Neocrocin A: a Novel Crocetin Glycoside with a Unique System for Binding Sugars Isolated from Gardenia Yellow

Yoshinori UEKUSA,[a,1)] Naoki SUGIMOTO,*[,b] Kyoko SATO,[b] Young Sook YUN,[a] Akira KUNUGI,[a] Takeshi YAMAZAKI,[b] and Ken-ichi TANAMOTO[b]

[a] *School of Life Science, Tokyo University of Pharmacy and Life Science; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan: and* [b] *National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.*
Received June 20, 2007; accepted August 29, 2007; published online September 3, 2007

A novel crocetin glycosyl ester, neocrocin A (2), was isolated from gardenia yellow. The structure of 2 was elucidated as that of an all-*trans*-crocetin β-D-gentiobiosyl β-D-glucopyranosyl-(1→6)-D-2-deoxy-glucopyranos-2-yl diester based on chemical and spectral data. The findings provide evidence that the binding system of crocetin glycosides is not limited to the anomeric position.

Key words *Gardenia jasminoides*; gardenia yellow; neocrocin A; crocetin glycoside; crocin

Crocin (**1**)[2)] is a digentiobiosyl all-*trans*-crocetin (8,8′-diapocarotene-8,8′-dioic acid) ester that is the major yellow pigment in gardenia yellow and saffron, which are extracts of *Gardenia jasminoides* fruits and *Crocus sativus* stigmas, respectively.[3,4)] Gardenia yellow and saffron consist of many minor pigments as well as some relatively abundant pigments which have been characterized as all-*trans*- and 13-*cis*-crocetin monoglucosyl ester,[2)] diglucosyl ester,[2)] monogentiobiosyl ester,[2,5)] glucosyl gentiobiosyl ester[2)] and gentiobiosyl neapolitanosyl ester.[5)] However, the structures of the other minor pigments have so far remained uncertain, and the binding systems for sugars have not previously been confirmed by detailed spectroscopic investigations, such as NMR analysis, after isolation. Here we report on the isolation and structural elucidation of a novel crocetin glycoside, neocrocin A (**2**), which has a unique binding system for sugars, based on spectral data and chemical derivatization (Fig. 1).

Results and Discussion

Gardenia yellow extracted from dried gardenia fruits was fractionated on a Diaion HP-20 column. The 60—70% methanol eluate was then concentrated and the residue was loaded into a preparative LC-MS system,[6)] led to the isolation of crocin (**1**) and neocrocin A (**2**).

Neocrocin A (**2**) was isolated as a red amorphous powder. The molecular formula of **2** was established as $C_{44}H_{64}O_{24}$, which was as the same as that of **1**, according to HR-ESI-MS (*m*/*z* 999.3707, $[M+Na]^+$, Calcd 999.3685), and the IR spectrum and UV/Vis absorption were similar to those of **1**. All-*trans*-crocetin dimethyl ester (**3**) and D-glucose were obtained, respectively, after the methanolysis and hydrolysis[7)] of **2**. These observations implied that **2** had the same carotenoid moiety, all-*trans*-crocetin, as the chromophore group, but that the binding system for glucoses differed from that of **1**. The ^{1}H- and ^{13}C-NMR spectra of **1** showed simple subduple signals, because **1** had C_2 structural symmetry. The ^{1}H-NMR spectra of **2** were similar to, but more complex than, those of **1**. Based on this comparison, we predicted that the C_2 structural symmetry was disrupted in **2** by the different binding system for glucoses at each end of crocetin, and that equilibrated isomerization could occur readily in the NMR solvent. The ^{1}H-NMR spectrum of **2** revealed a crocetin moiety (δ_H 6.49—7.31), anomeric doublets of β configuration (δ_H 5.38 (d, J=7.8 Hz), 4.53 (d, J=6.9 Hz), 4.13 (d, J=7.8 Hz), 4.15 (d, J=7.8 Hz)) and an anomeric doublet of α configuration (δ_H 5.06 (d, J=3.6 Hz)). Two anomeric protons (δ_H 4.53, 5.06) were shifted to a high magnetic field, and two oxymethines on H-2 (δ_H 4.39 (dd, J=3.7, 10.1 Hz), 4.53 (t, J=6.9 Hz)) were shifted to a low magnetic field, in comparison to those of **1**. This observation indicated the existence of a free hydroxyl group at an anomeric position on the glycosyl groups. Furthermore, HMBC correlations were observed between the H-2 of the α-glucoside and β-glucoside (glucose C) at δ_H 5.06 and δ_H 4.53, and the carbonyl carbons on the crocetin moiety at δ_C 167.92 and δ_C 167.11, respectively. Based on these chemical and spectral data, **2** was determined to be an all-*trans*-crocetin β-D-gentiobiosyl β-D-glucopyranosyl-(1→6)-D-2-deoxy-glucopyranos-2-yl ester. The NMR spectral data for **1** and **2** are summarized in Table 1.

Furthermore, to confirm the binding system for the sugars of neocrocin A (**2**), we firstly carried out peracylation of **2**. However, the sufficient quantity of peracetylated **2** was not obtained for the structure determination, because **2** was unstable more than crocin (**1**). Hence, after peracetylation of gardenia yellow, we isolated peracetylated crocin (**1a**) and an isomer of neocrocin A (**2a**). The other isomer of peracetylated **2** could not be isolated using preparative LC-MS because of overlapping with the peak of **1a**. The HR-ESI-MS spectra indicated that the molecular formula of both **1a** and **2a** was $C_{72}H_{92}O_{38}$. To compare the ^{1}H- and ^{13}C-NMR data between **2a** and **1a**, detailed 2D-NMR experiments were performed and the coupling constants were assigned using 1D-TOCSY. The chemical shifts and coupling constants of glu-

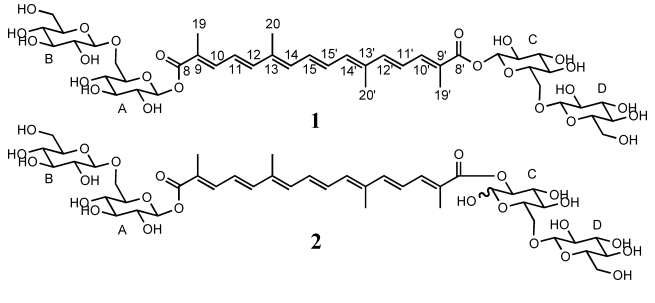

Fig. 1. Chemical Structures of Crocin (**1**) and Neocrocin A (**2**)

* To whom correspondence should be addressed. e-mail: nsugimot@nihs.go.jp

Table 1. ^{1}H (800 MHz)- and ^{13}C (200 Mz)-NMR Spectral Data[a] of Crocin (**1**) and Neocrocin A (**2**) (in DMSO-d_6/D_2O (9 : 1))

Crocin (**1**)[b]			Neocrocin A (**2**)[b]										
			α isomer						β isomer				
No.	δ_H	δ_C	No.	δ_H	δ_C	No.	δ_H	δ_C	No.	δ_H	δ_C	No.	δ_H	δ_C
8,8′		166.80	8		166.72	8′		167.92	8		166.72	8′		167.11
9, 9′		125.70	9		125.77	9′		126.63	9		125.77	9′		127.08
10, 10′	7.31 (d, 11.0)	140.50	10	7.31 (d, 11.4)	140.44	10′	7.27 (d, 11.0)	139.42	10	7.31 (d, 11.4)	140.44	10′	7.20 (d, 11.0)	138.80
11, 11′	6.62 (brt, 14.7)	124.40	11	6.63 (brt, 11.7)	124.35	11′	6.62 (brt, 13.1)	124.52	11	6.63 (brt, 11.7)	124.35	11′	6.62 (brt, 13.1)	124.52
12, 12′	6.77 (d, 14.7)	145.20	12	6.78 (d, 14.6)	145.16	12′	6.72 (d, 15.1)	144.40	12	6.78 (d, 14.6)	145.16	12′	6.72 (d, 15.1)	144.14
13, 13′		137.40	13		137.45	13′		137.35	13		137.45	13′		137.33
14, 14′	6.50 (brd, 7.8)	136.60	14	6.49 (brd, 8.2)	136.52	14′	6.49 (brd, 8.2)	136.15	14	6.49 (brd, 8.2)	136.52	14′	6.49 (brd, 8.2)	136.05
15, 15′	6.81 (brd, 7.8)	132.50	15	6.82 (brd, 8.2)	132.56	15′	6.82 (brd, 8.2)	132.32	15	6.82 (brd, 8.2)	132.56	15′	6.82 (brd, 8.2)	132.22
19, 19′	1.92 (s)	13.16	19	1.92—1.95[c]	13.08—13.48[c]	19′	1.92—1.95[c]	13.08—13.48[c]	19	1.92—1.95[c]	13.08—13.48[c]	19′	1.92—1.95[c]	13.08—13.48[c]
20, 20′	1.94 (s)	13.04	20			20′			20			20′		
Glucose A, C			Glucose A			Glucose C			Glucose A			Glucose C		
1	5.37 (d, 7.8)	95.00	1	5.38 (d, 7.8)	95.05	1	5.06 (d, 3.6)	89.58	1	5.38 (d, 7.8)	95.05	1	4.53 (d, 6.9)	94.80
2	3.18 (t, 7.8)	72.78	2	2.90—4.10[c]	68.00—77.00[c]	2	4.39 (dd, 3.7, 10.1)	74.87	2	2.90—4.10[c]	68.00—77.00[c]	2	4.53 (t, 6.9)	75.87
3	3.23 (t, 8.0)	76.51	3			3	2.90—4.10[c]	68.00—77.00[c]	3			3	2.90—4.10[c]	68.00—77.00[c]
4	3.20 (t, 8.0)	69.57	4			4			4			4		
5	3.38 (m)	76.70	5			5			5			5		
6	3.54 (m)	68.36	6			6			6			6		
	3.94 (d, 10.1)													
Glucose B, D			Glucose B			Glucose D			Glucose B			Glucose D		
1	4.13 (d, 7.8)	103.49	1	4.13 (d, 7.8)	103.60	1	4.13 (d, 7.8)	103.70	1	4.13 (d, 7.8)	103.60	1	4.15 (d, 7.8)	103.70
2	2.91 (t, 8.0)	73.88	2	2.90—4.10[c]	61.00—77.00[c]	2	2.90—4.10[c]	61.00—77.00[c]	2	2.90—4.10[c]	61.00—77.00[c]	2	2.92—4.10[c]	61.00—77.00[c]
3	3.08 (t, 8.0)	77.00	3			3			3			3		
4	2.99 (t, 8.0)	70.37	4			4			4			4		
5	3.01 (m)	77.23	5			5			5			5		
6	3.38 (m)	61.38	6			6			6			6		
	3.60 (d, 10.5)													

a) Chemical shifts are in ppm and coupling constants in Hz are in parentheses. *b*) Assignments were made using 1D-TOCSY, COSY, HMQC and HMBC. *c*) Signals were overlapped and could not be assigned.

Table 2. ^{1}H (800 Mz)- and ^{13}C (200 MHz)-NMR Spectral Data[a] of Peracetylated Crocin (**1a**) and Neocrocin A (**2a**) (in $CDCl_3$)

Peracetylated crocin (**1a**)[b]			Peracetylated neocrocin A (**2a**) (α isomer)[b]					
No.	δ_H	δ_C	No.	δ_H	δ_C	No.	δ_H	δ_C
8, 8′		166.09	8		166.07	8′		166.92
9, 9′		124.57	9		124.55	9′		125.02
10, 10′	7.33 (d, 12.4)	141.45	10	7.32 (d, 11.4)	141.45	10′	7.20 (d, 12.4)	140.46
11, 11′	6.54 (dd, 11.7, 14.9)	123.66	11	6.53 (dd, 11.5, 15.1)	123.71	11′	6.50 (dd, 11.7, 14.9)	123.63
12, 12′	6.68 (d, 14.5)	145.42	12	6.68 (d, 15.2)	145.42	12′	6.60 (d, 15.1)	144.85
13, 13′		138.00	13		136.96	13′		136.85
14, 14′	6.42 (d, 10.1)	136.95	14	6.41 (d, 9.7)	136.33	14′	6.39 (d, 10.1)	136.02
15, 15′	6.73 (dd, 2.1, 8.0)	131.83	15	6.71 (br dd)	131.84	15′	6.72 (br dd)	131.67
19, 19′	1.98 (s)	12.87	19	1.97 (s)	12.70	19′	1.91 (s)	12.80
20, 20′	1.99 (s)	12.73	20	1.98 (s)	12.90	20′	1.98 (s)	12.90
Glucose A, C			Glucose A			Glucose C		
1	5.74 (d, 8.2)	92.08	1	5.74 (d, 7.8)	92.08	1	6.73 (d, 3.7)	88.99
2	5.20 (t, 9.0)	70.29	2	5.20 (t, 9.0)	70.29	2	5.07 (dd, 3.9, 10.3)	69.63
3	5.27 (t, 9.4)	72.90	3	5.26 (t, 9.6)	72.90	3	5.55 (t, 9.9)	70.08
4	5.01 (t, 9.6)	68.42	4	5.01 (t, 9.8)	68.38	4	5.03 (t, 10.1)	68.47
5	3.83 (ddd, 2.4, 6.4, 10.5)	74.01	5	3.82 (m)	74.01	5	4.05 (m)	70.99
6	3.59 (dd, 5.8, 11.7)	67.68	6	3.59 (dd, 5.8, 11.7)	67.68	6	3.53 (dd, 6.0, 11.0)	67.96
	3.93 (dd, 2.3, 11.4)			3.92 (1H, br d)			3.93 (br d)	
Glucose B, D			Glucose B			Glucose D		
1	4.55 (d, 8.2)	100.65	1	4.55 (d, 8.2)	100.65	1	4.53 (d, 7.8)	100.85
2	4.98 (dd, 7.8, 9.6)	70.99	2	4.97 (t, 8.9)	70.99	2	4.97 (t, 8.9)	70.99
3	5.17 (t, 9.4)	72.90	3	5.17 (t, 9.4)	72.90	3	5.19 (t, 9.4)	72.76
4	5.05 (t, 10.2)	68.42	4	5.05 (t, 9.4)	68.43	4	5.07 (t, 9.9)	69.03
5	3.63 (ddd, 2.3, 4.6, 10.1)	71.98	5	3.63 (m)	71.98	5	3.67 (ddd, 2.4, 4.5, 10.0)	72.02
6	4.09 (dd, 2.3, 12.4)	61.88	6	4.09 (dd, 1.8, 12.4)	61.88	6	4.11 (dd, 2.1, 12.2)	61.88
	4.24 (dd, 4.6, 12.4)			4.23 (dd, 5.0, 12.3)			4.26 (dd, 4.6, 12.4)	
Acetyl	2.00—2.07 (s)	20.65—20.80		1.98—2.06 (s)	20.65—20.94			
		169.45—170.73			169.36—170.79			

a) Chemical shifts are in ppm and coupling constants in Hz are in parentheses. *b*) Assignments were made using 1D-TOCSY, COSY, HMQC and HMBC.

cose A and B of **2a** were identical to those of **1a**, and the HMBC correlations of the H-1 (δ_H 5.74) of glucose A to C-8 (δ_C 166.07) of crocetin, and the H-1 (δ_H 4.55) of glucose B to C-6 (δ_C 67.68) of glucose A, suggested the existence of a gentiobiosyl moiety connected to crocetin at the anomeric position, similar to in **1a**. By contrast, the chemical shifts and coupling constants of glucose C suggested that the anomeric position of glucose C was in the α configuration, and the HMBC correlations of the H-2 (δ_H 5.07) of glucose C to C-8′ (δ_C 166.07) of crocetin, and the H-1 (δ_H 4.53) of glucose D to C-6 (δ_C 67.96) of glucose C, supported the notion that another gentiobiosyl moiety was connected to the crocetin *via* the number 2 position on glucose C. The ^{1}H- and ^{13}C-NMR data for **1a** and **2a** are summarized in Table 2 and key correlations of **2a** are shown in Fig. 2. These observations provided evidence that neocrocin A (**2**) possessed a unique binding system for sugars.

As neocrocin A (**2**) isolated from gardenia yellow was a mixture of isomers along with crocin (**1**), we initially suspected that neocrocin A (**2**) was an artefact formed by crocin (**1**) *via* the rearrangement of the acyl moiety during the isolation process. However, the LC profile of gardenia yellow, especially the peak height of neocrocin A (**2**), remained unchanged after dissolving again with ethanol and water under acidic and basic conditions. We thus concluded that neocrocin A (**2**) was an original constituent of the *G. jasminoides* fruits. To our knowledge, this is the first published evidence that the binding system for the sugars of a crocetin glycosyl ester is not limited to the anomeric position in nature. We are further investigating the possibility that other types of crocetin glycoside might also exist in this fruit through an ongoing study.

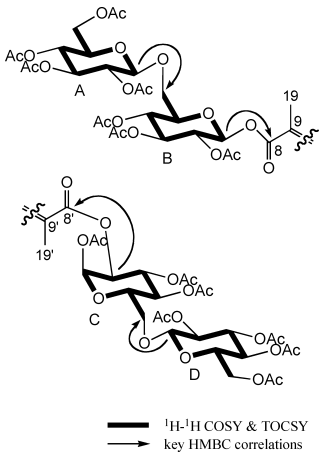

Fig. 2. Key Correlations of Peracetylated Neocrocin A (**2a**)

Experimental

General IR spectra were recorded on a JASCO FT/IR-4100. UV/Vis spectra were recorded on a JASCO UV-560. FAB-MS spectra were measured with JEOL JMS-700 system and ESI-MS spectra were measured with JEOL JMS-T100LC "AccuTOF". NMR spectra were recorded on JEOL JNM-ECA (800 MHz) and JNM-ECA (500 MHz). Diaion HP-20 was used for open-column chromatography. The preparative LC-MS system (Waters FractionLynx^{TM}MS auto purification system), which is described in our previous report,[6] was used for isolation of compounds on this research. The

solvents for LC-MS were purchased from Sigma-Aldrich Japan. All of the other chemicals were of reagent or HPLC grade and were used without further purification.

Plant Material The dried gardenia fruits, defined as the fruits of *Gardenia jasminoides*, were purchased from Uchida Wakanyaku Co., Ltd., Japan, in July 2004. The crude drug name was Gardeniae Fructus (Serial No.: VMAMQ), and the voucher specimen (GJ 72004) was deposited at Division of Food Additives in National Institute of Health Sciences.

Extraction and Isolation Gardenia yellow was extracted by stirring dried gardenia fruits (1 kg) with 50% aqueous ethanol at ambient temperature in the dark for 2 h. The part of extracted gardenia yellow (2.25 g) was subsequently fractionated on a Diaion HP-20 column (40 mm i.d.×250 mm) by successive elution with 0—100% MeOH. The 60—70% methanol eluate was then concentrated *in vacuo* and the residue was loaded into a preparative LC-MS system.[6] The conditions were as follows: column, Waters XTerra® Prep MS C_{18} (5 μm, 19 mm i.d.×100 mm); mobile phase, H_2O : MeOH= 58 : 42; flow rate, 10 ml/min; make-up liquid, MeOH 1.0 ml/min; injection volume, 200 μl; and detection and collection trigger, ESI (pos.) *m*/*z* 999.5. The electrospray source ran at a 3.0 kV capillary voltage, with 120 and 350 °C source and desolvation temperatures, respectively, and with 350 and 60 l/h desolvation and cone gases, respectively. The cone voltage was 50 V. Full-scan acquisition between *m*/*z* 100 and 2000 was performed at a scan speed of 0.3 s/scan, with a 0.1-s inter-scan delay. The solvent delivered to the electrospray interface was split in a 1 : 4 ratio, delivering around 200 μl/min to the interface. The peaks of crocin (**1**) (retention time (t_R) 15.3 min) and neocrocin A (**2**) (t_R 19.0 min) were each fractionated and concentrated *in vacuo* at <40 °C. The overall procedure was repeated approximately 25 times to afford 220 mg of crocin (**1**) and 18 mg of neocrocin A (**2**).

Crocin (1)[2] Red amorphous powder. IR (KBr) ν_{max} cm^{-1}: 1069, 1227, 1271, 1577, 1699, 2920, 3398. UV/Vis (MeOH) λ_{max} (log ε): 433 (4.49), 458 (4.46), 259 (3.65). HR-ESI-MS *m*/*z*: 999.3674 $[M+Na]^+$ (Calcd for $C_{44}H_{64}O_{24}Na$: 999.3685). ^1H (800 MHz)- and ^{13}C (200 MHz)-NMR spectral data: see Table 1.

Neocrocin A (2) Red amorphous powder. IR (KBr) ν_{max} cm^{-1}: 1023, 1227, 1270, 1578, 1702, 2918, 3375. UV/Vis (MeOH) λ_{max} (log ε): 432 (4.73), 457 (4.69), 258 (3.97). HR-ESI-MS *m*/*z* 999.3707 $[M+Na]^+$ (Calcd for $C_{44}H_{64}O_{24}Na$, 999.3685). ^1H (800 MHz)- and ^{13}C (200 MHz)-NMR spectral data: see Table 1.

Crocetin Dimethyl Ester (3) Methanolysis of **2** (10 mg) with 5% HCl methanol at room temperature for 50 h resulted in red amorphous powder (**3**, 3 mg) after purification by Silica gel column chromatography (EtOAc : hexane=2 : 3). ESI-MS *m*/*z*: 327 $[M-H]^-$. ^1H-NMR ($CDCl_3$, 500 MHz) δ: 1.98 (6H, s, H-20, 20′), 1.99 (6H, s, H-19, 19′), 3.75 (6H, s, MeO×2), 6.35 (2H, br d, *J*=10.0 Hz, H-14, 14′), 6.53 (2H, dd, *J*=11.0, 15.0 Hz, H-11, 11′), 6.60 (2H, d, *J*=15.0 Hz, H-12, 12′), 6.70 (2H, dd, *J*=3.0, 8.0 Hz, H-15, 15′), 7.28 (2H, dd, *J*=1.4, 11.0 Hz, H-10, 10′); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 12.87 (C-20, 20′), 12.99 (C-19, 19′), 51.92 (MeO×2), 123.88 (C-11, 11′), 126.48 (C-9, 9′), 131.40 (C-15, 15′), 135.43 (C-14, 14′), 136.79 (C-13, 13′), 138.97 (C-10, 10′), 143.84 (C-12, 12′), 169.02 (C-8, 8′).

Peracetylated Crocin (1a) and Neocrocine A (2a) Peracetylation of gardenia yellow (1.0 g) was carried out with Ac_2O/pyridine (cat. DMAP), affording peracetylated crocin (**1a**, 1.5 g) (t_R 26.0 min) and the a isomer of neocrocin A (**2a**, 7.4 mg) (t_R 27.9 min) using preparative LC/MS[6] under the following conditions: column, YMC J'sphere ODS-L80 (5 μm, 20 mm i.d.×250 mm); mobile phase, H_2O : MeCN=42 : 58; flow rate, 10 ml/min; make-up liquid, MeOH 1.0 ml/min; injection volume, 200 μl; detection and collection trigger, ESI (positive mode), *m*/*z* 1587.5. The overall procedure was repeated approximately 30 times. The β isomer of peracetylated **2** could not be isolated because of overlapping with the peak of **1a**.

1a red amorphous powder. IR (KBr) ν_{max} cm^{-1}: 1063, 1222, 1371, 1615, 1751, 2944. UV/Vis (MeOH) λ_{max} (log ε): 436 (4.95), 462 (4.92), 260 (4.05): HR-ESI-MS *m*/*z*: 1587.5156 $[M+Na]^+$ (Calcd for $C_{72}H_{92}O_{38}Na$: 1587.5164). **2a** (an α isomer): red amorphous powder. IR (KBr) ν_{max} cm^{-1}: 1038, 1223, 1375, 1637, 1752, 2945. UV/Vis (MeOH) λ_{max} (log ε): 438 (4.81), 460 (4.77), 260 (4.05). HR-ESI-MS *m*/*z* 1587.5192 $[M+Na]^+$ (Calcd for $C_{72}H_{92}O_{38}Na$: 1587.5164); ^1H (800 MHz)- and ^{13}C (200 MHz)-NMR spectral data of **1a** and **2a**: see Table 2.

References and Notes

1) Present address: *School of Food and Nutritional Sciences, University of Shizuoka; 52–1 Yada, Suruga, Shizuoka 422–8526, Japan*.
2) Calsteren M. V., Bissonnette M. C., Cormier F., Dufresne C., Ichi T., LeBlanc J. C. Y., Perreault D., Roewer I., *J. Agric. Food Chem.*, **45**, 1055—1061 (1997).
3) Carmona M., Zalacain A., Sánchez A. M., Novella J. L., Alonso G. L., *J. Agric. Food Chem.*, **54**, 973—979 (2006).
4) Ichi T., Higashimura Y., Katayama T., Koda T., Shimizu T., Tada M. J., *Jpn. Soc. Food Sci. Technol.*, **42**, 776—783 (1995).
5) Pfister S., Meyer P., Steck A., Pfander H., *J. Agric. Food Chem.*, **44**, 2612—2615 (1996).
6) Sato K., Sugimoto N., Ohta M., Yamazaki T., Maitani T., Tanamoto K., *Food Add. Contam.*, **20**, 1015—1022 (2003).
7) Kawahara N., Tamura T., Inoue M., Hosoe T., Kawai K., Sekita S., Satake M., Goda Y., *Phytochemistry*, **65**, 2577—2581 (2004).