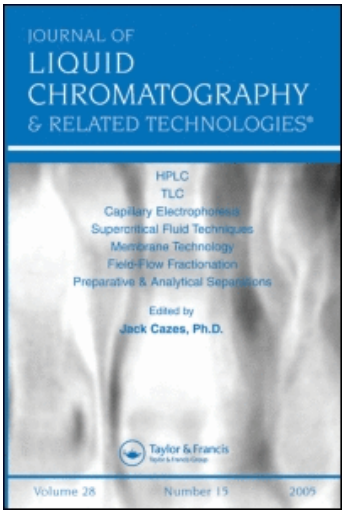

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An HPLC Method for the Analysis of Orange Color in Food Using β-Cryptoxanthin as an Indicator

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An HPLC Method for the Analysis of Orange Color in Food Using β-Cryptoxanthin as an Indicator

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ABSTRACT

An HPLC method for the analysis of orange color in food using β-cryptoxanthin as an indicator was established. Orange color was extracted from

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food samples with ether, and after evaporation of the extract, the residue was dissolved in methanol, to which 5% sodium hydroxide–methanol was then added. The mixture was occasionally stirred, then allowed to stand for 24 hr at room temperature, in a tightly sealed container shielded from light. Subsequently, distilled water was added and the pH of the mixture was adjusted to be below 4.5 using hydrochloric acid. It was then purified with a C18 cartridge before being subjected to HPLC analysis. The HPLC conditions were as follows: column, Tosoh TSK gel ODS-80Ts (5 μm, 4.6 × 150 mm); column temperature, 45°C; mobile phase, acetone–water (6 : 1); flow rate, 1.2 mL/min; detection wavelength, 460 nm. The average recoveries of the orange color were over 73.9% from juice and jelly fortified at the concentrations of 2–100 μg/g. The coefficients of variation were 10.7% or less. Twenty-two commercially available foods such as juice, candy, jelly, sherbet, and cake that had a label stating the use of orange color were analyzed, and the detected β-cryptoxanthin concentrations ranges from 0.011 to 0.275 μg/g.

Key Words: β-Cryptoxanthin; HPLC method; Orange food color; Saponification.

INTRODUCTION

Orange color is a natural food additive extracted from the fruit or rind of orange (*Citrus sinensis*) and the yellow-orange color is derived from β-cryptoxanthin and its esters, such as lauric and myristic acids (Fig. 1).[1–7] Commercially available orange colors are known to have different compositions of these color components depending on the material the orange color is extracted from, which makes the determination of orange color based on the analysis of the color components impossible, causing difficulty in developing a simple, rapid, and reliable method for the analysis of the orange color in foods.

In our previous study,[8] we developed a TLC method for the simultaneous analysis of carotenoid colors, including annatto extract, orange

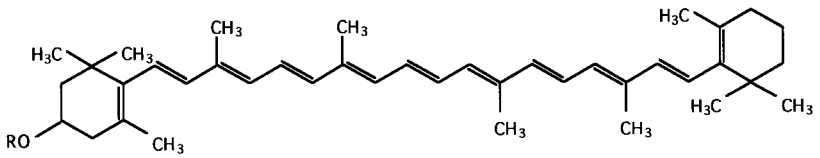

Figure 1. Structures of β-cryptoxanthin and its ester. β-Cryptoxanthin: R = H, β-cryptoxanthin palmitate: R = palmitoyl.

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color, gardenia yellow, paprika color, tomato color, marigold color, and β-carotene extracted from various foods, in which the extracted orange color was saponified and then purified using a C18 cartridge before being identified by reversed phase TLC/scanning densitometry. The main product of the saponification was identified as β-cryptoxanthin, which will be described later.

In the present study, an HPLC method for the analysis of orange color using β-cryptoxanthin, which is a main product of saponification, as an indicator was investigated.

EXPERIMENTAL

Samples

Foods available on the Japanese market, including juice, candy, jelly, sherbet, and cake were used.

Standards and Chemical Reagents

β-Cryptoxanthin from Extrasynthese (Lyon, France) was used as the β-cryptoxanthin standard, and orange color from San-Ei Gen FFI (Osaka, Japan) was used as the orange color standard. The C18 cartridges used in the study were Sep-Pak Vac C18 3cc (500 mg) from Waters (Milford, MA). All the other reagents were of analytical grade from Wako (Osaka, Japan) and Kanto Kagaku (Tokyo, Japan).

Analytical Conditions

TLC

The TLC plate was an RP-18F254S (Art. 15423, E. Merck, Darmstadt, Germany), and the solvent systems were (I) acetonitrile–acetone–*n*-hexane (11 : 7 : 2) and (II) acetone–water (9 : 1).

HPLC

The HPLC used in the study was from Hitachi, Ltd. (Tokyo, Japan): Detector, L-4200 UV-VIS; column oven, L-7300; autosampler, L-7200: pump, L-6000; integrator, D-2500. The HPLC conditions were as follows: column, TOSOH TSK gel ODS-80Ts (5 μm, 4.6 × 150 mm); column

temperature A, 40°C; column temperature B, 45°C; mobile phase A, acetonitrile–acetone–*n*-hexane (11 : 7 : 2); mobile phase B, acetone–water (6 : 1); flow rate, A: 0.8 mL/min, B: 1.2 mL/min; detection wavelength, 460 nm. The calibration curve was linear over the range of 0.05–40 μg/mL for β-cryptoxanthin.

LC/MS

The LC/MS used in the study was an Quattro II triple quadrupole tandem mass spectrometer (Micromass UK LTD., Altrincham, UK) equipped with a Z-spray API source. The LC/MS conditions were as follows: column, TOSOH TSK gel ODS-80Ts (5 μm, 4.6 × 150 mm); column temperature, 45°C; mobile phase, acetone–water (6 : 1); flow rate, 1.2 mL/min (sprit ratio = 1 : 5); ion source, ESI+; capillary voltage, 3.0 kV; cone voltage, 20 kV; ion source temperature, 100°C; desolvation temperature, 250°C.

Scanning Densitometer

The scanning densitometer used in the study was a CS-9000 from Shimadzu (Kyoto, Japan). The measurement conditions were as follows: wavelength scanning range, 370 − 700 nm; slit size, 0.4 × 0.4 mm^2; method, reflecting absorption.

Preparation of Test Solutions

A 100 mL of water was added to an aliquot of the sliced and homogenized food samples (150 g for juice, 50 g for jelly, and 20 g for candy, sherbet, and cake) and was blended with 30 mL of ether three times using a high speed blender. The sample was then centrifuged. The supernatant was evaporated to dryness under reduced pressure at 35°C and the residue was dissolved in 20 mL of methanol. It was then saponified in the following manner: after adding 2 mL of 5% sodium hydroxide (NaOH)–methanol solution, the mixture was placed in a tightly stoppered container, and allowed to stand for 24 hr at room temperature, occasionally stirred, and kept away from light. Subsequently, 20 mL of water was added and the pH of the mixture was adjusted to less than 4.5 using 1 mol/L hydrochloric acid. The mixture was loaded onto a C18 cartridge that had been activated with methanol and water (5 mL each) in advance. The cartridge was then washed with 10 mL of water, and the colors were eluted with 5 mL of acetone, and collected in a 5 mL volumetric flask. The obtained test solution was subjected to an HPLC analysis.

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RESULTS AND DISCUSSION

Saponification Conditions of Orange Colors

When an orange color standard before saponification was separated by reversed phase TLC, a number of overlapping spots were observed, and a satisfactory separation could not be obtained [Fig. 2(I)A and (II)A]. This was probably caused by containing a large number of esters of β-cryptoxanthin in the orange color. It is known that orange color is hydrolyzed into carotenoid and a fatty acid by saponification under mild conditions.[1,2] Thus, a orange color standard after saponification was subjected to TLC. The orange color standard after saponification was satisfactorily separated into a main spot having R_f value of 0.37 in the solvent system of acetonitrile–acetone-*n*-hexane (11 : 7 : 2) [Fig. 2(I)B] and a main spot having R_f value of 0.36 in the solvent system of acetone–water (9 : 1) [Fig. 2(II)B]. The main spot gave the same R_f value, color, and shape as the spot of the β-cryptoxanthin standard [Fig. 2(I)C and (II)C].

As described above, it was suggested that the orange color is hydrolyzed into carotenoid and a fatty acid by saponification under mild conditions. Next, the saponification conditions were carefully investigated, because we have not optimized the conditions in the previous study.[8] Various volumes (0.1, 0.2, 1.0, 2.0, 5.0, 7.0, and 10 mL) of 5% NaOH–methanol solution were added

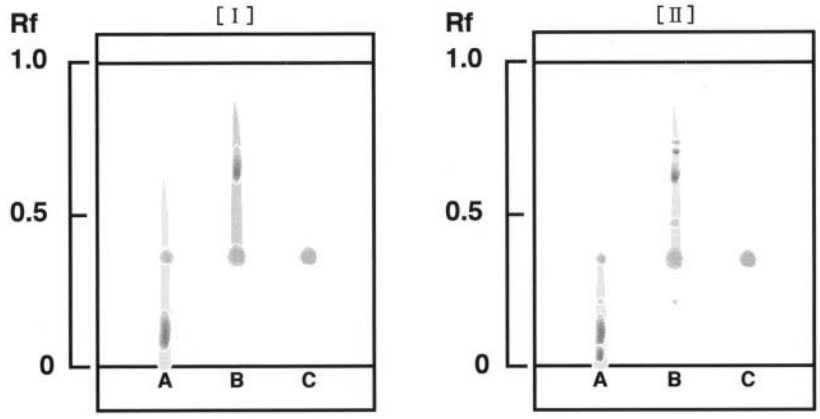

Figure 2. TLC chromatograms of orange color with and without saponification and β-cryptoxanthin. Plate: RP-18 F254S TLC (E. Merck, Art. 15423), solvent system (I): acetonitrile–acetone–*n*-hexane (11 : 7 : 2), and solvent system (II): acetone–water (9 : 1). A: Orange color before saponification, B: orange color after saponification, and C: standard of β-cryptoxanthin.

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to the orange color standard methanol solution (100 mg/100 mL) and the mixtures were placed in tightly sealed containers, and allowed to stand for 0.5, 1, 3, 6, 16, and 24 hr at room temperature, occasionally stirred and kept away from light, for saponification. The progress of saponification was observed by purification of the saponified orange color using a C18 cartridge followed by reversed phase TLC. When 0.2 mL of 5% NaOH–methanol solution was added, no change was observed by TLC even 24 hr after the initiation of saponification. Likewise, no change was observed when the mixture was allowed to stand for 3 hr even when 10 mL of 5% NaOH–methanol solution was added. Only slight and unclear changes in the spots were observed when the mixture was allowed to stand for 6 hr with 5% NaOH–methanol solution being added in an amount of over 1 mL, or when it was allowed to stand for 16 hr with 1 mL of 5% NaOH–methanol solution being added. In contrast, when the mixture was allowed to stand for 16 hr with 2 mL or more of 5% NaOH–methanol solution being added, or when it was allowed to stand for 24 hours with 1 mL or more of 5% NaOH–methanol solution being added, the spots were satisfactorily separated into a main spot and subsidiary spots, as shown in Fig. 2(I)B and (II)B.

Identification of the Main Product After Saponification of Orange Color

TLC/Scanning Densitometry

The separated spots of the saponified orange color standard on the TLC plates were then subjected to scanning densitometry. The visible absorption spectra were measured in the range of a 370–700 nm scanning wavelength, and excellent visible absorption spectra were obtained. The spectrum of the main spot of the orange color after saponification showed its maximum absorption wavelength at 455 nm, which identically matches the spectrum of the β-cryptoxanthin standard.

HPLC

Figure 3 shows chromatograms of the orange color before saponification, after saponification, and of the β-cryptoxanthin standard obtained by subjecting them to HPLC under the conditions described in Experimental section. As shown in Fig. 3A(1), the orange color standard before saponification showed a number of peaks in mobile phase A, while the orange color standard after saponification showed only one peak at the retention time of 4 min as shown in Fig. 3A(2). Thus, it is considered that sufficient saponification of the orange

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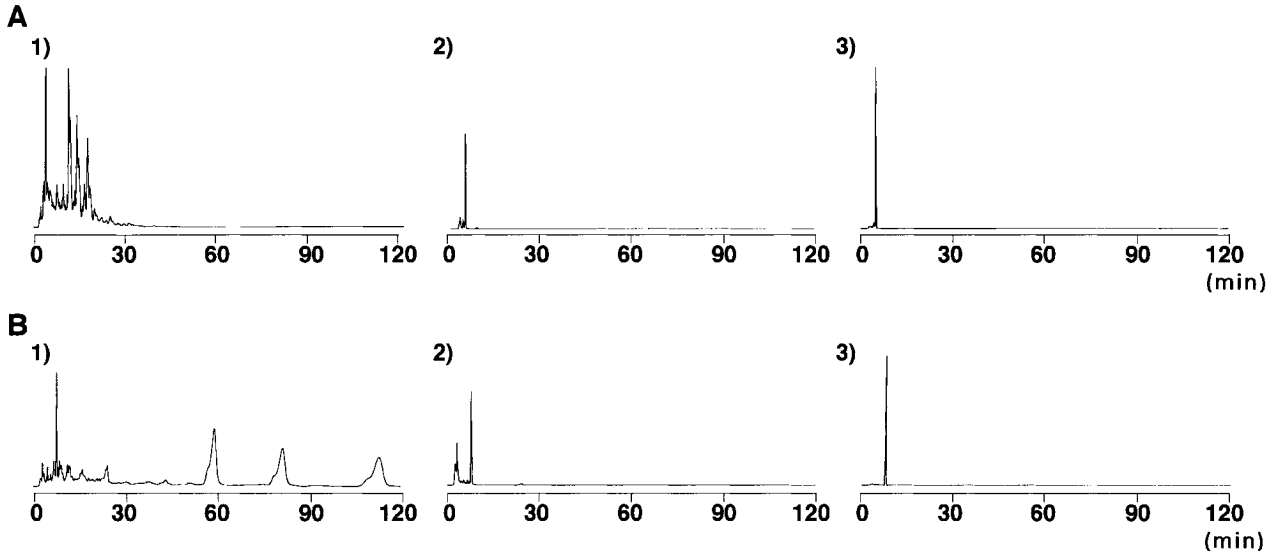

Figure 3. HPLC chromatograms of orange color with and without saponification and β-cryptoxanthin. Column: Tohsoh TSK gel ODS-80Ts (5 μm, 4.6 × 150 mm), column temperature A: 40°C, column temperature B: 45°C, detection: 460 nm, mobile phase A: acetonitrile–acetone–*n*-hexane (11 : 7 : 2), mobile phase B: acetone–water (6 : 1), flow rate A: 0.8 mL/min, and flow rate B: 1.2 mL/min. (1) Orange color before saponification, (2) orange color after saponification, and (3) standard of β-cryptoxanthin.

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color was carried out. The obtained product by saponification showed the same retention time as the β-cryptoxanthin standard as shown in Fig. 3A(3), however, the retention time was as short as 4 min. Therefore, another HPLC analysis was performed using mobile phase B so that the peak of the β-cryptoxanthin standard would appear at the retention time of around 7 min.

In mobile phase B, as shown in Fig. 3B(1), the orange color standard before saponification showed multiple peaks at the retention times between 2 and 110 min. In contrast, as shown in Fig. 3B(2), the chromatogram of the orange color after saponification showed its main peak clearly at the retention time of 7 min. The main peak was confirmed to be the main spot on the TLC described above by isolation of the saponified product. Moreover, the retention time of this peak completely agreed with that of the β-cryptoxanthin standard as shown in Fig. 3B(3).

LC/MS

The orange color standard, after saponification, and the β-cryptoxanthin standard were analyzed under the LC/MS conditions described in the Experimental section. Figure 4 shows the mass spectrum of the main peak of the orange color after saponification, taken at the top of the peak at the retention time of 7 min. $[M]^{+\cdot}$ was clearly observed at m/z 552, respectively, and the mass spectrum was found to be identical with the spectrum of the β-cryptoxanthin standard.

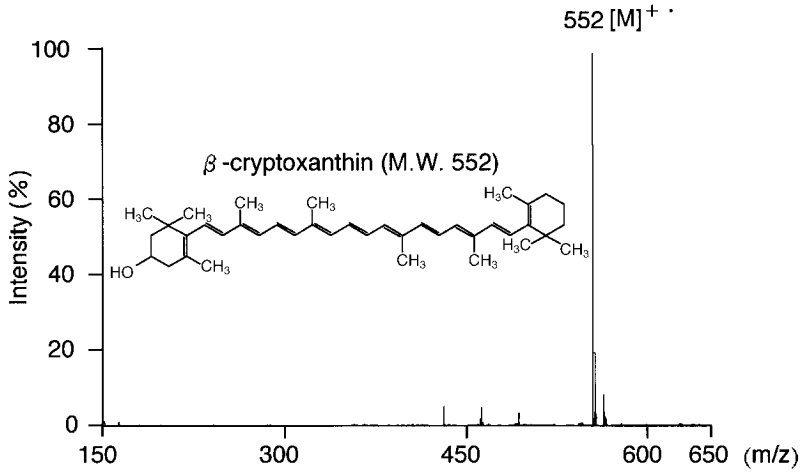

Figure 4. Mass spectrum of orange color with saponification under LC/MS conditions.

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Accordingly, the main product obtained after saponification of the orange color was confirmed to be β-cryptoxanthin.

β-Cryptoxanthin Obtained from the Orange Color Standard

Correlation and Reproducibility

The orange color standard was dissolved in 20 mL of methanol in the amounts of 0.1, 0.2, 0.5, 1, 2.5, 5, 10, and 20 mg. The β-cryptoxanthin concentrations in five samples for each concentration (40 samples in total) were determined under the HPLC conditions (mobile phase B) described in the Experimental section. These results are shown in Fig. 5. When the orange color concentration varied in the range of 0.005–1.000 mg/mL, the β-cryptoxanthin concentration was found to range from 0.05 to 16.93 μg/mL. A satisfactory correlation was obtained between the orange color concentration and the β-cryptoxanthin concentration with the coefficient of correlation being 0.999. In addition, excellent reproducibility with the coefficient of variation being 9.0% or less was obtained for each of the orange color concentrations.

Stability

The stability of β-cryptoxanthin obtained from the orange color standard was studied. Test solutions were prepared in the same manner as described in the Experimental section, using methanol solutions containing the orange

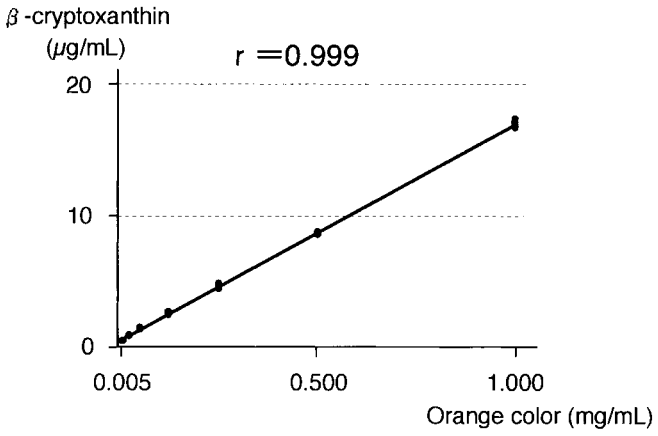

Figure 5. Correlation between orange color and β-cryptoxanthin concentrations.

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color in the amount of 0.005–1.000 mg/mL. The β-cryptoxanthin concentrations in the test solutions were determined by HPLC. The remaining refrigerated β-cryptoxanthin concentrations at −20°C, for each storage time with respect to the β-cryptoxanthin concentration immediately after purification (100%), were represented as percentages (Table 1). When the storage time was 6 hr, the remaining β-cryptoxanthin concentration was over 91% at any concentration, and over 79% even when the storage time was as long as 5 days. Thus, an excellent stability was obtained.

Application to Commercially Available Foods

Next, the present method was applied to the analysis of orange color in commercially available foods, since the above experimental results suggest that the method may be satisfactorily applicable to an analysis of the orange color in foods.

Recovery Test of Orange Color

Juice and jelly were selected as typical foods containing the orange color. After confirming that no orange color was detected in any of the foods, a recovery test was performed in the following manner. An orange color standard was added to each sample to make the concentration shown in Table 2. The orange color concentrations were determined five times, at each concentration level, from the β-cryptoxanthin concentrations as described in the Experimental section. Table 2 shows the average recoveries of the

Table 1. Stability of β-cryptoxanthin obtained from orange color.

Orange color (mg/L)	Remaining β-cryptoxanthin (%)						
	0 hr	6 hr	24 hr	48 hr	72 hr	96 hr	120 hr
			($n = 5$)				
0.005	100	91	89	88	86	80	79
0.010	100	92	91	87	87	87	87
0.025	100	93	92	92	90	90	88
0.050	100	98	92	91	90	88	85
0.100	100	97	96	96	91	91	92
0.250	100	99	98	96	94	93	92
0.500	100	98	97	95	93	92	92
1.000	100	95	95	94	91	91	91

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Table 2. Recovery of orange color from various foods.

Fortified concentration (μg/g)	Juice recovery (%) (CV, %)	Fortified concentration (μg/g)	Jelly recovery (%) (CV, %)
	($n = 5$)		
3.5	83.2 (9.8)	2.0	73.9 (10.7)
7.5	82.1 (3.2)	5.0	74.9 (10.0)
15.0	85.0 (9.9)	10.0	85.6 (9.9)
30.0	92.9 (3.4)	20.0	83.8 (6.0)
		50.0	80.7 (3.7)
		100.0	86.3 (2.2)

orange color from the various foods, with respect to the β-cryptoxanthin concentration obtained from the orange color standard (100%) and the coefficients of variation. The average recoveries from the juice and jelly were over 82.1% and 73.9%, respectively, and their coefficients of variation were 9.9% and 10.7% or less, respectively. Thus, excellent recoveries and reproducibility were obtained.

Analytical Results of Orange Color in Commercially Available Foods

The orange color concentrations in 22 commercially available foods that had a label stating the use of orange juice as orange color were determined three times each from the β-cryptoxanthin concentrations. The recoveries and coefficients of variation are shown in Table 3 and their typical chromatograms are given in Fig. 6. The detected β-cryptoxanthin concentrations were as follows: 0.025–0.119 μg/g from nine samples of juice, 0.011–0.048 μg/g from five samples of candy, 0.021–0.039 μg/g from four samples of jelly, and 0.042–0.275 μg/g from three samples of sherbet, and 0.043 μg/g from one sample of cake.

The coefficients of variation were 10.2% or less; thus, an excellent reproducibility was obtained. Accordingly, the present method is considered to be applicable to the analysis of orange color in commercially available foods. The limit of determination was 0.002 μg/g as β-cryptoxanthin.

We have developed an HPLC method for the analysis of orange color in food using β-cryptoxanthin as an indicator. β-Cryptoxanthin is obtained as the main product of saponification of the orange color followed by purification using C18 cartridges. Based on the results described above, it was confirmed that the present method is suitable for practical use.

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Table 3. Analytical results of orange color in food on the market.

		Determination[a]	
Food	*n*	Mean (μg/g)	CV (%)
Juice A	3	0.025	4.7
Juice B	3	0.029	3.4
Juice C	3	0.031	1.8
Juice D	3	0.031	4.6
Juice E	3	0.036	4.2
Juice F	3	0.036	5.8
Juice G	3	0.041	7.1
Juice H	3	0.054	8.8
Juice I	3	0.119	4.1
Candy A	3	0.011	10.2
Candy B	3	0.017	9.2
Candy C	3	0.038	1.5
Candy D	3	0.041	8.8
Candy E	3	0.048	2.1
Jelly A	3	0.021	3.4
Jelly B	3	0.022	9.1
Jelly C	3	0.024	4.2
Jelly D	3	0.039	1.5
Sherbet A	3	0.042	5.9
Sherbet B	3	0.045	10.1
Sherbet C	3	0.275	3.0
Cake A	3	0.043	0.0

[a]As β-cryptoxanthin.

CONCLUSIONS

An HPLC method for the analysis of orange color in food using β-cryptoxanthin as an indicator was developed and the following results were obtained.

It was confirmed that the main product obtained by saponification of a orange color standard followed by purification using a C18 cartridge was β-cryptoxanthin.

The β-cryptoxanthin obtained by the saponification of a orange color standard exhibited excellent reproducibility, and a high correlation was obtained between the β-cryptoxanthin and orange color concentrations.

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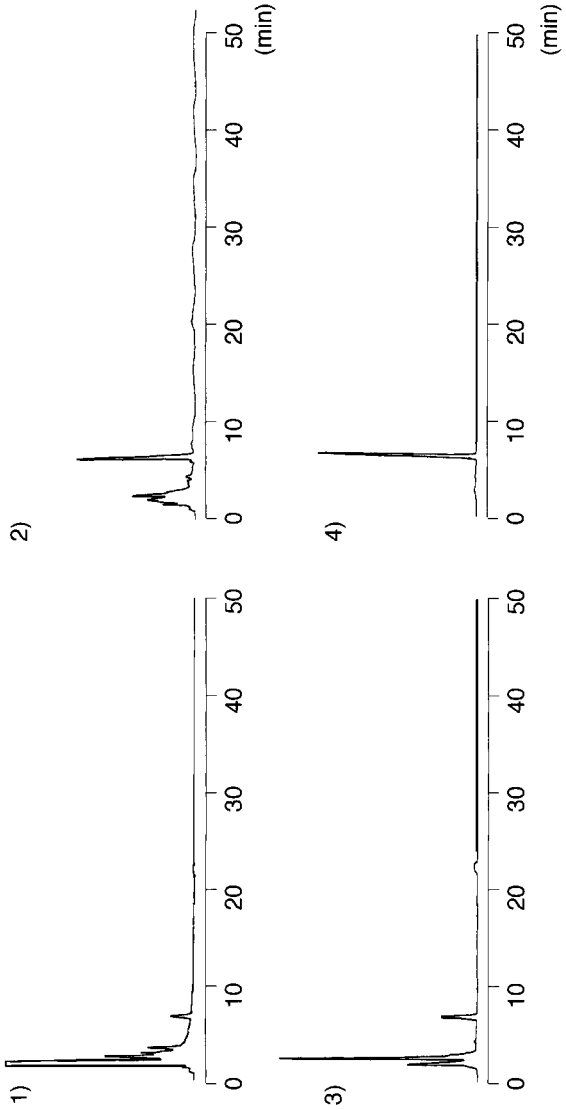

Figure 6. HPLC chromatograms of orange color in foods on the market and β-cryptoxanthin. Column: Tohsoh TSK gel ODS-80Ts (5 μm, 4.6 × 150 mm), column temperature: 45°C, mobile phase: acetone–water (6 : 1), flow rate: 1.2 mL/min, detection: 460 nm. (1) Candy, (2) jelly, (3) juice, and (4) standard of β-cryptoxanthin (1 μg/mL).

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Also, the refrigerated β-cryptoxanthin at -20°C remained stable for as long as 5 days.

The results of the recovery tests of the orange color in typical foods exhibited excellent recoveries and reproducibility. Thus, the present method using β-cryptoxanthin as an indicator for orange color was found to be applicable to an analysis of the orange color in commercially available foods.

Based on these findings, the present method was found to be useful for the quantitative analysis of the orange color in foods.

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