Simultaneous Determination of Sudan Dyes and Carotenoids in Red Pepper and Tomato Products by HPLC

Hussein G. Daood[1,*] and **Péter A. Biacs**[2]

[1]Central Food Research Institute, Herman Ottó út 15, H-1022 Budapest, Hungary and [2]Hungarian Office of Food Safety, Miklós tér 1, H-1035, Budapest, Hungary

Abstract

To simultaneously detect Sudan dyes and carotenoids in red pepper and tomato products, high-performance liquid chromatography (HPLC) methods with photodiode-array detection are developed and validated. The methods include the use of end-capped and nonend-capped adsorbents with a gradient elution system starting with water containing methanol. Water content of 9% in the starting mobile phase is found to be necessary to ensure sufficient separation of Sudan dyes and to avoid overlapping or interference with the carotenoids of considerable content. The data of the validation reveal the accuracy and precision of the developed methods. A limit of Sudan dyes detection of 1–5 µg/g in red pepper or tomato sauce could be approached. The methods provide excellent separation of the carotenoids from the unsaponified extracts of red pepper and the tomato products

Introduction

During the last few years there has been concern about the use of organic colorants in food and foods ingredients. Colorization or amending of the original color with Sudan dyes can make food unsafe because of the toxicity and carcinogen properties of such compounds (1,2). According to European Union (EU) regulations No. 178/2002 (4), the use of azo dyes such as Sudan derivatives in hot chili products and other food preparations does not comply with the EU food safety requirements. Therefore, the committee of the European Commission (EC) adopted a decision prohibiting the use of Sudan dyes as food colorant (EC No. 460/2003) (5).

To detect and determine Sudan dyes in foods, certain methods have been developed and applied. These methods include polarography (6), thin-layer chromatography (7), high-performance liquid chromatography (HPLC) with molecular-imprinted solid-phase extraction (SPE) (8), HPLC with atomic pressure chemical ionization–mass spectrometry (MS) (9), and, more recently, capillary HPLC–electrospray ionization (ESI) MS–MS (10,11) developed for the detection and quantitation of small quantities of Sudan I, II, II, and IV in hot chili tomato sauce and chili tomato with cheese sauce samples.

The objective of this work was to develop and validate an HPLC method for simple, fast, and simultaneous determination of carotenoids and Sudan dyes in tomato and red pepper products.

* Author to whom correspondence should be addressed: email h.daood@cfri.hu.

Experimental

Chemicals and samples

Sudan I, II, III, IV, and β-carotene (95%) were purchased from Sigma-Aldrich (Budapest, Hungary). All analytical-grade organic solvents used for extraction were from Reanal (Budapest, Hungary), whereas those of HPLC grade were obtained from Merck (Darmstadt, Germany). Stock solutions of Sudan dyes and standard carotenoids were prepared by dissolving 2 mg in 10 mL of methanol–acetonitrile–isopropanol (10:35:55) (eluent B in the gradient HPLC elution). All stock and working solutions were stored at refrigeration temperature in brown-colored glass vials. Tomato and red pepper products (sauce or paste) from different commercial batches were obtained from the local supermarkets.

HPLC–photodiode-array analysis

A Waters Alliance liquid chromatograph consisting of a Model 2695 Separation Module (gradient pump and autosampler) and a Model 2996 photodiode-array (PDA) detector (Waters, Milford, MA) was used. The HPLC instrument was operated by Empower 2000 software (Waters). HPLC separation of pigments from pepper products was performed on a Nucleosil 100 column (250- × 4.6-mm, 3 µm) (Machery Nagel, Düren, Germany) and those from tomato products were separated on a Hypersil Gold column (250- × 4.6-mm, 5 µm) (Thermo, Waltham, MA) using different gradient elution systems, as described in Table I.

Method validation

The limits of detection (LOD) and quantitation (LOQ) were measured by calculating the concentration of the analytes in µg/mL at a ratio of peak/noise of 3 and 10, respectively. Linearity was tested by plotting peak area versus concentration of the analytes starting from a concentration higher than the LOQ value. Precision, in terms of within-day (intraday) repeatability, expressed as relative standard deviation [percent relative standard deviation (%RSD)] was calculated from five measurements (extraction and HPLC analysis) of a well-homogenized red pepper and tomato paste spiked with Sudan dyes compounds. For the recovery test, 2 g of each product was spiked with known quantities of different dyes (1–10 µg) in triplicate, extracted, and then analyzed by HPLC.

Extraction of pigments

From red pepper products

The pigments were extracted using a previously described procedure (12) with slight modification. One gram of spiked or nonspiked paprika paste or sauce was crushed in a crucible mortar followed by gradual addition of 50 mL of methanol–acetone–dichloroethane (2:2:1) with careful mixing and transfer to an Erlenmeyer flask. The mixture was subjected to ultrasonic power using a water bath ultrasonic device (Tesla, Roznov, Czech Republic) for 2 min, followed by mechanical shaking for 15 min. The mixture was then filtered through type 640 MN filter paper, and the solvent was evaporated under vacuum at 40°C. The residues were dissolved in 5 mL of HPLC eluent B and filtered through a polytetrafluoroethylene (PTFE) 0.45-µm syringe filter before injection into the HPLC column.

From tomato products

Extraction of the pigment from spiked or nonspiked tomato sauce started with crushing 1 to 2 g in a crucible mortar and adding 20 mL methanol. In the validation experiments, known quantities of Sudan dyes (1–10 µg/g) were added before crushing. The mixture was left to stand for 2 min; the methanol fraction was carefully decanted to a 100-mL Erlenmeyer flask, and the residues were crushed again. To the residues, 60 mL of methanol–dichloroethane mixture (1:5) was added gradually with gentle mixing to ensure complete solubility of the pigment. The mixture was then transferred quantitatively to the Erlenmeyer flask where the methanol fraction was kept. After mechanical shaking for 15 min, a few drops of doubly distilled water were added to reduce the solubility of fat-soluble pigments in the methanol fraction. The less polar fraction (dichloroethane) was separated in a separatory funnel and passed through anhydrous sodium sulfate to a round-bottom flask. The solvent was then evaporated under vacuum at 40°C, the residues were redissolved in 3 mL chloroform, and the volume was completed to 10 mL with the HPLC eluent B.

Table I. Gradient Elution Systems Used for the Simultaneous Determination of Sudan Dyes and Carotenoids from Red Pepper and Tomato Products*

Time (min)	Flow (mL/min)	%A	%B	%C
Red pepper products, Nucleosil 100, C-18 (250 × 4.6 mm, 3 µm) end-capped column				
0.0	0.8	80.0	0.0	20.0
20.0	1.0	0.0	80.0	20.0
35.0	1.0	0.0	90.0	10.0
40.0	1.0	0.0	90.0	10.0
45.0	0.8	80.0	0.0	20.0
Tomato products, Hypersil Gold, C-18 (250 × 4.6 mm, 5 µm) nonend-capped column				
0.0	1.0	75.0	0.0	25.0
20.0	1.0	0.0	50.0	50.0
30.0	1.0	0.0	65.0	35.0
35.0	1.0	75.0	0.0	25.0

* A: 9% water in methanol, B: methanol–acetonitrile–isopropanol (10:35:55), and C: methanol.

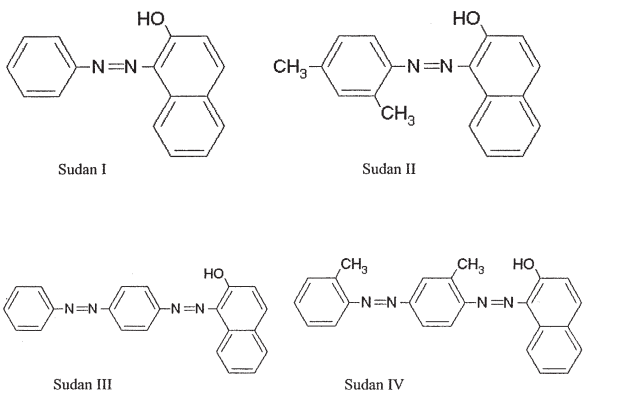

Figure 1. Structure of Sudan dyes.

Results and Discussion

Although Sudan dyes and carotenoids have different chemical structures (Figure 1), they have similar solubility and spectral characteristics (both are fat-soluble and have a maximum absorption wavelength between 400 and 600 nm). This makes it difficult to detect and determine each of them in the presence of the other in natural matrixes such as foods without selective separation by SPE or efficient liquid chromatography (LC). However, when existing alone or separated by chromatographic means, the azo dyes can be differentiated from the carotenoids on the basis of variations in the absorption spectrum of the individual colorant type.

In the HPLC separation, a nonaqueous gradient elution starting with methanol was first applied. The results showed that the most polar dye (Sudan I) elutes with a longer retention time than unesterified capsanthin and overlaps with antheraxanthin in the HPLC analysis of red pepper extract spiked with Sudan dyes. Sudan III and IV eluted from the column with retention times similar to those of the monoesters of red and yellow xanthophylls (chromatogram not shown). In order to increase the variation between the carotenoids and Sudan dyes in partitioning and retention on a reversed-phase column, water was added to

methanol (eluent A) at different proportions. With the addition of water, Sudan I, II, III, and IV eluted with shorter retention times, indicating that Sudan dyes are more hydrophilic than carotenoids, which have longer retention times when using the water-containing elution system. An initial water proportion of 9% in starting eluent A was found the best for Sudan dyes to appear in a parts of the chromatogram in which no carotenoid peaks are present (see Figure 2). In the extract of processed red pepper or spice paprika, a degradation product of some xanthophylls appears with a retention time close to that of Sudan I, though in fresh or processed products an epoxide of antheraxanthin elutes with the same retention time as Sudan II. Fortunately, these two compounds exist in minute quantities (less than 0.1 µg/g) and their spectral characteristics are considerably different from those of Sudan I and II (Table II). Sudan III and IV appear in the chromatogram without overlapping with carotenoids. It should be mentioned that red pepper products are colored with Sudan dyes or any other organic and inorganic dyes if the products have low color intensity (with low content of red-colored carotenoids). Therefore, in order to intensify their color, organic dyes are added at a concentration higher than 100 µg/g, which is well above the LOD and LOQ estimated in the method developed in this work. This statement was based on a separate experiment (not shown in this article), in which the color of faded spice red pepper (paprika) was improved to be faint red with Sudan I and IV at a concentration of 1 mg/g in the paprika.

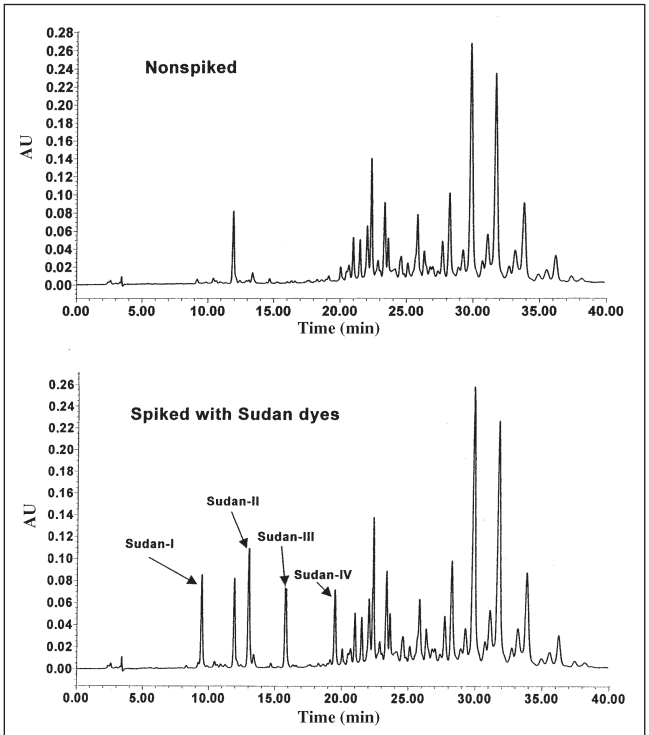

Figure 2. HPLC separation of carotenoids and Sudan dyes on a Nucleosil C-18, 3-µm column with gradient elution. See text for more details.

Table II. Spectral Characteristics of Sudan Dyes and Some Carotenoids Present in Red Pepper and Tomato Products as Determined by HPLC with Photodiode-Array Detector

Pigments	Maximum absorption wavelength
Sudan I	312, 421, 484, (492)
Sudan II	316, 421, 498, (509)
Sudan III	343, 506, (534)
Sudan IV	350, 515, (540)
Capsanthin	288, 478, (498)
Capsorubin	288, 484, (518)
Lycoxanthin	292, 445, 472, 501
cis-Lycopene epoxide	295, 360, 440, 464, 498
Lutein	288, 423,441, 472
Zeaxanthin	285, 427, 450, 476
Violaxanthin	270, 418, 440, 466

The developed HPLC method provided excellent separation of the individual components of the red pepper carotenoid pigment, which consists of four classes such as free (unesterified) xanthophylls, monoesters of yellow and red xanthophylls, carotenes (mainly β-carotene), and diesters of different xanthophylls. This approach is of special interest from the technological point of view because color stability and coloring capacity of red pepper products depends (to a high extent) not only on the color content but also on the ratio of mono–diesters, esterified–unesterified carotenoids, and red–yellow pigments (13–15)

In the case of tomato products, the method used for the separation of red pepper pigments was not suitable to separate sufficiently the major carotenoids such as lycopene β-carotene and their cis isomers from each other. Also, some Sudan dyes overlapped with the xanthophylls such as lycoxanthin and lycopene epoxide. Therefore, it was necessary to work out a new method that ensures better separation of the azo dyes from the carotenoids and the individual carotenoids from each other. For this purpose, a nonend-capped reversed-phase chromatographic

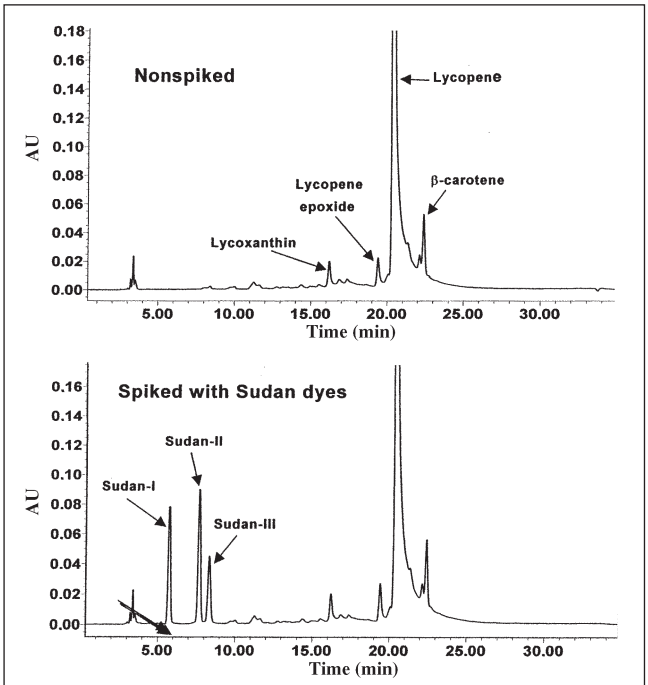

Figure 3. HPLC separation of carotenoids and Sudan dyes on a Hypersil Gold C-18, 5-µm column with gradient elution. See text for more details.

material (Hypersil Gold from Thermo) was selected as the stationary phase. Such a column separated efficiently the Sudan dyes with relatively short retention time without overlapping with the polar xanthophylls present in the tomato extract when the mobile phase gradient indicated in Table I was applied. The method was also capable to separate the most important tomato carotenoids, lycopene and β-carotene, as well as their derivatives (Figure 3). The existence of lycopene (less polar carotene) at a high concentration makes it difficult to get a clear solution from the tomato extract with any of the mobile phases used. For instance, with methanol or HPLC eluent B, a turbid suspension was obtained. Filtration through a Teflon (PTFE) filter aid resulted in a great loss of lycopene, which was seen in crystallized form on the filter. Injection of the suspension without filtration caused the major pigments to overlap and led to incorrect quantitative analysis. In order to avoid any overlapping between lycopene and β-carotene, the extract of tomato paste or sauce should be solubilized first in 3 mL chloroform and then diluted with HPLC eluent B.

Table III. Data of Method Validation for the HPLC Determination of Sudan Dyes in Red Pepper and Tomato Products

Sudan dye	LOD (µg/mL)	LOQ (µg/mL)	Recoveries: Spiked (µg/g)	Recoveries: Found (µg/g)	Recoveries: Recovery (%)	Precision %RSD
In red pepper products						
Sudan I	0.030	0.102	1	0.85 ± 0.02	85	2.4
			5	4.35 ± 0.13	87	3.0
Sudan II	0.032	0.120	1	0.95 ± 0.03	95	3.2
			5	4.90 ± 0.14	98	3.0
Sudan III	0.037	0.128	1	1.02 ± 0.03	102	2.9
			5	5.10 ± 0.17	102	3.3
Sudan IV	0.048	0.162	5	4.95 ± 0.12	99	2.4
			10	101.0 ± 0.22	101	2.2
In tomato products						
Sudan I	0.025	0.088	1	0.95 ± 0.02	92	2.2
			5	4.93 ± 0.12	93	2.4
Sudan II	0.026	0.090	1	0.98 ± 0.02	98	2.0
			5	4.96 ± 0.13	99	2.7
Sudan III	0.031	0.102	1	1.07 ± 0.03	107	2.8
			5	5.16 ± 0.17	103	3.3
Sudan IV	0.042	0.147	5	5.14 ± 0.11	103	2.1
			10	10.4 ± 0.28	104	3.0

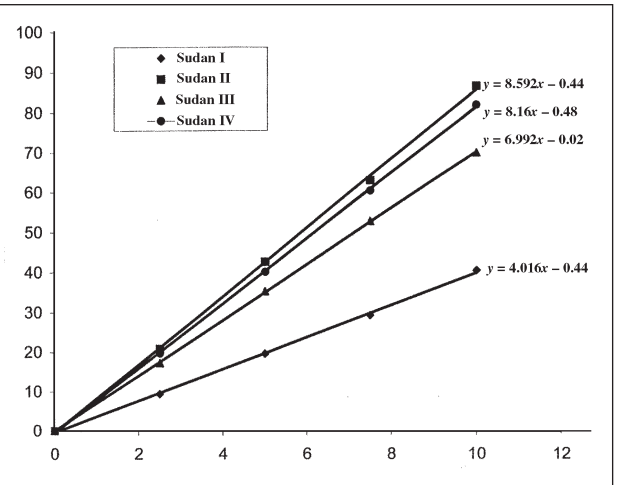

Figure 4. Calibration curves of Sudan dyes as determined by HPLC using a Nucleosil C-18, 3-µm column with gradient elution. See text for more details.

Results of validation

The use of a PDA detector starting with the maximum noise assisted in the rapid and accurate investigation of the LOD and LOQ. For this purpose, a solution containing 0.05 µg/mL of each Sudan dye was injected. The values of the LOD and LOQ listed in Table III are close to those reported by Tato and Bononi (8), who used LC–MS for the determination of Sudan I in chili sauce, but substantially higher than those recorded when LC–ESI-MS–MS was applied (10). The latter method is very sensitive and specified for the detection of minute quantities of Sudan I dyes in foodstuffs. However, the values estimated in the present work are low enough to make the analytical procedure quite accurate for the detection of food adulteration or color amendment with azo type red-color dyes

Method accuracy of LC–PDA detection was then tested in terms of precision. Under the conditions of the within-day repeatability experiment, RSD values between 2.1% and 3.3% were obtained. These values are typical for HPLC-based determinations and acceptable for routine detection purposes. Within-one-day repeatability results for both red pepper and tomato are reported in Table III.

Recovery functions were calculated to ascertain the influence of the matrix on the determination of all the examined Sudan dyes. In general, recovery of Sudan I was found to be lower than that of the other dyes (significant with $p = 0.01$ for red pepper and 0.05 for tomato product). This indicates that Sudan I is more sensitive to the conditions of extraction and HPLC determination than the other compounds. Also, its stability in tomato paste is higher than in red pepper paste, probably because of the effect of some preservatives added during processing of pungent red pepper paste or sauce. It was observed that the stability of Sudan dyes in the food matrix increases with the increase of the molecular weight and polarity of the azo dye. This is derived from the high recovery of the dyes having high molecular weight and relatively low polarity such as Sudan III and IV.

The plot of the peak area versus concentration of Sudan dyes measured by HPLC with end-capped conventional Nucleosil adsorbent (method used for red pepper) and detection at 490 nm showed good linearity with the correlation coefficient (R^2) ranging between 0.9996 and 0.9998 (Figure 4). Very similar results, but with slightly higher detector response were obtained when the nonend-capped adsorbent was used for the measurements with tomato product. These calibration curves can be used for the quantitation of Sudan dyes in different products of adul-

terated fruits and vegetables and also as internal standard materials in the qualitative analysis of carotenoids from different foods.

It should be mentioned that validation and evaluation of carotenoid determination by the developed methods is not included in the present article. These will be reported and discussed in detail in a separate paper to be submitted for publication.

Conclusion

Applying this procedure, a limit of Sudan dyes detection of 1–5 µg/g can be achieved for tomato or red pepper paste or sauce depending on the type and sensitivity of the detection means used. This limit is believed to be much lower than the concentration of azo-type dyes usually applied to effectively amend the color content and coloring capacity of bad quality processed tomato or red pepper.

Acknowledgments

The author would like to appreciate the financial support of the Hungarian Scientific Research Fund (OTKA) under Grant No. T 032889 and Ministry of Education under Grant No. OM-00190/2001 (NKFP-Hungária Aránya).

References

1. R.A. Lumbet, C. Connolly, R.E. Kouri, D.W. Nebert, and S.W. Bigelow. Biological effect of the Sudan dyes: role of the Ah cytosolic receptor. *Biochem. Pharmacol.* **32:** 3053–58 (1983).
2. M. Stiborová, E. Frei, H.H. Schmeiser, M. Wiessler, and J. Hradec. Detoxication products of the carcinogenic azodye sudan: 1 (solvent yellow 14) bind to nucleic acids after activation by peroxidase. *Cancer Lett.* **68:** 43–47 (1993).
3. M. Stiborová, H.H. Schmeiser, and E. Frei. Prostaglandin H synthase-mediated oxidation and binding of DANN of a detoxication metabolism of carcinogenic sudan I, 1-(phenylazo)-2,6-dihydroxy-naphthalene. *Cancer Lett.* **146:** 53–60 (1999).
4. EC regulation No. 178/2002. *Off. J. Eur. Union* **31:** (2002).
5. Commission Decision of 20 June 2003 on emergency measures regarding hot chilli products notified under document No. 460/2003. *Off. J. Eur. Union L.* **154**/114 (2003).
6. L.E. de la Cruz Yaguez, J.M. Pingarron Carrazon, and L.M. Polo Diez. Polarographic study of the 1-(2,4-dimethylphenylazo)-2-naphthol (Sudan-II) in hydroalcoholic medium. *Electrochem. Acta* **31:** 119–21 (1986).
7. P.N. Marshall. Thin-layer chromatography of Sudan dyes. *J. Chromatogr. A* **136:** 353–57 (1977).
8. F. Puoci, C. Garreffa, F. Iemma, R. Muzzalupo, U.G. Spizzirri, and N. Picci. Molecular imprinted solid phase extraction for detection of sudan I in food matrices. *Food Chem,* in press.
9. F. Tateo and M. Bononi. Fast determination of sudan I by HPLC/APCI-MS in hot chilli, spice, and oven-baked foods. *J. Agric. Food Chem.* **52:** 655–58 (2004).
10. F. Calbiani, M. Careri, I. Elviri, A. Mangia, and I. Zagnoni. Accurate mass measurements for the confirmation of Sudan azo-dyes in hot chili products by capillary liquid chromatography-electrospray tandam quadrupole orthogonal-acceleration time of flight mass spectrometry. *J. Chromatogr. A* **1058:** 127–35 (2004).
11. F. Calbiani, M. Careri, I. Elviri, A. Mangia, I. Pistara, and I. Zagnoni. Development and in-house validation of a liquid chromatography-electrospray-tandam mass spectrometry method for the simultaneous determination of Sudan I, Sudan II, Sudan III, and Sudan IV in hot chili products. *J. Chromatogr. A* **1042:** 123–30 (2004).
12. P.A. Biacs and H.G. Daood. High-performance liquid chromatography with photodiode-array detection of carotenoids, and carotenoid esters in fruits and vegetables. *J. Plant Physiol.* **143:** 520–25 (1994).
13. P.A. Biacs, H.G. Daood, A. Pavisa, and F. Hajdu. Studies on the carotenoid pigments of paprika. *J. Agric. Food Chem.* **37:** 350–53 (1989).
14. M.I. Mínguez-Mosquera and D. Hernero-Méndez. Changes in carotenoid sterification diring the fruit ripening of *Capsicum annuum* cv. Bola. *J. Agric. Food Chem.* **42:** 640–44 (1994).
15. D. Hernero-Méndez and M.I. Mínguez-Mosquera. Xanthophyll esterification accompanying carotenopid overaccumulation in chromoplst of *Capsicum annuum* ripening fruits is a constitutive process and useful for ripeness index. *J. Agric. Food Chem.* **48:** 1617–22 (2000).

Manuscript received March 2, 2005;
revision received August 2, 2005.