Analysis of Natural Coloring Matters in Food. 4. Methylation of Cochineal Color with Diazomethane for the Analysis of Food Products

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Methylation with ethereal diazomethane in THF was applied to detect cochineal color by TLC. A reddish orange spot at R_f 0.75 on a silica gel plate developed with chloroform–methanol–water (65:35:10, lower phase) as a solvent was derived from all of the cochineal color preparations when they were methylated. The chemical structure of the spot component employed as indicator compound of cochineal color was characterized as 3,6-di-*O*-methylcarminic acid methyl ester by physicochemical analysis, UV–vis, IR, ^{1}H and ^{13}C NMR, and MS. The cleanup of the crude extract from food product was carried out on a reversed-phase cartridge. The improved analytical procedure for individual food product was accurate, as results of recovery checks (91.2% for a jelly and 89.8% for a milk beverage). Indicator compound was observed from 23 of 65 food products analyzed by TLC, and its concentrations ranged from 0.9 to 137.7 μg/g by HPLC analysis.

INTRODUCTION

Considerable increment of public concern about the use of synthetic color additives in food products has led to an increase in the use of natural food colorants. Since Japanese food legislation on the statement of natural food additives on labels was enforced in 1991, public health agencies must construct simple and reliable methods for the detection of them, especially natural food colorants, in food products.

Cochineal color extracted from the dried female bodies of the scale insect (*Coccus cacti* L.) is a water-soluble reddish natural color and is widely in demand for the coloring of food products and cosmetics (*Merck Index*, 1989). The main coloring component is established as carminic acid (7-β-D-glucopyranosyl-9,10-dihydro-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-2-anthracenecarboxylic acid) (Ali and Haynes, 1959; Bhatia and Venkataraman, 1965; Fiecchi et al., 1981).

TLC and HPLC methods have been proposed for the analysis of cochineal color in food products (Ogiwara et al., 1980; Kanda et al., 1981; Hirata et al., 1983; Takatsuki et al., 1983; Nishizawa et al., 1985; Mercedes et al., 1989). Using TLC methods (Ogiwara et al., 1980; Kanda et al., 1981; Hirata et al., 1983), judgment was not achieved by the comparison with R_f values on a plate because of the appearance of several colored spots from cochineal color preparations and the nonuniformity of spots on the plate.

The aim of our research was to develop reliable TLC methods for the detection of natural coloring matters in food products. We have already reported practical TLC methods for gardenia yellow (Noda et al., 1983), cochineal color (Yamada et al., 1985), and lac color (Yamada et al., 1989). As for cochineal color, our strategy was to improve detection on a silica gel plate by masking polar functional groups such as carboxyl and phenolic hydroxyl groups of the color constituent (carminic acid) with diazomethane. In the previous paper (Yamada et al., 1985), the proposed TLC method was constructed with solvent extraction, cleanup on an Amberlite XAD-2 column, methylation, and detection of a methylated indicator compound on a silica gel plate. However, choosing the chemical structure of the indicator compound remained.

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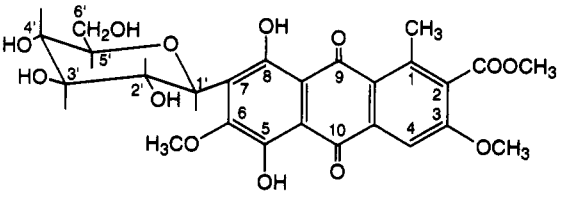

Figure 1. Chemical structure of indicator compound of cochineal color.

In the present work, we characterize the chemical structure of the indicator compound as 3,6-di-*O*-methylcarminic acid methyl ester represented in Figure 1. We also improve the previous TLC method and carry out a small-scale survey of cochineal color in food products by this method.

EXPERIMENTAL PROCEDURES

Food Samples. Sixty-five food products with labels stating that they contained natural coloring matters as ingredients were purchased from a local supermarket in 1991–1992. Details of the 65 food products are shown in Table III.

Chemicals and Materials. Common reagents were of analytical grade unless otherwise stated. Organic solvents were of pesticide residue grade or HPLC grade. Water was deionized and was distilled before use in all experiments. Six commercially available cochineal color preparations were purchased from five Japanese chemical manufacturers. A native cochineal color was prepared in the laboratory as follows: dried scale insects were ground and were extracted with 70% ethanol. Aqueous ethanol extract was evaporated to near dryness after washing with *n*-hexane. The concentrate was dried with a light of nitrogen (45% yield). A Sep-Pak Plus C_{18} cartridge (Millipore Corp., Milford, MA) was prewashed with 20 mL of 0.5% oxalic acid in methanol followed by washing until neutral. A silica gel coated TLC plate (silica gel 60 F_{254}, 0.25-mm thickness) and 70–230-mesh silica gel 60 ASTM for chromatography were purchased from E. Merck (Darmstadt, FRG). Sephadex LH-20 was purchased from Pharmacia (Uppsala, Sweden). Ethereal diazomethane was prepared as described by Arndt (1943a,b). Diazomethane is a highly toxic carcinogen and, under some conditions, explosive. It should be prepared carefully in fume hood.

Apparatus. Melting points were determined on a Yanaco (Kyoto, Japan) micro melting point apparatus and are uncorrected. UV–vis spectra were taken in methanol solution on a JASCO (Tokyo, Japan) Ubest-50 spectrophotometer. IR spectra were recorded with a Hitachi (Tokyo, Japan) 295 IR spectro-

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photometer using the sample mixed in KBr with ordinate scale for the region 4000–700 cm^{-1}. The ^1H and ^{13}C NMR spectra were measured in DMSO-d_6 or chloroform-d with a JEOL (Tokyo, Japan) JMN-GX400 spectrometer. Chemical shifts are expressed in δ (parts per million) values with internal tetramethylsilane. Significant ^1H NMR data are tabulated in the following order: number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), and coupling constants in hertz. The EI-MS were measured with a JEOL JMS-AX505W spectrometer using direct sample introduction. The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-6A pump, a Rheodyne (Cotati, CA) 7125 injector with a 100-μL loop, a Shimadzu SPD-6AV UV–vis detector, a Shimadzu RF-530 fluorometric detector, and a Hitachi D-2500 chromatointegrator.

Methods. *Isolation of Indicator Compound.* A commercial cochineal color preparation (1 g) was extracted with 200 mL of methanol twice. The methanol extract was dissolved in 500 mL of THF followed by methylation with 20 mL of ethereal diazomethane. The methylation was stopped by addition of 0.5 mL of acetic acid after standing for 5 min at room temperature. The reaction mixture was redissolved in 300 mL of chloroform and was washed with 200 mL of water. The chloroform phase was evaporated to near dryness after dehydration with anhydrous Na_2SO_4. The chloroform extract was subjected to a silica gel column (20 g, 3 cm i.d. × 13 cm length) eluted with 200 mL of chloroform and chloroform–methanol (C–M, 98:2), C–M (95:5), and C–M (90:10) mixtures, successively. Each step was divided into four fractions. Fractions containing indicator compound were combined by monitoring with silica gel TLC developed with chloroform–methanol–water (C–M–W, 65:35:10, lower phase) as a solvent. The resultant fraction was then chromatographed on a Sephadex LH-20 column (100 g, 3 cm i.d. × 60 cm length) eluted with methanol. Each 10-mL fraction was monitored with the same TLC as above. Finally, indicator compound was isolated with preparative HPLC using an RCM 25 × 10 pressure module system (Millipore). Preparative HPLC conditions were as follows: column, Nova-Pak HR C_{18} (6 μm, 25 mm i.d. × 100 mm length, Millipore); mobile phase, 30% acetonitrile in water; flow rate, 4.0 mL/min; detection, reflective index. Indicator compound was crystallized from ethyl acetate–methanol (8:2) as red-brown needles (yield 170 mg).

Acetylation of Indicator Compound. Crystalline indicator compound (100 mg) was dissolved in 50 mL of chloroform followed by acetylation with 1 mL of acetyl chloride for 12 h at room temperature. The acetylation was monitored with silica gel TLC developed with C–M–W (90:10:1). The reaction mixture was washed with 50 mL of water twice to remove excess reagent and resultant HCl. The chloroform phase was evaporated to dryness after dehydration with anhydrous Na_2SO_4. The acetyl derivative of indicator compound was isolated in the same manner as above; the preparative HPLC system employed 65% acetonitrile in water as a mobile phase. The acetyl derivative was crystallized from methanol as red-brown needles (yield 85 mg).

Sample Preparations. A juice or broken ice flavor (10 g) was centrifuged, if necessary. Transparent supernatant was subjected to a Sep-Pak C_{18} cartridge. A cartridge was washed until neutral. The color was eluted from a cartridge with 5 mL of methanol. The eluate was evaporated to dryness. The concentrate was redissolved in 10 mL of THF followed by methylation with 0.5 mL of ethereal diazomethane for 5 min at room temperature. Methylation was stopped by addition of 1 drop of acetic acid. The reaction mixture was concentrated under reduced pressure. The concentrate redissolved in methanol was analyzed with silica gel TLC developed with C–M–W (65:35:10, lower phase) and HPLC described below. A candy or jelly (10 g) was dissolved in 50 mL of 0.1% oxalic acid solution. The color was extracted with 30 mL of 1-butanol after removal of oily ingredients with 30 mL of n-hexane. The 1-butanol extract was redissolved in 5 mL of water. The cleanup on a cartridge and methylation were carried out in the same manners as above. A steamed rice cake (uiro, 10 g) was homogenized in 50 mL of 0.1% oxalic acid in methanol. The homogenate was stirred for 3 h, automatically. Transparent supernatant was evaporated to dryness after centrifugation. The concentrate redissolved in water was turned to the cleanup stage. A milk beverage or ice cream (50 g) was lyophilized. The residue was extracted with 50 mL of 0.1% oxalic acid in methanol after washing of the residue with 50 mL of n-hexane. Acidic methanol extract redissolved in water was turned to the cleanup stage. A ham or sausage (10 g) was homogenized in 100 mL of water. The homogenate was incubated for 2 h at 37 °C after additions of 100 mg of Pronase (6 units/mg, Boehringer, Mannheim, FRG) and 50 mg of sodium dodecyl sulfate. The incubation mixture was centrifuged, and the supernatant was washed with 100 mL of n-hexane. It was extracted with 50 mL of 1-butanol after addition of 100 mg of oxalic acid. The 1-butanol extract was redissolved in water and was turned to the cleanup stage.

Determination. Indicator compound in sample solution was determined under the following HPLC conditions: column, LiChrosorb (E. Merck) RP-18, 5 μm, 4 mm i.d. × 250 mm length; mobile phase, 30% acetonitrile in 0.1% H_3PO_4; flow rate, 1.0 mL/min; temperature, ambient. The eluates were monitored at 495 and 365 nm (excitation) and 565 nm (emission), successively. Working standard solutions at three different concentrations were injected into HPLC. The calibration curve constructed by plotting peak area vs weight was found to be linear in the range 20–300 ng. Amounts of indicator compound were calculated from the calibration curve.

Recovery. Precision in the whole analytical procedure was estimated by 10 trials using both a noncolored jelly (10 g) and a milk beverage (50 g) spiked with 0.1 mg of a commercial cochineal color preparation.

RESULTS AND DISCUSSIONS

TLC of Cochineal Color Methylated with Diazomethane. Six commercial preparations and a cochineal color prepared in the laboratory were methylated with ethereal diazomethane in THF. A reddish orange spot at R_f 0.75 was observed when reaction products were developed on a silica gel plate with C–M–W (65:35:10, lower phase). This conspicuous spot was also observed by the irradiation of long-wave UV light. It was derived from all of the commercial cochineal colors as well as the native preparation. Therefore, the starting compound to be methylated is not a byproduct of the manufacturing process but is an inherent component of the scale insect. The certification of cochineal color in food products can be easily achieved by the detection of this spot as indicator compound with TLC.

Chemical Structure of Indicator Compound. Physical properties of crystalline indicator compound were as follows: melting point, 293–295 °C; UV–vis λ_{max} nm (log ϵ), 220 (4.61), 272 (4.85), 473 (4.09), 498 (4.13), and 530 (3.93). The IR spectrum gave absorption bands at 3400, 2930, 2870, 1730, 1615, 1570, and 920 cm^{-1}, indicating the presence of hydroxy, methoxy, carboxyl ester, chelated quinone, and phenyl ring. In the EI-MS spectrum, a molecular ion peak at m/z 534 and intensive fragment peaks at m/z 516 ($M^+ - H_2O$), 425 ($M^+ - C_3H_9O_4$), 401 ($M^+ - C_5H_9O_4$, base peak), 385 ($M^+ - C_5H_9O_5$), and 372 ($M^+ - C_6H_{10}O_5$) were observed with percent intensities of 24, 19, 11, 100, 20, and 18, respectively. The high-resolution MS analysis of indicator compound showed the molecular formula to be $C_{25}H_{26}O_{13}$ (M^+ m/z found 534.1365; calcd 534.1374). This formula assumes the addition of three methyl groups to carminic acid ($C_{22}H_{20}O_{13}$, MW 492).

The ^1H and ^{13}C NMR spectra of indicator compound measured in DMSO-d_6 are given in Table I. The ^1H NMR spectrum showed signals caused by a methyl group (2.64, 3H, S), three methoxyl groups (3.90, 3.98, 4.02, each 3H, S), and an isolated aromatic proton (7.78, 1H, d, J = 4.9 Hz). The signals of two chelated phenolic protons (12.99, 1H, s; 14.04, 1H, d, J = 9.8 Hz) disappeared immediately when water-d_2 was added. The complex group of signals between δ 3.16 and 3.74 and at δ 4.17 and 4.73 was characteristic of the glucose moiety. The multiplet at δ 3.16–3.42 (3H) was attributed to protons 2′, 3′, and 5′ of glucose. The signals at δ 3.55 (1H, m) and 3.74 (1H, br)

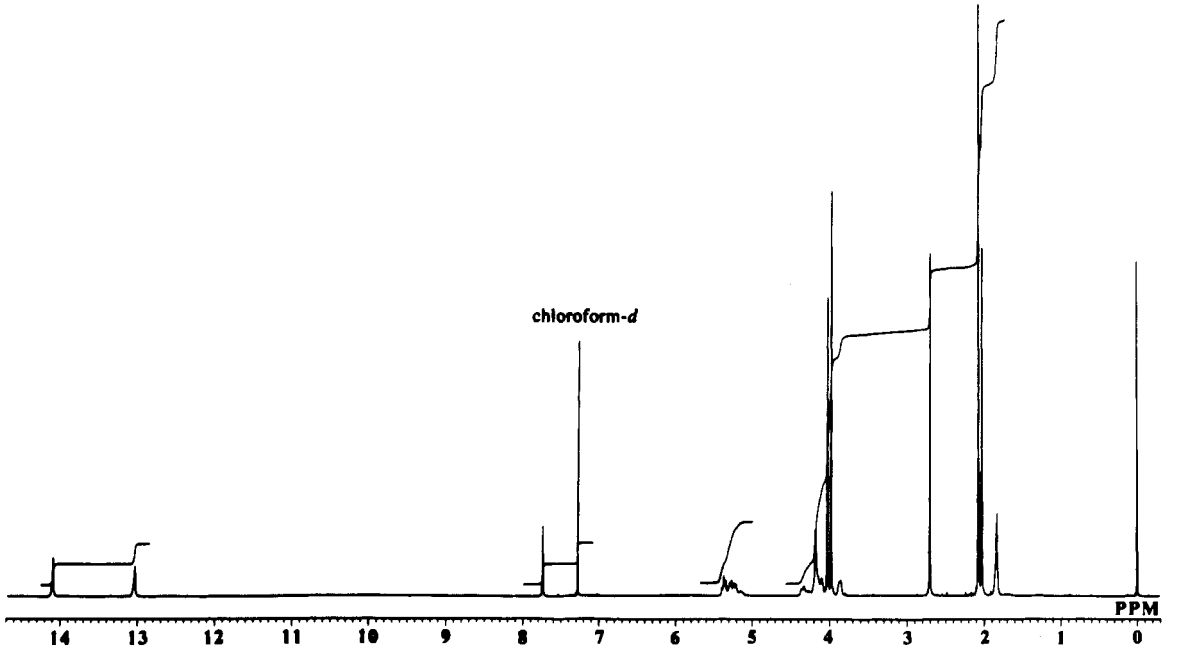

Figure 2. ^1H NMR spectrum of acetylated derivative of indicator compound.

Table I. ^1H and ^{13}C NMR Data of Indicator Compound

^1H NMR		^{13}C NMR	
1-CH_3	2.64 s	1-CH_3	19.75
2-$COOCH_3$	3.90 s	2-$COOCH_3$	166.55
3-OCH_3	3.98 s	2-$\bar{C}OOCH_3$	52.69
4	7.78 d, J = 4.9 Hz	3-OCH_3	61.44
5-OH	12.99 s	4	106.95
6-OCH_3	4.02 s	6-OCH_3	56.62
8-OH	14.04 d, J = 9.8 Hz	9	185.94 or 186.85
1′	4.73 d, J = 9.2 Hz	10	186.85 or 185.94
2′	3.16–3.74 m	1′	72.54
3′		2′	70.51
5′		3′	78.53
4′	4.17 m	4′	69.71
6′	3.55 m	5′	81.70
	3.74 br	6′	61.68
			108.45
			112.38
			124.38
			131.04
			132.23
			136.56
			140.15
			151.51
			155.88
			157.48
			159.02

were assigned to 6′ methylene protons. The multiplet at δ 4.17 (1H) was attributed to the 4′ proton. An anomeric proton signal observed at δ 4.73 (1H, d, J = 9.2 Hz) was characteristic of β configuration.

In the ^{13}C NMR studies, indicator compound consisted of 4 primary, 1 secondary, 6 tertiary, and 14 quaternary carbon atoms. Experiments carried out in DEPT 90 and 135 confirmed these data. Signals at δ 52.69, 56.62, and 61.44 were caused by three methoxyl carbons. Methyl, methine, carboxyl, and two carbonyl carbons (quinone) were observed at δ 19.75, 106.95, 166.55, 185.94, and 186.85, respectively. Six signals between δ 61.68 and 81.70 were assigned to glucose carbons. Assignments of proton and carbon signals of indicator compound were attained by several correlations of ^{13}C–^1H COSY and ^1H–^1H NOESY. However, signals of the other 11 quaternary carbons could not be assigned. Those spectral data supported the chemical structure of indicator compound as being 3,6-di-*O*-methylcarminic acid methyl ester as illustrated in Figure 1.

To obtain further support of the above chemical structure, indicator compound was acetylated with acetyl chloride in chloroform. The crystallization of acetyl derivative from methanol resulted in red-brown needles with mp 143–145 °C (dec). In the EI-MS, it gave the molecular ion peak at m/z 702 and intensive fragment peaks at m/z 642 ($M^+ - C_2H_4O_2$), 523 ($M^+ - C_6H_{11}O_6$), 480 ($M^+ - C_8H_{14}O_7$), 467 ($M^+ - C_{12}H_{11}O_5$, base peak), 401 ($M^+ - C_{13}H_{17}O_8$), and 395 ($M^+ - C_{12}H_{19}O_9$) with percent intensities of 84, 11, 25, 70, 100, 68, and 58, respectively, which meant the introduction of four acetyl groups to indicator compound.

The ^1H NMR spectrum measured in chloroform-*d* is shown in Figure 2. It exhibited new signals ascribed to four acetyl groups at δ 1.83 (3H, S), 2.03 (3H, S), and 2.07 (6H, S). Two chelated hydroxyl groups were not acetylated under the employed reaction conditions, because two signals at δ 13.03 and 14.09 (each 1H, s) were observed. Those spectral data strongly supported the assumptions that acetylation occurred on four residual hydroxyl groups of the glucose moiety and the chemical structure of indicator compound was 3,6-di-*O*-methylcarminic acid methyl ester.

Examination of Reaction Conditions. In the previous paper (Yamada et al., 1985), methanol was used as a reaction solvent; however, inconsistent results were observed in the course of quantitative studies. Reaction conditions were reexamined on solvents, time course, and temperatures. The use of 0.1 mg of a commercial cochineal color preparation, 10 mL of a solvent, and 0.5 mL of ethereal diazomethane was fixed in examination studies. As to reaction solvents, methanol, ethanol, acetone, acetonitrile, and THF were nominated in point of solubility of cochineal color. Table II shows the effect of reaction solvents on the yield of indicator compound. It was produced in all five reaction solvents examined; however, its yield depended on the solvent. THF showed the highest production among them. The yield in THF was 1.6-fold higher than in methanol. THF resulted in consistent and repeatable methylation.

Table II. Influence of Reaction Solvents on the Yield of Indicator Compound

solvent	yield[a] μg/g[b]	yield[a] %[c]	solvent	yield[a] μg/g[b]	yield[a] %[c]
methanol	235	62.8	acetonitrile	26	7.0
ethanol	328	87.7	THF	374	100.0
acetone	296	79.1			

[a] Mean for triplicate experiments. [b] μg/g of a commercial preparation. [c] Percentage of maximum yield found in THF.

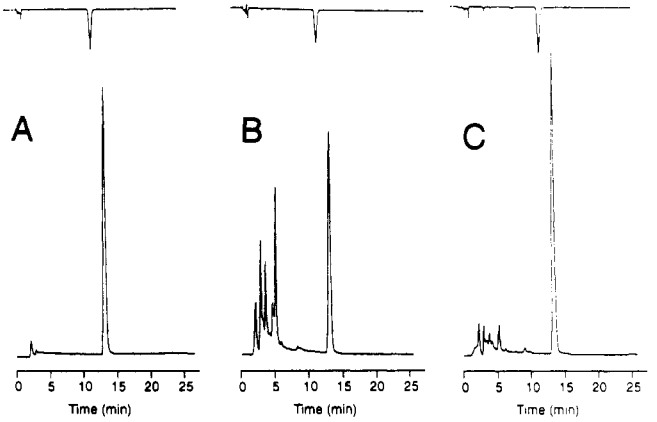

Figure 3. HPLC chromatograms of methylated products of food extracts: (A) indicator compound (25 μg/mL); (B) milk beverage (15 g of food/5 mL); (C) steamed rice cake (10 g of food/1.5 mL); (upper chromatogram) monitored at 495 nm; (bottom chromatogram) monitored at ex 365 nm and em 565 nm.

The methylation was observed for 20 min as a time course. The production curve of indicator compound crossed points of 321 (86%), 355 (95%), and 374 μg/g (100%) at 1, 2, and 3 min after the methylation was started, respectively. Then the curve held at a plateau until 20 min. The methylation was checked at 4 °C and room temperature. No significant differences of the yield were observed between the two temperature conditions. Methylation in THF for 5 min at room temperature was employed as reaction conditions on the basis of above findings.

Food Analysis. Instead of an Amberlite XAD-2 column used in the previous paper (Yamada et al., 1985), a Sep-Pak C_{18} cartridge was used for the cleanup of crude extracts from food products, especially removal of oxalic acid added at the extraction stage and organic acids as intact food components and/or ingredients. The cleanup process was essential to following methylation because of the protection of diazomethane from consumption by organic acids including oxalic acid. One hundred milligrams of cochineal color preparation in up to 50 mL of 2% oxalic acid was retained in a cartridge and was eluted from a cartridge with 5 mL of methanol, completely.

Reaction mixture obtained from food products according to the improved procedure offered a reddish orange spot at R_f 0.75 identical to indicator compound on a silica gel plate developed with C–M–W (63:35:10, lower phase), which spot was also detected by the irradiation of UV light. The use of cochineal color as a food color additive was easily certified by TLC. Figure 3A shows a typical HPLC chromatogram of indicator compound. The upper chromatogram was monitored at 495 nm, and the bottom was by fluorescence detection. The peak of indicator compound appeared at RT 13 min by both detections; however, the sensitivity of fluorescence detection was 10-fold higher than the vis under the employed HPLC conditions. Parts B and C of Figure 3 show HPLC chromatograms of a milk beverage and a steamed rice cake (uiro). Both food products offered the peak at the same RT as indicator compound. It was also certified that they were emphasized by cochineal color.

Table III. Determination of Indicator Compound in Food Products

item	N[a]	n[b]	concn,[c] μg/g
candy	24		
orange		2	3.3, 9.8
strawberry		2	41.5, 45.0
juice	4		
orange		2	8.6, 13.0
jelly	19		
orange		3	3.7–13.8
strawberry		5	17.3–36.6
milk beverage	5		
strawberry		2	7.4, 20.6
ice cream	4		
strawberry		2	0.9, 31.6
broken ice flavor	4		
strawberry		2	112.8, 137.7
steamed rice cake (uiro)	2		
cherry blossoms		1	4.0
loin ham	1	1	1.9
Vienna sausage	2	1	4.4

[a] Number of items surveyed. [b] Number of items detected cochineal color. [c] Results are expressed in μg/g of original food. Mean for triplicate experiments.

Table IV. Determination of Indicator Compound in Commercial Preparation

no.	source	form	concn,[a] μg/g
1	commercial	powder	374.0
2	commercial	powder	349.0
3	commercial	powder	307.0
4	commercial	powder	358.0
5	commercial	liquid	63.8
6	commercial	liquid	22.5
7	scale insect		188.2

[a] Results are expressed in μg/g of a preparation or dried body. Mean for triplicate experiments.

On recoveries through the whole analytical process, 91.2% (CV 2.1%) and 89.8% (CV 2.7%) were observed for a jelly and a milk beverage, respectively. The detection limit of indicator compound was approximately 10 ng (S/N = 5). When carminic acid is in excess of 0.1 μg/g food, definitely identifiable peaks can be caught by HPLC methods.

The determination of indicator compound in food products is summarized in Table III. It was detected from 23 of 65 food products, and its concentrations ranged from 0.9 to 137.7 μg/g food. It seems that cochineal color is used to improve the appearance of products such as strawberry, citrus, and cherry blossoms to the consumer.

The contents in commercial preparations are shown in Table IV. Four powder preparations were on the level of around 300 μg/g. Liquid preparation 5 was labeled that it was prepared by 5-fold dilution of preparation 4. Judging from the content in intact scale insect, powder preparations must be 2-fold concentrated under the manufacturing process.

Conclusions. This study demonstrated the application of methylation with ethereal diazomethane in THF for the detection of cochineal color in food products. The chemical structure of methylated indicator compound was characterized as 3,6-di-*O*-methylcarminic acid methyl ester by various spectroscopic data. Qualitative analysis is achieved by detection of indicator compound under usual TLC conditions. The HPLC method offers great selectivity, little interference by ingredients in food, and

methodology to perform quality control of cochineal color in manufacturing.

ABBREVIATIONS USED

TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; UV–vis, ultraviolet–visible; IR, infrared; NMR, nuclear magnetic resonance; EI-MS, electron impact mass spectrometry; DEPT, distortionless enhancement by polarization transfer; ^{13}C–^{1}H COSY, carbon-13 and proton correlation spectroscopy; ^{1}H–^{1}H NOESY, proton and proton nuclear Overhauser effect spectroscopy; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide.

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