Quantitative Determination of Carthamin in Carthamus Red by ^{1}H-NMR Spectroscopy

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Carthamus Red is a food colorant prepared from the petals of *Carthamus tinctorius* (Asteraceae) whose major pigment is carthamin. Since an authentic carthamin standard is difficult to obtain commercially for the preparation of calibration curves in HPLC assays, we applied ^{1}H-NMR spectroscopy to the quantitative determination of carthamin in commercial preparations of Carthamus Red. Carthamus Red was repeatedly extracted in methanol and the extract was dissolved in pyridine-d_5 containing hexamethyldisilane (HMD) prior to ^{1}H-NMR spectroscopic analysis. The carthamin contents were calculated from the ratios of singlet signal intensities at approximately σ: 9.3 derived from H-16 of carthamin to those of the HMD signal at σ: 0. The integral ratios exhibited good repeatability among NMR spectroscopic analyses. Both the intra-day and inter-day assay variations had coefficients of variation of <5%. Based on the coefficient of absorption, the carthamin contents of commercial preparations determined by ^{1}H-NMR spectroscopy correlated well with those determined by colorimetry, although the latter were always approximately 1.3-fold higher than the former, irrespective of the Carthamus Red preparations. In conclusion, the quantitative ^{1}H-NMR spectroscopy used in the present study is simple and rapid, requiring no carthamin standard for calibration. After HMD concentration has been corrected using certified reference materials, the carthamin contents determined by ^{1}H-NMR spectroscopy are System of Units (SI)-traceable.

Key words quantitative NMR; carthamin content; Carthamus Red; *Carthamus tinctorius* (Asteraceae)

Carthamus Red is a natural colorant, which is produced from the dried petals of *Carthamus tinctorius* L. (Asteraceae). It has been permitted for use and is distributed in Japan because it is accepted as a food additive on the List of Existing Food Additives prepared by the Ministry of Health, Labour and Welfare of Japan.[1] The major pigment in Carthamus Red is carthamin with two fully conjugated chalcone moieties,[2,3] whereas a minor red pigment was isolated and identified as the hydroxyethyl ether of carthamin[4] (Fig. 1A). Carthamin was reported to exhibit potent radical-scavenging and neuroprotective activities.[5] The carthamin content is an important index for the evaluation and/or validation of commercial preparations of Carthamus Red. An HPLC method was described for the quantitative determination of carthamin.[6] Nevertheless, a colorimetric index based on the color value has been used to validate the Carthamus Red preparations[7] because it is difficult to obtain commercially an authentic carthamin sample to prepare the calibration curves.

NMR spectroscopy is a powerful tool for structure elucidation and quantitative determination of organic molecules.[8] The basis of proton-specific quantitative NMR (qHNMR) spectroscopy is that the integral of the signal (area under the signal) is proportional to the number of protons producing the signal and that the signal intensity per proton is proportional to the molar amount of the compound. qHNMR spectroscopy is unique because it allows absolute quantification of organic compounds by comparing the signal intensity of a specific compound and that of an appropriate internal standard with known absolute purity, and the value obtained is theoretically traceable using the International System of Units (SI-traceable).[9,10] Compared with HPLC, qHNMR spectroscopy is advantageous because no standard compounds are required to prepare calibration curves. Furthermore, qHNMR spectroscopy is rapid and non-invasive, and in most cases it does not require laborious sample pre-cleaning steps.[11]

In the present investigation, we demonstrated that qHNMR spectroscopy can be effectively applied for the quantitative determination of carthamin in commercial preparations of Carthamus Red without an authentic carthamin sample.

Experimental

Isolation of Carthamin Carthamus Red (color value 8000; Yaegaki Bio-Industry, Inc., Himeji, Japan) was stirred with methanol at room temperature for 3 h. The methanol extract was applied to octadecylsilyl silica gel (ODS) column which was then eluted with 60% methanol containing 0.2% formic acid. Red pigment fraction thus obtained was purified by repeating ODS column chromatography to yield carthamin. Purity of carthamin was estimated to be 42.6±1.1% by qHNMR method.

Carthamus Red Preparations Commercial preparations of Carthamus Red were obtained from various suppliers (Table 1). The voucher samples were stored in the Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University. The color values of preparations were determined by a previously described method.[12]

Chemicals Pyridine-d_5 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hexamethyldisilane (HMD, Sigma-Aldrich Inc., St. Louis, MO, U.S.A.) was used as an internal standard for NMR spectroscopic analyses.

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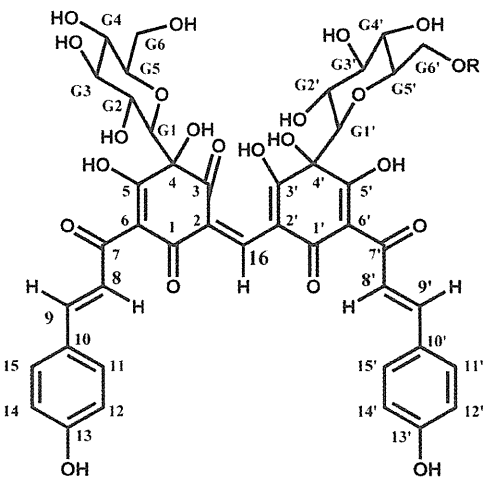
(A)



R=H: Carthamin
R=CH_2CH_2OH: Carthamin hydroxyethyl ether

(B)

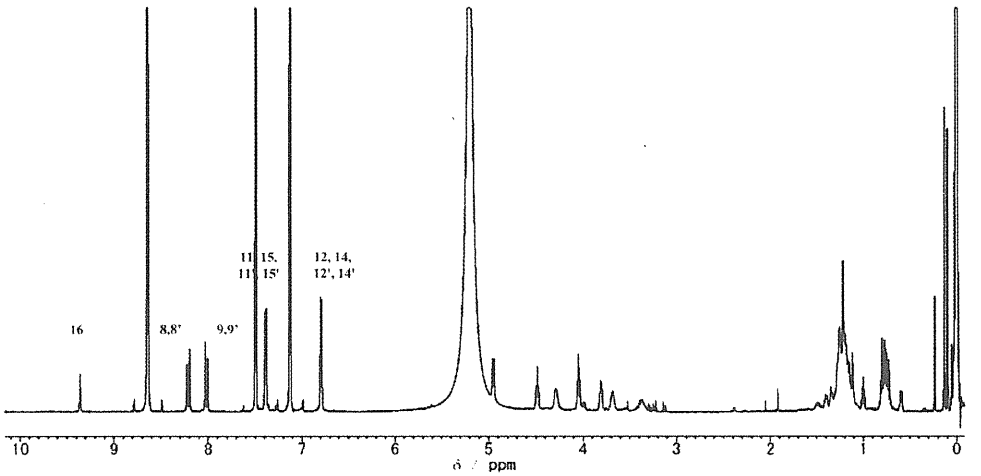


Fig. 1. (A) Chemical Structures of Carthamin and Carthamin Hydroxyethyl Ether and (B) ^{1}H-NMR Spectrum of Carthamin Prepared from Carthamus Red

Carthamin was dissolved in pyridine-d_5 containing hexamethyldisilane (HMD) as a NMR standard.

Table 1. Commercial Preparations of Carthamus Red Used in the Present Investigation

Carthamus Red	Lot No.	Color value	
		Claimed	Estimated[a]
Product No. 1	09A012	NA[b]	18.4±0.8
Product No. 2	90625	13.8	13.9±0.3
Product No. 3	90612	NA	0.143±0.02
Product No. 4	90701	7.43	8.19±0.99
Product No. 5	90528	0.455	0.354±0.02

a) Average±standard deviation from five independent measurements. *b*) Not available.

Potassium hydrogen phosphate (PHP; NMIJ CRM 3001-a), a certified reference material whose purity was certified to be 100.00±0.027%, was purchased from Wako Pure Chemical Industries, Ltd. 1,4-Bis(trimethylsilyl)benzene-d_4, a reference material for qHNMR spectroscopy whose purity was certified to be 99.8% according to the National Metrology Institute of Japan (NMIJ), was obtained from Wako Pure Chemical Industries, Ltd.

^{1}H-NMR Spectroscopy Apparatus and Parameters The ^{1}H-NMR spectra were recorded using a Bruker Avance 600 (600 MHz) spectrometer. NMR spectroscopy acquisition and processing were performed as previously described.[11] In brief, eight scans were performed for each sample with a 90° pulse and a 30-s pulse delay because high precision qHNMR spectra can be achieved when the pulse angle is 90° and the pulse delay time is greater than the quintuple spin-lattice relaxation time (>5*T_1). The chemical shifts are given as δ values (ppm) relative to the internal standard HMD. The start and end points were manually selected during the integration of the signal.

Determination of HMD Concentration in the qHNMR

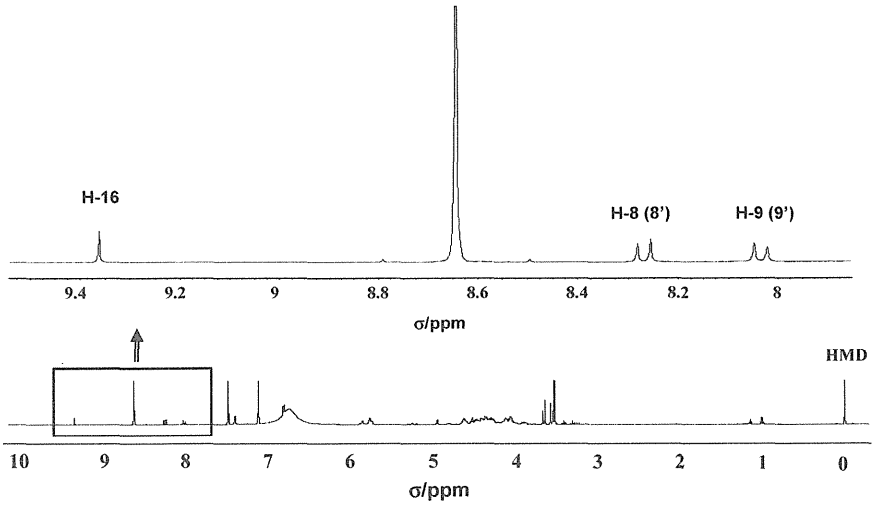


Fig. 2. ^{1}H-NMR Spectrum of the Methanol Extract of a Carthamus Red Preparation Carthamus Red Product No. 1 (Table 1) Was Sonicated in Methanol and the Methanol Extract Was Dissolved in Pyridine-d_5, Which Contained Hexamethyldisilane (HMD) as the Internal Standard

The top spectrum contains signals that correspond to the H-16, H-8 (8'), and H-9 (9') of carthamin.

Spectroscopy Reference Solution The qHNMR spectroscopy reference solution was essentially prepared as previously described.[13,14] In brief, to prepare the qHNMR spectroscopy reference solution, HMD (10 mg) was dissolved in pyridine-d_5 (25 mL) and the accurate concentration of HMD was determined by measuring the ^{1}H-NMR spectrum with the internal standard bisphenol A. Bisphenol A (5.00 mg) was dissolved in 1.00 mL of the qHNMR spectroscopy reference solution and subjected to ^{1}H-NMR spectroscopic analysis. HMD concentration in the qHNMR spectroscopy reference solution was calculated based on the ratio of signal intensity at δ: 0 (HMD) to that at δ: 1.6 (bisphenol A).

Determination of Carthamin by qHNMR Spectroscopy Carthamus Red (50 mg or 1 g) was sonicated in 5.0 mL methanol at room temperature for 30 min. The slurry was centrifuged at 3000 rpm for 10 min at room temperature. The extraction process was repeated thrice. Further extraction yielded nearly colorless supernatant. The supernatants were combined and concentrated to dryness *in vacuo*. The residue was dissolved in 1.00 mL of the qHNMR spectroscopy reference solution and subjected to NMR spectroscopic analysis. The carthamin concentration was calculated based on the ratio of peak integration at δ: 9.37 (H-16 of carthamin) to that at δ: 0 (HMD).

Spectrophotometric Determination of the Carthamin Contents and the Color Values of Carthamus Red Carthamus Red (10–40 mg, based on the preparation) was dissolved in 10 mL dimethylformamide (DMF). After preparing an appropriate dilution, absorbance of the solution was measured at 530 nm. The carthamin content of the Carthamus Red preparation was estimated on the basis of the reported absorption coefficient ($E_{1\%}$=992).[15] Color value of the preparation was calculated as the absorbance of a 10% solution.

Results and Discussion

Selection of a Target Signal for the qHNMR Spectroscopy Analysis of Carthamin We isolated carthamin from Carthamus Red and measured ^{1}H-NMR and ^{13}C-NMR. The ^{1}H-NMR spectrum is shown in Fig. 1B and NMR data are presented in Table 2. Signals from all the protons of the isolated carthamin were clearly detected as separate signals in the spectrum and their chemical shifts were in good agreement with those reported previously.[4] Then, we measured the ^{1}H-NMR spectrum of a Carthamus Red preparation (Product No. 1 in Table 1). The signals corresponding to H-16, H-8 (8') and H-9 (9') of carthamin were detected at σ: 9.37 (singlet), σ: 8.22 (doublet) and σ: 8.02 (doublet), respectively. The signal that corresponded to H-16 formed a clearly separated signal for all commercial preparations tested in the present investigation. The signals derived from H-8 (8') and/or H-9 (9')

Table 2. ^{1}H- and ^{13}C-NMR Spectral Data for Carthamin Isolated from Carthamus Red [a]

Proton number [b]	^{1}H-NMR data (ppm)	^{13}C-NMR data (ppm)
1, 1'	—	189.3
2, 2'	—	113.5
3, 3'	—	193.8
4, 4'	—	90.2
5, 5'	—	194.4
6, 6'	—	109.9
7, 7'	—	184.0
8, 8'	8.22 (d, J=15.6 Hz)	121.3
9, 9'	8.02 (d, J=15.6 Hz)	142.0
10	—	127.5
11, 15, 11', 15'	7.39 (d, J=8.4 Hz)	131.0
12, 14, 12', 14'	6.79 (d, J=8.4 Hz)	116.7
13, 13'	—	161.2
16	9.37 (s)	144.1
G1, G1'	4.96 (d, J=9.6 Hz)	86.9
G2, G2'	4.49 (t, J=9.0 Hz)	71.2
G3, G3'	4.05 (t, J=9.0 Hz)	83.1
G4, G4'	3.70 (brd, J=*ca.* 9 Hz)	80.2
G5, G5'	3.83 (t, J=7.4 Hz)	71.7
G6, G6'	4.31 (brd, J=*ca.* 10 Hz)	63.5
	4.08 (brd, J=*ca.* 10 Hz)	60.4

a) ^{1}H- and ^{13}C-NMR were measured at 600 MHz and 150 MHz, respectively, in pyridine-d_5. Hexamethyldisilane (HMD) was used as an internal standard for ^{1}H-NMR measurement. *b*) For proton number see Fig. 1.

Table 3. Ratios of Intensity per Proton of the Signals from Carthamin to That from Hexamethyldisilane (HMD)

Preparation	Target proton		
	H-16 [σ: 9.37 (singlet)]	H-8,8′ [σ: 8.22 (doublet)]	H-9,9′ [σ: 8.02 (doublet)]
Product No. 1	1.35±0.01[a)]	1.34±0.01	1.37±0.02
Product No. 2	1.14±0.04	1.10±0.01	ND[b)]
Product No. 4	0.360±0.017	ND	ND

a) Average±standard deviation from triplicate measurements on a single extract. *b*) Not determined due to the presence of interfering signals.

Table 4. Carthamin Contents of Commercial Preparations of Carthamus Red

Carthamus Red	Carthamin content (%)[a)]	
	qHNMR method	Colorimetric method
Product No. 1	13.6±0.06	18.1±0.6
Product No. 2	10.3±0.02	14.1±0.3
Product No. 3	0.0913±0.002	0.133±0.004
Product No. 4	5.58±0.07	7.45±0.23
Product No. 5	0.261±0.001	0.336±0.030

a) Average±standard deviation from five independent measurements.

overlapped with interfering signals in the methanol extracts of some preparations. The ratio of intensity of each signal per proton to the intensity of HMD was calculated. Regardless of the signal used for calculation, provided the signal was detected as a separate peak, the ratio was constant (Table 3). Based on these results, H-16 was selected as the target signal because it appeared in a relatively lower field, which usually contained fewer signals that may interfere with the accurate estimation of the signal intensity.

In the present investigation, HMD was used as an internal standard for the calibration of chemical shifts and the quantification of signal intensities in ^{1}H-NMR spectroscopic analyses. HMD is less volatile, so it is easier to weigh than tetramethylsilane (TMS). To determine the accurate HMD concentration in the qHNMR spectroscopy reference solution, the ^{1}H-NMR spectrum of Certified Reference Material (CRM)-grade bisphenol A was recorded in the qHNMR spectroscopy reference solution and the HMD concentration was calculated by comparing the signal integrated at δ: 0 from HMD with the methyl hydrogen signals of bisphenol A at δ: 1.63. At present, SI-traceable NMR spectroscopy standards such as 1,4-bistrimethylsilylbenzene (1,4-BTMSB)-d_4 and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS-d_6) are commercially available and can be used as qHNMR spectroscopy internal standards without calibrating the concentration using a second internal SI-traceable standard such as CRM grade bisphenol A. Therefore, we attempted to use 1,4-BTMSB-d_4 instead of HMD. When HMD was used to calibrate the chemical shift of 1,4-BTMSB-d_4, the proton signal from 1,4-BTMSB-d_4 appeared at δ: 0.1. The ^{1}H-NMR spectra of five commercial Carthamus Red preparations were measured using HMD as the standard and the spectra from two preparations contained signals at approximately δ: 0.1; thus, in the present investigation, 1,4-BTMSB-d_4 was not be used as the qHNMR spectroscopy standard.

Validation of qHNMR Spectroscopy Repeatability of measurement of the carthamin signal intensity at δ: 9.37 was assessed by recording five consecutive spectra on the same day (intra-day runs) using the same qHNMR spectroscopy assay solution from one Carthamus Red preparation (Product No. 1). The average ratio of the signal intensity of carthamin H-16 to that of HMD was 1.44±0.02 and the coefficient of variation was 1.5%. The reproducibility was estimated by performing six measurements of the ratio of signal intensity of carthamin H-16 to that of HMD either on the same day (intra-day runs) or different days (inter-day runs) using different samples of the identical Carthamus Red preparation (Product No. 1) and the identical experimental protocol. The coefficient of variation for the intra-day runs was 3.3% and that for the inter-day runs was 2.2%. Until the signal-to-noise ratio of the H-16 signal in the ^{1}H-NMR spectrum reached a minimum value of 5, the limit of quantification for carthamin was determined by analyzing serial dilutions of the solution from the commercial Carthamus Red preparation (Product No. 1). The limit was calculated as 0.214 mg/mL (0.235 mM) in the qHNMR spectroscopy reference solution. The ratio of the integrated signal corresponding to the H-16 of carthamin to that of HMD was proportional to the carthamin concentration of the qHNMR spectroscopy sample solution at 0.214–3.42 mg/mL. Because the extract prepared from as much as 1 g of Carthamus Red preparation was dissolved in 1 mL of the qHNMR spectroscopy reference solution, the detection limit of 0.214 mg/mL indicates that the minimum carthamin content in Carthamus Red preparation that can be determined by qHNMR spectroscopy is 0.02% (w/w).

For estimating recovery rate of carthamin by the present qHNMR method, 3.0 mg of carthamin was spiked into 300 mg Carthamus Red preparation (Product No. 1). Three sets of the spiked samples and three sets of the blank extract (without addition of carthamin) were subjected to qHNMR analysis. The recovery rate was determined to be 106%.

Determination of Carthamin Contents in Commercial Preparations of Carthamus Red The carthamin contents of five commercial Carthamus Red preparations distributed in Japan were quantitatively analyzed by qHNMR spectroscopy. In all the Carthamus Red preparations, the signal corresponding to the H-16 of carthamin was well separated from other signals and no interfering signals were detected at δ: 9.0–10.0 in the NMR spectrum (Fig. 2). Two preparations (products No. 3 and No. 5) had color values of <100 and their NMR spectra contained clear signals that corresponded to H-16 when the extracts were prepared starting from 1 g. The carthamin contents of these samples were determined to be in the range of 0.0913–13.6% (Table 2). Sato *et al.* reported that a proton signal (σ: 9.43) that corresponded to the H-16 of carthamin hydroxyethyl ether, a minor red pigment of Carthamus Red, was clearly distinguishable from that of carthamin.[4] Such a signal was not detected in any of the preparations in the present experimental conditions.

We colorimetrically estimated the carthamin contents of the Carthamus Red preparations based on the reported absorption coefficient of carthamin ($E_{1\%}$=992; at 530 nm in DMF).[14] Although carthamin contents estimated by colorimetry correlated well with those determined by qHNMR spectroscopy, the colorimetric-based values were always approximately 1.3-fold higher than those calculated by qHNMR spectroscopy, irrespective of the commercial products tested (Table 4). This

suggests that the carthamin standard used to determine the absorption coefficient was impure, and the reported absorption coefficient was underestimated. In addition, it is likely that some minor pigments that absorbed visible light at approximately 530 nm were present in the preparation. Nevertheless, the result also indicates that the absorption at 530 nm is a good index for comparing the relative carthamin contents among Carthamus Red preparations.

In conclusion, the carthamin content of the food colorant Carthamus Red can be determined by ^{1}H-NMR spectroscopy. The present method is simple and requires no carthamin standard to prepare the calibration curves. In addition, it quantifies the SI-traceable carthamin contents, if the concentration of NMR spectroscopic internal standard is determined using certified reference materials. It is interesting to note that the present method is likely to be applicable to chemical evaluation of Carthami Flos (dried flowers of *Carthamus tinctorius*).

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