Note

Kinetic Study of the Equilibration between Carminic Acid and Its Two Isomers Isolated from Cochineal Dye

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Carminic acid (CA) is a major component of cochineal dye used in food additives, cosmetics, and pharmaceuticals. CA and its isomers, 2-*C*-*α*-glucofuranoside and 2-*C*-*β*-glucofuranoside of kermesic acid (DCIV and DCVII, respectively), were isolated from cochineal dye and the equilibrium constants (*K*) between CA, DCIV and DCVII were investigated. DCIV was partially converted to CA and DCVII, and DCVII was converted to CA and DCIV, whereas CA was very stable and only very slightly converted to DCIV and DCVII. Most of the DCIV and DCVII was converted to CA under aqueous conditions. The kinetic rate constants (*k*) for the degradation of DCIV within the first day of incubation at 24°C was determined to be $0.901\,d^{-1}$ and for the degradation of DCVII it was determined to be $1.102\,d^{-1}$. The *k* value for the formation of CA from the remaining DCIV was calculated to be $0.146\,d^{-1}$ and for the formation of CA from the produced DCVII it was found to be $0.148\,d^{-1}$. The *K* values were calculated as 1.22×10^{-7}, 2.61×10^{-3} and 2.36×10^{-3} mol/L for CA, DCIV and DCVII, respectively. These findings will be helpful for ensuring the safety and for aiding the quality assurance of cochineal dye products.

Key words cochineal; kermesic acid; anomerization; glucofuranoside; kinetic rate constant; equilibrium constant

Cochineal is a natural red dye extracted from *Dactylopius coccus* Costa. Historically, this dye was used predominantly for coloring artwork and textiles,[1)] but currently it is also used worldwide in food additives, cosmetics and pharmaceuticals. Cochineal dye is a generally highly safe material, but occasionally induces harmful allergic reactions including anaphylaxis, urticarial and occupational asthma.[2–4)] Causative substances of the allergic reactions are not well-known,[5)] and the involvement of minor components of the dye in the reactions has still not been studied. In addition, the minor components in food additives, colored textiles and historical artwork generally have been required to identify the species and the geographical origin of the cochineal by using multivariate statistical methods such as principal components analysis, partial-least squares discriminant analysis and hierarchical cluster analysis.[1,6–10)]

The quality, insect species, and geographical origin of cochineal dye are typically evaluated using an HPLC equipped with a UV-Vis detector[6,7,11,12)] and a photodiode array detector[1,13–15)] to determine carminic acid (CA), the main constituent of cochineal, as well as minor components.

Minor components isolated from cochineal dye have been identified as flavokermesic acid, kermesic acid, 2-*C*-glucopyranoside of flavokermesic acid, 4-aminocarminic acid, 3-*O*-glucopyranoside of flavokermesic acid, and 3,4-dideoxycarminic acid.[11,14,15)] The two isomers of CA were identified as 2-*C*-*α*-glucofuranoside and 2-*C*-*β*-glucofuranoside of kermesic acid (DCIV and DCVII, respectively).[14–16)]

Stathopoulou *et al.* recently reported that DCIV and DCVII produce two isomeric forms *via* anomerization and both can be transformed to CA,[15)] although the mechanism of inter-conversion has not been clarified. The time course of the transformation between CA, DCIV and DCVII has not yet been clarified. Knowledge of the time-dependent changes in the minor components DCIV and DCVII is very important for establishing the safety and quality assurance of cochineal dye containing in foods and drugs.

In this study, we purified CA, DCIV and DCVII from a cochineal dye product used as a food additive and determined the kinetic rate constant (*k*) and the equilibrium constants (*K*) for the interconversion of CA, DCIV and DCVII under equilibrium conditions in aqueous solution.

Experimental

General Experimental Procedures ^{1}H-NMR spectra were measured using an ECA-800 spectrometer (JEOL Ltd., Tokyo, Japan) and the chemical shifts were referenced to tetramethylsilane (TMS, $\delta_H=0$ ppm) as an internal standard. IR spectra were measured using an FT/IR-4100 spectrometer (JASCO, Tokyo, Japan) and UV spectra were obtained using a V-630 spectrophotometer (JASCO). The high-resolution electrospray ionization time-of-flight mass spectra (HR-ESI-TOF-MS) were obtained using Xevo G2 Q-Tof mass spectrometer (Waters, Milford, MA, U.S.A.) in negative mode, where the $[M-H]^-$ peak was indicated as *m*/*z*. Optical rotations were measured with a P-1020 automatic digital polarimeter (JASCO).

Materials Cochineal dye was obtained from Alps Pharmaceutical Industry (Gifu, Japan). The components were isolated and purified using reagent grade chemicals purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

HPLC Conditions The analytical system comprised two

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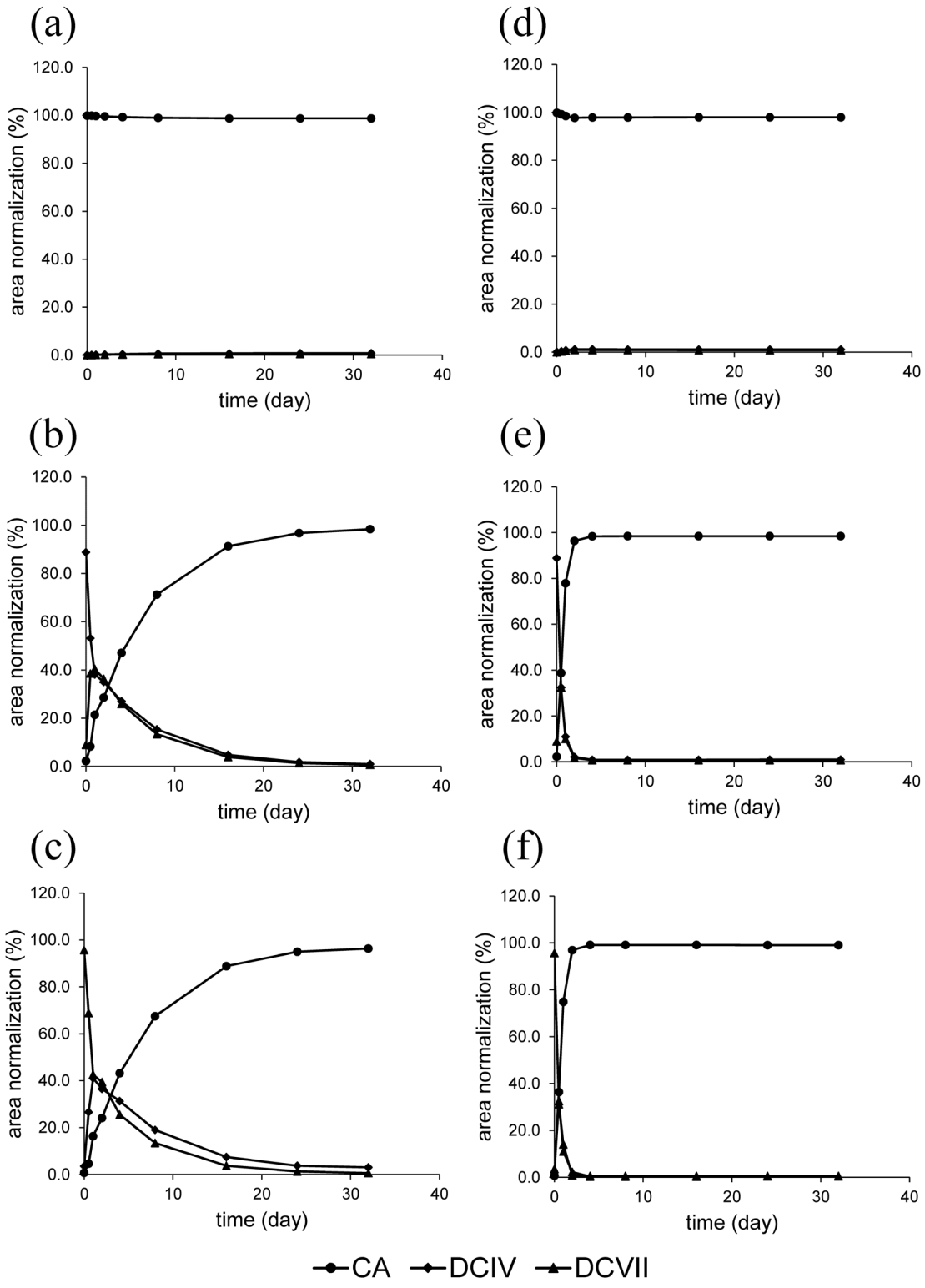

Fig. 1. Changes in the Concentrations of CA, DCIV and DCVII at 24°C (Left Side, (a)–(c)) and 40°C (Right Side, (d)–(f)), in Aqueous Solutions of (a) and (d) CA, (b) and (e) DCIV, and (c) and (f) DCVII

●: CA, ◆: DCIV, and ▲: DCVII.

LC-10ADVP pumps (Shimadzu, Kyoto, Japan), a SPD-10AVP UV-Vis detector, a CTO-10A column heater, and a Chromato-PRO integrator (Run Time Instruments, Kanagawa, Japan) and the separation conditions were as follows: column: Inertsil ODS-4 (4.6×250 mm, 5 μm, GL Science, Tokyo, Japan), flow rate: 1.0 mL/min, column temperature: 30°C, eluent: aqueous 0.05% phosphoric acid–methanol (55 : 45) and detection: UV 276 nm. The calibration curves for all three compounds (CA, DCIV and DCVII) were linear from 0.0016 to 1.0 mg/mL (r^2>0.999).

The preparative system comprised two LC-10AS pumps (Shimadzu), a SPD-10AV UV-Vis detector, a CTO-10A col-

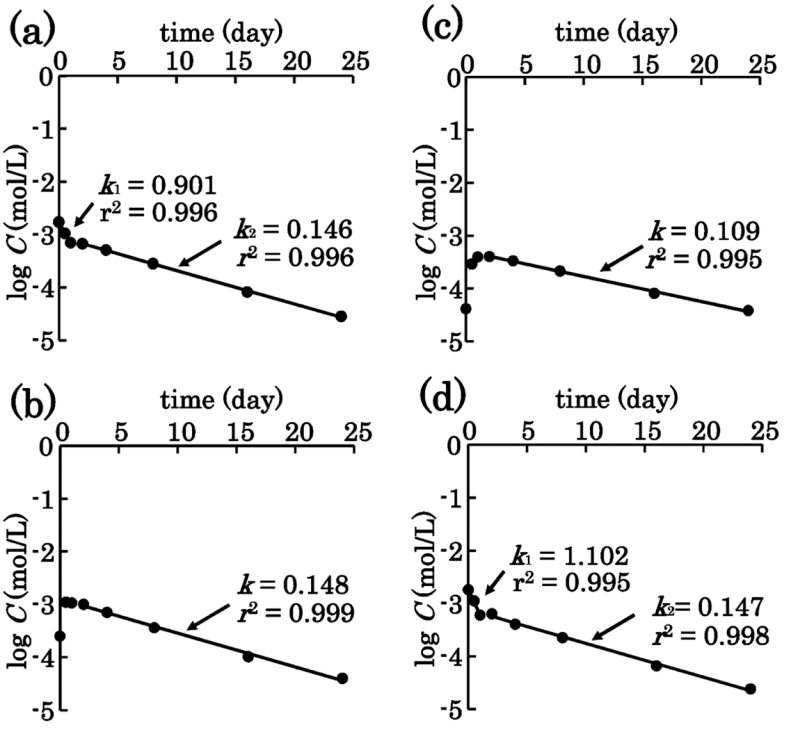

Fig. 2. Kinetic Curves for the Degradation of DCIV and DCVII

Kinetic curves showing the degradation of DCIV and DCVII in DCIV aqueous solution (left side) and DCVII aqueous solution (right side) at 24°C, (a) DCIV in DCIV aqueous solution, (b) DCVII in DCIV aqueous solution, (c) DCIV in DCVII aqueous solution and (d) DCVII in DCVII aqueous solution.

umn heater, and a C-R8A integrator (Shimadzu) and the separation conditions were as follows: column: Inertsil ODS-4 (14×250 mm, 5 μm, GL Science), flow rate: 5.0 mL/min, column temperature: 40°C, eluent: aqueous 0.1% formic acid–methanol (50 : 50) and detection: UV 254 nm.

Isolation and Purification CA Cochineal dye consisted of 94.0% CA, 2.4% DCIV and 1.2% DCVII at their compositions, respectively, by the HPLC analysis. A part of large amounts of CA was isolated at the purity of 97.8% before eluting DCIV and DCVII using the conventional column chromatography described below.

DCIV and DCVII Cochineal dye (1.5 g) dissolved in water (1.5 L) was subjected to reversed-phase column chromatography using a column (40×180 mm, width×height) filled with octadecylsilyl (ODS)-silica resin (YMC GEL ODS-A, 12 nm S-150 μm; YMC, Kyoto, Japan). The DCIV- and DCVII-containing fractions were obtained using aqueous 10% methanol after CA was eluted. After evaporating methanol from each fraction under vacuum at room temperature, the residue was re-dissolved in an aliquot of water and then subjected to preparative HPLC to obtain 11.1 mg of DCIV (84.4% purity) and 2.7 mg of DCVII (88.7% purity). The purity of these compounds was calculated from their peak areas in the HPLC chromatogram, respectively. These purified compounds were then subjected to structural analysis and kinetic studies. The obtained spectral data for DCIV and DCVII are described below:

DCIV

^{1}H-NMR (CD_3OD) δ: 2.78 (3H, s, CH_3), 3.67 (1H, dd, J=11.7, 5.6 Hz, H-6′b), 3.79 (1H, dd, J=11.8, 3.5 Hz, H-6′a), 4.01 (1H, dd, J=7.6, 2.7 Hz, H-4′), 4.08 (1H, ddd, J=9.1, 5.6, 3.5 Hz, H-5′), 4.23 (1H, dd, J=2.7, 1.0 Hz, H-3′), 4.26 (1H, dd, J=3.0, 1.1 Hz, H-2′), 5.36 (1H, d, J=2.9 Hz, H-1′), 7.71 (1H, s, H-5). IR (KBr) cm^{-1}: 3440, 2930, 1570. UV λ_{max} (H_2O) nm (ε): 278 (37540), 313 (11950), 491 (8310). HR-ESI-TOF-MS m/z: 491.0832 [Calcd for $C_{22}H_{19}O_{13}$ $(M-H)^-$, 491.0826]. $[\alpha]_D^{26}$ −94.0 (c=0.01, MeOH).

DCVII

^{1}H-NMR (CD_3OD) δ: 2.78 (3H, s, CH_3), 3.72 (1H, dd, J=11.5, 5.2 Hz, H-6′b), 3.82 (1H, dd, J=11.5, 3.0 Hz, H-6′a), 4.01 (1H, ddd, J=8.4, 5.3, 3.0 Hz, H-5′), 4.34 (1H, dd, J=3.4, 1.3 Hz, H-3′), 4.40 (1H, dd, J=8.5, 3.3 Hz, H-4′), 4.52 (1H, dd, J=2.9, 1.0 Hz, H-2′), 5.72 (1H, d, J=3.0 Hz, H-1′), 7.73 (1H, s, H-5). IR (KBr) cm^{-1}: 3450, 2920, 1630. UV λ_{max} (H_2O) nm (ε): 278 (26800), 310 (9090), 489 (6160). HR-ESI-TOF-MS m/z: 491.0832 [Calcd for $C_{22}H_{19}O_{13}$ $(M-H)^-$, 491.0826]. $[\alpha]_D^{26}$ −210.5 (c=0.02, MeOH).

Kinetic Study Aqueous solutions of CA, DCIV and DCVII were prepared at 2.0 mmol/L. Each sample solution was divided into aliquots and incubated at either 24 or 40°C for 0.5, 1, 2, 4, 8, 16 and 24 d, then a portion of each sample was subjected to analytical HPLC.

The k value was calculated according to the formula: $\log C_t = -kt/2.303 + \log C_0$ ($\ln C_t = -kt + \ln C_0$), where C_t corresponds to the concentration of the sample at t days after the start of the incubation, and C_0 is the concentration of the sample at the start of the incubation.

Results and Discussion

Kinetic studies on CA, DCIV and DCVII were conducted after isolating the compounds from cochineal dye product. Figure 1 shows the changes in the concentrations of CA, DCIV and DCVII in aqueous solution incubated at either 24°C (left side (a)–(c)) or 40°C (right side (d)–(f)). As shown in Fig. 1(a), CA was converted very slowly to DCIV and DCVII over time at 24°C, with 0.012 mmol/L DCIV and 0.019 mmol/L

DCVII present in the aqueous CA solution after 24 d. This result suggested that CA, DCIV and DCVII reached equilibrium in the aqueous CA solution at 24°C. As shown in Fig. 1(b), DCIV was partially degraded during incubation at 24°C, with CA and DCVII produced within one day, and then DCIV and DCVII were gradually converted to CA until equilibrium was established between the three compounds.

Figure 2 shows kinetic curves for the degradation of DCIV and DCVII in each aqueous solution at 24°C. The k_1 value for the degradation of DCIV within the first day of incubation at 24°C was determined to be 0.901 d^{-1} (Fig. 2(a)), the k_2 value for the formation of CA from the remaining DCIV was calculated to be 0.146 d^{-1} (Fig. 2(a)), and the k value for the formation CA from the produced DCVII was found to be 0.148 d^{-1} (Fig. 2(b)). Figure 1(c) shows that some DCVII was degraded, and CA and DCIV were produced in the aqueous DCVII solution within one day. DCIV and DCVII were then gradually converted into CA until equilibrium was attained between these three compounds. The k_1 value for the degradation of DCVII within the first day of incubation was determined to be 1.102 d^{-1} (Fig. 2(d)), the k value for the formation of CA from the produced DCIV was calculated as 0.109 d^{-1} (Fig. 2(c)), and the k_2 value for the formation of CA from the remaining DCVII was 0.147 d^{-1} (Fig. 2(d)). From the results shown in Figs. 1(a)–(c), the K values were calculated to be 1.22×10^{-7} mol/L for CA, 2.61×10^{-3} mol/L for DCIV, and 2.36×10^{-3} mol/L for DCVII, as summarized in Table 1.

At 40°C, DCIV (Fig. 1(e)) and DCVII (Fig. 1(f)) in aqueous solution both transformed into CA more rapidly than at 24°C and reached equilibrium within 2 d, where it was difficult to illustrate the figure on the kinetic curves. These results suggested that the rate of the equilibrium reaction depends on the temperature.

Table 1. Equilibrium Constants for Each Reaction at 24°C

Reaction	K (mol/L)
CA→DCIV+DCVII	1.22×10^{-7}
DCIV→CA+DCVII	2.61×10^{-3}
DCVII→CA+DCIV	2.36×10^{-3}

As described above, the degradation rates of DCIV and DCVII in aqueous solution were similar (0.901 d^{-1} for DCIV and 1.102 d^{-1} for DCVII). This result suggests that the anomerization reaction from the α- to β-form and the β- to α-form proceeds at similar rates after the dried purified sample is dissolved in water when DCIV is 2-*C*-α-glucofuranoside form and DCVII is 2-*C*-β-glucofuranoside form, respectively. Following anomerization, both DCIV generated from DCVII and DCVII generated from DCIV in aqueous solution are slowly converted into CA until equilibrium is achieved with the remaining original DCVII or DCIV and this conversion into CA occurs at a similar rate (in the DCIV solution: 0.146 d^{-1} for DCIV and 0.148 d^{-1} for DCVII, in the DCVII solution: 0.109 d^{-1} for DCIV and 0.147 d^{-1} for DCVII). The dynamic equilibrium between CA, DCIV and DCVII in aqueous solution is schematically illustrated in Fig. 3.

The K value for CA in aqueous solution is about 10^4 fold less than that for DCIV and DCVII, which suggests that CA is more stable than DCIV and DCVII. CA contains a glucopyranose moiety, whereas DCIV and DCVII contain a glucofuranose moiety. It has been reported that glucose is more stable in the pyranose form than in the furanose form because of the lower energy of its intermolecular hydrogen bonds,[17] and that in aqueous solution, glucose primarily exists in the pyranose form and not in the furanose form (≦1%) at equilibrium.[18] These observations may explain the difference in the equilibration rate between CA and its two isomers, DCIV and DCVII.

This study focused on assessing the equilibrium between *C*-glucofuranosides, reported only once previously,[19] although there are a couple of reports on *C*-glycosides, including *C*-glycoside flavonoids,[20] xanthones[21] and anthranoids.[22] The k and K values calculated in the present study may help clarify the structural changes that take place during anomerization between *C*-glucopyranoside and *C*-glucofuranoside.

Conclusion

CA and its two isomers, DCIV and DCVII, were isolated in highly purified form from cochineal dye using a series of reversed-phase chromatographic methods and their kinetic properties were evaluated. The degradation rates of DCIV

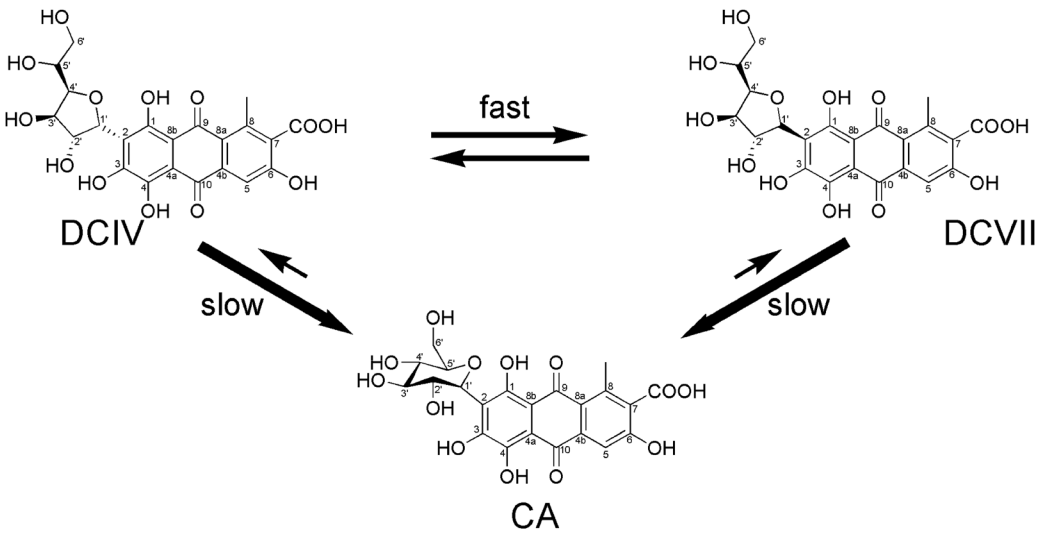

Fig. 3. Schematic Illustration of the Dynamic Equilibrium between CA, DCIV and DCVII in Aqueous Solution

and DCVII were similar in aqueous solution. The DCIV and DCVII produced in aqueous solution were slowly converted into CA until equilibrium was reached, and the remaining DCVII and DCIV were converted to CA at similar rates. The K for CA was about 10^4 fold less than that for DCIV and DCVII. These results suggested that CA is major component in every kind of raw material, and the influence to the quality and safety of products including cochineal dye is negligible even if DCIV and DCVII are contained much. This study of the time-dependent changes in concentration of the minor components DCIV and DCVII will be very helpful for establishing the safety of cochineal dye products and for their quality assurance.

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Conflict of Interest The authors declare no conflict of interest.

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