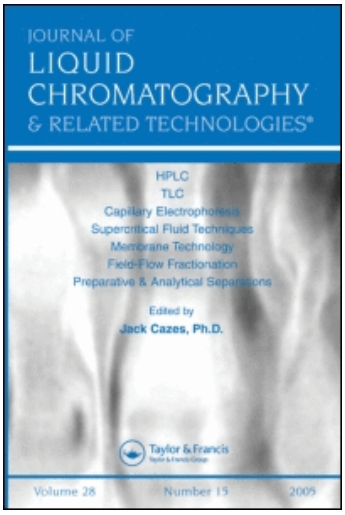

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Simultaneous Analysis of Carotenoid Colorings in Foods by Thin Layer Chromatography

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ABSTRACT

We established a simultaneous analysis method by TLC for carotenoid colorings (annatto extract, orange color, gardenia yellow, paprika color, tomato color, marigold color, and β-carotene) in foods. Reversed phase C_{18} TLC using the solvent systems of acetonitrile–acetone–*n*-hexane

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(11 : 7 : 2) and acetone–water (9 : 1), and normal phase silica gel TLC, using the solvent systems of *n*-hexane–diethyl ether–acetic acid (4 : 1 : 1) and benzene–ethyl acetate–methanol (15 : 4 : 1) yielded well-delineated spots with good separation. These TLCs were applied to the analysis of a total of 294 commercially available foods, and the Rf value of each color spot was evaluated under the four TLC conditions. The difference in the Rf value was slight between each color extracted from the food samples and the standard color, and the coefficient of variation was small, indicating excellent reproducibility. The present method is considered to be useful for the rapid analysis of the carotenoid colorings.

Key Words: Carotenoid colorings; TLC; Annato extract; Orange color; Gardenia yellow; Paprika color; Tomato color; Marigold color; β-carotene.

INTRODUCTION

Natural colorings derived from natural materials have a wider variety than synthetic colorings, so that they are frequently used in foods in Japan.[1] In terms of food sanitation, the establishment of accurate and rapid analysis methods for natural colorings is required. Especially, for the carotenoid colorings that are used in various foods,[2] simple, rapid, and simultaneous analysis methods should be established. Synthetic colors are generally analyzed by TLC, because TLC allows for faster analysis time and the simultaneous analysis of many samples. The establishment of a TLC method for carotenoid colorings may be necessary for rapid response to food sanitary surveillance. We previously reported the analysis methods for the annatto extract, turmeric oleoresin, orange color, gardenia yellow, cochineal color, paprika color, tomato color, marigold color, and lac color using reversed phase TLC with scanning densitometry.[3–8] However, in these methods, the extraction and TLC conditions differed among the colorings, and the rapid analysis of many of these samples was difficult. Furthermore, these methods needed a TLC scanning densitometer to identify the colors.

Therefore, we developed a simple, rapid, and simultaneous analysis method only by TLC without using any special instrumentation for the carotenoid colorings frequently used in Japan (annatto extract, orange color, gardenia yellow, paprika color, tomato color, marigold color, and β-carotene) (Fig. 1). In this paper, we describe a technique for the simultaneous analysis of carotenoid colorings in foods.

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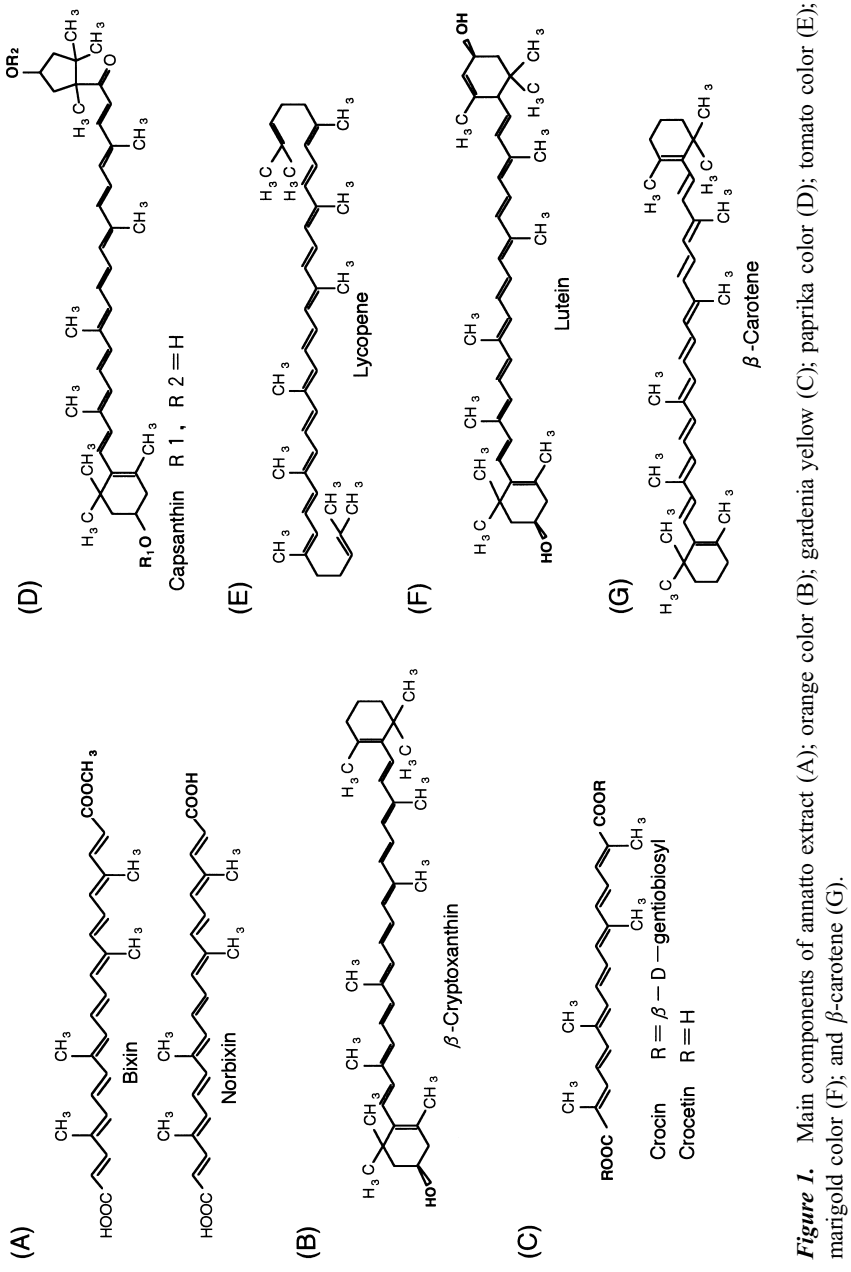

Figure 1. Main components of annatto extract (A); orange color (B); gardenia yellow (C); paprika color (D); tomato color (E); marigold color (F); and β-carotene (G).

EXPERIMENTAL

Samples

Foods available on the Japanese market, including confectionaries, pickles, ice cream/sherbet, seasonings, prepared dishes, refreshing drinks, fish paste foods, meat products, dairy products, and noodles were used. Among them, 26 food items contained the annatto extract, 38 had orange color, 21 had gardenia yellow, 79 had paprika color, 33 had tomato color, 24 had marigold color, and 73 contained β-carotene.

Standards and Reagents

The color standards used were capsanthin [Capsanthin (TLC)], β-carotene, and β-cryptoxanthin from Extrasynthese (Lyon, France), paprika color and bixin from Tokyo Kasei (Tokyo, Japan), crocetin from Sigma (St. Louis, MO), lycopene from Sigma-Aldrich (Steinheim, Germany), lutein from DHI Water and Environment (Hϕrsholm, Denmark), crocin (gardenia yellow) and norbixin (annatto extract) from Wako Pure Chemical Industries (Osaka, Japan), and the orange color, tomato color, and marigold color from San-EiGen F.F.I (Osaka, Japan). The C_{18} cartridges used in this study were Sep-Pak C_{18} Vac, 3 cc (500 mg) from Waters (Milford, MA). All other reagents used were of analytical grade from Wako Pure Chemical Industries and Kanto Chemical (Tokyo, Japan).

TLC Conditions

The TLC plates were RP-18F254S (Art 15389, Merck, Darmstadt, Germany) for TLC conditions (1) and (2) and silica gel 60F254 (Art 5808, Merck) for TLC conditions (3) and (4). The solvent systems were as follows: acetonitrile–acetone–*n*-hexane (11 : 7 : 2) for TLC condition (1), acetone–water (9 : 1) for TLC condition,[2] *n*-hexane–diethyl ether–acetic acid (4 : 1 : 1) for TLC condition,[3] and benzene–ethyl acetate–methanol (15 : 4 : 1) for TLC condition.[4]

Preparation of Test Solutions

Dairy drink samples (50 mL) were mixed with 3 volumes of acetone, stirred, and left for a while. The precipitate was removed and the supernatant (acetone layer) was evaporated. Juice samples (50 mL), other than the dairy drink samples and melted ice cream/sherbet samples (50 g), were used as purchased. The solid samples (50 g) were cut into small pieces. Each sample

was mixed with water or warm water (50 mL), and the colors were extracted with ether (50 mL). When no color was extracted from the samples using ether, and the aqueous layer was still colored, crocin was considered to be present in the sample. Therefore, this aqueous layer was used as the color extract solution and directly purified using C_{18} cartridges without the following saponification. After evaporation of the ether layer, 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 hours at room temperature, occasionally being stirred and kept away from light. Subsequently, 60 mL of water was added and the pH of the mixture was adjusted to 4.5 or less using 1 mol/L hydrochloric acid. The mixture was loaded into a C_{18} cartridge that had been previously activated with methanol and water (5 mL each). The cartridges were washed with 10 mL of water, and the colors were eluted using 20 mL of *n*-hexane, 5 mL of acetone, and 10 mL of methanol in this order. In the hexane fraction of the color solution, *β*-carotene, *β*-cryptoxanthin, bixin, and lycopene were eluted. Capsanthin, crocetin, norbixin, and lutein were eluted in the acetone fraction and crocin in the methanol fraction. The obtained color solutions were concentrated and used as the test solutions.

RESULTS AND DISCUSSION

TLC Conditions

We investigated the TLC conditions using two types of plates (C_{18} chemically bonded silica gel and silica gel) with various combinations and mixing ratios of the following solvents as the solvent systems: acetonitrile, acetone, ethanol, methanol, diethyl ether, petroleum ether, benzene, *n*-hexane, ethyl acetate, and acetic acid. We found two solvent systems each for the normal and reversed phase TLC, as shown in the experimental. Figures 2 and 3 show the separation under TLC condition (2) [acetone–water (9 : 1) for the reversed phase plate] and TLC condition (4) [benzene–ethyl acetate–methanol (15 : 4 : 1) for the normal phase plate], respectively. A, D, and G indicate orange, paprika, and marigold colors, respectively. Since these colors contain various esters, direct separation by TLC resulted in continuous spots, and satisfactory separation was not obtained. However, when TLC was performed after saponification, followed by clean up using C_{18} cartridges, the main spots were clearly observed (B, E, and H). These main spots were identical with the *β*-cryptoxanthin standard (C), capsanthin standard (F), and lutein standard (I) in terms of the Rf value and color tone. For tomato color (J), the main spot

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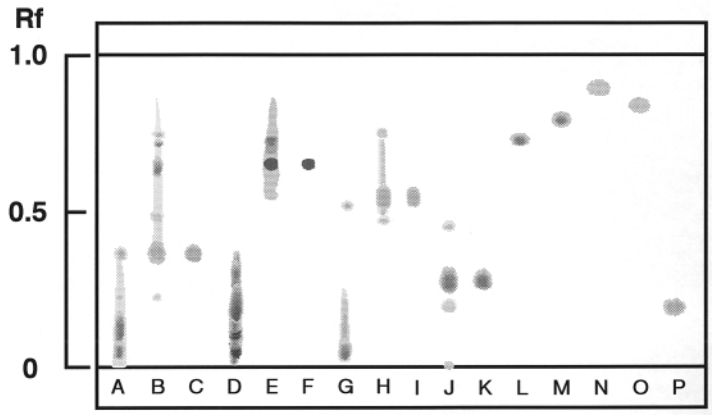

Figure 2. TLC of orange color, paprika color, marigold color, tomato color, annatto extract, gardenia yellow, and β-carotene. (A) Orange color, before saponification; (B) Orange color, after saponification; (C) β-cryptoxanthin; (D) Paprika color, before saponification; (E) Paprika color, after saponification; (F) Capsanthin; (G) Marigold color, before saponification; (H) Marigold color, after saponification; (I) Lutein; (J) Tomato color; (K) Lycopene; (L) Bixin; (M) Norbixin; (N) Crocin; (O) Crocetin; (P) β-carotene. TLC condition (2): Plate, RP-18F254S TLC (E. Merck, Art 15389; solvent system, acetone–water (9 : 1).

could be separated without saponification, and was identical with the lycopene standard (K) in terms of the Rf value and color tone. Bixin (L), norbixin (M), crocin (N), crocetin (O), and β-carotene (P) could also be detected as single spots without saponification. These spots showed good shapes and did not overlap each other, thus, showing good separation.

The Rf value of each color spot is shown in Table 1. Under each TLC condition, overlapping was not observed, and simultaneous separation was possible. Therefore, seven types of carotenoid colorings could be identified using the appropriate combinations of the above four TLC conditions with bixin and norbixin as indicators for the annatto extract, β-cryptoxanthin for the orange color, crocin and crocetin for the gardenia yellow, capsanthin for the paprika color, lycopene for the tomato color, lutein for the marigold color, and β-carotene for the β-carotene. In addition, among the other natural colorings, shrimp color, krill color, crab color, and phaffia color (main color, astaxanthin for each), and corn color (main color, zeaxanthin) as carotenoids, lac color (main color, laccaic acid) and cochineal color (main color, carmic acid) as quinones, and turmeric oleoresin as a diketone (main color, curcumin), showed Rf values or colors that differ from those of the seven carotenoid colorings in

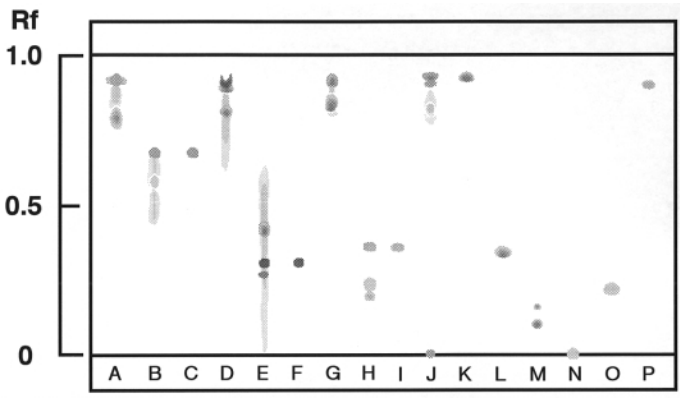

Figure 3. TLC of orange color, paprika color, marigold color, tomato color, annatto extract, gardenia yellow, and β-carotene. (A)–(P), see Fig. 2. TLC condition (4): plate, silica gel 60F254 TLC (E. Merck, Art 5808); solvent system, benzene–ethylacetate–methanol (15 : 4 : 1).

Table 1. Rf values of bixin, norbixin, β-cryptoxanthin, crocin, crocetin, capsanthin, lycopene, lutein, and β-carotene on TLC.

	Rf value			
Color	Solvent system 1[a]	Solvent system 2[a]	Solvent system 3[a]	Solvent system 4[a]
Bixin	0.80	0.73	0.38	0.35
Norbixin (Annatto extract)	0.83	0.80	0.37	0.10
β-cryptoxanthin (Orange color)	0.37	0.36	0.40	0.68
Crocin	0.00	0.89	0.00	0.00
Crocetin (Gardenia yellow)	0.86	0.84	0.39	0.22
Capsanthin (Paprika color)	0.50	0.64	0.26	0.32
Lycopene (Tomato color)	0.46	0.27	0.87	0.93
Lutein (Marigold color)	0.43	0.55	0.36	0.37
β-Carotene	0.34	0.18	0.89	0.91

[a]Solvent systems, see experimental section.

this method. Therefore, the seven carotenoid colorings are not misidentified as these colors when using the above four TLC conditions.

Application to Commercially Available Foods

The above investigation using standard colors suggested the applicability of this TLC method to commercially available foods, so we analysed a total of 294 food items described in the experimental (annatto extract, 26 items; orange color, 38; gardenia yellow, 21; paprika color, 79; tomato color, 33; marigold color, 24; and β-carotene, 73), using the four TLC conditions to evaluate the present method.

Identification by TLC

Based on the TLC chromatograms of the extract from foods under the four conditions as shown in Figs. 4–7, good separation of the color spots from the foods was obtained, and the Rf values and colors of these spots were identical with those of the standards, suggesting an accurate and simple identification.

In the present method, orange color, paprika color, and marigold color were identified using β-cryptoxanthin, capsanthin, and lutein, respectively, as indicators after sapanification of the color extract solution. The saponification

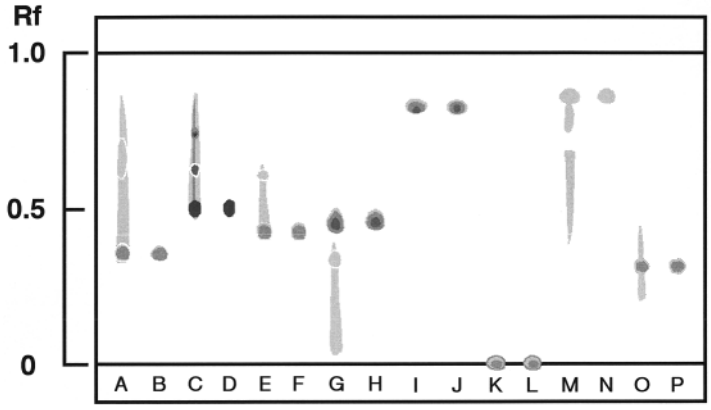

Figure 4. TLC of the extracts of various foods. (A) Jelly; (B) β-cryptoxanthin; (C) Korean pickles (kimuchi); (D) Capsanthin; (E) Juice; (F) Lutein; (G) Spaghetti sauce; (H) Lycopene; (I) Sausage; (J) Norbixin; (K) Pickles; (L) Crocin; (M) Noodle; (N) Crocetin; (O) Chocolate; (P) β-carotene. TLC condition (1): Plate, RP 18F254S TLC (E. Merck, Art 15389); solvent system, acetonitrile–acetone–*n*-hexane (11 : 7 : 2).

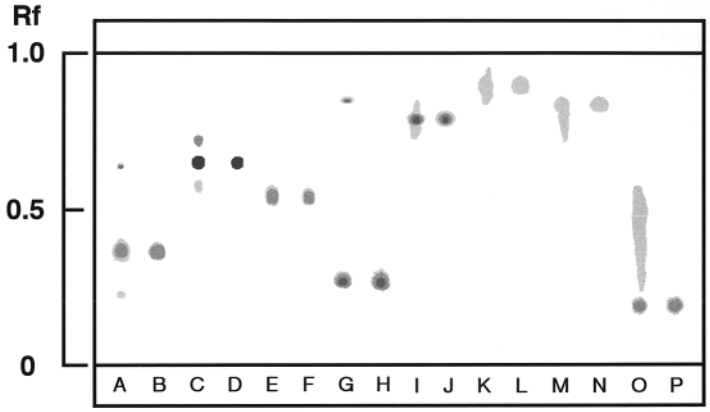

Figure 5. TLC of the extracts of various foods. (A)–(P), see Fig. 4; TLC condition (2), see Fig. 2.

step did not affect the spots of the other colors not requiring saponification (β-carotene and lycopene). Norbixin formed from bixin and crocetin from crocin due to the saponification, were indicators to identify the annatto extract and gardenia yellow, respectively, and did not interfere with their identification.

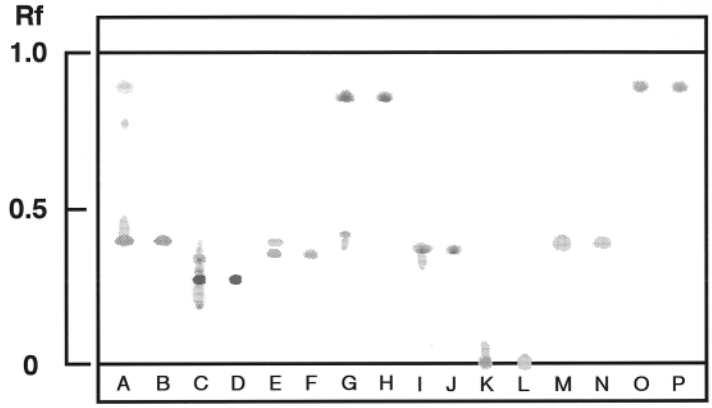

Figure 6. TLC of the extracts of various foods. (A)–(P), see Fig. 4. TLC condition (3): plate, silica gel 60F254 TLC (E. Merck, Art 5808); solvent system, *n*-hexane–ether–acetic acid (4 : 1 : 1).

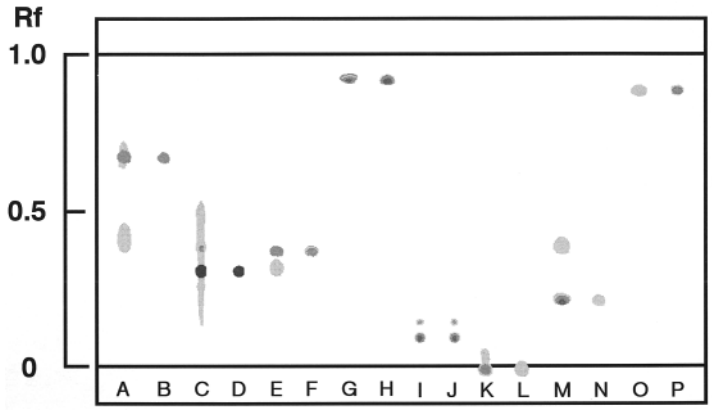

Figure 7. TLC of the extracts of various foods. (A)–(P), see Fig. 4; TLC condition (4); see Fig. 3.

Reproducibility of Rf Values by TLC

To evaluate the influences of co-existing substances from the food samples on the Rf values, the Rf values of the spots obtained by TLC were compared. The difference between the Rf value of the standard color and the Rf value of the color in the sample was expressed as the ratio between the Rf value of the color in the sample (Ra) and the Rf value of the standard color (Rs), and the reproducibility was evaluated according to the coefficient of variation of this ratio.[8] The results are shown in Table 2. Under the four TLC conditions, the mean Ra/Rs value and the coefficient of variation were 0.98–1.05 and $\leq 7.4\%$, respectively, for the annatto extract, 1.01–1.03 and $\leq 3.5\%$ for the orange color, 0.99–1.07 and $\leq 5.9\%$ for the gardenia yellow, 1.01–1.02 and $\leq 2.9\%$ for the paprika color, 0.99–1.02 and $\leq 6.1\%$ for the tomato color, 0.95–1.02 and $\leq 5.8\%$ for the marigold color, and 0.98–1.01 and $\leq 3.8\%$ for β-carotene. These results suggest that the color spots extracted from the samples appear nearly at the same positions as those of the corresponding standard colors, without being affected by co-existing substances from the sample, and that the identification of the colors is reliable and reproducible. Therefore, the method is considered to be sufficiently applicable to routine analyses at facilities such as the Centers of Public Health and the Food Inspection Office.

Table 2. Ra/Rs values of bixin, norbixin, β-cryptoxanthin, crocin, crocetin, capsanthin, lycopene, lutein, and β-carotene in foods on TLC.

		Solvent system 1[a]			Solvent system 2[a]			Solvent system 3[a]			Solvent system 4[a]		
Color	*n*	Rf value	Average Ra/Rs value[b]	C.V. (%)	Rf value	Average Ra/Rs value[b]	C.V. (%)	Rf value	Average Ra/Rs value[b]	C.V. (%)	Rf value	Average Ra/Rs value[b]	C.V. (%)
Bixin	—	0.80	—	—	0.73	—	—	0.38	—	—	0.35	—	—
Norbixin (Annatto extract)	26[c]	0.83	0.98	4.0	0.80	1.00	1.4	0.37	1.02	4.2	0.10	1.05	7.4
β-cryptoxanthin (Orange color)	38[d]	0.37	1.01	2.9	0.36	1.01	1.8	0.40	1.03	3.5	0.68	1.01	3.1
Crocin	8[e]	0.00	—	—	0.89	0.99	0.6	0.00	—	—	0.00	—	—
Crocetin (Gardenia yellow)	13[f]	0.86	1.03	2.3	0.84	0.99	0.9	0.39	1.07	5.9	0.22	1.03	5.3
Capsanthin (Paprika color)	79[g]	0.50	1.01	2.4	0.64	1.02	2.9	0.26	1.01	2.6	0.32	1.01	2.5
Lycopene (Tomato color)	33[h]	0.46	0.99	1.0	0.27	1.01	2.9	0.87	1.02	6.1	0.93	1.00	1.8

(continued)

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Table 2. Continued.

Color	*n*	Solvent system 1[a] Rf value	Solvent system 1[a] Average Ra/Rs value[b]	Solvent system 1[a] C.V. (%)	Solvent system 2[a] Rf value	Solvent system 2[a] Average Ra/Rs value[b]	Solvent system 2[a] C.V. (%)	Solvent system 3[a] Rf value	Solvent system 3[a] Average Ra/Rs value[b]	Solvent system 3[a] C.V. (%)	Solvent system 4[a] Rf value	Solvent system 4[a] Average Ra/Rs value[b]	Solvent system 4[a] C.V. (%)
Lutein (Marigold color)	24[i]	0.43	1.02	1.0	0.55	1.02	3.2	0.36	0.95	5.8	0.37	0.99	2.4
β-carotene	73[j]	0.34	0.99	2.4	0.18	0.98	3.8	0.89	1.01	2.6	0.91	1.00	2.2

[a]Solvent systems, see experimental section.
[b]Ratio of Rf (sample)/Rf (standard).
[c]Seasoning, chewing gum, sausage, chocolate, etc.
[d]Juice, sherbet, jelly, marmalade, etc.
[e]Pickles, seasoning, candy.
[f]Pickles, seasoning, candy, noodle.
[g]Pickles, sherbet, candy, potato chips, etc.
[h]Juice, spaghetti sauce, ketchup, etc.
[i]Noodle, juice.
[j]Candy, juice, jelly, chocolate, etc.

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CONCLUSIONS

We developed a simultaneous analysis method using TLC for seven types of carotenoid colorings (annatto extract, orange color, gardenia yellow, paprika color, tomato color, marigold color, and β-carotene) in foods and obtained the following results:

1. Reversed phase C_{18} TLC using the solvent systems of acetonitrile–acetone–*n*-hexane (11 : 7 : 2) and acetone–water (9 : 1) and normal phase silica gel TLC using solvent systems of *n*-hexane–diethyl ether–acetic acid (4 : 1 : 1) and benzene–ethyl acetate–methanol (15 : 4 : 1), yielded well-delineated spots and good separation.

2. As indicators, bixin and norbixin for the annatto extract, β-cryptoxanthin for the orange color, crocin and crocetin for the gardenia yellow, capsanthin for the paprika color, lycopene for the tomato color, lutein for the marigold color, and β-carotene for β-carotene were effective for the analysis of these carotenid colorings from foods.

3. A method using C_{18} cartridges was useful for clean up of the seven types of carotenoid colorings.

4. The reproducibility of the Rf value by this method was evaluated using a total of 294 commercially available food items (annatto extract, 26 items; orange color, 38; gardenia yellow, 21; paprika color, 79; tomato color, 33; marigold color, 24; and β-carotene, 73). The difference in the Rf value was slight between each color from the food samples and the corresponding standard colors, and the coefficient of variation was small, thus, showing excellent reproducibility.

The present method, using only TLC without special instruments is useful for readily and rapidly identifying seven types of carotenoid colorings in foods, i.e., the annatto extract, orange color, gardenia yellow, paprika color, tomato color, marigold color, and β-carotene.

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