JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 2975−2981 **2975**

Identification of Betalains from Petioles of Differently Colored Swiss Chard (*Beta vulgaris* L. ssp. *cicla* [L.] Alef. Cv. Bright Lights) by High-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry

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The betalain pattern of differently colored Swiss chard (*Beta vulgaris* L. ssp. *cicla* [L.] Alef. cv. Bright Lights) was investigated for the first time. Nineteen betaxanthins and nine betacyanins were identified by RP-HPLC and positive ion electrospray mass spectrometry, co-injection experiments with semisynthetic reference compounds, and standards derived from authentic plant material, respectively. Histamine−betaxanthin and alanine−betaxanthin were found to be novel betaxanthins, which to the best of our knowledge have not been reported as natural compounds until now. Furthermore, tyramine−betaxanthin (miraxanthin III) and 3-methoxytyramine−betaxanthin, which to date were known only from families other than the Chenopodiaceae, were detected for the first time in colored Swiss chard. The betacyanin pattern of purple petioles was composed of betanin, isobetanin, betanidin, and isobetanidin. Although phyllocactin was present in only trace amounts, further acylated structures such as betanidin−monoferuloyl-5-*O*-β-diglucoside and lampranthin II, accompanied by their corresponding C_{15}-epimers, were identified. In addition, quantification of betalains and CIE *L*C*h*° measurements were performed with the colored extracts to correlate the visual appearance with the respective pigment patterns. Besides the novel phytochemical findings, the present study is useful for the evaluation of betalainic Swiss chard as a potential coloring foodstuff.

KEYWORDS: Chenopodiaceae; *Beta vulgaris*; colored Swiss chard; betalains; betaxanthins; acylated betacyanins; coloring foodstuff

INTRODUCTION

For coloring foodstuff, an annual growth rate of 10−15% is predicted for the European market until 2008 (*1*). This forecast reflects the disapproval of synthetic food colorants among consumers and the industry's increasing endeavors to use natural colorants for food production. Chlorophylls, carotenoids, anthocyanins, and betalains are the most important natural pigment classes. The latter, comprising the red-purple betacyanins and the yellow betaxanthins, can be found in only 13 families of the plant order Caryophyllales (*2*) and also in some genera of the Basidiomycetes (*3*, *4*). In contrast to anthocyanins, betalains keep their color properties over a wide pH range from pH 3 to pH 7 (*5*), which makes them interesting for coloring low-acid products. Until now, only red beet preparations have been commercially exploited, although there are other potential edible betalain-containing sources such as *Amaranthus* sp. (*6*), cactus fruits (*7*, *8*), yellow beet (*7*), and colored Swiss chard. Hitherto, very little scientific research has been conducted on the betalain pigment composition of Swiss chard. This may possibly be ascribed to the more widespread use of white-stemmed cultivars because the colored ones are less known among consumers.

A preliminary investigation (*9*) on betalainic Swiss chard was performed using simple analytical methods. Betanin has been identified as the main compound accompanied by glutamine−betaxanthin (vulgaxanthin I) by applying thin-layer chromatography. Therefore, the present study aimed at investigating the betacyanin and betaxanthin composition of differently colored Swiss chard in detail, applying high-performance liquid chromatography and mass spectrometry. Besides screening the pigment patterns of purple, red-purple, yellow-orange, and yellow petioles, their betalain contents were investigated by correlation of photometric and HPLC data. Furthermore, to obtain their corresponding *L*C*h*° values, color measurements of the extracts were performed.

MATERIALS AND METHODS

Plant Material. Swiss chard (*Beta vulgaris* L. ssp. *cicla* [L.] Alef. cv. Bright Lights; Chenopodiaceae) (**Figure 1**) was purchased in 2003 from a local grower (Pommerenke, Steinheim am Albuch, Germany). Cv. Bright Lights is a mixed cultivar that produces differently colored plants. It was harvested in July, 20 weeks after sowing, and visually

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10.1021/jf035491w CCC: $27.50
Published on Web 04/14/2004

Figure 1. Purple, red-purple, yellow-orange, and yellow Swiss chard (*B. vulgaris* L. ssp. *cicla* [L.] Alef. cv. Bright Lights), together with a white-stemmed cultivar.

separated into purple, red-purple, yellow-orange, and yellow petioles. After washing, the leaves were removed from the petioles and the latter were sealed in polyacrylamide−polyethylene bags under reduced pressure before storage at −26 °C until further processing.

For the isolation and preparation of reference substances, the following plant materials were used. Concentrated juice from red beet (*B. vulgaris* L. ssp. *vulgaris*; Chenopodiaceae) was obtained from Ernteband (Winnenden, Germany). Cactus pear (*Opuntia ficus-indica* [L.] Mill. cv. Gialla; Cactaceae) was from Italy and purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose; Cactaceae) from Israel. Feathered amaranth (*Celosia argentea* var. *plumosa* [Burvenich] Voss; Amaranthaceae) with yellow inflorescences and purple midday flowers (*Lampranthus* sp.; Aizoaceae) were purchased from a local supplier.

Solvents and Reagents. Reagents and solvents were purchased from VWR (Darmstadt, Germany) and were of analytical or HPLC grade. Amino acids and amines were from Fluka (Buchs, Switzerland). Deionized water was used throughout.

Cryogenic Grinding and Pigment Extraction. To avoid enzymatic reactions during comminution of the plant material, liquid nitrogen was added to the petioles during grinding in a model 38BL41 Waring blender (Waring Products, Torrington, CT) additionally equipped with an outlet for pressure release at the top. The resulting powder was stored at −26 °C until pigment extraction.

For pigment analysis, 150 g of the colored powder was extracted by the addition of 0.6 L of 60% aqueous methanol containing 50 mM sodium ascorbate. After 10 min of shaking under ice cooling, the colored solution was separated from the plant material by filtering through a Büchner funnel with a filter paper (Schleicher & Schuell, Dassel, Germany) under reduced pressure. To achieve complete pigment extraction of the plant material, the residue was rinsed with 100% methanol. The extract was concentrated in vacuo at 30 °C, resuspended in 50 mL of purified water, and stored at −26 °C. For HPLC analyses, photometric quantification, and color measurements, filtered (0.45 μm) aqueous extracts were used.

Preparation of Betalamic Acid. Juice from cactus pear (*O. ficus-indica* [L.] Mill. cv. Gialla) was desalted as described earlier (*7*). Indicaxanthin was isolated from the desalted juice by semipreparative HPLC and hydrolyzed at pH 11.3−11.5 by the addition of 25% NH_4OH to obtain betalamic acid. The hydrolysis was controlled spectrophotometrically with a UV−vis spectrometer (Perkin-Elmer, Überlingen, Germany) at 424 nm (*10*). At the point of maximum absorption, the reaction was stopped by adding trifluoroacetic acid (TFA) until a pH of 9 was reached.

Partial Synthesis of Betaxanthins. The synthesis of betaxanthins was modified according to a method described previously (*7*). For each betaxanthin, the respective amino compound (10−20-fold molar excess) was predissolved in 50 μL of 0.1 N HCl, and after vortexing, 750 μL of the alkaline betalamic acid solution was added. The vortexed mixture was concentrated in vacuo at 30 °C, and the remainder was resuspended in 1 mL of purified water. For further purification, the betaxanthin solution was applied to a Chromabond 1000 mg C_{18}-reversed phase cartridge (Macherey & Nagel, Düren, Germany) after washing the minicolumn with 5 volumes of 100% methanol and subsequent conditioning with 5 volumes of acidified water (TFA, pH 2). Purification was achieved by rinsing with 2 volumes of acidified water (pH 2), and final elution of the betaxanthin was performed with 100% methanol. The solution thus obtained was adjusted to pH 5−7 by the addition of 1.5 M NH_4OH and reduced in vacuo at 30 °C. The residue was redissolved in 1 mL of purified water. Prior to analysis, the semisynthetic standards were filtered (0.2 μm) and stored at −26 °C. All betaxanthin standards were checked for identity by LC-MS analysis.

Extraction and Isolation of Reference Substances. Lampranthin II and isolampranthin II were identified by co-injection of an aqueous extract of *Lampranthus* sp. For this purpose, purple petals of *Lampranthus* sp. were ground in a mortar after the addition of liquid nitrogen, and the resulting powder was extracted with 80% aqueous methanol. After continuous stirring for 2 h, the purple solution was separated from the plant material by paper filtration (Schleicher & Schuell) and concentrated in vacuo at 30 °C. The residue was redissolved in purified water and filtered (0.45 μm) before analysis. For the co-injection experiments phyllocactin was isolated from a depectinized red-violet pitaya juice from *H. polyrhizus* (Weber) Britton & Rose (*8*) using semipreparative HPLC. Because betaxanthin yields resulting from the condensation of betalamic acid with both dopamine and 3-methoxytyramine were low, an aqueous extract of yellow *C. argentea* var. *plumosa* (*11*) was used for HPLC assignment of these compounds. The yellow plant material was frozen by adding liquid nitrogen and pulverized in a mortar. The homogenized material was extracted with 80% aqueous methanol containing 50 mM sodium ascorbate. After 30 min of stirring, the extract was passed through a

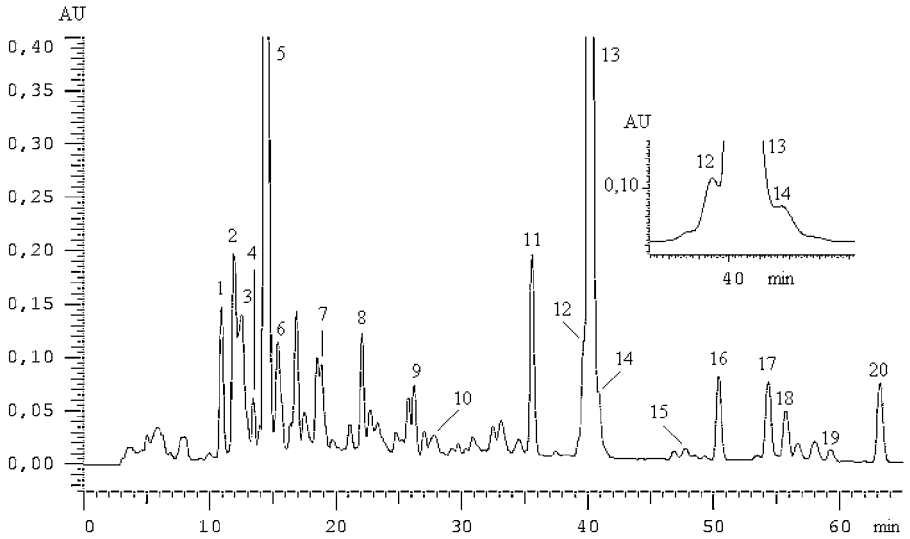

Figure 2. HPLC separation of betaxanthins from yellow Swiss chard petioles monitored at 470 nm (peak assignment is given in **Table 1**).

filter paper (Schleicher & Schuell), and the filtrate was concentrated in vacuo at 30 °C. The residue was resuspended in purified water and filtered (0.45 μm) prior to analysis. All reference substances were checked for identity by means of LC-MS.

Semipreparative High-Performance Liquid Chromatography (HPLC). For semipreparative isolation of indicaxanthin and phyllocactin, respectively, an HPLC system (Bischoff, Leonberg, Germany) consisting of an LC-CaDI 22-14 control unit, two HPLC compact pumps, connected with an SPD 10 AV *VP* UV−vis detector (Shimadzu, Tokyo, Japan), and a dynamic mixing chamber (Knauer, Berlin, Germany) equipped with Bischoff McDAcq 32 software was used. Separation was achieved on a 250 × 21.2 mm i.d. (5 μm) semipreparative C_{18}-AQUA column (Phenomenex, Torrance, CA) operating at room temperature, at a flow rate of 9 mL/min and a pressure of 70 bar.

As described earlier (*12*), the mobile phase consisted of 0.5% formic acid in water (v/v, eluent A) and a mixture of MeCN in water of 50:50 (v/v, eluent B). Starting with 84% A in B, a linear gradient was followed to 72% A in B at 14 min and then to 0% A in B in 2 min before re-equilibration to starting conditions. Aliquots of 700 μL of the respective juices were injected, and monitoring was performed at 476 and 538 nm for indicaxanthin (t_R = 16.0 min) and phyllocactin (t_R = 19.5 min), respectively.

HPLC Analyses. The HPLC system (Merck, Darmstadt, Germany) was equipped with an L-7200 autosampler, a D-7000 interface module, an L-7100 pump, an L-7350 column-oven with a Peltier cooling module, and an L-7400 UV−vis detector. An analytical scale 250 × 4.6 mm i.d. Atlantis dC_{18}-reversed phase column with a particle size of 5 μm (Waters, Wexford, Ireland), fitted with a 4 × 3.0 mm i.d. C_{18}-ODS security guard column, was used for pigment analyses, operating at a constant temperature of 25 °C and a flow rate of 1 mL/min.

The betaxanthin and betacyanin compositions of differently colored extracts were studied with 1% formic acid in water (v/v, eluent A) and a mixture of MeCN in water of 80:20 (v/v, eluent B) by applying different elution conditions. Betaxanthins were separated starting isocratically with 100% A for 2 min at a pressure of 115 bar, followed by a linear gradient from 0 to 20% B in 60 min and a subsequent linear gradient from 20 to 100% B in 5 min. Separation of betacyanins was accomplished beginning with 2% B in A at 0 min at a pressure of 117 bar, followed by a linear gradient to 33% B in A in 30 min. Betaxanthins and betacyanins were monitored and quantified at 470 and 538 nm, respectively.

LC-MS Analyses. The separation of betalains was performed on a series 1100 HPLC (Agilent, Waldbronn, Germany) equipped with ChemStation software, a G1322A degasser model, a G1312A binary gradient pump model, a G1329/1330A autosampler model, a G1316A column-oven model, and a G1315A diode array detector model. The HPLC system was connected in series with a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an electrospray ionization source operated in the positive mode. Nitrogen was used as dry gas at a flow rate of 12 L/min and a pressure of 70 psi. The nebulizer temperature was set to 365 °C. Using helium as the collision gas (1.2×10^{-5} mbar), collision-induced dissociation spectra were obtained with a fragmentation amplitude of 1.2 V (MS/MS). HPLC conditions were the same as described above.

Photometric Quantification of Betalains. The aqueous pigment extracts were diluted with McIlvaine buffer (pH 6.0, citrate−phosphate) to obtain absorption values of $0.8 \leq A \leq 1.0$ at their respective absorption maxima. The betalain content (BC) was calculated by applying the equation proposed in ref *13*: BC (mg/L) = ($A \times$ DF $\times$ MW $\times$ 1000/ϵl), where A is the absorption value at the absorption maximum, DF the dilution factor, and l the path length (1 cm) of the cuvette. For quantification of betacyanins, the molecular weight (MW) and molar extinction coefficient (ϵ) of betanin [MW = 550 g/mol; ϵ = 60000 L/mol·cm in H_2O; λ = 538 nm; (*14*)] were applied. Betaxanthins were quantified by applying a mean molar extinction coefficient (*15*) and the molecular weight of glutamine−betaxanthin (vulgaxanthin I; MW = 339 g/mol; ϵ = 48000 L/mol·cm in H_2O; λ = 480 nm). All measurements were performed in triplicate using a UV−vis spectrometer (Perkin-Elmer, Überlingen, Germany) equipped with UVWinLab V 2.85.04 software (Perkin-Elmer Instruments, Norwalk, CT).

Color Measurements. For color analyses, a UV−vis spectrometer (see above) was used applying a UVWinLab V 2.85.04 and Wincol V 2.05 color software (Perkin-Elmer Instruments). By diluting the aqueous pigment extracts with McIlvaine buffer (pH 6.0, citrate−phosphate), the absorption was normalized to 1.00 ± 0.05 at the respective maxima and visible spectra (380−780 nm) were monitored in 1 cm path length cuvettes. Chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$] and hue angle [$h° = \arctan(b^*/a^*)$] were calculated from CIE a^* and b^* values using illuminant D_{65} and a 10° observer angle. All determinations were performed in duplicate.

RESULTS AND DISCUSSION

Identification of Betalains. *Betaxanthins*. Through improvement of a previously described method (*7*), separation of the complex betaxanthin mixture of colored Swiss chard stems was achieved (**Figure 2**), resulting in the identification of 19 betaxanthins (**Table 1**; **Figure 3**). The yellow betalains were identified both by their UV−vis and mass spectrometric characteristics and by co-injection experiments with reference compounds. The application of a new stationary phase afforded delayed retention times of the most polar betaxanthins, and thus a better separation from equally eluting phenolic compounds abundant in Swiss chard (*16*) was accomplished. Moreover, the addition of previously applied trifluoroacetic acid to extend

Table 1. Identification and Distribution of Betalains in Differently Colored Swiss Chard[a]

peak[b]	betalain	yellow	yellow-orange	red-purple	purple	t_R (min)	λ_{max}[c] (nm)	*m/z* $[M + H]^+$
	betaxanthins (bx)							
1	histidine-bx	+[d]	+[d]	+[d]	tr[d]	11.0	472	349
2	asparagine-bx	+[d]	+[d]	+[d]	+[d]	12.0	470	326
3	serine-bx	+	+[d]	+	+	12.6	468	299
4	histamine-bx	+[d]	tr[d]	tr[d]	tr[d]	13.5	468	305
5	glutamine-bx	++	++	++	++	14.5	469	340
6	glycine-bx	+[d]	+[d]	+[d]	–	15.4	468	269
7	glutamic acid-bx	+	+[e]	+[d,e]	tr[d]	18.9	469	341
8	alanine-bx	+	+	+	tr	22.1	468	283
9	γ-aminobutyric acid-bx	+	tr[e]	tr	tr	26.2	459	297
10	proline-bx	+	tr[d]	–	–	27.8	478	309
11	unknown	+	+	+	+	35.6	(482)[f]	345
12	tyrosine-bx	+	+	tr	tr	39.7	472	375
13	dopamine-bx	++	++	++	++	40.2	461	347
14	valine-bx	+	tr	tr	tr	40.9	470	311
15	tyramine-bx	tr[e]	tr[d,e]	–	–	47.8	461	331
16	3-methoxytyramine-bx	+	+	+	tr	50.4	464	361
17	isoleucine-bx	+	tr	tr	tr[d]	54.4	470	325
18	leucine-bx	+	tr	tr	tr[d]	55.8	470	325
19	phenylalanine-bx	tr	tr[d]	–	–	59.3	472	359
20	tryptophan-bx	+	tr[d]	tr[d]	tr[d,e]	63.2	473	398
	betacyanins							
I	betanin	tr	++	++	++	15.1	534	551
I′	isobetanin	tr[d,e]	+	+	++	16.5	534	551
II	phyllocactin	–	–	–	tr	18.1	534	637
III	betanidin	–	tr[d]	+	+	18.4	540	389
III′	isobetanidin	–	–	tr[d,e]	+[d]	20.2	540	389
IV	betanidin-monoferuloyl-5-*O*-β-diglucoside	–	+	tr[d]	+	26.1	(326, 535)[g]	889
IV′	isobetanidin-monoferuloyl-5-*O*-β-diglucoside	–	tr[d]	tr[d,e]	+	26.8	(325, 535)[g]	889
V	lampranthin II	–	+	++	+	29.1	326, 534	727
V′	isolampranthin II	–	tr	+	+	29.6	329, 535	727

[a] Contents were evaluated by area units as follows: –, not detectable; tr, traces, for areas <300,000; + for areas >300,000; ++, for areas >3,000,000 (injection volume = 80 μL). [b] Peak assignment and retention times refer to **Figures 2** and **4**, respectively. [c] Absorption maxima of reference substances determined on the Agilent system. [d] Although co-injection with reference substances matched with the respective compounds, the mass signal obtained from the Swiss chard extract was not unambiguous. [e] Although co-injection experiments were successful, the absorption maximum of the respective compound in the Swiss chard extract was not unambiguous. [f] Absorption maximum recorded from a yellow extract. [g] Absorption maxima determined from a purple extract.

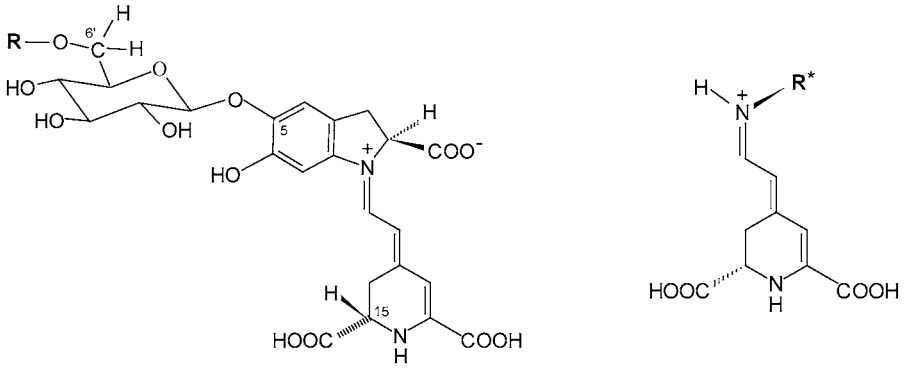

Figure 3. Basic structures of betanin-type betacyanins (left; R = H for betanin and isobetanin or R = ferulic acid for lampranthin II and isolampranthin II, respectively) and betaxanthins (right; **R*** = amino acid or amine).

pigment retention was dispensable, thereby improving the sensitivity of the mass spectrometric detection. Despite these advancements, some *m/z* signals of early-eluting betaxanthins still overlapped with those of coeluting colorless phenolics and were therefore judged as not unambiguous (**Table 1**).

Dopamine−betaxanthin (miraxanthin V) and glutamine−betaxanthin (vulgaxanthin I) represented the major betaxanthins in colored Swiss chard petioles. The presence of the latter betaxanthin is consistent with the finding of a preliminary investigation on a reddish Swiss chard cultivar (*9*). Both compounds have already been identified in hairy root cultures (*17*) and hypocotyls (*7*) of yellow beet. Interestingly, the proportions of these major components varied with the color of the Swiss chard petioles. Whereas in yellow petioles dopamine−betaxanthin and glutamine−betaxanthin accounted for 37 and 34% of the whole peak area of all identified betaxanthins at 470 nm, they added up to 28 and 42% in yellow-orange, 41 and 29% in red-purple, and finally 55 and 25% in purple petioles, respectively (data not shown).

The adducts of betalamic acid with serine, γ-aminobutyric acid, valine, isoleucine, and phenylalanine have very recently been detected in yellow beet (*7*), and the presence of the same set of betaxanthins in colored Swiss chard supports the close phylogenetic relationship between beet root and Swiss chard varieties (*18*).

In addition, histidine−betaxanthin (muscaaurin VII), asparagine−betaxanthin (vulgaxanthin III), glycine−betaxanthin (portulacaxanthin III), glutamic acid−betaxanthin (vulgaxanthin II), proline−betaxanthin (indicaxanthin), tyrosine−betaxanthin (portulacaxanthin II), leucine−betaxanthin (vulgaxanthin IV),

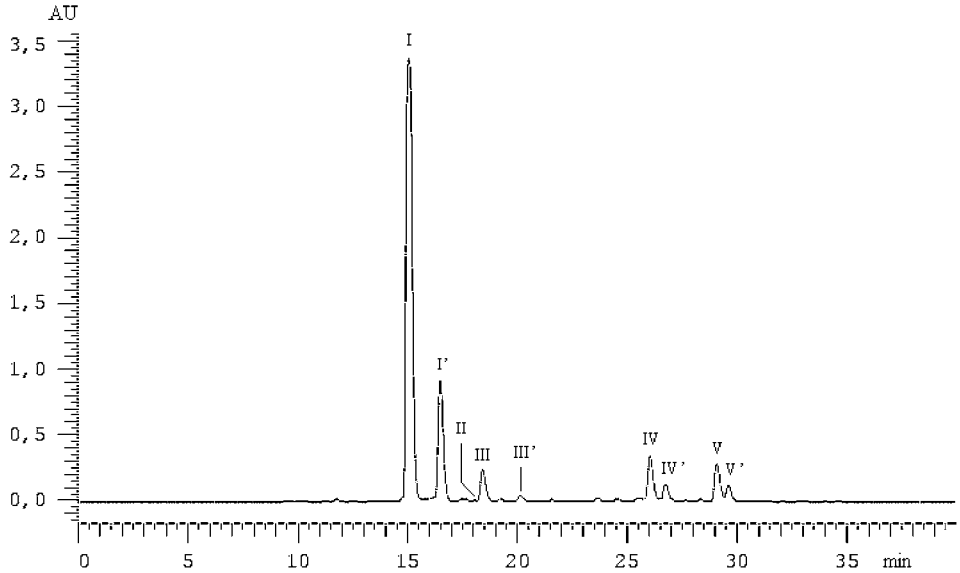

Figure 4. HPLC separation of betacyanins from purple Swiss chard petioles monitored at 538 nm (peak assignment is given in **Table 1**).

and tryptophan−betaxanthin, all of which have already been detected in the Chenopodiaceae (*7*, *15*, *19*−*21*), were found to contribute to the broad betaxanthin pattern of colored Swiss chard petioles, as well (**Table 1**).

Tyramine−betaxanthin (miraxanthin III) and 3-methoxytyramine−betaxanthin have never been found previously in the Chenopodiaceae. Although the former was detected earlier in flowers of *Mirabilis jalapa* L. (Nyctaginaceae) (*22*−*24*), the latter has only recently been found in extracts of inflorescences from *C. argentea* varieties (Amaranthaceae) (*11*). For the biosynthesis of 3-methoxytyramine−betaxanthin two possibilities were proposed, namely, methylation of dopamine−betaxanthin or methylation of dopamine and subsequent condensation of the resulting 3-methoxytyramine with betalamic acid (*11*). The latter pathway was assumed to be more likely because the corresponding amino compound 3-methoxytyramine could also be detected in the same plant material at comparable levels (*11*). Because tyramine−betaxanthin and 3-methoxytyramine−betaxanthin are present in yellow and yellow-orange Swiss chard, a third biosynthetic pathway may be considered regarding tyramine−betaxanthin as a potential precursor of the methoxylated betaxanthin.

Two further betaxanthins yielded *m*/*z* values of 283 and 305, corresponding with adducts of betalamic acid with alanine and histamine, respectively. This assumption was confirmed by co-injection experiments and mass spectrometric analyses with reference compounds. To the best of our knowledge, adducts of betalamic acid with histamine and alanine (**Table 1**) have never before been described as naturally occurring betaxanthins (*22*, *23*).

Another yellow compound (λ_{max} = 482 nm), which yielded an *m*/*z* value of 345 (**11**) (**Table 1**; **Figure 2**), could be detected. Co-injection experiments with semisynthetic 2-phenylglycine−betaxanthin and 4-(aminomethyl)benzoic acid−betaxanthin did not match with the endogenous compound in question. Further studies remain to be done to identify compound **11**.

Betacyanins. Whereas nine betacyanins were identified in purple Swiss chard stems, only trace amounts could be detected in the yellow ones (**Table 1**). Because the acylated betacyanins required less polar elution conditions than applied for betaxanthin separation, a different gradient system had to be established (see Materials and Methods). As described for betaxanthins, betacyanins were identified by means of LC-MS, their spectroscopic characteristics, and cochromatography.

Betanin (betanidin-5-*O*-β-glucoside) and isobetanin (isobetanidin-5-*O*-β-glucoside) (**Figure 3**), together with their corresponding aglycons betanidin and isobetanidin, were identified in extracts of purple Swiss chard both by co-injection of red beet juice and by their characteristic mass signals $[M + H]^+$ of *m*/*z* 551 and 389, respectively. Betanin and isobetanin represented the major compounds, accounting for 66 and 14%, respectively, of the total peak area at 538 nm. The occurrence of betanin and isobetanin was in agreement with previously obtained results (*9*), but not even traces of prebetanin could be detected in the present study. Surprisingly, minute amounts of phyllocactin [betanidin-5-*O*-(6′-*O*-malonyl)-β-glucoside], the typical pigment of fruits and flowers from the Cactaceae (*23*, *25*), were identified in purple Swiss chard petioles.

Further acylated structures were found in purple, red-purple, and yellow-orange Swiss chard stems as indicated by their late retention times (**Table 1**). An additional absorption maximum at ∼330 nm pointed to hydroxycinnamic acid conjugates (*26*−*28*). Mass spectrometric analyses yielded pseudomolecular ions $[M + H]^+$ of two pigment pairs with *m*/*z* 889 (**IV**/**IV′**) and *m*/*z* 727 (**V**/**V′**), respectively (**Figure 4**). Fragmentation in the MS^2 mode afforded *m*/*z* 551 (betanin) and *m*/*z* 389 (betanidin) for both pigment pairs. The *m*/*z* loss of 338 (889 − 551) matched with a feruloyl-glucose moiety, whereas the mass difference of 176 (727 − 551) provided evidence for acylation with ferulic acid. With reference to a previous paper (*29*), the two betacyanins with retention times of 26.1 and 26.8 min (**IV**/**IV′**), identical *m*/*z* of 889, and corresponding absorption characteristics were tentatively assigned to betanidin-monoferuloyl-5-*O*-β-diglucoside and its C_{15}-epimer (**Table 1**). Interestingly, another fragment (*m*/*z* 727) was obtained in the MS^2 mode, matching with a mass loss of a single glucose moiety (889 − 727), thus indicating that the glucose moiety directly attached to the betanidin structure is acylated with ferulic acid. Because no reference substances could be made available from plant material, more detailed identification was not possible.

The second pigment pair (**V**/**V′**) showed later retention times (29.1 and 29.6 min) but similar absorption maxima. Its mass spectrometric data and collision-induced fragmentation pattern gave evidence of a monoferuloyl derivative of betanin (*30*).

Table 2. Betalain Contents and Color Characteristics[a] of Betalainic Swiss Chard Extracts

characteristic value	purple	red-purple	yellow-orange	yellow
betaxanthins[b]	24.3 ± 0.3	24.9 ± 0.1	22.0 ± 0.3	49.7 ± 0.4
betacyanins[b]	51.1 ± 0.8	25.7 ± 0.1	11.6 ± 0.1	
betaxanthins[c]	16.7	20.3	19.9	49.7
betacyanins[c]	58.7	30.3	13.7	
betalains[d]	75.4	50.6	33.6	49.7
*L**	60.9 ± 0.1	62.0 ± 0.1	71.5 ± 0.0	85.3 ± 0.2
*C**	55.5 ± 0.1	50.6 ± 0.1	51.0 ± 0.3	62.6 ± 0.3
h°	350.3 ± 0.0	30.4 ± 0.0	58.1 ± 0.2	88.2 ± 0.0

[a] *L**, lightness; *C**, chroma; *h*°, hue angle (means of duplicate determinations ± standard deviation at a constant absorbance of 1.00 ± 0.05 at λ_{max}). [b] Values (mg/kg of fresh weight) obtained by photometric quantification (means of triplicate determinations ± standard deviation). [c] Values (mg/kg of fresh weight) were calculated by correction of the photometric determinations ([b]) considering the proportions of betaxanthins and betacyanins. [d] Betalain content (mg/kg of fresh weight) obtained by summing up the corrected values.

Through co-injection of an extract from purple petals from *Lampranthus* sp. (*31*), the presence of lampranthin II together with its C_{15}-epimer was confirmed (**Table 1**; **Figure 3**). Until now, lampranthin II has only been detected in purple midday flowers (*Lampranthus* sp.) (*31*), pokeberries (*Phytolacca americana*) (*27*), cell cultures of *B. vulgaris* L. ssp. *vulgaris* var. *conditiva* (*32*), and ice plants (*Mesembryanthemum crystallinum*) (*29*).

The identification of feruloylated betacyanins in the present work corresponds with an earlier study (*33*) showing Swiss chard to be rich in ferulic acid-*O*-*β*-D-glucoside. The latter was assumed to serve as an acyl donor in the biosynthesis of acylated betacyanins (*32*, *34*, *35*).

Quantification of Betalains and Color Measurements. The betacyanin and betaxanthin contents determined by spectrophotometric measurements (**Table 2**) were corrected by the respective area proportions of betacyanins (538 nm) and betaxanthins (470 nm) obtained by HPLC determination to achieve more precise values (*5*). Purple petioles contained the highest betalain concentrations (75.4 mg/kg of fresh weight), followed by red-purple (50.6 mg/kg of fresh weight) and yellow stems (49.7 mg/kg of fresh weight), with lowest values in yellow-orange Swiss chard petioles (33.6 mg/kg of fresh weight) (**Table 2**). Whereas betacyanin contents were maximal in purple petioles, contents of red pigments declined from red-purple toward yellow stems, with only trace amounts of betacyanins in the latter. Except for maximum values in yellow petioles, the betaxanthin content was on a similar level for yellow-orange, red-purple, and purple petioles. Thus, color variation in Swiss chard must be mainly ascribed to varying betacyanin levels.

Finally, CIE *L***C***h*° measurements were performed with the differently colored Swiss chard extracts after normalization to a constant absorbance value at the respective λ_{max} values. Hue angle (*h*°) is expressed on a color wheel, where 0°/360° = red-purple, 90° = yellow, 180° = green, and 270° = blue. The *h*°-shift from 88.2 (yellow stems) to 350.3 (purple stems) was in accordance with the visual appearance of the respective Swiss chard stems (**Table 2**; **Figure 1**). Chroma values (*C**) expressing color brilliance were highest for yellow, followed by purple extracts, and were lowest for red-purple and yellow-orange stems. These data indicate that *C** values tend to reach their maxima in solutions with a predominance of either betaxanthins or betacyanins. As expected, *L** values declined with increasing betacyanin contents; that is, solutions became darker.

The broad range of tonalities of the differently colored Swiss chard petioles render this underestimated vegetable interesting for future exploitation of betalains for food-coloring purposes. Because breeding programs on red beet were successful in considerably increasing the betalain content (*36*), similar potential can be suspected for Swiss chard. In light of most recent reports on the potential bioactivities of betalains (*1*, *37*, *38*), the present study may contribute to extend the knowledge on betalainic foodstuffs.

ACKNOWLEDGMENT

We are grateful to Dr. E. Götz (Institute of Botany, Hohenheim University) for the identification of *Lampranthus* sp. and to Dipl.-Ing. J. Meier (Institute of Food Technology, Hohenheim University) for taking the photograph for Figure 1. We thank Ernteband (Winnenden, Germany) for providing red beet juice concentrate and Pommerenke (Steinheim am Albuch, Germany) for data on Swiss chard cultivation.

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Received for review December 19, 2003. Revised manuscript received March 5, 2004. Accepted March 5, 2004.

JF035491W