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Journal of Chromatography B, 859 (2007) 192–200

JOURNAL OF CHROMATOGRAPHY B

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Quantification of fat-soluble vitamins in human breast milk by liquid chromatography–tandem mass spectrometry

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Received 2 April 2007; accepted 19 September 2007
Available online 17 October 2007

Abstract

Sensitive quantification method for fat-soluble vitamins in human breast milk by liquid chromatography–tandem mass spectrometry was developed. Vitamins A, D and E were extracted from 10.0 mL of breast milk after saponifying by basic condition. Vitamin K derivatives were extracted from 3.0 mL of breast milk after lipase treatment. The corresponding stable isotope-labeled compounds were used as internal standards. For the determination of vitamin D compounds, derivatization with a Cookson-type reagent was performed. All fat-soluble vitamins were determined by liquid chromatography–tandem mass spectrometry in the positive ion mode. The detection limits of all analytes were 1–250 pg per 50 μL. The recoveries of fat-soluble vitamins were 91–105%. Inter-assay CV values of each vitamin were 1.9–11.9%. The mean concentrations of retinol, vitamin D_3, 25-hydroxyvitamin D_3, α-tocopherol, phylloquinone and menaquinone-4 were 0.455 μg/mL, 0.088 ng/mL, 0.081 ng/mL, 5.087 μg/mL, 3.771 ng/mL, and 1.795 ng/mL, respectively ($n = 82$). This method makes possible to determine fat-soluble vitamins with a wide range of polarities in human breast milk. The assay may be useful for large-scale studies.
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Keywords: Vitamin A; Vitamin D; Vitamin E; Vitamin K; Fat-soluble vitamins; Liquid chromatography–tandem mass spectrometry; Breast milk

1. Introduction

International agencies and health organizations of various countries recommend breast-feeding as the preferred method of infant feeding for the first several months of life and thereafter as long as is beneficial to the relationship between mother and infant [1]. Term infants nursed by nutritionally adequate mothers are provided with sufficient energy and the proper profile of nutrients to support normal growth and development during the first 6 months except for vitamins D and K in the immediate newborn period [2,3].

There have been reports of clinical rickets in breast-fed infants, especially nursed by mothers who restrict their intake of vitamin D-rich foods (i.e. strict vegetarians) [4]. Also, in countries where climate or custom lead to low levels of exposure of the child or the mother to sunlight, infant serum concentrations of 25-hydroxyvitamin D [25(OH)D] may be sub-optimal [5,6]. In previous reports, the concentrations of vitamin D_3 (cholecalciferol, D_3), 25-hydroxyvitamin D_3 [25(OH)D_3], 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2$$D_3$] and 1α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2$$D_3$] in human breast milk were 0.03–0.12, 0.28–0.34, 0.04–0.28 and

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doi:10.1016/j.jchromb.2007.09.023

0.005–0.02 ng/mL, respectively [7–9]. These results indicate that most antirachitic activity is attributable to D_3 and $25(OH)D_3$, and the transfer of vitamin D and its metabolites from plasma to milk is limited.

Vitamin K is also a possible problem for breast-fed infants. Transplacental transfer of vitamin K is minor and infants are born with low tissue stores. Human breast milk contains only a low concentration of vitamin K and there is strong evidence of increased incidence of late haemorrhagic disease in breast-fed infants [10]. It was reported that the vitamin K_1 (phylloquinone, PK) concentration of human breast milk ranges from 1 to 9 ng/mL [11,12]; however, there are few reports associated with other important vitamin K derivatives, vitamin K_2 (menaquinone, MK), contents [13].

Regional or individual nutritional problems with other fat-soluble vitamins in breast-fed infants are also reported. In the developing world, subclinical vitamin A deficiency has been observed in infants fed with breast milk. Several reports have indicated an association between the vitamin A content of the diet in pregnancy and lactation, and vitamin A concentration in breast milk. The vitamin A content of milk in poorer populations in developing countries such as India, Ceylon and Jordan, where intake is marginal, is lower than in North America and Europe [14]. Meanwhile, there has been no report of problems with vitamin E for breast-fed infants.

In this manner, surveys of the concentrations of fat-soluble vitamins in human breast milk have important implications for the promotion of breast-feeding. However, there are several problems in sensitivity, specificity and accuracy with determination of fat-soluble vitamins in breast milk. Especially, it is difficult to determine vitamin D compounds using standard assay methods such as HPLC with ultraviolet detection [15], competitive protein binding assay (CPBA) [16], radio immunoassay (RIA) [17] and enzyme immunoassay (EIA) [18], because concentrations of them are markedly low. In this study, we have developed a high-sensitive quantification method of fat-soluble vitamins in human breast milk using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Also, we applied this method to a nutrition survey for lactating mothers.

2. Experimental

2.1. Chemicals and reagents

Retinyl palmitate and β-carotene were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). D_3, vitamin D_2 (ergocalciferol, D_2), $25(OH)D_3$ and 25-hydroxyvitamin D_2 [$25(OH)D_2$] were obtained from Duphar B.V. (Amsterdam, The Netherlands). α-Tocopherol (α-Toc), PK, MK-4 and MK-7 were kindly provided by Eisai Pharmaceuticals (Tokyo, Japan). Four internal standards, d_6-retinyl acetate, d_6-β-carotene, d_7-D_3, and d_6-α-Toc were synthesized as described below. d_6-$25(OH)D_3$, [$^{18}O_2$]-PK, [$^{18}O_2$]-MK-4 and [$^{18}O_2$]-MK-7 were synthesized as described in our previous reports [19,20]. The chemical structures of the internal standards are shown in Fig. 1.

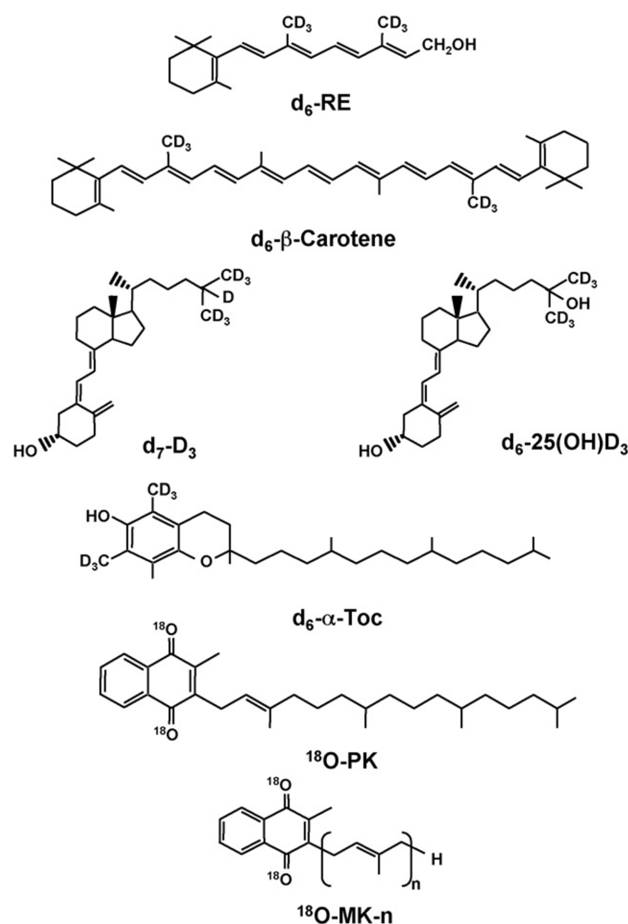

Fig. 1. Chemical structures of internal standards.

2.2. Synthesis of internal standards

The structure of the target compounds were confirmed by ^1H NMR and MS. The 500 MHz and 300 MHz ^1H NMR spectra were measured on a Varian VXR-500 and VXR-300, respectively. All compounds were dissolved in 0.3 mL of deuterated chloroform ($CDCl_3$, Merck, Darmstadt, Germany). Chemical shifts are given in ppm (δ) using tetramethylsilane (TMS) as the internal standard. Mass spectra were obtained using M-4100 (Hitachi, Tokyo, Japan).

2.2.1. d_6-Retinyl acetate and d_6-β-carotene

d_3-Labeled β-ionone **2** was prepared from *N*-methoxy-*N*-methyl-3-(2,6,6-tri-methylcyclohexenyl)propanoic acid amide **1** [21] by treatment with methyl-d_3-magnesium iodide and was then converted to the corresponding d_3-β-ionylidenacetaldehyde **3** by the previously reported method [22]. Polyene chain elongation of d_3-aldehyde **3** by a standard method [17] gave d_3-retinal **4**. Reductive coupling of d_3-retinal **4** with lithium aluminum hydride and titanium tetrachloride [23] afforded d_6-β-carotene **5**. The spectral data of d_6-β-carotene

were as follows: ^{1}H NMR (300 MHz, $CDCl_3$) δ; 1.03 (12H, s), 1.40–1.52 (4H, m), 1.58–1.64 (4H, m), 1.63 (6H, s), 1.93 (6H, s), 2.02 (4H, t, J = 7.0 Hz), 6.10–6.20 (6H, m), 6.25 (2H, d, J = 12.0 Hz), 6.35 (2H, d, J = 15.0 Hz), 6.62 (2H, dd, J = 15.0, 12.0 Hz), 6.66 (2H, d, J = 12.0 Hz); HRMS calcd for [$C_{40}H_{50}D_6$] 542.4752, found 542.4752. The Horner–Emmons reaction of d_3-β-ionylidenacetaldehyde **3** with triethylphosphonoacetate proceeded smoothly to afford a vinylogous ester, which was easily converted to the *N*-methoxy-*N*-methyl-5-trideuteriomethyl-7-(2,6,6-trimethylcyclo-hexenyl)heptanoic acid amide **6** by the reaction of *N*,*O*-dimethylhydroxylamine hydrochloride. Treatment of the *N*-methoxy-*N*-methyl amide **6** with methyl-d_3-magnesium iodide afforded d_6-C_{18}-ketone **7**, which was then transformed into the corresponding d_6-retinyl acetate **9** *via* ethyl d_6-retinoate **8**. The spectral data of d_6-retinyl acetate were as follows: ^{1}H NMR (300MHz) δ; 1.02 (6H, s), 1.40–1.44 (2H, m), 1.55–1.64 (2H, m), 1.70 (3H, s), 2.01 (2H, t, J = 7.0 Hz), 2.05 (3H, s), 4.71 (2H, d, J = 7.5 Hz), 5,60 (1H, t, J = 7.5 Hz), 6.09 (1H, d, J = 12.0 Hz), 6.12 (1H, d, J = 16.0 Hz), 6.18 (1H, d, J = 16.0 Hz), 6.27 (1H, d, J = 15.5 Hz), 6.63 (1H, dd, J = 15.5, 12.0 Hz); HRMS calcd for [$C_{22}H_{26}D_6O_2$] 334.2773, found 334.2781.

2.2.2. d_7-D_3

d_7-D_3 was synthesized as illustrated in Scheme 1 (See supplementary material). The synthesis of 1-(isopentylsulfonyl) benzene-d_7 **11** from phenyl vinyl sulfone and 2-iodopropane-d_7 followed the method of Miyabe et al. [24]. Condensation of the CD-ring portion (**10**) with the side-chain moiety (**11**) using *n*-butyllithium as a base in the presence of hexamethylphosphoramide furnished a mixture of C-23 epimeric sulfones, *tert*-butyldimethyl(octahydro-7α-methyl-1-((*R*)-6-methyl-4-(phenylsulfonyl)heptan-2-yl)-1*H*-inden-4-yloxy)silane-d_7 **12** as a previous report [25]. Desulfonylation with sodium amalgam in a buffered mixture of methanol and tetrahydrofuran (THF) produced *tert*-butyldimethyl(octahydro-7α-methyl-1-((*R*)-6-methylheptan-2-yl)-1*H*-inden-4-yloxy)silane-d_7 **13**. Removal of the protecting group in **13** with toluenesulfonic acid afforded octahydro-7α-methyl-1-((*R*)-6-methylheptan-2-yl)-1*H*-inden-4-ol-d_7 **14**. The resulting secondary alcohol was oxidized with tetrapropylammonium perruthenate and 4-methylmorpholine *N*-oxide to give octahydro-7α-methyl-1-((*R*)-6-methylheptan-2-yl)inden-4-one-d_7 **15**. Bromomethylenation of **15** furnished the requisite CD-ring synthon, (*E*)-4-(bromomethylene)-octahydro-7α-methyl-1-((*R*)-6-methylheptan-2-yl)-1*H*-indene-d_7 **16**. The coupling reaction of the A-ring enyne (**17**) which was obtained according to the reported method [25] with the CD-ring portion (**16**) catalyzed by tetrakis(triphenylphosphine)palladium and triethylamine in toluene under reflux for 2 h, followed by deprotection with *tetra*-butylammonium fluoride in THF gave a diastereomer mixture (3α and 3β) of deuterated D_3 **18a–18b**. 3β-Deuterated D_3 (**18b**, d_7-D_3) was used as an internal standard. The spectral data of 3α and 3β deuterated D_3 were as follows: (18a) ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.55 (3H, s), 0.92 (3H, d, J = 6.5 Hz), 1.11–1.68 (16H, m), 1.82–1.92 (2H, m), 1.96–2.02 (2H, m), 2.15 (1H, m), 2.27 (1H, dd, J = 8.5, 13.0 Hz), 2.40 (1H, m), 2.58 (1H, dd, J = 4.0, 13.0 Hz), 2.82 (1H, dd, J = 4.0, 12.5 Hz), 3.88 (1H, m), 4.84 (1H, m), 5.06 (1H, m), 6.04 (1H, d, J = 11.5 Hz), 6.24 (1H, d, J = 11.0 Hz); HRMS calcd for [$C_{27}H_{37}D_7O$] 391.3824, found 391.3832 (18b) ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.54 (3H, s), 0.92 (3H, d, J = 6.5 Hz), 1.04–1.68 (16H, m), 1.82–1.92 (2H, m), 1.96–2.02 (2H, m), 2.18 (1H, m), 2.29 (1H, dd, J = 7.5, 13.5 Hz), 2.40 (1H, m), 2.58 (1H, dd, J = 3.0, 13.0 Hz), 2.82 (1H, dd, J = 4.0, 12.5 Hz), 3.93 (1H, m), 4.82 (1H, m), 5.05 (1H, m), 6.03 (1H, d, J = 11.5 Hz), 6.24 (1H, d, J = 11.5 Hz); HRMS calcd for [$C_{27}H_{37}D_7O$] 391.3824, found 391.3822.

2.2.3. d_6-α-Toc

d_6-α-Toc was synthesized as illustrated in Scheme 2 (See supplementary material). Minor modifications gave the desired d_6-α-Toc **20** from γ-tocopherol as previously reported in the method by Hughes et al. [26]. d_6-α-Toc **20** was prepared from γ-Tocopherol **19** by treatment with $SnCl_2$, DCl (35% in D_2O) and $(CD_2O)_n$ in isopropyl ether under reflux for 2.5 h. The spectral data of d_6-α-Toc were as follows: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.83 (3H, s), 0.84 (3H, s), 0.85 (3H, s), 0.88 (3H, s), 1.01–1.28 (16H, m), 1.48–1.61 (4H, m), 1.57 (3H, s), 1.78-1.82 (3H, m), 2.10 (3H, s), 2.60 (2H, t, J = 6.0 Hz), 4.17 (s, 1H). HRMS Calcd for [$C_{29}H_{44}D_6O_2$] 436.4181, found 436.4174.

2.3. Standards and sample preparation

2.3.1. Preparation of retinol and d_6-retinol

Retinol (RE) and d_6-RE solutions were prepared by saponifying retinyl palmitate and d_6-retinyl acetate before use. Forty microgram of retinyl palmitate and d_6-retinyl acetate were dissolved in 1.5 mL of pyrogallol–ethanol (7%, w/v). After the addition of 0.5 mL of NaCl solution (1%, w/v) and 0.8 mL of KOH solution (60%, w/v), the mixture was incubated at 70 °C for 30 min. RE and d_6-RE were extracted with hexane-diethyl ether (90:10, v/v), evaporated under reduced pressure, and the residue was dissolved with 2-propanol. Concentrations of RE and d_6-RE were determined spectrophotometrically using a molar extinction coefficient, ε = 52,480. The purity was checked by high-performance liquid chromatography (HPLC) consisting of an LC-10AD$_{VP}$ pump, a SIL-10AD$_{VP}$ auto injector, a CTO-10AD$_{VP}$ column oven set to 35 °C, and an RF-10A$_{XL}$ fluorescence detector set to an excitation wavelength of 340 nm and an emission wavelength of 460 nm (Shimadzu, Kyoto, Japan). Separation was performed on a Capcellpak C18 UG120 (4.6 mm × 250 mm, Shiseido Co. Ltd., Tokyo, Japan) eluted with ethanol:H_2O (95:5) at a flow rate of 0.4 mL/min.

2.3.2. Sample collections

Human breast milk samples were collected from March 2005 to October 2006 from 82 lactating mothers aged 18–39 years (30.8 ± 4.5 years) at 3–265 d (49.1 ± 57.6 d) post-partum living in Japan. Written informed consent was obtained from each subject prior to enrollment in this study according to the conditions of the Helsinki Declaration and approved by the ethics committee of our university. Approximately 50 mL of human breast milk was collected by manual expression at an interme-

diate time during suckling and immediately frozen at −20 °C. Before extraction of fat-soluble vitamins, frozen breast milk was thawed and sonicated in ice water twice for 15 min. For the developmental work on the assay, pooled human breast milk prepared by mixing breast milk from seven healthy subjects was used.

2.3.3. Extraction of fat-soluble vitamins except for vitamin K derivatives

Ten milliliters of breast milk samples was placed in a 50-mL screw-top vial. After the addition of 50 μL of internal standard solution (d_6-RE, d_6-β-carotene, d_7-D_3, d_6-25(OH)D_3 and d_6-α-Toc, 50 ng/50 μL ethanol each), 20 mL of pyrogallol–ethanol (7%, w/v), 6 mL of NaCl solution (1%, w/v) and 10 mL of KOH solution (60%, w/v), the mixture was incubated at 70 °C for 60 min. Then, the mixture was transferred to a 200-mL of separating funnel containing 38 mL of NaCl solution (1%, w/v) and fat-soluble vitamins were extracted twice with 30 mL of hexane-ethyl acetate (9:1, v/v), washed with water, and dehydrated with Na_2SO_4. The eluate was evaporated under reduced pressure, and the residue was dissolved with 2.5 mL of hexane-ethyl acetate (9:1, v/v). For the determination of RE, β-carotene and α-Toc, 1.0 mL of 2.5 mL was evaporated, and the residue was dissolved with 100 μL of ethanol, 50 μL of which was subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS). For determination of vitamin D (D) and 25(OH)D, another 1.5 mL was concentrated and purified by normal phase HPLC. HPLC was carried out using a model 600 pump and a model 996 photodiode array detector (Waters Associates, Milford, MA). Elution was performed on a Zorbax SIL column (4.6 mm × 250 mm, Agilent, Santa Clara, CA), using hexane-2-propanol-methanol (88:10:2, v/v/v), at a flow rate of 1.0 mL/min. The eluates corresponding to D_3 and D_2 (D fraction, 3.5–5.0 min) and 25(OH)D_3 and 25(OH)D_2 (25D fraction, 5.0–8.0 min) were collected.

2.3.4. DMEQ-TAD derivatization of D and 25(OH)D

DMEQ-TAD derivatization was performed according to the method of Higashi et al. [27]. D and 25(OH)D fractions were dried and then dissolved in 150 μL of ethyl acetate containing DMEQ-TAD (60 μg). The mixture was kept at room temperature for 30 min, then an additional reagent (60 μg/150 μL of ethyl acetate) was added and the entire mixture was further kept at room temperature for 1 h. After the addition of 1.5 mL of EtOH to decompose excess reagent, the solvent was evaporated and the residue was dissolved in 100 μL of acetonitrile, 30 μL of which was subjected to LC–MS/MS.

2.3.5. Extraction of vitamin K derivatives

Three milliliters of breast milk samples was placed in a 50 mL screw-top vial. After the addition of internal standard solution ([$^{18}O_2$]-PK, [$^{18}O_2$]-MK-4 and [$^{18}O_2$]-MK-7, 25 ng/25 μL ethanol each), 12 mL of phosphate buffer (pH 7.7) and 0.3 g of lipase, the mixture was incubated at 37 °C for 90 min with stirring. Then, 12 mL of ethanol was added and vitamin K derivatives were extracted twice with 12 mL of hexane. The mixture was shaken for 5 min before centrifuging at 3000 rpm for 5 min. The extracts were combined and evaporated under reduced pressure, and the residue was dissolved with 3 mL of hexane. The resultant extract was passed through a Sep-Pak Vac silica cartridge (Waters, Milford, MA, USA) that was washed with 10 mL of hexane. Vitamin K derivatives were eluted with 5 mL of hexane-diethyl ether (97:3). The eluate was evaporated under reduced pressure, and the residue was dissolved with 200 μL of ethanol, 50 μL of which was subjected to LC–MS/MS.

2.4. LC–MS/MS

2.4.1. Apparatus

The HPLC system consisted of a SCL-10ADvp system controller, two LC-10ADvp pumps, a DGC-14A automatic solvent degasser, a SIL-10ADvp auto injector, and a CTO-10ADvp column oven set to 35 °C (Shimadzu). The HPLC system was coupled to an API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source. Analyst (Ver. 1. 3. 2; Applied Biosystems/MDS SCIEX) was used for data acquisition and analysis.

2.4.2. Chromatographic conditions

Separations were performed on a Capcellpak C18 UG120. For the determination of RE, β-carotene, α-Toc and vitamin K derivatives, a solvent system consisting of an isocratic solvent A (methanol–H_2O, 90:10, v/v) in 10 min and then a linear gradient from 0 to 90% acetonitrile in 30 min was used (Condition 1). The injection volumes of standard and sample solutions were 50 μL. For the determination of DMEQ-TAD derivatives of D and 25(OH)D, a solvent system consisting of a mixture of acetonitrile and H_2O (30:70, v/v) in 5 min and then a linear gradient from 30 to 95 % acetonitrile in 30 min was used (Condition 2). The injection volumes of standard and sample solutions were 30 μL. Acquisition settings were optimized by the infusion of a 1 μg/mL solution of each compound at a rate of 20 μL/min as shown in Table 1. Curtain gas (8 psi), nebulizer gas (8 psi), collision gas (4 psi), nebulizer current (2 μA) and ion source temperature (400 °C) were identical for all analytes. The mass spectrometer was operated in the positive ion mode. All analytes were detected in the MS/MS-multiple reaction monitoring (MRM) with unit resolution at both Q1 and Q3.

2.5. Statistical analysis

All statistical analyses were performed using JMP statistical software (version 5.0.1 J: SAS Institute Inc, Cary, NC, USA). For cross-sectional analyses, simple regression analysis was performed.

3. Results and discussion

3.1. Acquisition settings and chromatography

The mass spectrometer was used in MRM mode to optimize selectivity and sensitivity. The selected molecular transitions are listed in Table 1. MRM chromatograms of human breast milk sample for the determination of RE, β-carotene and α-Toc in Condition 1 are shown in Fig. 2. Under these conditions, MRM

Table 1
Retention times, molecular weights and optimized instrument settings

Analyte	Retention time (min)	MW	Transitions, m/z		DP[a] (V)	FP[a] (V)	CE[a] (V)	CXP[a] (V)
			Parent ion	Product ion				
Condition 1								
RE	9.73	286.5	269.1	213.4	21	80	19	14
d_6-RE	9.48	292.5	275.2	192.4	16	70	19	14
β-Carotene	87.01	536.9	537.6	177.2	31	100	27	12
d_6-β-Carotene	86.24	542.9	543.6	180.2	31	110	25	12
α-Toc	38.72	430.7	430.4	165.2	51	180	43	10
d_6-α-Toc	38.44	436.7	436.5	171.2	56	180	41	10
PK	49.15	450.7	451.5	187.1	41	140	33	12
$[^{18}O_2]$-PK	49.13	454.7	455.4	191.2	41	140	33	12
MK-4	32.72	444.7	445.5	187.3	21	80	31	12
$[^{18}O_2]$-MK-4	32.66	448.7	449.4	191.2	26	100	31	12
MK-7	86.74	649.0	649.7	187.2	41	150	47	12
$[^{18}O_2]$-MK-7	86.67	653.0	653.7	191.1	36	130	43	12
Condition 2								
DMEQ-TAD-D_3[b]	36.46	729.9	730.5	468.3	61	200	35	32
DMEQ-TAD-D_2[b]	36.43	742.0	742.6	468.3	56	170	35	34
DMEQ-TAD-d_7-D_3[b]	36.30	737.0	737.6	468.2	56	210	33	8
DMEQ-TAD-25(OH)D_3[b]	22.21	745.9	746.5	468.1	61	210	37	16
DMEQ-TAD-25(OH)D_2[b]	21.92	758.0	758.5	468.2	56	180	37	16
DMEQ-TAD-d_6-25(OH)D_3[b]	22.08	752.0	752.5	468.1	56	190	39	16

[a] DP, declustering potential; FP, focusing potential; CE, collision energy; CXP, collision cell exit potential.
[b] The retention times of the derivatives are those of the 6*S*-isomer.

provided high specificity for all compounds, and no crosstalk interference with d_6-labeled internal standards was observed. The linearity of calibration curves of RE (up to 62500 ng/mL, $r^2 = 0.9993$), α-tocopherol (up to 62500 ng/mL, $r^2 = 0.9998$) and β-carotene (up to 2500 ng/mL, $r^2 = 0.9989$) was confirmed (data not shown). LC–MS/MS MRM chromatograms of human breast milk sample for the determination of PK, MK-4 and MK-7 in Condition 1 are shown in Fig. 3. Vitamin K derivatives were successfully detected without interruption of co-eluting compounds in breast milk and interference of their ^{18}O-labeled internal standards. The linearity of calibration curves of PK (up to 2500 ng/mL, $r^2 = 1.0000$), MK-4 (up to 2500 ng/mL, $r^2 = 0.9998$) and MK-7 (up to 2500 ng/mL, $r^2 = 1.0000$) was confirmed (data not shown). LC–MS/MS MRM chromatograms of human breast milk samples after DMEQ-TAD derivatization for the determination of D_3, D2, 25(OH)D_3 and 25(OH)D_2 in Condition 2 are shown in Fig. 4. In the reaction with DMEQ-TAD, vitamin D compounds produce two C6-epimeric derivatives. In the case of D_3, D_2, 25(OH)D_3 and 25(OH)D_2, the 6*S* isomer was the main product (6*S*:6*R* = 3:1). Thus, 6*S* isomer was used for the determination of each vitamin D compound. The linearity of calibration curves of the DMEQ-TAD derivatives of D_3 (up to 50 ng/mL, $r^2 = 0.9999$), D_2 (up to 50 ng/mL, $r^2 = 1.0000$), 25(OH)D_3 (up to 50 ng/mL, $r^2 = 0.9999$) and 25(OH)D_2 (up to 50 ng/mL, $r^2 = 0.9991$) was confirmed (data not shown).

At present, standard assay methods are HPLC with fluorescence detection for RE [28], tocopherol [29] and vitamin K derivatives [30] and HPLC with visible detection for β-carotene [31]. For the determination of vitamin D compounds, HPLC with ultraviolet detection [15], competitive protein binding assay (CPBA) [16], radio immunoassay (RIA) [17] and enzyme immunoassay (EIA) [18] are used widely; however, in the comprehensive evaluation of the nutritional status of fat-soluble vitamins, each vitamin needs to be measured separately. Our proposed method, including two extraction methods and sensitive LC–MS/MS detection using stable isotope-labeled internal standards, makes it possible to determine the principal fat-soluble vitamins in breast milk which contain more interfering compounds compared to plasma or serum samples. Vitamin K cannot be extracted along with other fat-soluble vitamins because of its lability under basic condition. In addition, D and 25(OH)D could be measured by LC–MS/MS after DMEQ-TAD derivatization with the equivalent of only 6 mL of breast milk. DMEQ-TAD, a fluorescence-labeling reagent, is highly sensitive and stable for conjugated dienes. Recently, DMEQ-TAD has been used for the derivatization of vitamin D metabolites to improve ionization efficiency of LC–MS/MS with APCI [27]. In this study, DMEQ-TAD derivatization enhanced the measurement sensitivity of D and 25(OH)D by about 40 times.

3.2. Sensitivity, recovery and reproducibility

The detection limits of RE, β-carotene, α-Toc, PK, MK-4, MK-7, DMEQ-TAD-D_3, DMEQ-TAD-D_2, DMEQ-TAD-25(OH)D_3 and DMEQ-TAD-25(OH)D_2 based on a signal-to-noise ratio of 3:1, were 1–250 pg per injection (Table 2). The recoveries of fat-soluble vitamins were about 91–105%. Inter-assay CV values of each vitamin calculated by measurements of pooled human breast milk were 1.9–11.9%. The sensitivity and overall recovery combined with reproducibility allowed the measurement of fat-soluble vitamins containing vitamins A, D, E and K with 10 mL of breast milk.

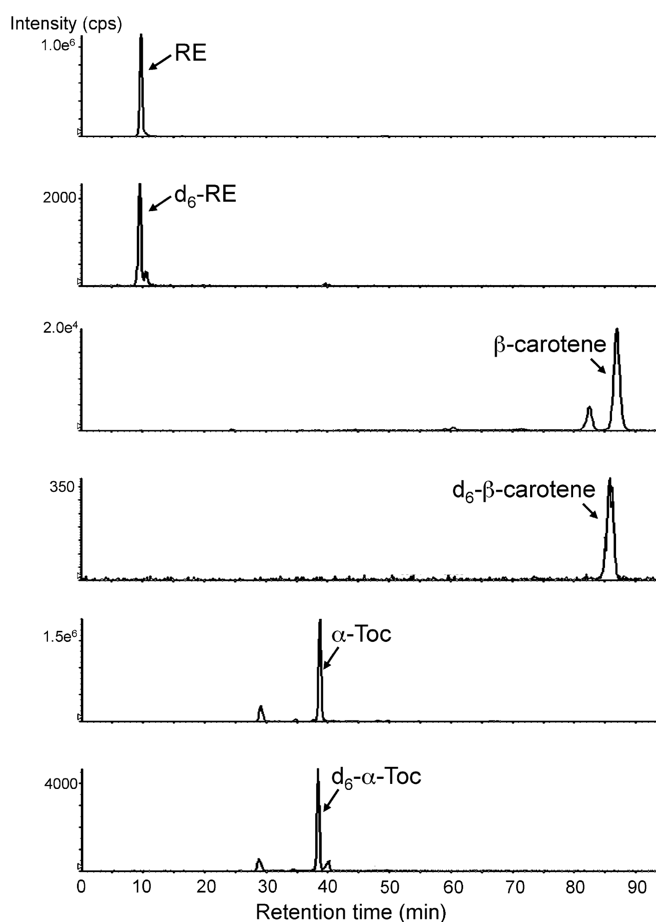

Fig. 2. LC–MS/MS MRM chromatograms of human breast milk sample for determination of RE, β-carotene and α-Toc in Condition 1. The concentrations of RE, β-carotene and α-Toc in this sample are 0.244, 0.055 and 2.131 μg/mL, respectively.

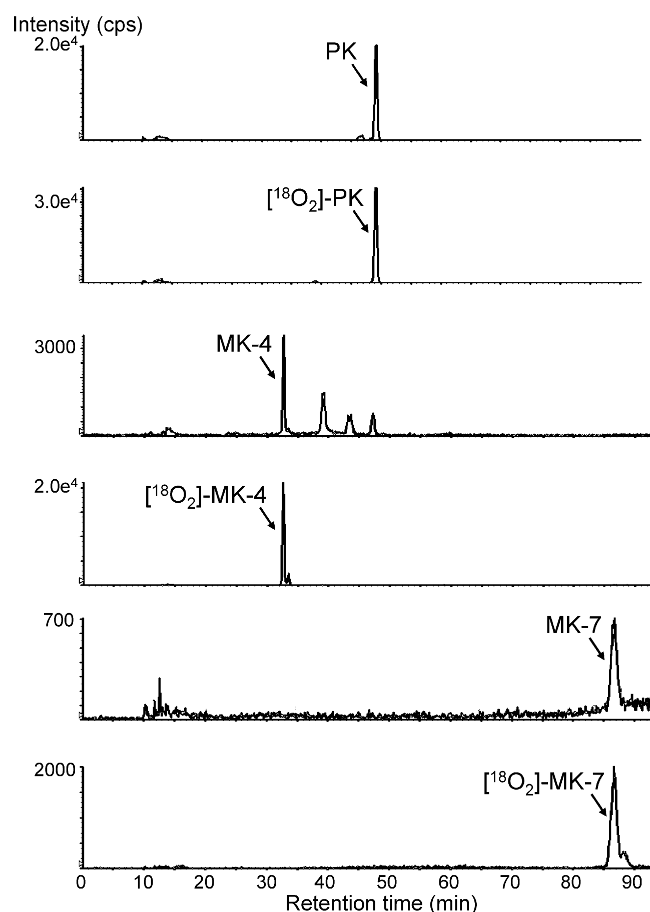

Fig. 3. LC–MS/MS MRM chromatograms of human breast milk sample for determination PK, MK-4 and MK-7 in Condition 1. The concentrations of PK, MK-4 and MK-7 in this sample are 1.628, 1.462 and 0.344 ng/mL, respectively.

3.3. Concentration of fat-soluble vitamins in human breast milk

This method was applied to breast milk samples obtained from 82 Japanese lactating mothers. The mean concentration of RE, β-carotene, D_3, D_2, 25(OH)D_3, 25(OH)D_2, α-Toc, PK, MK-4 and MK-7 of 82 lactating mothers were 0.455 (range, 0.097–1.783; median, 0.406) μg/mL, 0.062 (range, 0.002–0.375; median, 0.045) μg/mL, 0.088 (range, 0.010–1.116; median 0.061) ng/mL, 0.078 (range, 0–1.300; median, 0.021) ng/mL, 0.081 (range, 0.023–0.172; median, 0.078) ng/mL, 0.003 (range, 0–0.012; median 0.003) ng/mL, 5.087 (range, 0.387–35.664; median 3.590) μg/mL, 3.771 (range, 0.953–12.382; median 3.481) ng/mL, 1.795 (range, 0.720–4.750; median 1.611) ng/mL and 1.540 (range,

Table 2
Accuracy of measurement of fat-soluble vitamins

	Detection limit (pg)	Recovery[a]		Inter-assay	
		Mean ± SD (%)	CV (%)	Mean ± SD (mL^{-1})	CV (%)
RE	50	104.7 ± 7.5	7.2	0.489 ± 0.029 μg	7.2
β-Carotene	250	97.4 ± 7.0	8.2	0.027 ± 0.001 μg	3.8
α-Toc	100	96.3 ± 5.5	5.7	2.839 ± 0.058 μg	2.1
PK	10	97.5 ± 8.3	8.6	0.383 ± 0.014 ng	3.7
MK-4	10	99.4 ± 7.9	8.0	0.206 ± 0.004 ng	1.9
MK-7	80	97.1 ± 5.6	5.7	0.117 ± 0.011 ng	9.6
DMEQ-TAD-D_3	1	97.5 ± 3.8	3.9	0.040 ± 0.002 ng	5.4
DMEQ-TAD-D_2	1	105.0 ± 4.7	4.5	0.014 ± 0.002 ng	11.9
DMEQ-TAD-25(OH)D_3	2	93.9 ± 3.0	3.1	0.117 ± 0.005 ng	4.0
DMEQ-TAD-25(OH)D_2	1	90.9 ± 8.8	9.7	0.006 ± 0.001 ng	9.8

[a] Calculated by measurements of pooled human breast milk spiked with fat-soluble vitamins: RE, 20 μg; β-carotene, 0.6 μg; D_3, 20 ng; D_2, 20 ng; 25(OH)D_3, 20 ng; 25(OH)D_2, 20 ng; PK, 30 ng; MK-4, 20 ng; MK-7, 10 ng/20 mL of human breast milk.

Table 3
Concentration of fat-soluble vitamins in human breast milk*

Post-partum days	Total ($n = 82$) Mean ± SD	0–10 ($n = 8$) Mean ± SD	11–30 ($n = 43$) Mean ± SD	31–90 ($n = 18$) Mean ± SD	91–180 ($n = 8$) Mean ± SD	181–270 ($n = 5$) Mean ± SD
Age (years)	30.8 ± 4.5	27.6 ± 6.3^{a}	32.0 ± 3.6^{a}	30.3 ± 4.5^{a}	30.4 ± 5.6^{a}	28.6 ± 4.3^{a}
RE (μg/mL)	0.455 ± 0.264	1.026 ± 0.398^{a}	0.418 ± 0.138^{b}	0.384 ± 0.145^{b}	0.359 ± 0.219^{b}	0.267 ± 0.117^{b}
β-Carotene (μg/mL)	0.062 ± 0.063	0.188 ± 0.112^{a}	0.059 ± 0.037^{b}	0.033 ± 0.023^{b}	0.033 ± 0.031^{b}	0.043 ± 0.048^{b}
D_3 (ng/mL)	0.088 ± 0.128	0.075 ± 0.046^{a}	0.103 ± 0.169^{a}	0.079 ± 0.056^{a}	0.075 ± 0.079^{a}	0.035 ± 0.016^{a}
D_2 (ng/mL)	0.078 ± 0.156	0.129 ± 0.076^{a}	0.073 ± 0.199^{a}	0.066 ± 0.084^{a}	0.014 ± 0.005^{a}	0.181 ± 0.099^{a}
25(OH)D_3 (ng/mL)	0.081 ± 0.037	0.072 ± 0.047^{a}	0.085 ± 0.038^{a}	0.084 ± 0.034^{a}	0.068 ± 0.037^{a}	0.073 ± 0.041^{a}
25(OH)D_2 (ng/mL)	0.003 ± 0.002	0.007 ± 0.003^{a}	0.003 ± 0.002^{b}	0.003 ± 0.002^{b}	0.003 ± 0.003^{b}	0.003 ± 0.001^{b}
α-Toc (μg/mL)	5.087 ± 5.042	16.590 ± 9.635^{a}	4.079 ± 1.795^{b}	3.911 ± 1.798^{b}	3.296 ± 1.962^{b}	2.454 ± 1.045^{b}
PK (ng/mL)	3.771 ± 2.166	5.122 ± 2.561^{a}	3.938 ± 2.450^{a}	3.528 ± 1.454^{a}	2.294 ± 1.220^{a}	3.409 ± 1.462^{a}
MK-4 (ng/mL)	1.795 ± 0.732	2.561 ± 1.207^{a}	1.802 ± 0.664^{b}	1.785 ± 0.553^{ab}	1.195 ± 0.343^{ab}	1.510 ± 0.419^{b}
MK-7 (ng/mL)	1.540 ± 2.298	3.044 ± 2.901^{a}	1.675 ± 2.732^{a}	0.798 ± 0.746^{a}	1.363 ± 1.292^{a}	0.917 ± 0.916^{a}
Fat** (mg/mL)	28.89 ± 11.65	24.92 ± 11.55^{a}	32.64 ± 11.52^{a}	30.24 ± 7.91^{a}	21.39 ± 14.12^{a}	20.72 ± 10.08^{a}

* Means in the same row bearing different superscripts differ significantly ($p < 0.05$) by Tukey–Kramer HSD test.
** Measured by Röse–Gotlieb method.

0.074–15.861; median 1.001) ng/mL, respectively (Table 3). The mean concentration of α-Toc was the highest of the compounds measured in this study as in the plasma concentrations of normal subjects. The mean concentrations of vitamins D and K were low in breast milk as reported previously [7–12]. In particular, the concentrations of 25(OH)D in breast milk were markedly low compared to plasma concentrations of 25(OH)D, which is the most abundant circulating metabolite of vitamin D with a concentration of 20–50 ng/mL in normal subjects.

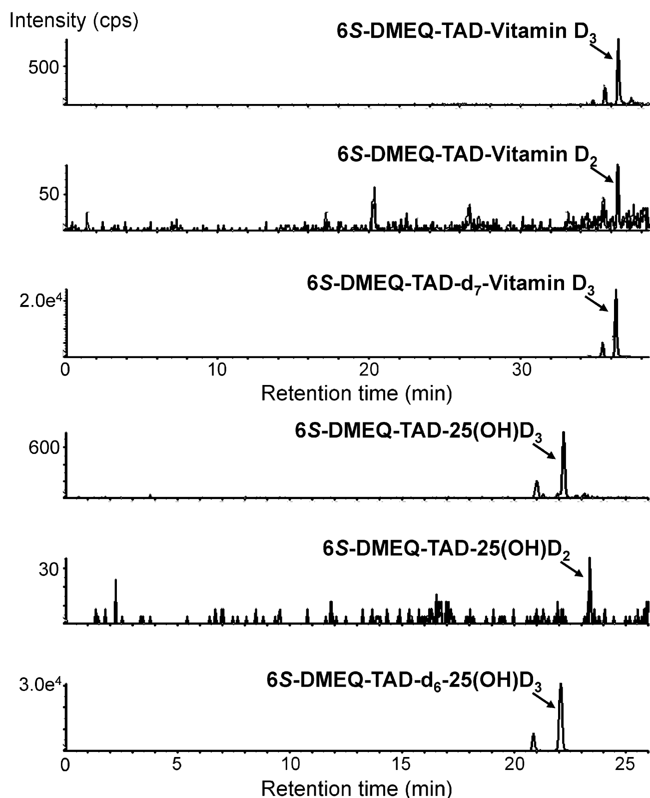

Fig. 4. LC–MS/MS MRM chromatograms of human breast milk sample after DMEQ-TAD derivatization for determination of D_3, D_2, 25(OH)D_3 and 25(OH)D_2 in Condition 2. The concentrations of D_3, D_2, 25(OH)D_3 and 25(OH)D_2 in this sample are 0.058, 0.005, 0.103 and 0.002 ng/mL, respectively.

Thus, the distributions of each fat-soluble vitamin in breast milk might be different.

Next, the subjects were stratified into five groups by post-partum days: 0–10, 11–30, 31–90, 91–180 and 181–270 days. The concentrations of many fat-soluble vitamins in human breast milk showed a tendency to decrease as the post-partum days passed. The concentrations of RE, β-carotene, 25(OH)D_2 and α-Toc in breast milk in the 0–10 day post-partum group were significantly higher than those of the other groups ($p < 0.05$). Fat concentrations were not significantly different between each group. The relations between the concentrations of each fat-soluble vitamin in breast milk and the other parameters are shown in Table 4. Post-partum days correlated significantly and negatively with the concentrations of RE, β-carotene, α-Toc and MK-4 in breast milk. Generally, vitamins A and E are high in colostrum, and decreased and stable in mature breast milk. Sakurai et al. [32] reported that the concentrations of RE, β-carotene and α-Toc in breast milk decreased as the duration of lactation increased; however, clear correlations between the concentration of D_3 and the stage of lactation were not observed. Kojima et al. [13] demonstrated that PK and MK-4 concentrations in breast milk were high in colostrum and decreased during the course of lactation. Taken together, these results suggest that the concentrations of MK-4 out of vitamin K derivatives in breast milk were influenced by the stage of lactation as well as vitamins A and E.

The concentrations of RE correlated significantly and positively with those of β-carotene, 25(OH)D_2, α-Toc, PK, MK-4 and MK-7. The concentrations of β-carotene and α-Toc were also positively correlated with 25(OH)D_2 and vitamin K derivatives. It should be noted that correlations between the concentrations of D_3 and D_2 or 25(OH)D_3 and 25(OH)D_2 were observed; however, correlations between D and 25(OH)D were not found. Therefore, the rate of secretion of vitamin D compounds from plasma to breast milk might be different depending on their polarity. Positive correlations were observed among the concentrations of vitamin K derivatives. In addition, fat concentrations also correlated significantly and positively with 25(OH)D_3, α-Toc, PK and MK-4. These results suggest that the

Table 4
Relation between concentrations of each fat-soluble vitamin in human breast milk[a]

	RE		β-Carotene		D_3		D_2		25(OH)D_3	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	−0.1528	0.1706	−0.0996	0.3735	0.2188	**0.0483**	0.1941	0.0806	0.2004	0.0710
Post-partum days	−0.3472	**0.0014**	−0.2530	**0.0218**	−0.1187	0.2881	0.0745	0.5058	−0.1021	0.3615
RE	–	–	–	–	–	–	–	–	–	–
β-Carotene	0.7588	**<.0001**	–	–	–	–	–	–	–	–
D_3	0.0294	0.7931	0.0349	0.7554	–	–	–	–	–	–
D_2	−0.0017	0.9879	0.0602	0.5908	0.7984	**<.0001**	–	–	–	–
25(OH)D_3	0.0548	0.6248	0.2208	**0.0462**	0.1490	0.1815	−0.0967	0.3875	–	–
25(OH)D_2	0.2794	**0.0110**	0.4132	**0.0001**	−0.0889	0.4269	−0.0824	0.4617	0.2748	**0.0125**
α-Toc	0.7957	**<.0001**	0.7702	**<.0001**	0.0659	0.5562	0.0401	0.7205	0.1146	0.3054
PK	0.4081	**0.0001**	0.4711	**<.0001**	0.0273	0.8075	−0.0218	0.8458	0.3324	**0.0023**
MK-4	0.5541	**<.0001**	0.5142	**<.0001**	0.2725	**0.0132**	0.1882	0.0905	0.2022	0.0685
MK-7	0.3264	**0.0028**	0.4773	**<.0001**	−0.0463	0.6795	−0.0039	0.9725	0.1025	0.3596
Fat	0.1442	0.2481	0.0929	0.4583	0.0840	0.5027	−0.1460	0.2422	0.4596	**0.0001**

	25(OH)D_2		α-Toc		PK		MK-4		MK-7	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	−0.2127	0.0550	−0.1433	0.1990	0.0599	0.5927	−0.0196	0.8615	−0.0700	0.5320
Post-partum days	−0.0462	0.6806	−0.2819	**0.0103**	−0.1803	0.1051	−0.2853	**0.0094**	−0.1110	0.3210
α-Toc	0.3989	**0.0002**	–	–	–	–	–	–	–	–
PK	0.2838	**0.0098**	0.5050	**<.0001**	–	–	–	–	–	–
MK-4	0.2059	0.0634	0.6545	**<.0001**	0.5751	**<.0001**	–	–	–	–
MK-7	0.1338	0.2307	0.4036	**0.0002**	0.3722	**0.0006**	0.2460	**0.0259**	–	–
Fat	0.1565	0.2095	0.2638	**0.0323**	0.5514	**<.0001**	0.5095	**<.0001**	0.0870	0.4873

[a] Values listed in bold face showed significant correlation.

concentrations of fat may have a considerable effect on those of vitamins E and K in breast milk. In contrast, factors other than fat, such as binding protein, may contribute to the concentrations of vitamins A and D in breast milk.

4. Conclusions

Here, we show a quantification method for fat-soluble vitamins in breast milk by LC–MS/MS. The present method maximizes the sensitivity and selectivity of the latest generation of tandem mass spectrometry and derivatization technique for the measurement of representative compounds of fat-soluble vitamins in human breast milk. The assay includes vitamins with a wide range of polarity, and this method has the advantage of low sample volume requirement. This method can apply the measurement of fat-soluble vitamins in other biological samples such as plasma, and may be useful for large-scale studies and the setting of Dietary Reference Intakes of fat-soluble vitamins.

Acknowledgements

We gratefully thank Eisai Co., Ltd. for providing α-Toc and vitamin K standards. We also thank Aiko Shimamoto, Yoshika Takechi, Hitomi Takeuchi, Yoshiaki Hirase and Yuri Uchino for their excellent technical assistance. We also acknowledge Dr. Atsuko Takeuchi and Dr. Chisato Tode for their technical advice. This work was supported in part by a Grant-in-aid for Comprehensive Research on Cardiovascular Diseases and the Research on the Dietary Reference Intakes in Japanese from the Ministry of Health, Labor and Welfare of Japan, and a Grant-in-aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2007.09.023.

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