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Isomer Separation of *trans*-Astaxanthin, 9-*cis*-Astaxanthin and 13-*cis*-Astaxanthin by Ligand Exchange Chromatography

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The astaxanthin molecule has two geometric isomers, *cis* or *trans* on account of the double bond from the polyene chain. The separation of these isomers is quite difficult using ordinary techniques. The conditions of ligand exchange chromatography for stereoisomer separation were examined using an astaxanthin stereoisomer. A C_{18} column was used with a mobile phase consisting of an acetonitrile-chloroform-methanol chiral mobile phase additive solution (containing different concentrations of L-serine and copper sulfate) at flow rate of 0.5 mL/min. The effects of different kinds and concentrations of ligands, bivalent ligand ions and organic modifier were evaluated. The astaxanthin isomers could be obtained from heat-treating the astaxanthin standard, which were confirmed by the ultraviolet-visible and nuclear magnetic resonance spectroscopy.

Keywords: Astaxanthin, Carotenoid, Separation, Geometric isomer, Extraction.

INTRODUCTION

Astaxanthin is a carotenoid red pigment present in microalgae, yeast, salmon, trout, krill, shrimp and crayfish. The compound belongs to xanthophyll class of carotenoids and has a chemical name of 3,3'-dihydroxy-β,β-carotene-4,4'-dione [1]. Astaxanthin has many physiological activities, such as antitumor [2], anti-inflammation, anticancer [3] and powerful antioxidant, than other carotenoids owing to its unique molecular structure [4]. Astaxanthin is a hydrocarbon of 40 carbon atoms and a ring structure at both end joined by a chain of conjugated double bonds or a polyene system. The presence of hydroxyl and carbonyl groups in each ionone ring explains some of the features of astaxanthin, such as the ability to be esterification, a more polar nature and a high antioxidant capacity [5]. Geometric isomers, *cis* or *trans*, are formed because each double bond from the polyene chain has two configurations. All-*trans* natural astaxanthin is isomerized easily to *cis*-*trans* by increasing the temperature, exposure to light, or the presence of acid [6,7]. Fig. 1 shows the structure of *trans*, 9-*cis* and 13-*cis*-astaxanthin. In one study, the antioxidant activity of *trans*, 9-*cis* and 13-*cis*-astaxanthin was examined using the free radical DPPH [2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl, free radical] scavenging activity test, lipid peroxidation, ROS (reactive oxygen species) generation in human neuroblastoma SH-SY5Y cells and collagen degradation induced by lipid hydroperoxides. As result, the *cis* isomer astaxanthin has higher antioxidant activity than the *trans* isomer [8]. Therefore, the extraction, separation and determination of the stereoisomers of astaxanthin are necessary to assess the health benefits of astaxanthin [3]. The development of extraction methods, such as solvent extraction using the variable organic solvents [9-11], supercritical fluid extraction [12,13] and oil extraction [14,15], for astaxanthin from nature sources has attracted considerable attention. On the other hand, few methods have been developed to separate the astaxanthin isomers. Generally, to separate the isomers, the chromatographic method is more effective than the others.

Ligand exchange chromatography is a liquid chromatography technique that can provide complete and reliable separation of stereoisomers of the most important classes of natural and synthetic compounds, such as amino acids, hydroxy acids and, amino alcohols [16]. Over the last few years, many amino acids, such as L-leucine, L-phenylalanine, L-serine, L-alanine, L-histidine and L-proline have been used as ligand agents. Different ligand ions, such as Mg(II), Fe(II), Zn(II) and Cu(II) ions in chiral ligand-exchange chromatography have been shown to have a considerable effect on the overall chromatographic performance [17,18]. Chiral ligand-exchange chromatography involves the formation of labile ternary metallic complexes in the mobile and/or stationary phase. These complexes can separate the enantiomers of a sample [19].

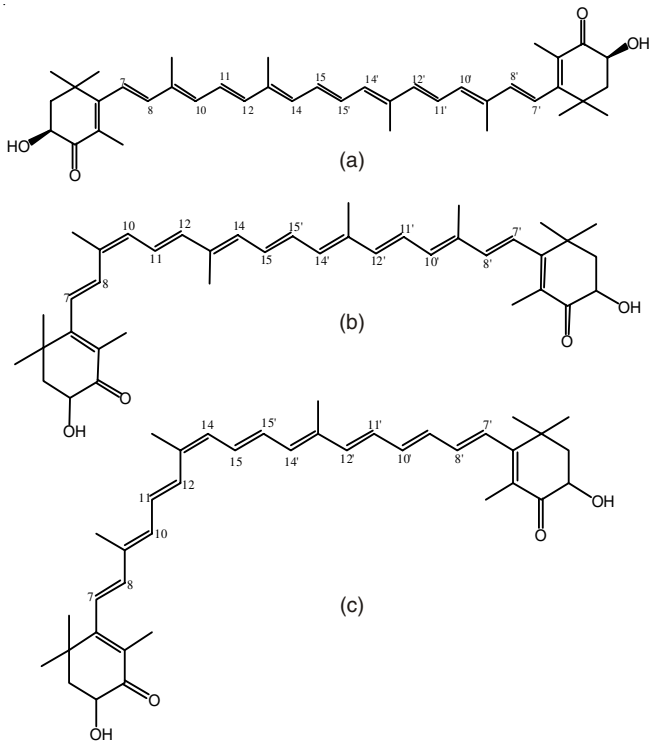

Fig. 1. Molecular structures of astaxanthin (a) *trans*-astaxanthin, (b) 9-*cis*-astaxanthin and (c) 13-*cis*-astaxanthin

Generally, chiral ligand-exchange chromatography uses chiral compounds separation. On the other hand, this study examined the separation of stereoisomers, which are geometric isomers. The appropriate HPLC conditions were developed for the separation and detection of *trans*, 9-*cis* and 13-*cis*-astaxanthin using mobile phase additives on a conventional C_{18} column. L-serine was used as a ligand agent and Cu^{2+} was used as the ligand ion. The effects of different separation conditions, such as the kinds and concentration of ligands, organic modifier and pH of the mobile phase were evaluated.

EXPERIMENTAL

The *trans*-astaxanthin (> 96 %) standard and chloroform for the NMR reference standard was obtained from Sigma. L-Leucine, L-phenylalanine, L-serine, L-alanine, L-histidine and L-proline were purchased from Sigma (St Louis, Mo, USA). The purity of all ligands was greater than 98 %. Copper sulfate pentahydrate, zinc sulfate heptahydrate, iron sulfate heptahydrate (Extra Pure grade). Methanol, chloroform, acetone and acetonitrile were supplied by Duksan Pure Chemical Co., Ltd. (Ansan, Korea). All chemicals and reagents were of HPLC grade. Distilled water was filtered through a vacuum pump (Division of Millipore, Waters, USA) and filter (HA 0.45, Division of Millipore, USA) prior to use.

Chiral ligand exchange system: High performance liquid chromatography (HPLC) was performed using a Waters 1525 Binary HPLC pump (Waters, Milford, MA, USA), a Waters 2489 UV/visible detector and a Rheodyne injection valve (IDEX Health & Science, Oak Harbor, WA, USA) (20 µL sample loop). The analytical column (250 mm × 4.6 mm i.d.) was packed with a C_{18} stationary phase (particle size 5 µm, rstech, Daejeon, Korea). A solution of the acetonitrile-chloroform-methanol chiral mobile phase additive (CMPA) consisted of a 10 mmol/L L-serine mixed with 5 mmol/L cupric sulfate in water. The mobile phase consisted of an acetonitrile-chloroform-methanol-chiral mobile phase additive solution (71:22:4:3 v/v). The flow rate of the mobile phase was set to 0.5 mL/min. The UV wavelength was set to 476 nm. The retention factor was calculated from the equation, $k = (t - t_0)/t_0$, where t and t_0 (min) are the retention times of the analyte and unretained solutes, respectively. The separation factor (α) was calculated from the equation, $\alpha_1 = k_1/k_2$, where k_1 and k_2 are the retention factors of *trans*-astaxanthin, 9-*cis*-astaxanthin and 13-*cis*-astaxanthin, respectively. The resolution was calculated from the equation, $R_1 = 2(t_{trans} - t_{9cis})/(w_{trans} + w_{9cis})$ and $R_2 = 2(t_{9cis} - t_{13cis})/(w_{9cis} + w_{13cis})$, where t_{trans} and t_{9cis}, t_{13cis} are the retention times of *trans*-astaxanthin, 9-*cis*-astaxanthin and 13-*cis*-astaxanthin respectively and w_{trans}, w_{9cis} and w_{13cis} (min) are the baseline peak widths of the enantiomers.

Sample treatment: 9-*cis* and 13-*cis*-astaxanthin cannot be obtained from the standard compounds directly. On the other hand, all-*trans* astaxanthin can be isomerized easily to the *cis*-isomer under temperature, light, oxygen or acid. Therefore, 9-*cis* and 13-*cis*-astaxanthin were obtained from the *trans*-astaxanthin standard. Astaxanthin was thermal transmuted using the procedure reported by Holtin *et al.* [25]. A stock standard solution of astaxanthin was prepared in acetone. Astaxanthin solution (1 mL) was mixed with petroleum ether (25 mL) and this solution was reflux at 70 °C for 12 h. After thermal transmutation, astaxanthin diastereomers was collect and the solvent was removed by vacuum evaporation at 40 °C and the mixture was dissolved in methanol-dichloromethane (75:25, v/v). The reaction solution was injected into the HPLC for separated of astaxanthin isomer. The three isomers of the astaxanthin were collected manually.

Preparation for the identification of isomer astaxanthin: The retention time of 9-*cis* and 13-*cis*-astaxanthin was monitored from the isomerization standard by HPLC because there was no standard compound. The mobile phase was removed by vacuum evaporation at 40 °C and only *trans*, 9-*cis* and 13-*cis*-astaxanthin remained. For UV-visible analysis, separate stock solutions of *trans*, 9-*cis* and 13-*cis*-astaxanthin were prepared in a mixture of methanol-dichloromethane (75:25, v/v). The ^{1}H NMR spectra were determined on a Varian ^{UNIT}INOVA 400 MHz spectrometer. For NMR analysis, a stock solution of *trans*, 9-*cis* and 13-*cis*-astaxanthin was collected in chloroform (NMR reference standard) and this solution was transferred to a NMR tube for analysis.

RESULTS AND DISCUSSION

Ligand exchange mechanism: Mobile phase additives were used to analyze the retention behaviour in reverse phase HPLC. Retention related the stability of the ligand complex and metal ion complex immobilized on a chromatographic stationary phase. The use of an optically active counter-ion often results in the formation of diastereomeric ion pairs which can be easily separated on conventional reversed-phase columns [20]. The stereoisomers can be separated on a C_{18} column due to the different interactions between the complex and stationary phase. Any difference in the stability constant of the interaction will result in different retention factors [21]. Fig. 2 shows the

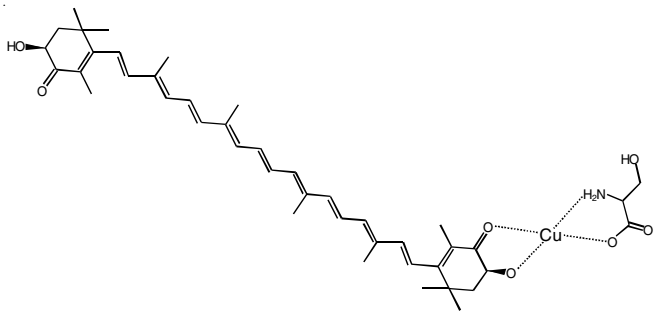

Fig. 2. Proposed structure of ligand complex of astaxanthin, L-serine and Cu^{2+}

possible structure of the ternary complex. A ligand is a molecule that binds to a central metal ion to form a coordination complex. The ligand forms a coordinate bond that provides an unshared electron pair to the central metal ion.

The astaxanthin molecule has two asymmetric carbon atoms located at the 3 and 3' positions of the benzenoid rings on either end of the molecule. Unlike the pigments that belong to the family of xanthophylls, astaxanthin has two carbonyl groups, two hydroxyl groups and eleven conjugated ethylenic double bonds [22]. Astaxanthin has two to four relevant ionizable functional groups: a hydroxyl group and a ketone group of the benzenoid rings on either end of the molecule. These groups allow the chelation interaction with various cations to take place. The astaxanthin stereoisomer, bivalent copper cation and L-serine could form two types of ternary complexes with different configurations.

Effects of the ligand: To examine the effects of different ligands for stereoisomer separation, L-leucine, L-phenylalanine, L-serine, L-alanine, L-histidine and L-proline were used as the ligand. As shown in Table-1, L-serine (R_1 = 2.39, R_2 = 1.86) provided a better resolution than the other ligand, such as L-leucine, L-phenylalanine, L-alanine, L-histidine and L-proline (R_1 = 1.61, R_2 = 1.22). R_1 is the resolution of *trans*-astaxanthin to 9-*cis*-astaxanthin, R_2 is the resolution of 9-*cis*-astaxanthin to 13-*cis*-astaxanthin. Therefore, L-serine was selected as the ligand for stereoisomer separation. According to Krustulovic *et al.* [23] and Gorog *et al.* [24], a ligand should have two or more functional groups to form a cheliform complex and should have a larger group to produce a space exclusion function. In addition, the ligand indicated that the chiral ligand for the stereoisomer should possess a larger group to produce a space exclusion function and also should possess certain lipophilia to be retained by the reverse stationary phase. The well separated *trans*, 9-*cis*- and 13-*cis*-astaxanthin contained chiral mobile phase additive in mobile phase (Fig. 3). The

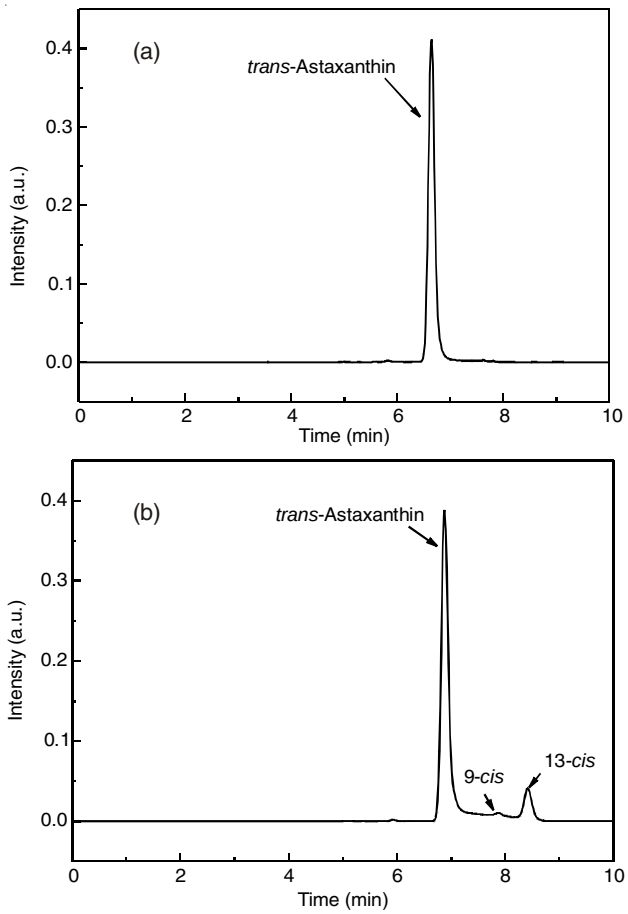

Fig. 3. Chromatogram of astaxanthin stereoisomer separation (flow rate: 0.5 mL/min, column: C_{18} (4.6 × 250 mm, 5 µm), UV: 476 nm, injection volume: 10 µL, standard concentration: 0.5 mg/mL, (a) Mobile phase: acetonitrile-chloroform-methanol-water, 71:22:4:3 (v/v/v), (b) Mobile phase: acetonitrile-chloroform-methanol-chiral mobile phase additive, 71:22:4:3 (v/v/v)

ligand complex is the formation of different forms due to the astaxanthin isomer, resulting in increased retention and separation of *trans*-, 9-*cis* and 13-*cis*-astaxanthin.

The effects of different L-serine concentrations in the mobile phase on the stereoisomer separation was examined in the ranging, 0 to 12 mmol/L and the results are shown in Fig. 4. The value of k and α increased with increasing L-serine concentration from 0 to 2 mmol/L and then remained constant at approximately 2 mmol/L. Increasing the ligand concentration in the mobile phase could result in the formation of more ligand complexes. These complexes can then be distributed on the stationary phase, which would increase the retention and separation of the stereoisomer separation.

TABLE-1
EFFECT OF DIFFERENT LIGANDS ON THE SEPARATION OF STEREOISOMER

Different ligands	Retention factor			Resolution (R)		Selectivity (α)	
	k_t	k_{9cis}	k_{13cis}	R_1	R_2	α_1	α_2
L-leucine	0.36	0.56	0.67	2.18	1.37	1.55	1.19
L-phenylalanine	0.48	0.69	0.81	2.37	1.72	1.43	1.17
L-serine	0.21	0.38	0.48	2.39	1.86	1.84	1.25
L-alanine	0.37	0.56	0.67	2.14	1.59	1.53	1.19
L-histidine	0.25	0.45	0.59	2.04	1.61	1.82	1.30
L-proline	0.45	0.66	0.78	1.61	1.22	1.47	1.18
Not including ligand	0.32	0.49	0.59	1.65	1.11	1.54	1.19

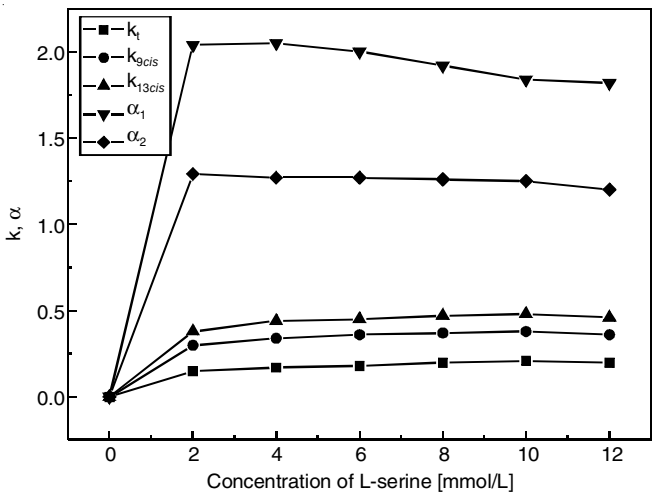

Fig. 4. Effect of L-serine concentration in mobile phase

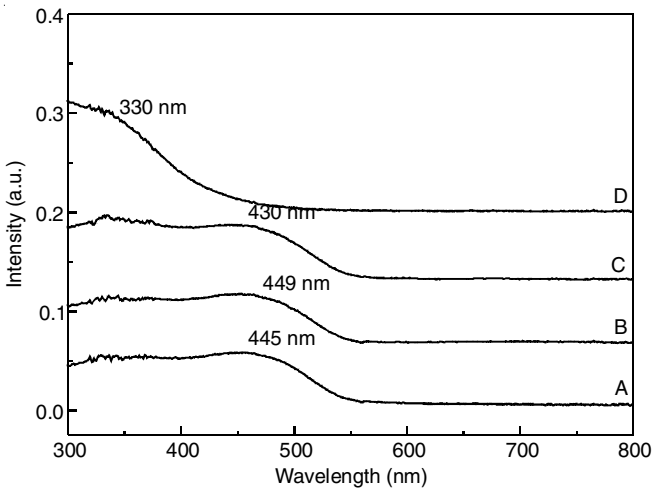

Fig. 5. UV-visible spectra of *trans*-astaxanthin, 9-*cis*-astaxanthin, 13-*cis*-astaxanthin isomer in methanol-dichloromethane (a) standard, (b) *trans*-astaxathin, (c) 9-*cis*-astaxanthin, (d) 13-*cis*-astaxanthin)

Iron, copper and zinc have similar chemical characteristics because they are in the same period of the periodic table. To examine the effects of different divalent ions for stereoisomer separation, Fe^{2+}, Cu^{2+} and Zn^{2+} were used as the central metal ion. The solution of chiral mobile phase additive (CMPA) consisted of 10 mmol/L L-serine mixed with 5 mmol/L Fe^{2+}, Cu^{2+} and Zn^{2+} in water (100 mL), respectively. The chiral mobile phase additive level only 3 % in the mobile phase (Table-2).

TABLE-2
EFFECT OF DIFFERENT DIVALENT IONS ON THE SEPARATION OF STEREOISOMER

Divalent ions	Retention factor			Resolution (R)		Selectivity (α)	
	k_t	k_{9cis}	k_{13cis}	R_1	R_2	α_1	α_2
Fe^{2+}	0.20	0.35	0.44	1.91	1.31	1.80	1.23
Cu^{2+}	0.21	0.38	0.48	2.39	1.86	1.84	1.25
Zn^{2+}	0.15	0.30	0.39	1.81	1.02	2.02	1.28

Effects of organic modifier: Acetonitrile, methanol and chloroform were used as the organic modifiers in the mobile phase to examine the effect of different organic solvents. When acetonitrile was used, cupric sulfate was not dissolved completely in the mobile phase. Therefore, acetonitrile was unsuitable. When chloroform was used, white gel-like sediment was formed in the mobile phase, which is believed to be copper(I) chloride (CuCl). The CuCl is a white solid that is sparingly soluble in water, alcohol and acetone, but quite soluble in concentrated hydrochloric acid and ammonia water. The formation of CuCl expected as a result of a reaction of chloroform and copper sulfide. Therefore, sediment formative chloroform was unsuitable for use as a modifier. When methanol was used as the organic modifier, only a low resolution was observed, whereas water gave better resolution and retention ability.

Analysis of UV-visible and NMR: Fig. 5 shown, UV-visible spectra of the individual isolated isomers collected at different retention time. The optical absorption bands related electronic excitations in conjugated polyene chain of astaxanthin. A difference in the shape of the UV/visible spectrum was obtained only for the 13-*cis*-astaxanthin arrangement; 13-*cis*-astaxanthin don't have a peak around 400 nm compared to the other peak. However, have maximum at 330 nm (*cis*-peak). 9-*cis*-astaxanthin has a maximum at 430 nm with a smaller *cis*-peak at 330 nm. Hence, this fraction is attributed to 9-*cis*-astaxanthin which is having terminal bend when compared with the 13-*cis*-isomer. The all-*trans* ketocarotenoid exhibited an absorption maximum at 445 nm. The peak for 9-*cis*-astaxanthin exhibited a hypochromic shift of 15 nm relative to the absorption maxima of the all-*trans* astaxanthin standard.

^{1}H NMR spectroscopy was used to provide the geometrical arrangements in carotenoid research [24]. The formation of a *cis* bond results in characteristic shift differences compared to the all-*trans* compound. Therefore, attention was directed to the chemical shift of the double bonds [25]. Fig. 6a shows the ^{1}H NMR spectrum of *trans*-astaxanthin. The protons 7/72 (1.220 ppm), 10/102 (1.255 ppm) and 14/142 (1.238 ppm) present a multiplet generated by the three overlapping doublets. The spectrum of *trans*-astaxanthin has an overlapping multiplet for the protons due to the centrosymmetric of *trans*-astaxanthin. On the other hand, *cis*-astaxanthin had the largest shift compared to the *trans* spectrum due to non-centrosymmetric structure of *cis*-astaxanthin. Table-3 lists the chemical shifts of *trans*, 9-*cis* and 13-*cis*-astaxanthin. In addition, Δδ shows the difference between the *cis* and *trans* spectra ($\Delta\delta = \delta_{cis} - \delta_{trans}$). In case of *cis*-astaxanthin, the spectrum was shifted more to the right than the *trans* isomer and 7', 8', 10', 11', 12', 14' and 15' peaks were observed (Fig. 6b-c). 9-*cis* are concave protons (8 and 11) that appeared at a lower field. The convex protons (10 and 12) appeared at a higher field depending on their positions for isomerization. The quartet of the concave proton 8 (3.725 ppm) was strongly affected, with a shift difference δΔ of 0.018ppm and proton 11 (2.166 ppm) was less influenced and shows a low field shift with a δΔ value of -0.002 ppm. On the other hand, the convex protons 10 (1.254 ppm) and 12 (3.707 ppm) were shifted to a lower field, with Δδ values of -0.001 ppm (10) and -0.016 ppm (12). The protons far from the *cis* bond were almost unaffected by the stereochemical behaviour. The 13-*cis* bond was located in the area close to the molecule (15 and 15'). The 13-*cis* spectrum was shifted more to the right than the 9-*cis* isomer. As a result, the protons were shifted to a lower field.

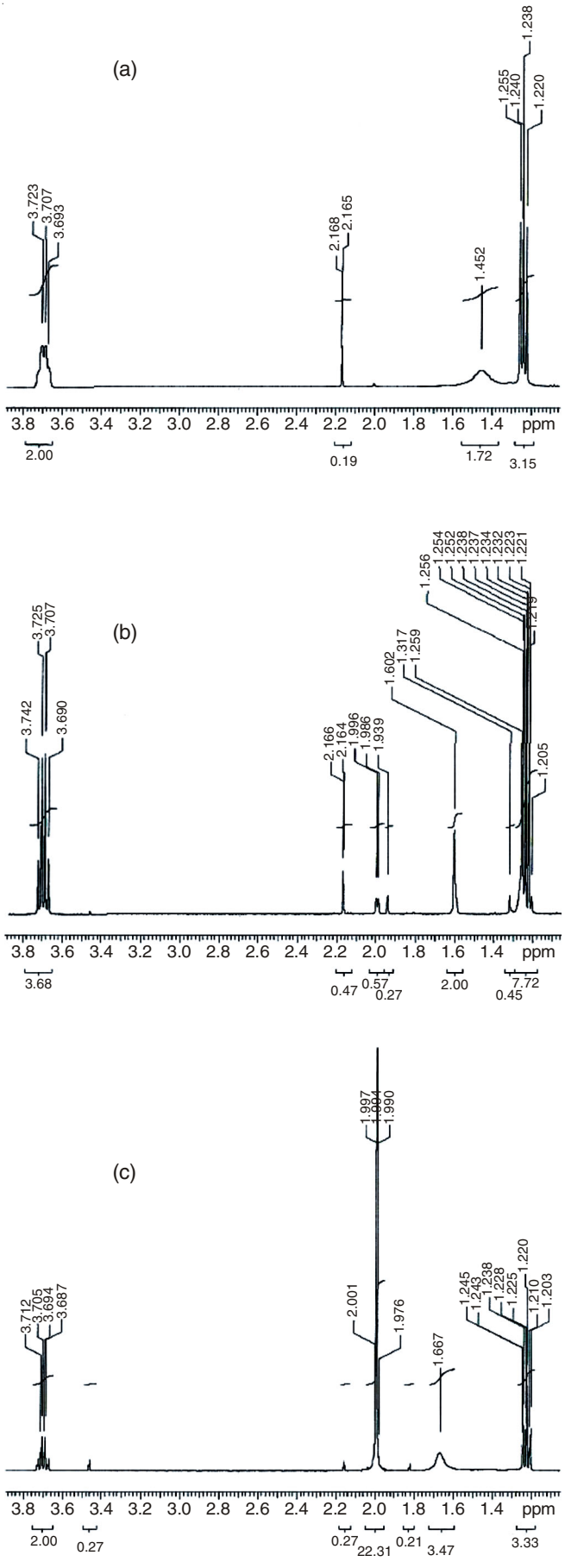

Fig. 6. ^{1}H NMR spectra (400 MHz) of isomer astaxanthin (a) *trans*-astaxanthin, (b) 9-*cis*-astaxanthin, (c) 13-*cis*- astaxanthin

TABLE-3
CHEMICAL SHIFTS δ OF *trans*, 9-*cis* AND 13-*cis*-ASTAXANTHIN ISOMERS

Proton	*trans* δ (ppm)	9-*cis* δ (ppm)	Δδ (ppm)	13-*cis* δ (ppm)	Δδ (ppm)
7	1.220	1.219	-0.001	1.203	-0.017
7'	–	1.223	0.003	1.220	0.000
8	3.707	3.725	0.018	3.687	-0.020
8'	–	3.690	-0.017	3.705	-0.002
10	1.255	1.254	-0.001	1.245	-0.010
10'	–	1.252	-0.003	1.243	-0.012
11	2.168	2.166	-0.002	1.994	-0.174
11'	–	1.996	-0.172	2.001	-0.167
12	3.723	3.707	-0.016	3.694	-0.029
12'	–	3.742	0.019	3.712	-0.011
14	1.238	1.237	-0.001	1.238	0.000
14'	–	1.238	0.000	1.225	-0.013
15	2.165	2.164	-0.001	2.154	-0.011
15'	–	1.939	-0.226	1.997	-0.168

Conclusion

This study examined the effects of different types and concentrations of ligands, bivalent ligand ions and organic modifier. Under the optimal conditions, The solution of the chiral mobile phase additive (CMPA) consisted of 2.0 mmol/L L-serine mixed with 5 mmol/L cupric sulfate in water. The mobile phase consisted of an acetonitrile-chloroform-methanol-chiral mobile phase additive solution (71: 22:4:3 v/v). Baseline separation of the *trans*, 9-*cis* and 13-*cis*-astaxanthin was achieved with a resolution of R_1= 2.39, R_2=1.86 in less than 10 min. A combination of one- and two-dimensional proton NMR spectroscopy and UV-visible also allowed unambiguous separation of the geometrical isomers present.

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