) from red beet (Chenopodiaceae) belonging to the C_3 plants (Nobel, 1991) appeared to be promising by determination of $^{13}C/^{12}C$ as well as $^{2}H/^{1}H$ ratios of betacyanins.

Based on these assumptions, the present study aimed at the differentiation of plant sources for the production of betacyanin colouring foodstuffs by isotope ratio determination. For this purpose, betalain purification from red beets of different provenance and purple pitaya, respectively, was achieved by combining different column chromatography techniques with semi-preparative HPLC. Subsequently, the particular $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios of betanin and isobetanin were assessed by EA-C/P-IRMS. Additionally, $\delta^{13}C_{V\text{-}PDB}$ values of blends from purple pitaya and red beet were determined. To the best of our knowledge, this is the first investigation into isotope ratio determination of pigments to prove adulteration. Furthermore, no isotope data have been reported for betalains so far.

2. Materials and methods

2.1. Plant material

Purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose, Cactaceae) fruits were purchased from Israel and stored at −18 °C. Fruits were thawed prior to manually squeezing. The obtained juice was paper filtered (Schleicher & Schuell, Dassel, Germany), flushed with nitrogen and kept at −40 °C until used. Red beet (*Beta vulgaris* L. ssp. *vulgaris,* 'Garden Beet Group', Chenopodiaceae; Lange, Brandenburg, & De Bock, 1999) juices were prepared by dilution of two commercial red beet juice concentrates. The German concentrate (69°Bx) was from Ernteband (Winnenden, Germany), while the French sample (69°Bx) was provided by Diana Végétal (Antrain, France). Both concentrates originated from red beets grown in the respective cultivation area.

2.2. Solvents and reagents

Reagents and solvents were purchased from VWR (Darmstadt, Germany) and were of analytical or HPLC grade. Sephadex LH-20 was from Amersham (Uppsala, Sweden), while red beet pigment extract (TCB 0397, betanin) was obtained from ABCR (Karlsruhe, Germany). Deionised water was used throughout.

2.3. Removal of phenolic substances (red beet juice)

Three millilitres of red beet juice concentrate diluted to single juice strength (1 + 6, w/w) were applied on a Sephadex LH-20 column (350 × 15 mm i.d.) pre-equilibrated with purified water. While most phenolics remained adsorbed until eluted with 100% methanol, elution of betalains was achieved using deionised water. At a flow rate of 1 ml/min, two betalain-containing fractions could be separated visually. The first fraction eluting after 30–40 min was concentrated in vacuo and used for further purification by semi-preparative HPLC.

2.4. Removal of pectic substances (purple pitaya juice)

Precipitation of pectic substances in purple pitaya juice was carried out by the addition of ethanol as described earlier (Stintzing et al., 2002).

2.5. Blending of red beet and purple pitaya juice

Red beet juice concentrate was diluted to yield the same concentration of betanin and isobetanin as present in purple pitaya juice, i.e. identical peak area sums of betanin and isobetanin monitored at 535 nm by analytical HPLC were adjusted. Pitaya juice (13°Bx) blends containing relative proportions of 20%, 40% and 60% red beet juice dilution (2°Bx, v/v), respectively, were prepared and subsequently purified as described for purple pitaya juice (see Sections 2.4 and 2.6).

2.6. Semi-preparative high-performance liquid chromatography (HPLC)

Isolation of betanin and isobetanin from a commercial red beet pigment extract, pre-purified purple pitaya and red beet juices as well as from pitaya-beet blends by semi-preparative HPLC was carried out as described earlier (Stintzing, Conrad, Klaiber, Beifuss, & Carle, 2004). While betanin and isobetanin fractions isolated from the juice blends were combined, the stereoisomers of the remaining samples were kept separately. After concentration in vacuo at room temperature, the samples were repeatedly washed with deionised water and re-concentrated to gently remove residual acid. The resulting betacyanin concentrates were checked for purity (see Section 2.7), lyophilised and stored in a sealed tube at −40 °C until EA-C/P-IRMS analysis (see Section 2.8).

2.7. Analytical HPLC

Purity of betanin and isobetanin samples was checked by analytical HPLC. For this purpose, an HPLC-system (Merck, Darmstadt, Germany) equipped with an auto sampler L-7200, an interface module D-7000, a pump L-7100, a column oven L-7350 with a Peltier cooling module, and a diode array detector L-7450A was applied. Separation was achieved at 25 °C on an analytical scale Luna RP18(2)-column (250 × 3.0 mm i.d; 5 μm; Phenomenex, Torrance, CA, USA) fitted with a C_{18} ODS security guard column (4 × 3.0 mm i.d.; Phenomenex, Torrance, CA, USA). The mobile phase A consisted of 5% (v/v) formic acid in water, MeCN was used as B. At a flow rate of 1 ml/min, the first 5 min were performed isocratically with 100% A, followed by a linear gradient from 0% B to 16% B in 30 min, and from 16% B to 100% B in 5 min. Monitoring was performed at 280 and 538 nm for simultaneous detection of phenolic substances and betacyanins. Purity of betanin and isobetanin was determined at 280 nm.

2.8. Elemental analyser-combustion/pyrolysis-isotope ratio mass spectrometry (EA-C/P-IRMS)

For isotope ratio analysis, a Delta^{plus} XL (Finnigan MAT, Bremen, Germany) isotope ratio mass spectrometer coupled with elemental analysers operating in the combustion and pyrolysis modes was applied. In general, sixfold determinations were carried out with 0.25 and 1 mg pigment isolate for carbon and hydrogen measurements, respectively. Determination of $^{13}C/^{12}C$ ratios was performed in the combustion mode with an oxidative reactor (quartz tube with tungsten oxide, quartz wool and copper fillings; Euro Vector EA 3000, Milano, Italy; reactor temperature 1000 °C; column: Porapak QS, 2 m × 5 mm i.d.; oven temperature: 60 °C). The reactor used for $^{2}H/^{1}H$ ratio determination (pyrolysis mode) consisted of an outer ceramic tube and an inner glass carbon tube filled with nickelised carbon and glass carbon pieces (HT Sauerstoff Analysator, HEKAtech, Wegberg, Germany; reactor temperature: 1460 °C; column: molecular sieve, 5′, 1 m × 5 mm i.d.; oven temperature: 85 °C). Daily system checks were carried out by measuring reference samples with known $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios. Stability checks of the used reference gases were continuously performed by measuring International Atomic Energy Agency (IAEA, Vienna, Austria) standards with defined $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios (for $^{13}C/^{12}C$ IAEA-CH7 and for and $^{2}H/^{1}H$ IAEA-CH7, NBS 22 oil, and V-SMOW, respectively).

The isotope ratios were expressed in per mil (‰) deviation relative to the V-PDB and V-SMOW international standards. Results were calculated as follows (for $\delta^{2}H$, the corresponding formula is valid): $\delta^{13}C_{V\text{-}PDB}$ [‰] = $[(R_{sample} - R_{V\text{-}PDB})/R_{V\text{-}PDB} \times 100]$ with R = isotope ratio $^{13}C/^{12}C$ (corresponding to hydrogen: $^{2}H/^{1}H$).

For $^{13}C/^{12}C$ and $^{2}H/^{1}H$ measurements, calibration of the IRMS was performed with certified CO_2

($\delta^{13}C_{V\text{-}PDB} = -24.9 \pm 0.2‰$) and H_2 ($\delta^2H_{V\text{-}SMOW} = -255 \pm 10‰$) reference gases (Messer Griesheim, Frankfurt, Germany). Standard deviations were ±0.1‰ and ±5‰ for $\delta^{13}C_{V\text{-}PDB}$ and $\delta^2H_{V\text{-}SMOW}$ determinations, respectively.

3. Results and discussion

3.1. Purification of betanin and isobetanin

Betanin and isobetanin from purple pitaya juice were obtained by precipitation of pectic substances and consecutive semi-preparative HPLC as previously described by Stintzing et al. (2002, 2004). Since phenolic substances in red beet juice could not satisfactorily be separated from the betacyanins by semi-preparative HPLC, additionally, gel chromatography on Sephadex LH-20 was required. Applying this resin not only separation of betalains and phenolic substances, but also betalain fractionation was possible. Two major fractions could be separated visually, the first one containing betanin and isobetanin in nearly equal shares, together with minor amounts of vulgaxanthin I (HPLC data not shown). The removal of phenolic substances, betacyanidins and other constituents of the original juice improved semi-preparative HPLC separation and allowed application of higher injection volumes. The purities of the different pigment samples are listed in Table 1. Although betanin purities exceeded 90% for purple pitaya and the German red beet sample, only about 81% purity was obtained for the French red beet juice concentrate, while purities of isobetanin samples ranged between 87% and 91%. Applying the same purification procedure, isolation of betanin and isobetanin from a commercial red beet pigment extract yielded purities higher than 94%.

3.2. Isotope ratios of betanin and isobetanin from red beet and purple pitaya

Carbon and hydrogen ratios of betanin and isobetanin from red beet and purple pitaya are compiled in Table 2. Irrespective of their origin, both betanin and isobetanin from red beet showed $\delta^{13}C_{V\text{-}PDB}$ values ranging from −26.9‰ to −27.7‰, consistent with carbon isotope ratios of −24‰ to −32‰ generally reported for C_3 plants (Winkler & Schmidt, 1980). Although remarkable impurities of the betanin sample derived from French red beet were monitored by analytical HPLC, its $\delta^{13}C_{V\text{-}PDB}$ value corresponded well with those obtained for the purer betanin samples derived from German red beet and a commercial red beet pigment extract, respectively. Compared to the red beet samples, betanin and isobetanin from purple pitaya were characterised by higher $\delta^{13}C_{V\text{-}PDB}$ values, thus indicating less pronounced isotope fractionation during CO_2 fixation. Like in red beet samples, both betanin isomers from purple pitaya exhibited quite similar carbon isotope ratios of −17.0‰ and −17.7‰, respectively, concordant with $\delta^{13}C_{V\text{-}PDB}$ values of −12‰ to −30‰ and −12‰ to −22‰ previously reported for CAM plants (Winkler & Schmidt, 1980) and other members of the Cactaceae family, respectively (Bender, Rouhali, Vines, & Black, 1973; Weckerle, Bastl-Bormann, Richling, Hör, Ruff, & Schreier, 2001; Winter & Holtum, 2002).

In addition to carbon isotope ratios, also $\delta^2H_{V\text{-}SMOW}$ values were reported to differ between C_3 and CAM plants with the latter exhibiting a greater trend for deuterium enrichment if grown under the same conditions (Ting, 1985). In this study, betanin and isobetanin from red beet samples showed $\delta^2H_{V\text{-}SMOW}$ values from −32‰ to −34‰, while betanin and isobetanin from purple pitaya exhibited only slightly enriched values of −17‰ and −14‰, respectively. These data can be hardly compared to previously reported $\delta^2H_{V\text{-}SMOW}$ values of CAM plant bulk material, selected fractions analysed therefrom and even defined plant metabolites because hydrogen isotope equilibria are governed by multiple factors: Climatic conditions during plant growth, but also differences of hydrogen pools available, metabolic turnover rates, let alone biosynthetic branching events that will determine the final hydrogen isotope ratio (Schmidt, 2003; Schmidt, Werner, & Eisenreich, 2003). Additionally, sample treatment during workup (e.g., rinsing with water) has been reported to alter the hydrogen isotope ratio due to hydrogen exchange (Sternberg, Deniro, & Johnson, 1984).

As proposed by Sternberg et al. (1984), $\delta^{13}C_{V\text{-}PDB}$ were plotted against $\delta^2H_{V\text{-}SMOW}$ values (Fig. 2) to illustrate the differences in isotope ratio composition of betanin and isobetanin from red beet and purple pitaya: two clusters of data points, representing red beet and purple pitaya, respectively, could be distinguished, thus allowing a preliminary differentiation of the particular plant pigment origin.

Table 1
Purities of betanin and isobetanin isolates from red beet and purple pitaya determined at 280 nm

Plant source	Origin	Betacyanin	Betanin content (%)	Isobetanin content (%)	Other impurities (%)
Purple pitaya	Israel	Betanin	92.8	1.7	5.5
		Isobetanin	3.3	86.6	10.1
Red beet	Germany	Betanin	94.6	2.6	2.8
		Isobetanin	3.6	88.9	7.5
Red beet	France	Betanin	80.8	2.3	16.8
		Isobetanin	4.0	90.8	5.2
Red beet	Commercial sample	Betanin	94.7	0.7	4.6
		Isobetanin	1.5	94.3	4.2

Table 2
Isotope ratios of betanin and isobetanin isolates from red beet and purple pitaya

Plant Source	Origin	Betacyanin	$\delta^{13}C_{V\text{-}PDB}$ (‰)	$\delta^{2}H_{V\text{-}SMOW}$ (‰)
Purple pitaya	Israel	Betanin	-17.0 ± 0.2	-17 ± 3
		Isobetanin	-17.7 ± 0.1	-14 ± 2
Red beet	Germany	Betanin	-27.3 ± 0.2	-32 ± 5
		Isobetanin	-27.7 ± 0.2	-35 ± 5
Red beet	France	Betanin	-27.7 ± 0.1	-32 ± 2
		Isobetanin	-27.5 ± 0.2	-32 ± 4
Red beet	Commercial sample	Betanin	-26.9 ± 0.1	n.d.[a]
		Isobetanin	-27.6 ± 0.2	n.d.[a]

[a] n.d., not determined.

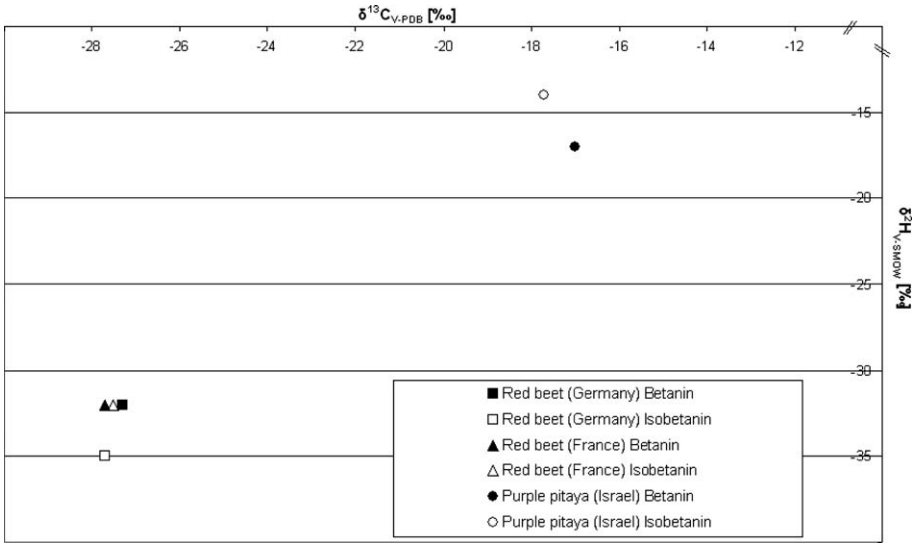

Fig. 2. Relationship between $\delta^{2}H_{V\text{-}SMOW}$ and $\delta^{13}C_{V\text{-}PDB}$ values of betanin and isobetanin isolates from red beet and purple pitaya.

Table 3
Purities determined at 280 nm and carbon isotope ratios of purple pitaya-red beet blends

Content of red beet-derived betanin/isobetanin in purple pitaya juice (%)	Betanin content (%)	Isobetanin content (%)	Impurities (%)	$\delta^{13}C_{V\text{-}PDB}$ (‰)
20	66.8	32.6	0.6	-15.8 ± 0.8
40	62.7	37.1	0.2	-19.6 ± 0.2
60	64.2	35.5	0.3	-21.8 ± 0.7

3.3. Carbon isotope ratio of blends from purple pitaya and red beet

To simulate adulterations, blends from purple pitaya with red beet were prepared by commixing diluted red beet juice concentrate and purple pitaya juice. The resulting admixtures contained 20%, 40% and 60% of beet-derived betanin and isobetanin, respectively. Since betanin and isobetanin isolates were shown to exhibit identical carbon isotope ratios (Table 1), separate measurement of the stereoisomers was waived. Purity exceeding 99% was achieved for all samples (Table 3). As expected, depletion in the heavy carbon isotope increased with incrementing red beet contents of the samples.

4. Conclusion

In the present study, determination of the carbon isotope ratios of betanin and isobetanin has been demonstrated to be a feasible approach for authenticity evaluation of products derived from purple pitaya and red beet, respectively. Additionally, hydrogen isotope data supported pigment source differentiation by plotting of carbon and hydrogen isotope ratios in one chart. Since isotopic composition of betanin and isobetanin samples was identical, separation of isomers for isotope determination was shown to be dispensable. In addition, the expensive and time-consuming purification procedure may become unnecessary by coupling a liquid chromatograph to a stable

isotope ratio mass spectrometer. Only very recently, such a system has been applied to IRMS-analysis of amino acids and carbohydrates (Krummen, Hilkert, Juchelka, Duhr, Schlüter, & Pesch, 2004). This technique would allow analysis of a broad range of samples, including different varieties, provenances and harvest periods of red beet and purple pitaya, thus providing more isotope data for unambiguous detection of pitaya product adulteration. Reliable proof of pitaya juice adulteration, however, was only possible when at least 40% of the betacyanin fraction was beet-derived (Tables 2 and 3). Since the present investigation was based on the same pigment concentration, this translates into 6% red beet juice addition to purple pitaya juice on a °Bx basis.

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