Isolation and Determination of Anthocyanins in Seed Coats of Black Soybean (*Glycine max* (L.) Merr.)

Myoung-Gun Choung,*,† In-Youl Baek,† Sung-Taeg Kang,† Won-Young Han,† Doo-Chull Shin,† Huhn-Pal Moon,† and Kwang-Hee Kang‡

National Yeongnam Agricultural Experiment Station, Rural Development Administration, 1085, Naidong, Milyang, 627-803, Gyeongnam, Republic of Korea, and College of Natural Resources, Yeungnam University, Kyongsan, 712-749, Republic of Korea

Anthocyanin pigments in seed coats of black soybean (*Glycine max* (L.) Merr.) were extracted with 1% HCl−CH_3OH, and the crude anthocyanin extract was purified by Shepadex LH-20 and Lichroprep RP-18 open-column chromatography. Three major anthocyanins were isolated, and their chemical structures were identified by spectroscopic methods (UV−visible, FABMS, ^1H and ^{13}C NMR, and by TLC). The complete structures of these anthocyanins were elucidated as delphinidin-3-glucoside, cyanidin-3-glucoside, and petunidin-3-glucoside. Among them, petunidin-3-glucoside was identified as a new anthocyanin in black soybeans. On the basis of RP-HPLC with a UV−vis detector, the contents of delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, and total anthocyanins in seed coats of 10 black soybeans were found in the ranges of 0−3.71, 0.94−15.98, 0−1.41, and 1.58−20.18 mg/g, respectively. The results obtained in this study imply that the seed coats of black soybean can be used as a good source for cyanidin-3-glucoside and delphinidin-3-glucoside.

Keywords: *Black soybean; Glycine max; anthocyanins; petunidin-3-glucoside*

INTRODUCTION

Anthocyanins are intensely colored, water-soluble pigments that can be obtained from various organs such as fruits, vegetables, roots, tubers, bulbs, legumes, and cereals (*1*−*4*). They have been used widely as a natural coloring agents in the food and pharmaceutical industries (*1, 2*). However, it is well-known that the anthocyanins are considerably less stable than synthetic pigments: they easily lose color if they undergo structural transformation (*1, 5*). For this reason, establishment of their stability has been the research subject of many food scientists (*5*). Furthermore, anthocyanins are also known to have pharmaceutical effects (*1, 3, 5*). They have been used in the treatment of various circulatory disorders (*6*) and inflammatory disease (*7*). Recently, Waterhouse (*8*) reported that the antioxidant properties of anthocyanins may reduce the risk of coronary heart disease.

Soybeans have various seed coat colors, such as yellow, green, brown, or black. Such color pigmentations are due to anthocyanins, chlorophyll, and various combinations of breakdown products of these pigments (*9, 10*). Black soybeans, which have been widely utilized as food and as material for Oriental medicine, contain anthocyanins in the seed coat (*11*). The black pigmentation is due to accumulation of anthocyanins in the epidermis palisade layer of the seed coat (*9*).

The presence of anthocyanins in soybeans was first reported by Nagai (*12*), and subsequently several groups (*13*−*17*) have identified different anthocyanins in different black soybean varieties. Kuroda et al. (*13*) and Manabe et al. (*14*) found that cyanidin-3-glucoside was the major anthocyanin of black soybean seed coats, whereas Yoshikura et al. (*15*) and Taylor (*16*) identified another anthocyanin, delphinidin-3-glucoside, in the black-seeded variety, and pelargonidin-3-glucoside in the reddish-buff seed coats of T236 soybeans.

Despite many reports, research on the exact composition and contents of anthocyanins in the seed coats of different black soybean varieties is limited. An understanding of the composition and contents of anthocyanins in the black soybean may aid in their further utilization as new anthocyanin resource materials. Therefore, the objectives of this study were to isolate and identify the types of anthocyanins in the seed coats of black soybean, and to determine their contents in black soybean varieties.

MATERIALS AND METHODS

Plant Materials. Ten soybean varieties with black seed coats, Heugchong, Geomjeong 1, Tawon, Tanbaguro, Cheongja, Peking, Milyang 95, Geomjeongol, IT 180220, and YJ 100-1, were selected for use in this study. The black soybeans were grown at the experimental field of National Yeongnam Agricultural Experiment Station, Milyang, South Korea, in 1999.

After harvest, the seeds were cleaned in distilled water to remove extraneous matters and subsequently dried at 105 °C for 2 h. The dried seeds were stored at 4 °C until they were used. For structural identification of anthocyanin in black soybean seed coats, Milyang 95 seeds, which have been identified as containing three kinds of anthocyanins in this laboratory, were used. On the other hand, 10 black soybean varieties were used for determination of anthocyanin contents.

Chemicals. Methanol, formic acid, water, and Lichroprep RP-18 (40−63μm) resin were purchased from Merck Chemical Co. (Germany). Tetramethylsilane (TMS), CD_3OD, DCl, and

* To whom correspondence should be addressed. Telephone: 82-55-350-1223. Fax: 82-55-353-3050. E-mail: cmg7004@rda.go.kr.

† National Yeongnam Agricultural Experiment Station.

‡ Yeungnam University.

10.1021/jf010550w CCC: $20.00
Published on Web 11/17/2001

Sephadex LH-20 resin were obtained from Sigma Chemical Co. (St. Louis, MO). A 0.45 μm membrane filter was purchased from Waters Co. (Milford, MA). A microcrystalline cellulose F flexible TLC plate was obtained from J. T. Baker (Germany). All laboratory chemicals used in this study were of reagent grade.

Isolation of Anthocyanins from Black Soybean Seed Coat. The seed coats of Milyang 95, a breeding line of black soybean, were peeled manually. The separated seed coats (50 g) were extracted three times with 1000 mL of 1% HCl− 40% CH_3OH at 4 °C for 24 h. The combined extracts were filtered and concentrated at 30 °C in vacuo. The crude anthocyanin extract was loaded onto a Sephadex LH-20 column (700 mm × 30 mm i.d.). The column was then eluted stepwise from 1% HCl−H_2O (200 mL), 1% HCl−10% CH_3OH (200 mL), 1% HCl−20% CH_3OH (200 mL), and 1% HCl−30% CH_3OH (400 mL). From the Sephadex LH-20 column chromatography, the three red-pigment-containing fractions were isolated from the crude anthocyanin extract. Each fraction was collected and concentrated at 30 °C in vacuo. Each fraction was further purified by RP-18 (300 mm × 30 mm i.d.) open column chromatography.

RP-18 open-column chromatography was carried out by stepwise elution with 1% HCl−H_2O (200 mL), 1% HCl−10% CH_3OH (200 mL), 1% HCl−20% CH_3OH (200 mL), 1% HCl−30% CH_3OH (200 mL), and 1% HCl−40% CH_3OH (200 mL). From RP-18 open-column chromatography of each fraction, black soybean anthocyanin **1** (fraction 1), **2** (fractions 1 and 2), and **3** (fraction 3) were purified. To determine the purity, the purified anthocyanins were analyzed by RP-HPLC using a Tosoh ODS-120T column (150 × 4.6 mm i.d., Japan). The flow rate was set at 0.6 mL/min by isocratic elution, using H_2O−CH_3OH−HCOOH (75:20:5) with monitoring at 530 nm, and the column temperature was set at 30 °C.

Determination of Anthocyanin Contents in Seed Coats of Black Soybean. To determine the anthocyanin contents, seed coats of each black soybean varietywere peeled manually. The separated seed coats (0.1 g) were extracted with 30 mL of 1% HCl−40% CH_3OH at 4 °C for 24 h. The 10 extract samples were analyzed by RP-HPLC. Prior to analysis, all samples were filtered through a 0.45 μm membrane filter.

Instrumentation and Conditions. UV−vis absorption spectra of purified anthocyanins were recorded on a Shimadzu Model 5840 spectrophotometer in 0.5% HCl−50% CH_3OH. The fast atom bombardment mass spectra (FABMS) were recorded on a JEOL JMS-AX 505WA with glycerol as the matrix. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured on a Varian Unity Plus 500 NMR instrument in 0.5% DCl−CD_3OD containing tetramethylsilane (TMS) as the internal standard. The HPLC system was composed of an L-6200 intelligent pump, an L-4250 UV−visible variable wavelength detector, and a D-2500 integrator (Hitachi). Injections were carried out with a Rheodyne 7725i injector equipped with a 20 μL loop. The column was a Tosoh ODS-120T (150 × 4.6 mm i.d., Japan), and the flow rate was set at 0.6 mL/min with isocratic elution, using H_2O−CH_3OH−HCOOH (75:20:5) with monitoring at 530 nm, and the column temperature was set at 25 °C. For protection of the analytical column, a Nova-Pak C_{18} guard insert column (Waters, Milford, MA) was used.

The anthocyanin contents were calculated by HPLC peak areas compared with external standard calibration curves. The linear standard calibration curves ($r \geq 0.999$) were generated by injecting 0.05 μg to 1μg of purified three anthocyanins in 20 μL of 1% HCl−40% CH_3OH. The minimum detectable concentration for anthocyanin standards is 500 ng/mL.

Thin-Layer Chromatography (TLC) Method. TLC was carried out using microcrystalline cellulose F flexible TLC plates (layer thickness, 0.1 mm) using the solvents HCOOH−HCl−H_2O (1:1:2) and *n*-Butanol−HOAc−H_2O (4:1:5, upper phase). The sample spots on the chromatogram were detected with the use of a UV lamp (254 and 365 nm) and the naked eye.

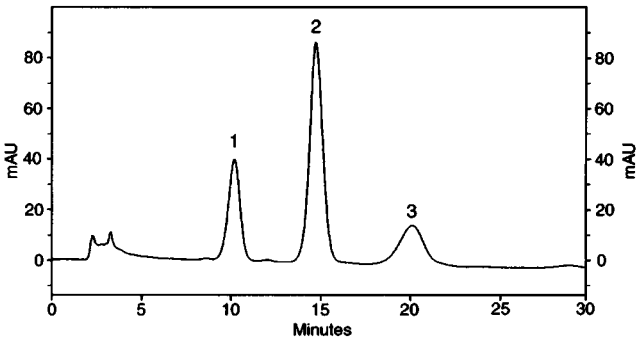

Figure 1. HPLC chromatogram of anthocyanins obtained from Milyang 95. Peak 1, delphinidin-3-glucoside; peak 2, cyanidin-3-glucoside; peak 3, petunidin-3-glucoside.

RESULTS AND DISCUSSION

HPLC Chromatogram. The HPLC chromatogram of seed coats extract obtained from Milyang 95 revealed three anthocyanin peaks (Figure 1). In the previous studies, only one or two anthocyanins were reported (*13*−*17*). However, in this study, we observed that black soybean seed coats of Milyang 95 contain three anthocyanins. In this experiment using RP-HPLC, the three peaks of anthocyanins eluted at 9.9, 14.5, and 20.1 min, respectively.

Identification of Anthocyanins. Further characterization of anthocyanins in the seed coats of Milyang 95, based on Cellulose F TLC and UV−vis spectral analysis, is presented in Table 1. The results of UV−vis characterizations using purified anthocyanins showed a maximum absorbance at 520−530 nm with E_{440}/E_{vis} of 26−30%, indicating a 3-glucoside of an anthocyanidin (*18*). The ratio of E_{uv}/E_{vis} (71−67%) and E_{acyl}/E_{vis} (8−10%) indicated that the purified anthocyanins were simple anthocyanin structures without complex acylation (*18*). The TLC and UV−vis spectral data agree with data from previously reported literature (*4*, *5*, *19*).

Considering FABMS (Table 1) and $^1H/^{13}C$ NMR (not shown) spectral data, the purified anthocyanins were identified as delphinidin-3-glucoside (**1,** R_1=OH, R_2=OH) (*20*, *22*, *23*), cyanidin-3-glucoside (**2,** R_1=OH, R_2=H) (*21*−*24*), and petunidin-3-glucoside (**3,** R_1=OCH_3, R_2=OH) (*25*) as shown in Figure 2.

Although the two anthocyanins delphinidin-3-glucoside and cyanidin-3-glucoside were reported to be present in the black-seeded soybean variety (*13*−*17*), this is the first report of petunidin-3-glucoside in black soybean.

Yoshida et al. (*17*) reported the structures and contents of anthocyanins from 11 species of edible legumes. In their study, only the black turtle (*Phaseolus vulgaris*), which is cultivated in North America, contained petunidin-3-glucoside, and all other black soybeans (*Glycine* legume) contained only cyanidin-3-glucoside or cyanidin-3-glucoside combined with delphinidin-3-glucoside. On the other hand, Taylor (*16*) identified another anthocyanin as a pelargonidin-3-glucoside in the reddish-buff seed coats of T236 soybean line. However, the T236 is not a black soybean but a reddish-colored soybean, although it is grouped with the *Glycine* legumes.

The difference between anthocyanin classes obtained in this study and previous studies (*13*−*17*) is considered mainly from the material used in the studies. Thus, it is believed that different compositions of anthocyanin in black soybean (*Glycine* legume) seed coats exist in the natural condition. Unfortunately, direct comparisons

Table 1. Chromatographic and Spectral Properties of Purified Anthocyanin Obtained from the Seed Coats of Milyang 95

	TLC (R_f)[a]			UV−vis characterization[d] (in 0.5% HCl−50% CH_3OH)			
anthocyanin	FHW[b]	BAW[c]	FABMS (m/z)	λ max(nm)	E_{uv}/E_{vis} (%)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)
1	0.26	0.28	465 $[M]^+$	279, 530	71	11	27
2	0.44	0.38	449 $[M]^+$	282, 520	69	10	30
3	0.36	0.35	479 $[M]^+$	279, 529	67	8	26

[a] Absorbent, microcrystalline cellulose F. [b] FHW, HCOOH−HCl−H_2O, 1:1:2. [c] BAW, *n*-butanol−HOAc−H_2O, 4:1:5, upper phase. [d] E_{uv}, extinction coefficient of maximum absorption peak in UV region; E_{vis}, extinction coefficient of maximum absorption peak in visible region; E_{acyl}, extinction coefficient at 330 nm; E_{440}, extinction coefficient at 440 nm.

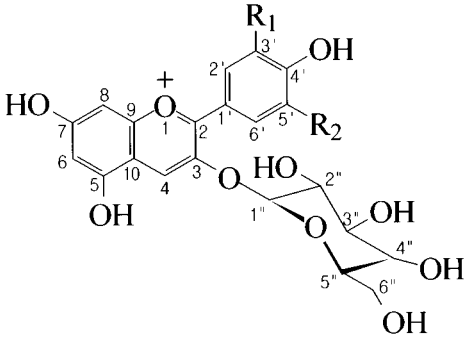

Figure 2. Chemical structures of anthocyanins in seed coats of black soybean. Delphinidin-3-glucoside (**1,** R_1=OH, R_2=OH); cyanidin-3-glucoside (**2,** R_1=OH, R_2=H); petunidin-3-glucoside (**3,** R_1=OCH_3, R_2=OH).

Table 2. Comparison of Anthocyanin Contents in Seed Coats of Ten Black Soybean Varieties

	anthocyanin content (mg/g)				
variety[a]	1	2	3	total	CV (%)[b]
Heugchong	0.64	0.94		1.58	2.96
Geomieong 1		4.50		4.50	3.61
Tawon	1.30	2.93	1.03	5.26	3.25
Tanbaguro	0.89	5.46		6.35	1.28
Cheongja	1.56	5.30	0.31	7.16	4.33
Peking		7.88		7.88	4.13
Milyang 95	1.98	6.45	1.41	9.83	4.05
Geomjeongol	2.78	7.36	0.47	10.62	3.57
IT 180220	3.71	14.80	0.30	18.81	3.22
YJ 100-1	3.21	15.98	0.99	20.18	3.35

[a] n = 3 for all samples. [b] CV, Coefficient of variation.

between the results of this study and previous studies (*13*−*17*) are impossible because of the lack of common materials.

Determination of Anthocyanin Contents in Seed Coats of Black Soybean. The anthocyanin contents in seed coats of black soybean, on the basis of HPLC peak area with monitoring at 530 nm, are presented in Table 2. A preponderance of cyanidin-3-glucoside was observed in all the black soybeans. Among the 10 black soybean varieties, Geomjeong 1 and Peking contained only cyanidin-3-glucoside (Figure 3B). Petunidin-3-glucoside was not detected in Heugchong and Tanbaguro, although they contained both cyanidin-3-glucoside and delphinidin-3-glucoside (Figure 3A).

Cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside contents in seed coats of 10 black soybean varieties were in the ranges 0.94−15.98, 0−3.71, and 0−1.41 mg/g, respectively. The total anthocyanin contents in seed coats of black soybeans ranged from 1.58 to 20.18 mg/g. The total anthocyanin content of YJ 100-1 was about 12 times higher than that of Heugchong.

Using HPLC, Yoshida et al. (*17*) analyzed the anthocyanin contents in 26 kinds of colored legumes that were

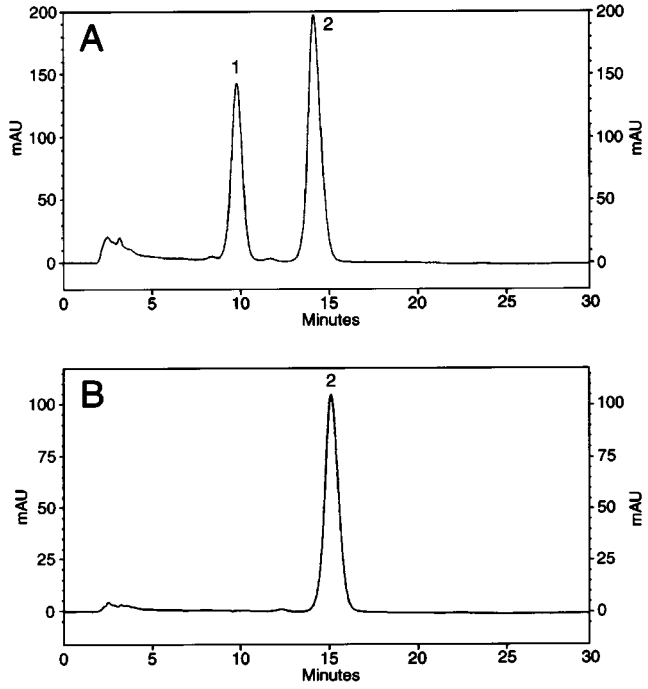

Figure 3. HPLC chromatograms of anthocyanins obtained from Heugchong (A) and Geomjeong 1 (B).

of different species or from different production districts. It was reported that the anthocyanin contents of *Vigna*, *Phaseous*, and *Glycine* legumes were 0.003−26, 0.5−5.2, and 4.1−20.4 mg/g, respectively, with *Glycine* having the highest value among the legumes. Especially in black soybeans, the anthocyanin contents of *Glycine soja* (15.3−20.4 mg/g) were about four times higher than that of *Glycine max* (4.1 mg/g) (*17*). However, this may not hold true for all *Glycine max* because only one variety was analyzed in their study.

However, we used 10 black soybean varieties (*Glycine max*), and the range of anthocyanin varied from 1.58 to 20.18 mg/g. This results suggest that black soybeans (*Glycine max*) can be an abundant source of anthocyanin for food colorants endowed with multiple functions such as antioxidant properties and anti-carcinogenic and immunostimulating effects (*17*, *22*). Further studies on determination of anthocyanin contents in seed coats of diverse black soybean germplasms are now in progress and future research will aim at investigating the physiological activities of black soybean anthocyanins.

ACKNOWLEDGMENT

We express sincere thanks to Dr. S. H. Kim, Korea Basic Science Institute Taegu Branch, for the FABMS and NMR measurements.

LITERATURE CITED

(1) Petri, G.; Krawczyk, U.; Kery, Á. Spectrophotometric and chromatographic investigation of bilberry anthocyanins for quantification purposes. *Microchem. J.* **1997**, *55*, 12−23.
(2) Bridle, P.; Timberlake, C. F. Anthocyanins as natural food colours − selected aspects. *Food Chem.* **1997**, *58*, 103−109.
(3) Mazza, G.; Miniati, E. Legumes. In *Anthocyanins in Fruits, Vegetables, and Grains*. CRC Press, Inc. : Boca Raton, FL, **1993**; pp 254−255.
(4) Renault, J. H.; Thépenier, P.; Zéches-Hanrot, M.; Men-Olivier, L. L.; Durand, A.; Foucault, A.; Margraff, R. Preparative separation of anthocyanins by gradient elution centrifugal partition chromatography. *J. Chromatogr. A.* **1997**, *763*, 345−352.
(5) Fiorini, M. Preparative high-perfomance liquid chromatography for the purification of natural anthocyanins. *J. Chromatogr. A.* **1995**, *692*, 213−219.
(6) Bettini, V.; Fiori, A.; Martino, R.; Mayellaro, R.; Ton, P. Study of the mechanism whereby anthocyanosides potentiate the effect of catecholamines on coronary vessels. *Fitoterapia* **1985**, *54*, 67−72.
(7) Vincieri, F. F.; Romani, A.; Baldi, A.; Mulinacci, N.; Alberti, M. B. Analysis HPLC of anthocyanins present in fluid extracts from *Malva sylvestris* L. flowers and leaves. *Bull. Liaison−Groupe Polyphenols* **1992**, *16*, 339−342.
(8) Waterhouse, A. L. Wine and heart disease. *Chem. Ind.* **1995**, 338−341.
(9) Todd, J. J.; Vodkin, L. O. Pigmented soybean (*Glycine max*) seed coats accumulate proanthocyanidins during development. *Plant Physiol.* **1993**, *102*, 663−670.
(10) Carlson, J. B.; Lersten, N. R. Reproductive morphology. In *Soybeans: Improvement, Production and Uses*, 2nd ed.; Wilcox, J. R., Ed.; ASA, CSSA, and SSSA: Madison, WI, 1987, pp 126−127.
(11) Chung, I. M. Test of antioxidative activity on Korean native black soybean. *Daesan Nonchong* **1998**, *6*, 23−30.
(12) Nagai, I. A genetico-physiological study of the formation of anthocyanins and brown pigments in plant. *Tokyo Univ. Coll. Agric. J.* **1921**, *8*, 1.
(13) Kuroda, C.; Wada, M. The colouring matter of kuromame. *Proc. Imp. Acad.* (Tokyo) **1933**, *9*, 17.
(14) Manabe, T.; Kubo, S.; Kodama, M.; Bessho, Y. Prevention of discoloration in red parts of white peach. III. Some properties of an anthocyanin pigment isolated from black soybean peels. *Nippon Shokuhin Kogyo Gakkaish* **1965**, *12*, 472.
(15) Yoshikura, K.; Hamaguchi, Y. Anthocyanins of the black soybean. *Eiyo To Shokuryo* **1969**, *22*, 367.
(16) Taylor, B. H. Environmental and chemical evaluation of variations in hilum and seed coat colors in soybean. M.S. Thesis, University of Arkansas, Fayetteville, AR, 1976.
(17) Yoshida, K.; Sato, Y.; Okuno, R.; Kameda, K.; Isobe, M.; Kondo, T. Structural analysis and measurement of anthocyanin from colored seed coats of *Vigna*, *Phaseolus*, and *Glycine* lugumes. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 589−593.
(18) Fossen, T.; Andersen, Ø. M.; Øvstedal, D. O.; Pedersen, A. T.; Raknes, Å. Characteristic anthocyanin pattern from onions and other *Allium* spp. *J. Food Sci.* **1996**, *61*, 703−706.
(19) Andersen, Ø. M.; Viksund, R. I.; Pedersen, A. T. Malvidin 3-(6-acetylglucoside)-5-glucoside and other anthocyanins from flowers of *Geranium sylvaticum*. *Phytochemistry* **1995**, *38*, 1513−1517.
(20) Pale, E.; Nacro, M.; Vanhaelen, M.; Vanhaelen, F. R. Anthocyanins from bambara groundnut (*Vigna subterranea*). *J. Agric. Food Chem.* 1**997**, *45*, 3359−3361.
(21) Fossen, T.; Andersen, Ø. M. Acylated anthocyanins from leaves of the water lily, *Nymphaea* × *Marliacea*. *Phytochemistry* **1997**, *46*, 353−357.
(22) Tsuda, T.; Ohshima, K.; Kawakishi, S.; Osawa, T. Antioxidative pigments isolated from the seeds of *Phaseolus vulgaris* L. *J. Agric. Food Chem.* **1994**, *42*, 248−251.
(23) Terahara, N.; Callebaut, C.; Ohba, R.; Nagata, T.; Kameyama, M. O.; Suzuki, M. Triacylated anthocyanin from *Ajuga reptans* flowers and cell cultures. *Phytochemistry* **1996**, *42*, 199−203.
(24) Wang, H.; Nair, M. G.; Iezzoni, A. F.; Strasburg, G. M.; Booren, A. M.; Gray, J. Quantification and characterization of anthocyanin in balaton tart cherries. *J. Agric. Food Chem.* **1997**, *45*, 2556−2560.
(25) Kidøy, L.; Nygard, A.; Andersen, Ø. M.; Pedersen, A. T.; Aksnes, D. W.; Kiremire, B. T. Anthocyanins in fruits of *Passiflora edulis* and *P. suberosa*. *J. Food Comp. Anal.* **1997**, *10*, 49−54.

Received for review April 27, 2001. Revised manuscript received September 6, 2001. Accepted September 8, 2001.

JF010550W