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Determination of lycopene, α-carotene and β-carotene in serum by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry with selected-ion monitoring

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Abstract

A selected-ion monitoring (SIM) determination of serum lycopene, α-carotene and β-carotene by an atmospheric pressure chemical ionization mass spectrometry (APCI–MS) was developed. A large amount of serum cholesterols disturbed the SIM determination of carotenoids by contaminating the segment of interface with the LC–MS. Therefore, separation of carotenoids from the cholesterols was performed using a mixed solution of methanol and acetonitrile (70:30) as the mobile phase on a C_{18} column of mightsil ODS-5 (75 mm×4.6 mm I.D.). The SIM determination was carried out by introducing only the peak portions of carotenoids and I.S. (squalene) by means of an auto switching valve. In the positive mode of APCI–MS, lycopene, α-carotene and β-carotene were monitored at m/z 537 and I.S. was monitored at m/z 411. This method was linear for all analytes in the range of 15–150 ng for lycopene, 7–70 ng for α-carotene and 25–50 ng for β-carotene. The detection limit of LC–APCI–MS-SIM for carotenoids was about 3 ng per 1 ml of serum ($S/N=3$). The repeatabilities, expressed as C.V.s, were 10%, 8.4% and 5.3% for lycopene, α-carotene and β-carotene, respectively. The intermediate precisions, expressed as C.V.s, were 11. 2%, 8.8% and 6.5% for lycopene, α-carotene and β-carotene, respectively.

Keywords: Lycopene; Carotene

1. Introduction

Physiological effects of β-carotene, such as anti-cancer [1,2] and anti-oxidative actions [3], on the human body have been reported. A number of coexisting substances and many kinds of carotenoids are contained in the serum. Accordingly, a precise assay of serum carotenoids is an important factor evaluating their in vivo effectiveness.

Currently, analysis of serum carotenoids is performed by an electrochemical detector [4–6] or an ultraviolet–visible absorbance detector [6–16] after separating them with high-performance liquid chromatography (LC). The electrochemical detector can analyze carotenoids with high sensitivity. However, its specificity in identifying the carotenoid peak is low and the detection of other coexisting substances can occur simultaneously with the detection of the carotenoids. In addition, stains adhering to the electrodes lower its sensitivity. The ultraviolet–visible absorbance detector enables determination with

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good reproducibility, but the specificity in detecting peaks is also inadequate.

On the other hand, detection by mass spectrometry with selected-ion monitoring (SIM) is based on structural information, hence, the use of LC–MS-SIM is usually considered to be unaffected by the presence of coexisting substances. Up to the present, reports on the analysis of β-carotene have demonstrated the use of an LC–MS apparatus with particle beam [17], continuous-flow FAB [18], electrospray [19] and atmospheric pressure chemical ionization (APCI) [20]. Although no quantitative analyses were carried out, the results have demonstrated the feasibility of generating linear calibration graphs for MS quantitation of carotenoids.

Previously, we developed a method of determination for lycopene, α-carotene and β-carotene in vegetable juice by LC–APCI–MS-SIM [21]. However, when analyzing serum carotenoids under the same analytical conditions as in the previous report, in which methanol was used as the mobile phase, some serum constituents were eluted together with the analytes. As these constituents contaminated the interface of LC–MS by adhering to it, noise peaks appeared on the SIM chromatograms. Accordingly, the measurement of analytes by SIM with LC–MS could not be continued.

The objectives of this work were (a) to ascertain and identify the serum constituents that elute simultaneously with carotenoids from the analytical column and soil the interface of LC–MS, (b) to separate carotenoids from the serum constituents disturbing the analysis of carotenoids on LC chromatograms, and (c) to develop a method for the determination of serum lycopene, α-carotene and β-carotene by LC–APCI–MS with SIM using an internal standard method.

2. Experimental

2.1. Apparatus

2.1.1. LC–APCI–MS

The LC–APCI–quadrupole MS was a computer-controlled LC/MS system model M-1200H (Hitachi, Tokyo, Japan) which consisted of a Hitachi L-7100 pump (pump A), a Hitachi L-6200 pump (pump B), a Hitachi L-7300 column oven, a Hitachi L-4000 UV detector and an SPV-N-4A auto switching valve (GL Science, Tokyo, Japan).

2.1.2. Analytical conditions

LC analyses were carried out on a Mightsil RP-18 (75 mm×4.6 mm I.D.; 5 μm particle size, Kanto, Tokyo, Japan). Elution was carried out isocratically with a mobile phase consisting of methanol–acetonitrile (70:30) at a flow-rate of 1 ml min^{-1} with a column oven temperature at 35°C. Analytes were monitored with a UV detector at a wave length of 450 nm. The injection volume was 30 μl. SIM determinations were carried out in the positive mode. The MS voltages for drift, multiplier and needle were 40 V, 1900 V and 3000 V, respectively. The MS temperatures for desolvator, nebulizer and aperture were 200°C, 400°C and 120°C, respectively. Lycopene, α-carotene and β-carotene were monitored at m/z 537, and squalene ((internal standard) I.S.) was monitored at m/z 411.

2.1.3. Maintenance of the APCI-interface

An iron needle was used as the corona discharge needle [22,23]. This needle was replaced each day at the same time, as the APCI interface was cleaned.

2.2. Reagents

2.2.1. Chemicals

Lycopene, α-carotene and β-carotene were obtained from Sigma (St. Louis, MO, USA). Squalene, used as an internal standard, and 3,5-di-*tert*.-butyl-4-hydroxytoluene (BHT) were obtained from Tokyo Chemical Industry (Tokyo Japan). Unless otherwise stated, all organic solvent contained BHT at the concentration of 30 μg ml^{-1} except for the LC mobile phase.

Serum: lyophilized serum (Dade Moni–Trol I·X) was obtained from Dade International (Miami, FL, USA). This freeze-dried serum was reconstituted with 10.0 ml of HPLC-grade water.

2.2.2. Stock standard solutions

Lycopene, α-carotene and β-carotene (5 mg) were each dissolved in 1 ml of dichloromethane. These solutions were diluted in hexane to the volume of 50 ml, and were used as the stock standard solutions.

The concentrations of stock carotenoid solutions were determined as follows: a 2 ml volume of the stock standard solution was diluted in hexane without BHT to the volume of 50 ml. The concentrations of these solution were determined from published absorption coefficients [13], i.e., $E_{1\%}$ (cm^{-1}) in hexane as a solvent: lycopene, 3450 at 472 nm, α-carotene, 2800 at 444 nm and β-carotene, 2592 at 452 nm.

Due to rapid degradation of lycopene, the solution of lycopene was prepared immediately before use.

2.3. Internal standard (I.S.) solution

Squalene was dissolved in ethanol to give the concentration of 2 μg ml^{-1}.

2.4. Preparation of serum extract for LC–MS analysis

Aliquots of serum (0.5 ml) were pipetted into a brown centrifuge tube and combined with 1.0 ml of I.S. solution. The samples were vortex-mixed for 15 s. One ml of hexane was added, and the samples were vortex-mixed for 1 min. The samples were then centrifuged for about 3 min at 1000 *g*, and the supernatants were removed and placed in brown glass vials. This extraction process was repeated, and the supernatant was removed and combined with that of the first. The combined extracts were evaporated under a stream of nitrogen, and reconstituted in 50 μl of ethanol. The reconstituted extracts were then ultrasonically agitated for 30 s to ensure dissolution and aliquots of this solution (30 μl) were injected to the LC–MS apparatus.

3. Results and discussion

3.1. APCI–MS of carotenoids and I.S.

In the positive measurement mode of the APCI, the protonated quasi-molecular ion $(M+H)^+$ is usually observed. Lycopene, α-carotene and β-carotene are geometric isomers as shown in Fig. 1. A strong quasi-molecular ion was observed at *m*/*z* 537. The LC–MS analytical conditions were the same as previously [21], except for differences in the mobile phases. In the previously reported study [21], using methanol as the mobile phase, in addition to the quasi-molecular ion, an unknown ion at *m*/*z* 567 was detected. However, when a mixed solution of methanol and acetonitrile (70:30) was used as a mobile phase, the quasi-molecular ion alone was observed (Fig. 2).

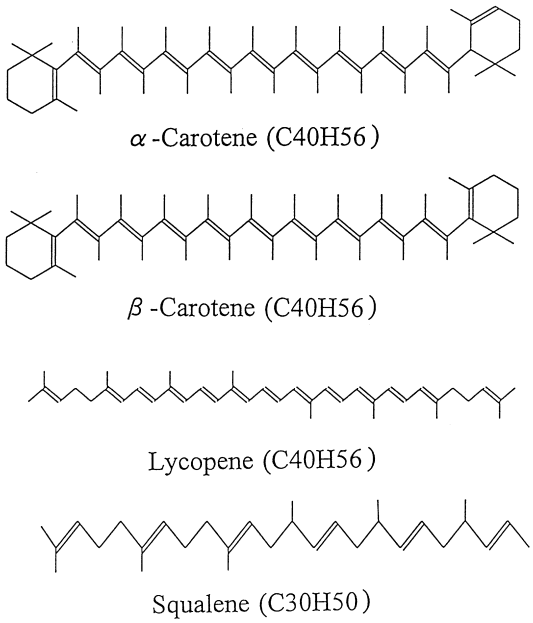

Fig. 1. Structure of carotenoids and squalene [internal standard (I.S.)].

Squalene used as the internal standard exhibited a quasi-molecular ion at *m*/*z* 411 (Fig. 2).

3.2. Identification of serum constituents disturbing the LC–APCI–MS analysis for carotenoids

In the previously reported study [21], carotenoids in vegetable juice were determined using methanol as the mobile phase. When lycopene, α-carotene and β-carotene in a hexane extract of serum were assayed under the previous analytical conditions, acceptable chromatograms were not obtained except for the first injection of the hexane extract (Fig. 3a). If the same serum specimen was analyzed successively, SIM chromatograms with good reproducibility were not obtained (Fig. 3c). In the UV chromatograms monitored parallel to the analysis shown in Fig. 3a, the presence of an unknown large peak with

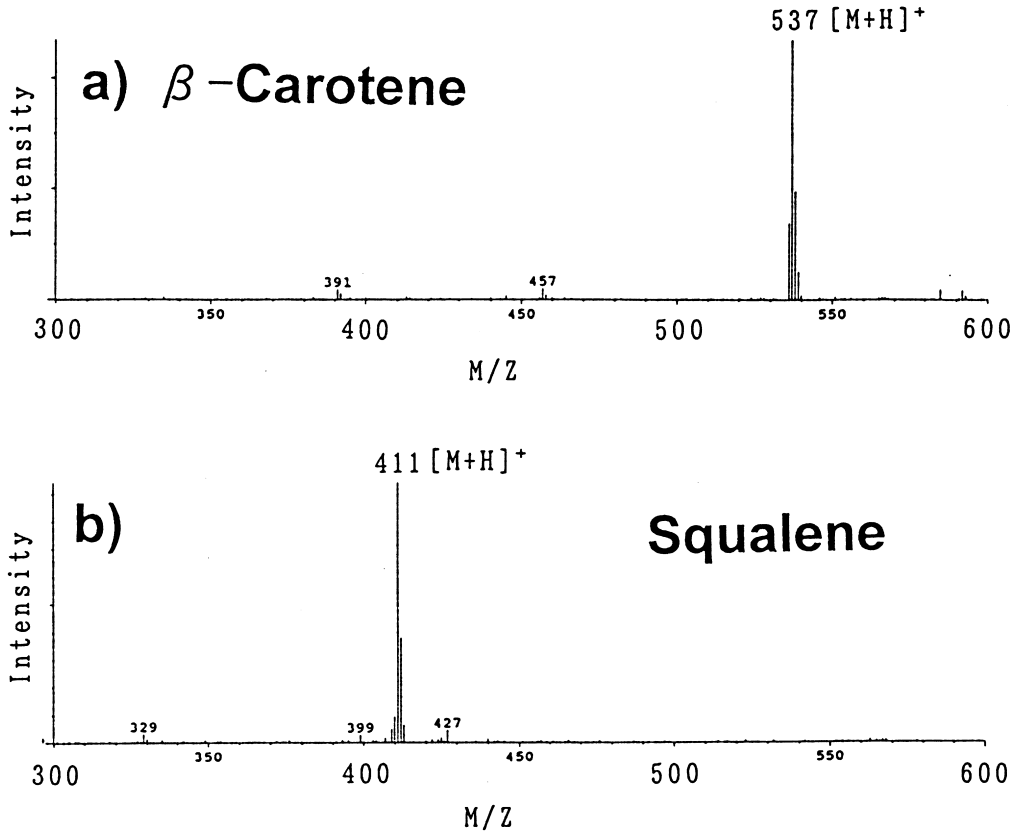

Fig. 2. LC–APCI–MS spectra of β-carotene (a) and squalene (b) when the mixed solution of methanol and acetonitorile (70:30) was used as a mobile phase for the proposed LC–MS conditions.

a retention time of 14–17 min was recognized (Fig. 3b). The corresponding APCI spectrum of this peak showed a strong ion at m/z 369. It was reported that cholesterols and cholesterol esters exhibited a large dehydrated ion at m/z 369 when measuring them in positive modes with LC–MS [21,24].

A large amount of serum cholesterols was extracted into hexane together with the carotenoids and adhered to the interface; they were eluted gradually and detected as a noise peak by a mass spectrometer. Therefore, it appeared that an acceptable chromatogram for determination could not be obtained. It was clarified that cholesterol analogue in serum interfered with the LC–MS-SIM of carotenoids in serum.

In order to separate analytes from cholesterols extracted from the serum, the conditions of the mobile phase were investigated. The increase in the ratio of acetonitrile to methanol in the mobile phase prolonged the retention time of cholesterols (Fig. 4). It was suggested that this prolongation was due to higher solubilities of cholesterols in methanol than in acetonitrile. A mixed solution of methanol and acetonitrile (70:30) was selected as the mobile phase.

We performed the SIM determination by introducing only part I, which contains peaks of carotenoids and I.S., into the interface by using an auto switching valve and discarding part II (Fig. 5a,b). This method of introducing the analytes into the interface separating serum cholesterol in the hexane extracts appeared to be indispensable to the LC–MS-SIM for giving favorable results with good reproducibility (Fig. 5c,d).

Squalene, which is the last metabolite preceding sterol ring formation in the biosynthetic cholesterol

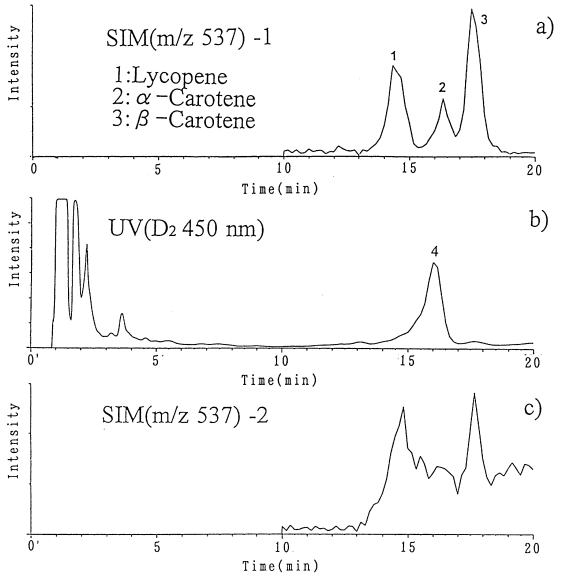

Fig. 3. Selected-ion monitoring (SIM) chromatograms of carotenoids in control serum when methanol was used as the mobile phase. Initial SIM analysis of carotinoids (a) and of the UV chromatogram (b). SIM chromatogram performed successively next to the initial analysis (c). Peaks: 1=Lycopene, 2=α-carotene, 3=β-carotene, 4=unknown (analogues of cholesterol).

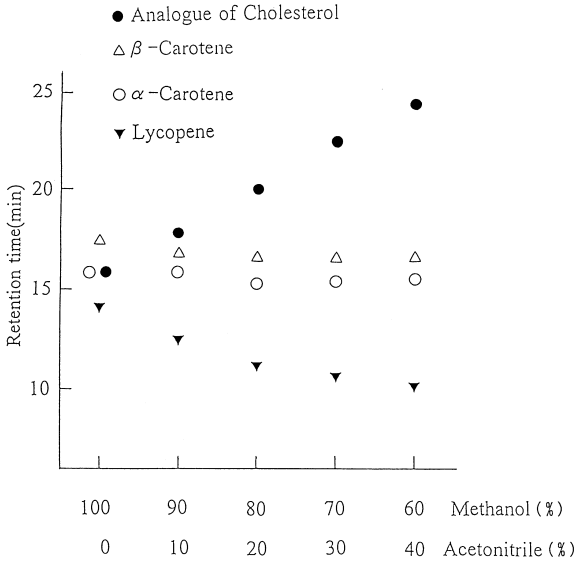

Fig. 4. Separation of the peaks between carotenoids and analogues of cholesterol based on the change of composition of mobile phase. (●) Analogues of cholesterol, (△) β-carotene, (○) α-carotene, (▼) lycopene.

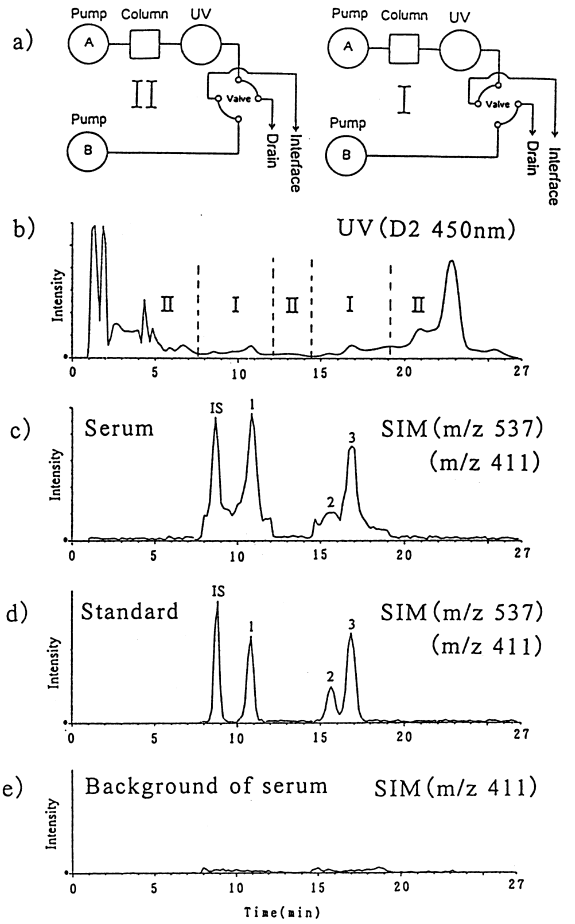

Fig. 5. Loading method of analytes to interface using an auto switching valve (a), UV chromatogram (D_2 450 nm) of the extracts from control serum (b), selected-ion monitoring (SIM) chromatogram of the control serum (c), SIM chromatogram of the standard of carotenoids and I.S. (d), and SIM (*m*/*z* 411) chromatogram obtained without adding I.S. to the control serum (e). Peaks: I.S.=squalene, 1=lycopene, 2=α-carotene, 3=β-carotene.

pathway, is found in human skin surface lipid [25,26]. However, few reports describing the existence of squalene in human serum can be found in literature. Therefore, we carried out the measurement of serum squalene by this proposed method without adding I.S. (squalene) to the serum.

The SIM chromatogram at *m*/*z* 411 did not have a squalene peak (Fig. 5e), hence, we used squalene as the internal standard for the determination of serum carotenoids.

3.3. Linearity and detection limit

Lycopene, α-carotene and β-carotene in amounts respectively, of 2000 ng, 700 ng and 2500 ng were taken out from the stock standard solution, mixed together in a 10-ml brown volumetric flask, and adjusted to volume with hexane. This hexane solution was used as a mixed standard solution. Aliquots of 0.1, 0.2, 0.5 and 1.0 ml of the mixed standard solution were evaporated respectively in nitrogen gas together with 1 ml of the internal standard solution. Next, 50 μl of ethanol was added to the residual substances and the residues were dissolved completely by the use of ultrasonic agitation. A 30 μl aliquot of this solution was injected to the LC–APCI–MS. The calibration curves were constructed based on the peak area ratio between the carotenoids and I.S., respectively.

This method was linear for all analytes in the range of 15–150 ng for lycopene, 7–70 ng for α-carotene and 25–250 ng for β-carotene. The calibration curves of lycopene, α-carotene and β-carotene passed through the origin. The correlation coefficient of the calibration curves were 0.999 for lycopene, 0.992 for α-carotene and 0.998 for β-carotene. The detection limit of LC–APCI–MS-SIM for carotenoids was about 3 ng per 1 ml of serum ($S/N=3$).

3.4. Precision of the proposed method

Spiking solution, which contained 52 ng of lycopene, 13 ng of α-carotene and 61 ng of β-carotene in 1 ml of the I.S. solution simultaneously, was prepared from the appropriate amount of the stock standard solution by combining them and diluting the mixture with I.S. solution. A 1 ml aliquot of this solution was added to 0.5 ml of serum, then lycopene, α-carotene and β-carotene were determined by the proposed method ($n=4$).

The recovery rates of lycopene, α-carotene and β-carotenes were about 90% (Table 1).

The repeatabilities obtained by repeating the determination four time successively are shown in Table 2. The intermediate precision for each of the analytes is also shown in Table 3. As for the precisions of determination, in every case, lycopene yielded a larger C.V. value than α-carotene and β-carotene, which might have been due to higher instability of lycopene than α-carotene and β-carotene.

Table 2
Repeatability of selected-ion monitoring of carotenoids in control serum by LC–APCI–MS (ng ml^{-1})

	Lycopene	α-Carotene	β-Carotene
1	164	39	140
2	181	48	156
3	136	43	154
4	159	48	139
$\bar{X}$(ng ml^{-1})	160	45	147
C.V.[a] (%)	10	8.4	5.3

[a]coefficient of variation.

Table 3
Intermediate precision of the selected-ion monitoring of carotenoids in control serum by LC–APCI–MS (ng ml^{-1})

	Lycopene	α-Carotene	β-Carotene
1	179	49	160
2	155	43	149
3	151	42	142
4	170	45	156
5	133	38	135
6	140	40	140
$\bar{X}$ (ng ml^{-1})	155	43	147
C.V.[a] (%)	11.2	9.0	6.6

[a]coefficient of variation.

Table 1
Recoveries of carotenoids in control serum by the proposed method

	Carotenoids in serum (ng 0.5 ml^{-1})	Added (ng)	Observed (ng)				Recovery (%) (Mean±S.D.)
Lycopene	81	52	137	135	120	124	92±13.2
α-Carotene	22	13	32.5	34.6	34.3	32.9	89±6.8
β-Carotene	74	61	136	131	140	136	102±5.2

The repeatability and intermediate precision of β-carotene were 5.3% and 6.6% respectively. The C.V. values were comparable with those obtained by Hasegawa [5] who determined β-carotene with an electrochemical detector after separating it with a column-switching HPLC.

4. Conclusions

If a hexane extract of a serum specimen was introduced directly into LC–APCI–MS, materials eluting in large amounts together with carotenoids from the analytical column contaminated part of the APCI–MS interface and noise peaks appeared on the SIM chromatogram. Accordingly, it was not possible to continue with SIM measurement. For this reason, by examining and elucidating the following items, a method for the determination of serum lycopene, α-carotene and β-carotenes by LC–APCI–MS-SIM was developed.

(1) The substances existing in large amounts in the serum and eluting together with analytes from the analytical column, thereby causing the disturbance of their determination were cholesterols and their esters.

(2) By using a mixed solution of methanol and acetonitrile (70:30) as the mobile phase, lycopene, α-carotene and β-carotenes could be separated from cholesterol analogues on the LC chromatograms.

(3) By introducing a portion of the analytes alone into an interface using an auto switching valve, the determination of carotenoids by LC–MS–SIM with good reproducibility, was possible.

(4) Squalene was used as the internal standard. Since squalene is a commercially available reagent and gave a large quasi-molecular ion at m/z 411 in the positive mode, it was suitable as the internal standard for the analysis of carotenoids by LC–APCI–MS–SIM.

References

[1] L.E. Hansson, O. Nyren, R. Bergstrom, A. Work, A. Lindgren, J. Baron, H.O. Adami, Int. J. Cancer. 57 (1994) 638.
[2] W.J. Blot, J. Nat. Can. Inst. 85 (1993) 1483.
[3] J.P. Allard, D. Royall, R. Kurian, R. Karpela, K.N. Jeejeebhoy, Am. J. Clin. Nutr. 59 (1994) 884.
[4] T. Murata, H. Tamai, T. Morinobu, M. Manago, A. Takenaka, H. Takenaka, M. Mino, Lipids 27 (1992) 840.
[5] H. Hasegawa, Vitamins (Jpn.) 71 (1997) 131.
[6] W.A. MacCrehan, E. Schonber, Clin. Chem. 33 (1987) 1585.
[7] F. Khachik, G.R. Beecher, M.B. Goli, W.R. Lusby, C.E. Daitch, Methods Enzymol. 213 (1992) 205.
[8] W.G. Rushin, G.L. Catignal, S.J. Schwartz, Clin. Chem. 36 (1990) 1986.
[9] W. Stahl, A.R. Sundquist, M. Hanusch, W. Schwarz, H. Sies, Clin. Chem. 39 (1993) 810.
[10] Z. Zaman, P. Fielden, P.G. Frost, Clin. Chem. 39 (1993) 2229.
[11] A.L. Sowell, D.L. Huff, P.R. Yeager, S.P. Caudill, E.W. Gunter, Clin. Chem. 40 (1994) 411.
[12] B.L. Lee, S.C. Chua, H.Y. Ong, C.N. Ong, J. Chromatogr. 581 (1992) 41.
[13] K.S. Epler, R.G. Ziegler, E. Craft, J. Chromatogr. 619 (1993) 37.
[14] M.H. Bui, J. Chromatogr. B 654 (1994) 129.
[15] J.B. Thomas, M.C. Kline, S.B. Schiller, P.M. Ellerbe, L.T. Sniegoski, D.L. Duewer, K.E. Sharpless, Fresenius J. Anal. Chem. 356 (1996) 1.
[16] J. Steghens, A.L. Kappel, E. Riboli, C. Collombel, J. Chromatogr. B 654 (1994) 129.
[17] F. Khachik, G.R. Beecher, M.B. Goli, W.R. Lusby, J.C. Smith, Anal. Chem. 64 (1992) 2111.
[18] R.B. Van Breemen, H.H. Schmitz, S.J. Schwartz, Anal. Chem. 65 (1993) 965.
[19] R.B. Van Breemen, Anal. Chem. 67 (1995) 2004.
[20] R.B. Van Breemen, C. Huang, Y. Tan, L.C. Sander, A.B. Schilling, J. Mass Spec. 31 (1996) 975.
[21] T. Hagiwara, T. Yashuno, K. Funayama, S. Suzuki, Food Hyg. Soc. Jpn. 38 (1997) 211.
[22] T. Hagiwara, M. Nishijima, J. Mass Spectrom. Soc. Jpn. 43 (1995) 365.
[23] T. Hagiwara, M. Nishijima, S. Suzuki, J. Mass Spectrom. Soc. Jpn. 44 (1996) 249.
[24] A. Takatsu, S. Nishi, Bio. Mass Spectrom. 22 (1993) 247.
[25] K. Imaeda, K. Ohsawa, T. Watanabe, Bunnseki Kagaku 36 (1987) T140.
[26] K. Aitzetmuller, J. Koch, J. Chromatogr. 145 (1978) 195.