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Journal of Chromatography A, 829 (1998) 393–399

JOURNAL OF CHROMATOGRAPHY A

Short communication

Semi-quantification of carotenoids by high-performance liquid chromatography: saponification-induced losses in fatty foods

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Received 21 April 1998; received in revised form 5 October 1998; accepted 9 October 1998

Abstract

Reversed-phase liquid chromatography, with a non-linear gradient and photodiode array detection (350–550 nm range), has been used to analyse fat-cured crude sausage (''Sobrassada'') in which the main ingredient is paprika. Saponification is seen to produce the underestimation of some carotenoids. The effects of saponification on carotenoid stability were characteristic of each individual carotenoid and depended on food type. Saponification produced a greater underestimation of capsanthin in a cured fatty food like Sobrassada, than in powdered paprika used as an ingredient in Sobrassada, 46% and 22%, respectively. In contrast, saponification produced a similar underestimation of β-carotene in this cured fatty food as in powdered paprika, 49% and 48%, respectively.

Keywords: Saponification; Carotenoids

1. Introduction

Carotenoids are one of the most abundant group of natural pigments because most living plants synthesise it [1] as a protector against photo-oxidative processes [2], and they are constituents of chromoplasts. The most important sources of carotenoids for humans are fruits and vegetables. However, various carotenoid extracts and/or fruits rich in carotenoids are now being used in the food industry to colour foods, thus, these foods may also contain important quantities of carotenoids. The carotenoid content of fruits and vegetables varies greatly in amount, depending on species, variety, time of the year and degree of ripeness [3,4]. The nutritional importance of carotenoid comes mainly from the provitamin A activity of β-carotene, β-cryptoxanthin, and others [1,5]. The importance of provitamin A in the prevention of xerophthalmia [6] is evident and at present the β-carotene relationship with atherosclerosis and cardiovascular disease is an object of study [7], particularly its inhibitory effect on LDL oxidation [8]. These new roles of carotenoids in health make their quantitative determination in foods important.

Carotenoids in vegetables are predominantly esterified by fatty acids [9], thus the identification of high-performance liquid chromatography (HPLC) peaks of these carotenoid esters can be difficult [10]. In addition, the degree of esterification can be a function of the number of hydroxyls present in the xanthophylls. In order to simplify the separation,

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00872-3

most methods for quantitative carotenoid analysis have a previous phase of extract saponification [11]. However, it is important to know whether or not saponification affects the quantification of individual carotenoids in particular, some of which are naturally occurring vitamin A precursors.

Photodiode array detection which records the spectral and chromatographic profiles simultaneously has enormously simplified the routine analysis of carotenoids from biological samples [12]. The photodiode array detection enables the spectra of each individual peak of the chromatogram to be recorded. These photodiode array detection features could allow carotenoid separation without sample saponification.

In the present work we show that sample saponification produces important deviations in the results of food carotenoid determination. At the same time, we propose a method of gradient HPLC to avoid this problem in a cured fatty food. The method has been applied to fat cured crude sausage (''Sobrassada'') carotenoid separation. Sobrassada is a typical sausage of Mallorca (Mediterranean area), which may be considered as having a high fat and carotenoid food content. This sausage is basically made from pork meat and fat, and a characteristic spice, paprika, which provides the high carotenoid content.

2. Experimental

2.1. Samples

The samples were obtained from Sobrassada maker Enterprises, Francisco Tejedor Garcia (Felanitx, Spain) and SEMAR (Llucmajor, Spain). The sausages were made according to Sobrassada of Mallorca specific original denomination with pork meat and fat, additives and condiments, 28%, 64% and 8%, respectively (5.6% powdered paprika). The sausage ripening was carried out at 15°C in a temperature-controlled room. The analyses of samples were made at 30 days of ripening, after a fall in pH.

2.2. Pigment extraction and saponification

The method of Folch et al. [13] was applied for the extraction of fat component of the sausage until it was colourless. Extraction solvents were cloroform–methanol (2:1) with 0.01% butylated hydroxyanisole (BHA, Merck-Schuchardt, Munich, Germany). The solvent was removed from two aliquots of extract under a N_2 stream in the dark. Then one of these samples was saponified with 10% of KOH–methanol with 0.01% BHA as antioxidant at 50°C for 5 min [14]. The free carotenoids were extracted with ethyl ether, washed free of alkali with distilled water, dried over anhydrous sodium sulphate, and the solvent was removed under a N_2 stream. Two sample extracts without solvent, saponified and non-saponified, were resuspended in acetone with 0.01% BHA as antioxidant and canthaxanthin as chromatographic internal standard.

2.3. Pigment purification and standards

Canthaxanthin was obtained from Hoffmann-La Roche. β-Carotene was purchased from Sigma (St. Louis, MO, USA). Capsanthin and capsorubin were purified as follows: a saponified extract of paprika (*Capsicum annum* L. Var. Bola) was purified by thin-layer chromatography (TLC) using methanol–acetone–petroleum ether (3.5:20:76.5) [14] as the solvent. The capsanthin and capsorubin bands were scraped off the silica gel plate and purified a second time by TLC using methanol–acetone–petroleum ether–ethanol (3.5:20:66.5:10) [11] as the solvent. The concentrations of capsanthin and capsorubin were calculated based on the $E^{1\%}_{1\ cm}$ value of 2072 in benzene at 483 nm and 2200 in benzene at 489 nm, respectively [15].

2.4. High-performance liquid chromatography

The chromatographic system consisted of a Waters Associates 600E multisolvent delivery system, an automatic injector Waters Associates Satellite WISP and a Waters Associates 996 photodiode array detector. The detector signals were transferred to an NEC computer under the control of chromatographic software Waters Associates Millennium 2010 version 1.10. The HPLC system was equipped with a Hewlett-Packard reversed-phase C_{18} Spherisorb ODS-2 (5 μm, 250×3.9 mm I.D.) column. To

protect the column, a pre-column of the same material was used (10×4 mm I.D.).

2.5. HPLC separation

The eluent composition was: (A) acetone–water (100:50), and (B) acetone–water (100:5). The gradient program was as follows:

Time (min)	Eluent A (%)	Eluent B (%)	Curve
5	100	0	
5	50	50	Convex
30	0	100	Concave
40	0	100	

Other chromatographic conditions were as follows: flow-rate 1 ml/min, injection volume: 20 μl, internal standard: canthaxanthin (1.5 μg/ml) and detection: 350–550 nm.

2.6. Peak identification and quantification

Peak identification of free pigments was based on the comparison of retention times, spectra in HPLC with those of known standards, confirmed by sample supplementation with the available standards capsanthin, capsorubin, β-carotene (*cis*, *trans*) and zeaxanthin, and by comparison with separation patterns reported by other authors [10,14,16–18]. Peak identification of esterified pigments was based on the comparison of spectra in HPLC with free standard pigments, and also with separation patterns reported by other authors [2,19–23].

Canthaxanthin was used as internal standard. In order to carry out quantitative analyses of the data, a chromatogram of maximum absorbances at each elution time was recorded. The relative detector responses (area ratios) of capsanthin, capsorubin and β-carotene with respect to canthaxanthin were determined. The slope of the curve (response factor) was used for the calculations. *cis*-Capsanthin and capsanthin esters were calculated as capsanthin. Capsorubin esters were calculated as capsorubin. *cis*-β-Carotene was calculated as β-carotene. Unidentified pigments, free and esters, anteraxanthin, violaxanthin, zeaxanthin+lutein, cryptocapsin and β-cryptoxanthin were calculated as β-carotene.

2.7. Statistics

All data are presented as group mean values±standard error of the means (S.E.M.s) of five different determinations. Differences between samples were assessed by one-way variance (ANOVA). The analyses were performed with DBASE IV and SPSS-X packages on a VAX8820 computer.

3. Results and discussion

Carotenoid determination of saponified or non-saponified Sobrassada extracts have been carried out on a reversed-phase (C_{18}) column. The chromatograms shown in Fig. 1 correspond to non-saponified (a) and saponified (b) Sobrassada extract. The peak assignation is shown in Table 1. When we take into consideration both chromatograms (saponified and non-saponified) 44 peaks can be resolved and classified in three different groups: (a) carotenoid peaks present in both saponified and non-saponified extracts (simple carotenoids), (b) carotenoid peaks present in non-saponified extracts and absent in saponified extracts (esterified carotenoids), and (c) carotenoid peaks present in saponified extracts and absent in non-saponified extracts (carotenoids naturally present only in the esterified form and/or obtained during saponification). Two kinds of esterified carotenoid peaks can also be differentiated in function of the elution time: (a) esterified carotenoid peaks eluting before β-carotene, and (b) esterified carotenoid peaks eluting after β-carotene. The first kinds of esterified carotenoid presents some non-esterified hydroxyl groups. The saponification treatment of the carotenoid extracts hydrolyses all carotenoid esters and allows the detection of some simple carotenoids such as cryptocapsin and other minority non identified peaks which were not detected in the non-saponified extract.

For quantification we used canthaxanthin as the internal standard and individual response factors were obtained using purified capsanthin, capsorubin and β-carotene standards, respectively. The relative standard deviation (R.S.D.) – about 5% – was similar to other methods described in literature [14,19]. The carotenoid content of Sobrassada consisted approximately of 68% capsanthin, 7% cap-

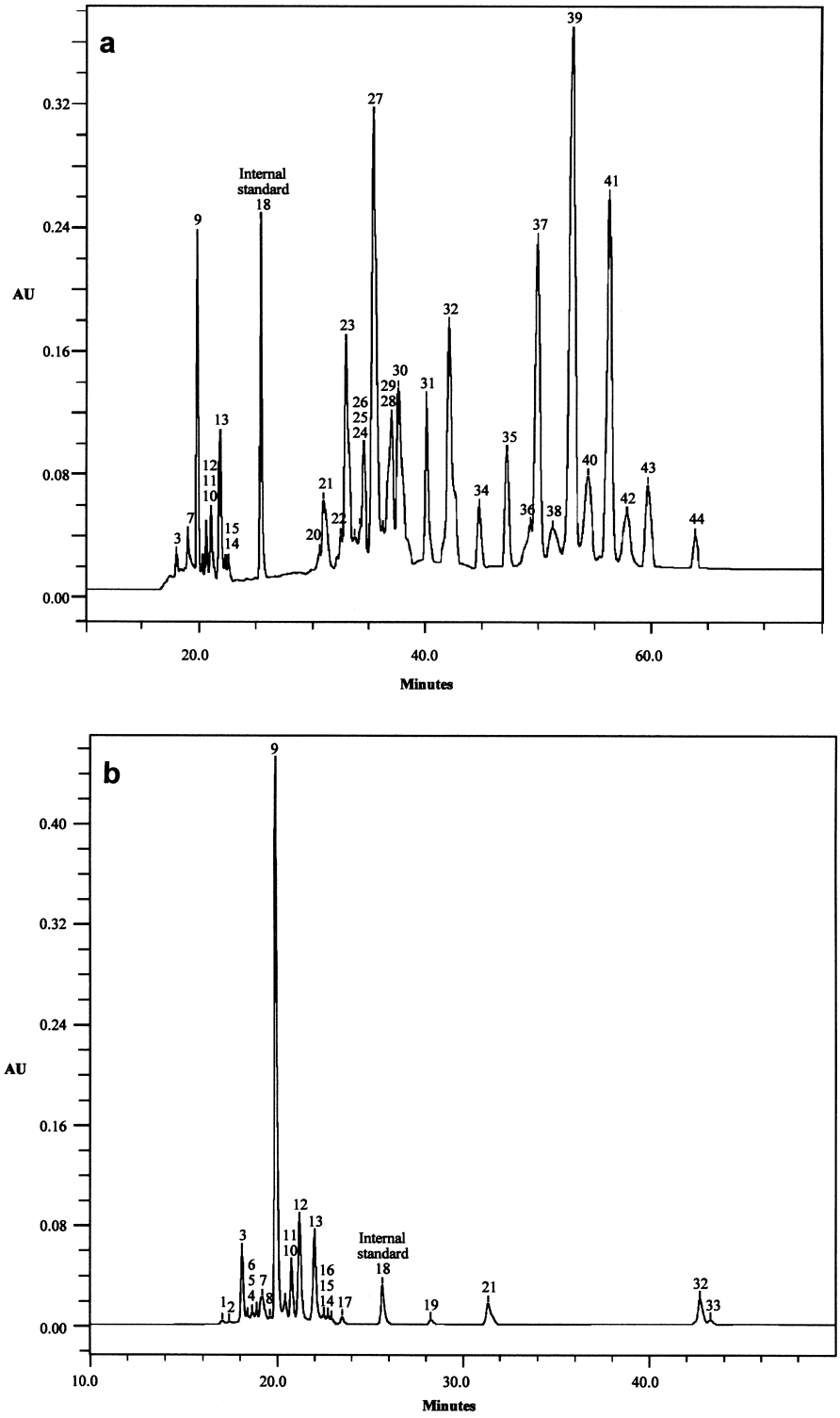

Fig. 1. Reversed-phase HPLC separation of (a) Sobrasada extract and (b) saponified Sobrasada extract in a Spherisorb C_{18} column, at maximum absorbances at each point in time. For peak numbers, see Table 1.

Table 1
Carotenoid composition of non-saponified and saponified Sobrassada extract and retention time for each pigment resolved

Peak No.	Pigment	t_R^a (min)	Carotenoid composition (μg/g d.w.)[b]	
			Non-saponified extract	Saponified extract
1	Unidentified free 1	17.1	n.d.	0.26±0.02
2	Unidentified free 2	17.4	n.d.	0.25±0.02
3	Capsorubin	18.1	0.29±0.04	7.4±0.4
4	Unidentified free 3	18.4	n.d.	0.49±0.06
5	Unidentified free 4	18.6	n.d.	0.65±0.04
6	Unidentified free 5	18.9	n.d.	0.67±0.03
7	Violaxanthin	19.2	0.41±0.09	2.2±0.1
8	Unidentified free 6	19.6	n.d.	0.36±0.03
9	Capsanthin	19.9	6.8±0.3	60±3
10	Anteraxanthin	20.4	0.13±0.02	1.5±0.1
11	*cis*-Capsanthin	20.8	1.1±0.1	7.7±0.5
12	Unidentified free 7	21.2	0.74±0.06	5.7±0.3
13	Lutein and zeaxanthin	22.0	1.7±0.1	5.0±0.3
14	Unidentified free 8	22.5	0.12±0.03	0.44±0.04
15	Unidentified free 9	22.7	0.12±0.03	0.48±0.05
16	Unidentified free 10	22.9	n.d.	0.18±0.07
17	Unidentified free 11	23.5	n.d.	1.1±0.1
18	Cantaxanthin, I.S.	25.8		
19	Cryptocapsin	28.4	n.d.	0.65±0.02
20	Unidentified monoester 1	30.8	0.15±0.07	n.d.
21	β-Cryptoxanthin	31.5	1.3±0.1	1.8±0.1
22	Capsorubin monoester	32.6	0.44±0.12	n.d.
23	Capsanthin monoester 1	33.0	10±1	n.d.
24	Unidentified monoester 2	33.9	0.24±0.10	n.d.
25	Unidentified monoester 3	34.3	0.33±0.10	n.d.
26	Capsanthin monoester 2	34.6	4.9±0.4	n.d.
27	Capsanthin monoester 3	35.5	22±1	n.d.
28	Unidentified monoester 4	36.5	0.30±0.13	n.d.
29	Capsanthin monoester 4	37.1	8.1±0.4	n.d.
30	Lutein–zeaxanthin monoester 1	37.7	4.1±0.3	n.d.
31	Lutein–zeaxanthin monoester 2	40.3	2.3±0.1	n.d.
32	β-Carotene	42.8	5.1±0.2	2.3±0.1
33	*cis*-β-Carotene	43.4	n.d.	0.31±0.03
34	Capsanthin diester 1	45.0	2.2±0.1	n.d.
35	Capsorubin diester	47.4	3.6±0.1	n.d.
36	Unidentified diester 1	49.5	0.72±0.11	n.d.
37	Capsanthin diester 2	50.2	15±0.3	n.d.
38	Unidentified diester 2	51.5	0.74±0.33	n.d.
39	Capsanthin diester 3	53.3	30±1	n.d.
40	Unidentified diester 3	54.8	2.7±0.2	n.d.
41	Capsanthin diester 4	56.7	20±0.3	n.d.
42	Unidentified diester 4	58.4	1.4±0.1	n.d.
43	Capsanthin diester 5	60.2	4.6±0.2	n.d.
44	Unidentified diester 5	64.6	0.54±0.06	n.d.

[a] Retention time of each peak.
[b] Data are given as mean±standard error of the mean (n=5, n.d.: not detected).

sorubin, 5% lutein+zeaxanthin, 3% β-carotene, 2% violaxanthin, 1.8% β-cryptoxanthin, 1.5% anteraxanthin, 0.6% cryptocapsin, and 10% unidentified pigments, although these percentages varied depending on the effects of extract saponification (see Table 2). The xanthophylls mainly occur in the esterified

Table 2
Saponification effect on some carotenoid quantification of Sobrassada and powdered paprika

Pigment (μg/g d.w.)	Sobrassada			Powdered paprika		
	Non-saponified extract	Saponified extract	%[b]	Non-saponified extract	Saponified extract	%[b]
Capsanthin total	125±2	68±3[†]	46±2	1117±18	873±15[‡]	22±3[#]
Capsorubin total	4.3±0.2	7.4±0.4[†]	−73±12	49±1	92±1[‡]	−89±4
β-Carotene total	5.1±0.2	2.6±0.2[‡]	49±3	51±1	27±1[‡]	48±1
β-Cryptoxanthin[a]	1.3±0.1	1.8±0.1[‡]	−44±13	11±1	20±0.4[‡]	−82±10
Zeaxanthin+Lutein total[a]	8.1±0.3	5.0±0.3[†]	39±2	40±1	82±0.2[‡]	−105±6[#]

[a] Quantified with the response factor of the β-carotene as μg of pigment/g of dry weight.
[b] Percentage of the difference between non-saponified extract and saponified extract.
Significant differences: * $P<0.05$, [†] $P<0.01$, [‡] $P<0.001$ non-saponified extract versus saponified extract and [×] $P<0.05$, [°] $P<0.01$, [#] $P<0.001$ powdered paprika versus Sobrassada percentage.

form (93% of capsanthin and capsorubin, and 78% of zeaxanthin+lutein). The red pepper carotenoid composition is influenced by several factors such as fruit ripening [21,23] and variety [17,24]. The semi-quantitative Sobrassada carotenoid composition is in accordance with the powdered paprika variety composition used for Sobrassada elaboration and also with a low influence of Sobrassada ripening on their individual carotenoid composition.

Rapid and advanced spectra analysis provided by photodiode array system makes the identification of carotenoid pigments easier. During analysis, chromatographic software acquires data covering the spectra range between 350 nm and 550 nm. This detection method is able to quantify each peak at its own maximum absorption. At the same time, it is possible compare peak spectra with others in order to identify simple carotenoids and their ester derivatives, and also the retention time correspondent to each peak in the saponified or non-saponified chromatogram. In addition, the chromatogram and spectra of suitable carotenoid standards allow the peak assignation to be confirmed and to serve as an indication of the position of other carotenoids described in the literature. It is noticeable that from the spectra of free carotenoids we can identify esterified forms of the same carotenoid in some cases. In fact, we have been able to verify that physalien, which correponds to the last chromatogram peak at retention time of 64.6 min (zeaxanthin dipalmitate obtained from Extrasynthese, Lyon, France) presents the same spectrum as free zeaxanthin, which elutes at 22.0 min. This is advantageous because the acid moiety of carotenoid esters is variable [10,14] therefore it can also change the retention time of carotenoid esters. Five capsanthin diesters and four capsanthin monoesters were identified. However, some esters of capsorubin, zeaxanthin, lutein and β-cryptoxanthin could not be identified, as indicated by their higher levels in saponified than in non-saponified extracts (Table 2). With the present method, capsorubin, zeaxanthin+lutein and β-cryptoxanthin were underestimated in the non-saponified samples, because some esters of these carotenoids were not able to be identified. In this case, in order to use the method with non-saponified samples it would be necessary to obtain individual carotenoid ester standards in order to identify the correspondent chromatogram peaks.

On the other hand, saponification of carotenoid extracts underestimated the individual carotenoid content of the foods, as shown in Table 2. The value of carotenoid content in the non-saponified extract was calculated from the sum of correspondent individual free and esterified carotenoids. These effects of saponification on carotenoid stability were characteristic of each individual carotenoid and also of food type. Capsorrubin and β-cryptoxanthin increased (negative values) by saponification of the extract as indicated above. In powdered paprika the loss of capsanthin by saponification was significantly lower than in Sobrassada, while β-carotene loss was similar in both food types. It is noticiable that we detect a loss of zeaxanthin+lutein by saponification in Sob-

rassada while in powdered paprika saponification produces a high increase of its determination. This different pattern can be explained if we take into account both a high distribution of zeaxanthin and lutein between different forms of esters at low concetration even in paprika, together with a higher rate of loss of carotenoids in Sobrassada than in paprika.

The conditions of saponification used in the present paper are representative of general conditions (see Refs. [11,14]). The methods used for carotenoid determination usually have a saponification step and are applied to the determination of carotenoids in very high carotenoid content samples, such as red peppers, paprika [14], or leaves [2]. These papers do not describe a loss of carotenoids by saponification [2,14], however some loss of total carotenoid content has been described in paprika [19].

Many differences could be explained by taking into account that the loss of carotenoid during saponification depends on the food type. Our results show that the loss of carotenoid content is higher in Sobrassada than in paprika. In accordance with Baranyai et al. [19], a higher loss can be expected in fatty-foods and after industrial or storage processes than in non-fat foods or fresh food. The lack of suitable standards does not allow the vitamin A activity of the samples to be calculated, however in paprika have been described only six carotenoids precursors of vitamin A, that is β-carotene, β-cryptoxanthin, cryptocapsin, mutatochrome, α-carotene and β-carotene 5′,6′-monoepoxide [5]. The effects of saponification on the determination of these individual carotenoids, lead us to conclude that the methods using saponification to determine vitamin A content underestimate the vitamin A content in a fatty ripened food, Sobrassada. The method proposed in this case is to determine β-carotene content, the main active provitamin A pigment, without extract saponification. But the other provitamin A precursors detected, cryptocapsin and β-cryptoxanthin have to be determined in a saponified extract because in the non-saponified extract their real value can be underestimated. However, the results can be improved by the use of cryptocapsin and β-cryptoxanthin esters as standards, in which case the method could be applied without saponification.

Acknowledgements

This work was supported by grants PB92-0748 C04-01 and C04-02, and PB94-1178 from Spanish Government (DGICYT), and grant from Conselleria d'Agricultura, Pesca i Alimentació of Balearic Island Government.

References

[1] O. Isler (Ed.), Carotenoids, Birkhäuser Verlag, Basel, 1971, p. 11.
[2] J. de Las Rivas, J.C.G. Milicua, R. Gomez, J. Chromatogr. 585 (1991) 168.
[3] A.R. Mangels, J.M. Holden, G.R. Beecher, M.R. Forman, E. Lanza, J. Am. Diet. Assoc. 93 (1993) 284.
[4] J.K. Chug-Ahuja, J.M. Holden, M.R. Forman, A.R. Mangels, G.R. Beecher, E. Lanza, J. Am. Diet. Assoc. 93 (1993) 318.
[5] J.C. Bauernfeind, J. Agric. Food Chem. 20 (1972) 456.
[6] WHO, WHO/UNICEF/ Helen Keller International/IVAGG meeting, Technical Report Series No. 672, 1982.
[7] H. Gerster, Int. J. Vit. Nutr. Res. 61 (1991) 277.
[8] I. Jialal, E.P. Norkus, L. Cristal, S.M. Grundy, Biochim. Biophys. Acta 1086 (1991) 134.
[9] T. Philip, W.W. Nawar, F.J. Francis, J. Food Sci. 36 (1971) 98.
[10] C. Fisher, J.A. Kocis, J. Agric. Food Chem. 35 (1987) 55.
[11] G. Britton, Methods Enzymol. 111 (1985) 113.
[12] P.M. Bramley, Phytochem. Anal. 3 (1992) 97.
[13] J. Folch, M. Lees, G.H. Sloane, J. Biol. Chem. 226 (1957) 497.
[14] G.K. Gregory, T.-S. Chen, T. Philip, J. Food Sci. 52 (1987) 1071.
[15] G. Britton, in: B.V. Charlwood, D.V. Banthorpe (Eds.), Methods in Plant Biochemistry, Vol. 7, Academic Press, London, 1991, pp. 473.
[16] B. Czinkotai, H.G. Daood, P.A. Biacs, F. Hajdu, J. Liq. Chromatogr. 12 (1989) 2707.
[17] P.A. Biacs, H.G. Daood, T.T. Huszka, P.K. Biacs, J. Agric. Food Chem. 41 (1993) 1864.
[18] P.A. Biacs, H.G. Daood, J. Plant Physiol. 143 (1994) 520.
[19] M. Baranyai, Z. Matus, J. Szabolcs, Acta Aliment. 11 (1982) 309.
[20] Z. Matus, J. Deli, J. Szabolcs, J. Agric. Food Chem. 39 (1991) 1907.
[21] J. Deli, Z. Matus, J. Szabolcs, J. Agric. Food Chem. 40 (1992) 2072.
[22] M.I. Mínguez-Mosquera, D. Hornero-Méndez, J. Agric. Food Chem. 41 (1993) 1616.
[23] M.I. Mínguez-Mosquera, D. Hornero-Méndez, J. Agric. Food Chem. 42 (1994) 38.
[24] M.J. Reeves, J. Food Sci. 52 (1987) 1047.