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Short communication

Rapid high-performance liquid chromatographic method for the simultaneous determination of retinol, α-tocopherol and β-carotene in human plasma and low-density lipoproteins ☆

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Abstract

A reversed-phase HPLC method with diode-array detection was used to simultaneously determine retinol, α-tocopherol and β-carotene in human plasma and low-density lipoproteins. An aliquot of sample was de-proteinized with ethanol containing α-tocopherol acetate as internal standard, and the analytes were extracted twice with hexane. The solvent was evaporated to dryness under a stream of nitrogen and the residue was redissolved in methanol to be injected directly into the HPLC system. A multiple solvent system based on methanol, butanol and water at a flow-rate of 2 ml/min and held at 45°C provided clear separation of these compounds in only 8 min. The method showed good linearity, precision and accuracy for all compounds. Owing to its simplicity, this method may be useful in routine clinical and epidemiological work.

Keywords: Vitamins; Retinol; α-Tocopherol; β-Carotene

1. Introduction

The assessment of the nutritional status of some vitamins is normally carried out by measuring their levels in serum or plasma [1].

''In vitro'' studies of some retinoids, carotenoids and tocopherols have shown their effectiveness against certain types of cancer [2,3]. Likewise, some epidemiological studies have shown an inverse relationship between either high consumption or high plasma concentrations of these compounds and the incidence of cancer [4,5]. Moreover, α-tocopherol is the predominant antioxidant for low-density lipoprotein (LDL), thus preventing the incidence of atherosclerosis and cardiovascular diseases [6–9]. However the hypothesis of a significant protective role ''in vivo'' should be tested because most of these substances act synergically [3]. Therefore further

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studies are required to clarify the role of these compounds, and it is essential to have simple and rapid methods available for the measurement of these antioxidants in a routine manner.

Many methods have been reported for the simultaneous determination of vitamins A and E in serum or plasma, because they are present in high enough concentrations to be detected easily [10–13]. However the determination of these vitamins together with carotenoids is complicated. For these reason most of the reported methods involve complex high-performance liquid chromatography (HPLC) conditions, for example double instead of single column separation [14] or more than one HPLC line connected to several detectors [15–19]. These conditions are useful to increase the selectivity and sensitivity of the procedure but are costly and complicated for routine analysis.

HPLC methods with ultraviolet–visible detectors are currently the most commonly used [3,11,12], as fluorimetric and colorimetric methods are hampered by interferences [1].

In this study we describe a rapid reversed-phase (RP) HPLC procedure for the simultaneous determination of vitamins A and E and β-carotene in plasma and lipoproteins. This method might be useful in routine assessment because it saves solvents and chromatographic time.

2. Experimental

2.1. Equipment

HPLC separation was performed with a Hewlett-Packard liquid chromatographic system (Waldbronn, Germany) equipped with a HP-1050 pump system, and a Rheodyne Model 7125 injector (Cotati, CA, USA) with a final loop volume of 500 μl. A HP-1040M photodiode-array detector was also used. The data were stored and processed by HPLC Chemstation (Dos Series, Hewlett-Packard). The column was a Tracer Extrasil ODS-2 (150×4.0 mm I.D., 5 μm particle size) (Tracer Analítica, Barcelona, Spain) protected by a guard cartridge system (Tracer, C_{18}, 5 μm).

2.2. Reagents and standards

All standard compounds were purchased from Sigma (St. Louis, MO, USA). The stock standard solutions of retinol (0.5 mg/ml), α-tocopherol (0.5 mg/ml) and α-tocopherol acetate (2 mg/ml) were prepared in ethanol and the stock standard solution of β-carotene in hexane (0.5 mg/ml). Working standard solutions were prepared in methanol from the stock standard solutions each week, and were stored at −20°C in amber-colored bottles for no longer than a month. A calibration graph was prepared just before analysis. Ultra-pure water generated by the Milli-Q system (Millipore, Bedford, MA, USA) was used. HPLC-grade methanol, *n*-hexane and 1-butanol were obtained from SDS (Peypin, France). All other reagents of analytical grade, such as absolute ethanol, were obtained from Panreac (Barcelona, Spain).

2.3. Sample collection and storage

Blood samples from 10 healthy male volunteers (aged 25 to 65 years) on non supplemented diets were obtained by venipuncture and collected in EDTA-containing tubes. The study was done in accordance with the Helsinki Declaration of 1975, as revised in 1989. A local ethics committee approved the protocol. All volunteers could be considered healthy on the basis of physical examination and standard biochemical and hematological test. Subjects had an average mass of 75±13.47 kg and had a body mass index of 25±3.1 kg/m^2.

Plasma was pooled and LDL was isolated by two-step sequential flotation ultracentrifugation [20]. Isotonic saline solution (1 ml; d=1006 kg/l) containing 1.091 mmol/l EDTA was layered on top of plasma (2 ml) in centrifuge tubes (Beckman polycarbonate tubes of 5 ml). The tubes were centrifuged in a Beckman XL-70 ultracentrifuge with a Beckman 50.4 rotor at 227 000 *g* for 18 h at 4°C. The very-low-density lipoprotein (VLDL) fraction was separated by aspiration. The plasma (3 ml) was placed in a centrifuge tube (Kontron polyallomer tubes of 13 ml) containing 0.1155 g of KBr. Isotonic saline solution (3 ml; d=1.21 kg/l) containing 1.091 mmol/l EDTA and 2.704 mol/l KBr was layered on

top of the plasma. Tubes were centrifuged in a Kontron 41.14 rotor at 215 000 *g* for 20 h at 4°C. The LDL-containing middle layer was aspirated. Protein content was determined by The Red Pyrogallol Method (Sigma).

Plasma and LDL samples were stored in amber polypropylene tubes at −80°C for a short period (1–2 weeks) before analysis. Analytes were stable for at least 28 months [16].

2.4. Sample preparation

A 1-ml aliquot of ethanol containing internal standard (25 μg/ml) was added to 1 ml of sample (plasma or LDL) in an amber polypropylene tube. After vortex mixing, the mixture was extracted twice with 2 ml of *n*-hexane. The mixing must be uniform and sufficiently vigorous to disrupt the precipitated particles in 60 s. The tubes were centrifuged at 3000 *g* for 5 min and the upper phase was carefully transferred to another polypropylene tube. The supernatant was evaporated at room temperature under a stream of nitrogen and the residue was reconstituted in 150 μl of methanol. Samples were kept at −20°C before injection but they only remained stable for 2 weeks.

2.5. Chromatographic conditions and quantification

The method involved one linear gradient and two isocratic steps. Solvent A was methanol, solvent B was Milli-Q water and solvent C was butanol. We began with A–B–C (92:3:5) for 3 min and went on to A–C (92:8) in 1 min. The composition was then held for 5 min and the system returned to the initial conditions. After 10 min the system was ready for a new injection. The analytical column was maintained at 45°C and the elution was performed at a flow-rate of 2 ml/min. A 100-μl aliquot of sample was injected. Optimum wavelengths were selected for the detection of retinol (325 nm), α-tocopherol (292 nm) and β-carotene (450 nm).

To determine the compounds in the sample, the working standard solutions were always analyzed along with the samples, and peak-area ratios were used for calculations following the internal standard method.

3. Results and discussion

3.1. Preparation of working standards and standards curves

The preparation of standards and the processing of the sample were performed in a darkened room with a red safety light to avoid the oxidation of the analytes during the process.

To check their concentration the standard solutions must be injected into the HPLC system and monitored at the appropriate wavelength periodically.

3.2. Sample preparation

The major concern is to be able to extract these compounds from a complex matrix, which is the lipoprotein particle. Therefore it is recommended to use internal standards that have extraction and chromatographic properties similar to those of the analytes. The use of α-tocopherol acetate as the only internal standard did not affect the accuracy and precision of the assay, so the use of more internal standards was not necessary [21].

The vortex-mixing step after the addition of hexane is probably the most critical manipulation of the assay. Mixing must be uniform and sufficiently vigorous to disrupt the precipitated lipoprotein particles.

Another key step is the collection of the upper phase after centrifugation because it has been shown that if some of the lower phase is collected the consequent chromatogram is not good.

3.3. HPLC procedure

The choice of the injection solvent was carefully studied in a previous report [22]. Methanol was used because it was the major component in the mobile phase. Although β-carotene is significantly less soluble in methanol than in other solvents, this did not pose any problem since very low concentrations were used.

Lee et al. [1] proposed a method in which the steps of drying and reconstituting were avoided. The analytes were extracted with butanol–ethyl acetate and injected directly into the HPLC system. Although this method appears to be rapid, it had a serious drawback because of the precipitation of the proteins of the sample in the HPLC column.

Likewise, in another same report [22], various mobile phases were studied in order to optimize the simultaneous elution of both components. Since the analytes were insoluble in water, the primary constituent in the mobile phase should be a weak organic solvent with low viscosity, which limited the choices to methanol and acetonitrile. Methanol has been recommended in several reports [11,23] because separations using acetonitrile or acetonitrile-based solvents have generally resulted in lower recoveries than those using methanol or methanol-based solvents. In order to achieve the desired retention and to increase solubility and selectivity, stronger organic modifiers such as tetrahydrofuran, ethyl acetate and chloroform were tested but butanol was found to be the most suitable since peak distortions did not occur.

3.4. Identification and quantification

All the analytes were well separated in only 8 min. Compounds were identified on the basis of retention time by comparison with standard solutions (Fig. 1) and through spectroscopic analysis. Figs. 2 and 3 show chromatograms of plasma and LDL samples.

For quantitative analysis, the calibration graph was calculated by linear regression. A constant amount of internal standard (150 μg/ml) was added to increasing analyte concentrations. The peak-area ratio of each compound (retinol, α-tocopherol or β-carotene) to α-tocopherol acetate (y) versus the mass of standard of each compound (x) was linear in the range tested (Table 1).

With regard to the suitability of this method, a limit of detection (LOD) of 11.5 ng and a limit of quantification (LOQ) of 23.0 ng for α-tocopherol ensured good sensitivity according to the USP criteria [24]. This was also observed for retinol (LOD=11.0 ng, LOQ=22.0 ng) and β-carotene (LOD=15.5 ng, LOQ=31.0 ng).

The within-run precision was measured by calculating the standard deviation and relative standard deviation (RSD) of 10 replicate analyses of the three compounds in a plasma sample. To evaluate the between-run precision, 10 analytical runs were carried out on consecutive days. The intra- and inter-laboratory precision obtained (Table 2) were consistent with the acceptable precision proposed by Horwitz [25] for analyte concentrations of the order of μg/ml.

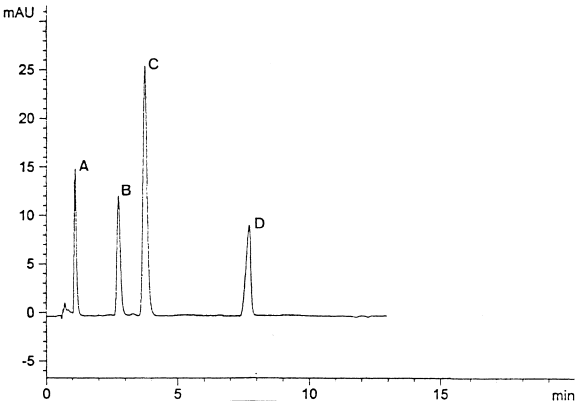

Fig. 1. HPLC chromatogram of retinol (A), α-tocopherol (B), α-tocopherol acetate (C) and β-carotene (D) standards.

The standard addition method was used to test accuracy. A standard of each compound was added to three aliquots of sample at three concentration levels in order to calculate recovery. Three determinations were performed for each addition level. Results are shown in Table 3.

Table 4 shows the mean levels of antioxidants in the volunteers tested.

This method is easy to perform and allows the identification and quantification of vitamins A and E and β-carotene present in plasma or lipoprotein samples. Therefore it is suitable for routine laboratory work and is also useful for epidemiological research. The method avoids the use of multiple detectors and has good linearity, precision and accuracy.

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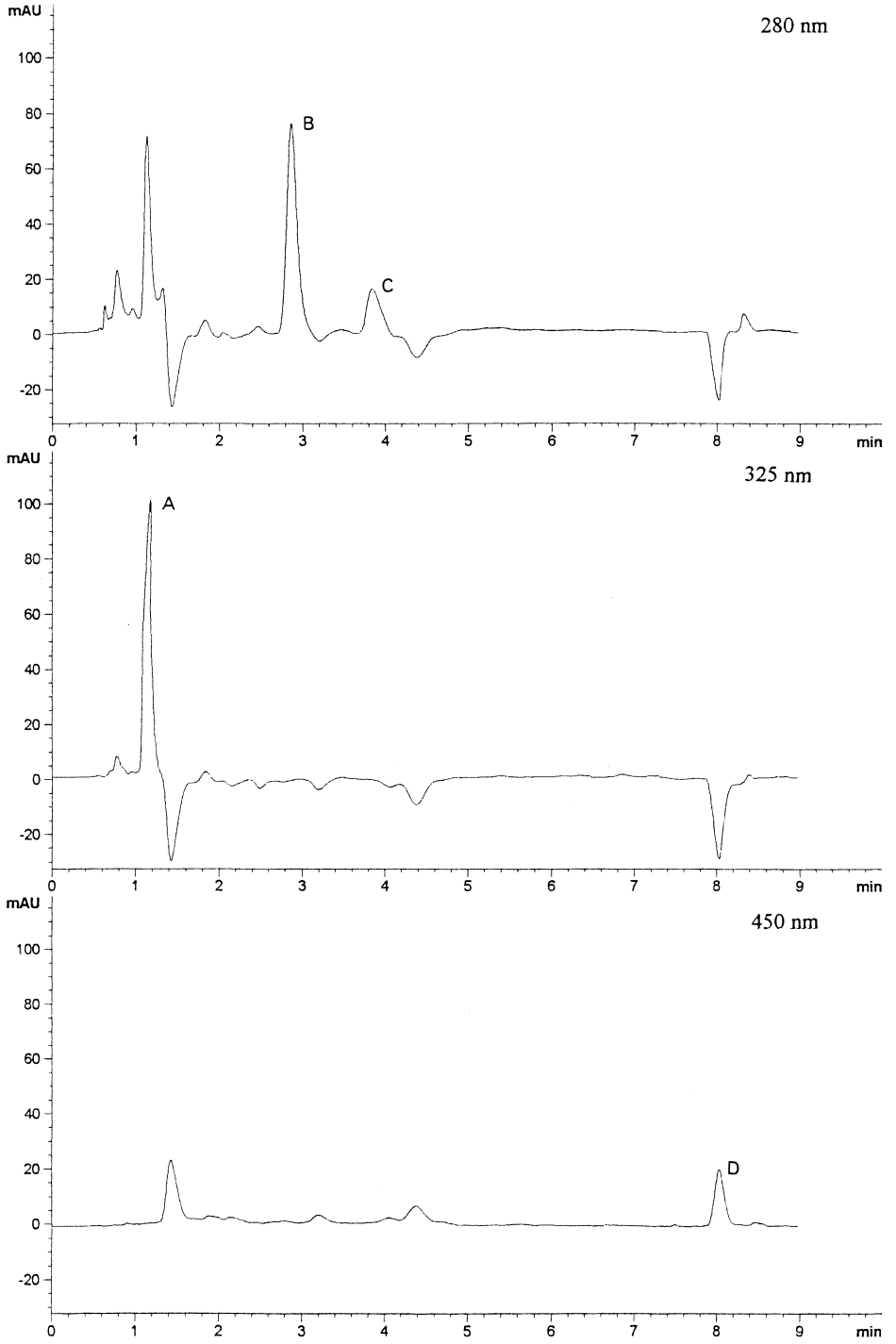

Fig. 2. Chromatogram of a plasma sample: retinol (A) at 325 nm, α-tocopherol (B) and α-tocopherol acetate (C) at 280 nm and β-carotene (D) at 450 nm.

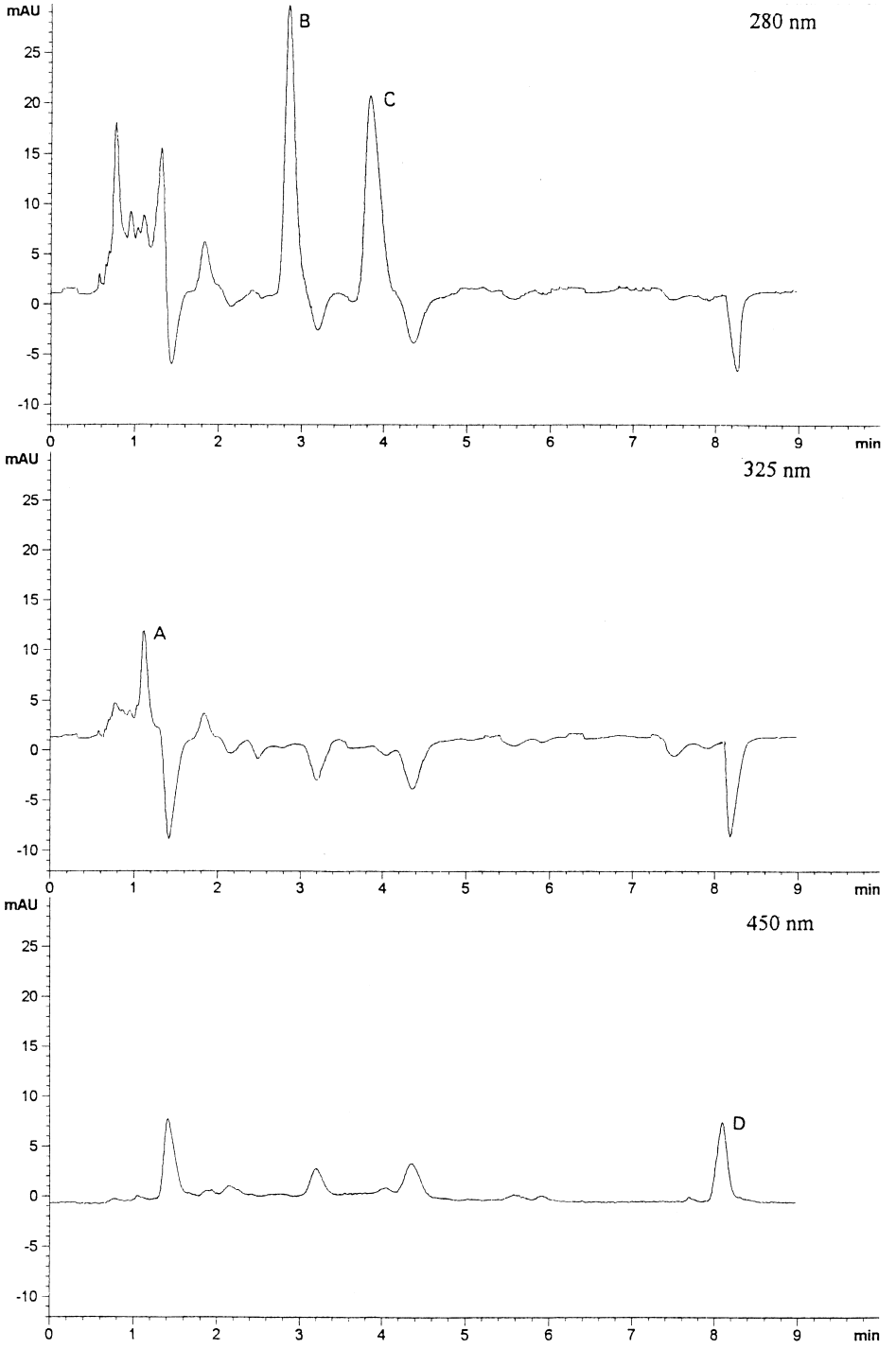

Fig. 3. Chromatogram of an LDL sample: retinol (A) at 325 nm, α-tocopherol (B) and α-tocopherol acetate (C) at 280 nm and β-carotene (D) at 450 nm.

Table 1
Linearity

Compound	Concentration range (μg/ml)	Mean linear regression (LR)	Coefficient of correlation (*r*)
Retinol	0.1–2	$y=0.691x-0.048$	0.998
α-Tocopherol	5–25	$y=0.039x-0.015$	0.998
β-Carotene	0.1–2	$y=0.470x-0.022$	0.997

y=Concentration (μg/ml); x=peak-height ratio (analyte/I.S.).

Table 2
Precision of the assay ($n=10$)

Compound	Within-day assay		Between-day assay	
	Mean (μg/ml)	RSD (%)	Mean (μg/ml)	RSD (%)
Retinol	0.37	2.5	0.38	3.7
α-Tocopherol	13.17	5.4	13.26	7.8
β-Carotene	0.24	4.8	0.23	6.9

Table 3
Results of the recovery studies in a plasma sample

Compound	Initial content (μg/ml)	Added (μg/ml)	Mean recovery (%)
Retinol	0.37	0.1	94.3
		0.2	95.7
		0.5	96.7
α-Tocopherol	13.17	5	102.8
		10	101.2
		15	103.5
β-Carotene	0.24	0.1	102.4
		0.2	104.5
		0.5	105.2

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Table 4
Mean levels of antioxidants in plasma and LDL ($n=10$)

Compound	Plasma (μg/ml)	LDL (μg/mg protein)
Retinol	0.52±0.05	0.10±0.01
α-Tocopherol	20.74±6.16	7.93±2.02
β-Carotene	0.36±0.14	0.10±0.01

Values are means±SD.

References

[1] B.L. Lee, S.C. Chua, H.Y. Ong, C.N. Ong, J. Chromatogr. B 581 (1992) 41.
[2] Alpha-Tocopherol and β-Carotene Cancer Prevention Study Group, New Engl. J. Med 330 (1994) 1029.
[3] B. Olmedilla, F. Granado, I. Blanco, E. Rojas-Hidalgo, Food Chem. 45 (1992) 205.
[4] K.W. Miller, N.A. Lorr, C.S. Yang, Anal. Biochem. 138 (1983) 340.
[5] N. Fotouchi, M. Meydani, M.S. Santos, S.N. Meydani, C.H. Hennekens, J.M. Gaziano, Am. J. Clin. Nutr. 63 (1996) 553.
[6] C. Fuller, I. Jialal, Am. J. Clin. Nutr. 60 (Suppl.) (1994) 1010s.
[7] M. Mancini, V.J. Parfitt, P. Rubba, Can. J. Cardiol. 11 (Suppl. G) (1995) 105G.
[8] H.M.G. Princen, W. Van Duyvenvoorde, R. Buytennek, A. van der Laarse, G. van Poppel, J.A.G. Leuren, V.W.M. van Hinsberg, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 325.
[9] A.G. Olsson, X.M. Yuan, Curr. Opin. Lipidol. 7 (1996) 374.
[10] W.J. Driskell, J.W. Neese, C.C. Bryant, M.M. Basha, J. Chromatogr. B 231 (1982) 439.
[11] D.W. Nierenberg, D.C. Lester, J. Chromatogr. B 345 (1985) 275.
[12] M.J. González-Corbella, N. Lloberas-Blanch, A.I. Castellote-Bargalló, M.C. López-Sabater, M. Rivero-Urgell, J. Chromatogr. B 660 (1994) 395.

[13] Y. Gobel, C. Schaffer, B. Koletzko, J. Chromatogr. B 668 (1997) 57.
[14] Y.M. Peng, M.J. Xu, D.S. Alberts, J. Natl. Cancer Inst. 78 (1987) 95.
[15] R.K. Aaran, T. Nikkari, J. Pharm. Biomed. Anal. 6 (6–8) (1988) 853.
[16] N.E. Craft, E.D. Brown, J.C. Smith Jr., Clin. Chem. 34 (1) (1988) 44.
[17] B. Olmedilla, F. Granado, E. Rojas-Hidalgo, I. Blanco, J. Liq. Chromatogr. 13 (8) (1990) 1455.
[18] A.B. Barua, H.C. Furr, D. Janick-Buckner ckne, J.A. Olson, Food Chem. 46 (1993) 419.
[19] K.S. Epler, R.G. Ziegler, N.E. Craft, J. Chromatogr. B 619 (1993) 37.
[20] R.M. Lamuela-Raventós, M.I. Covas, M. Fitó, J. Marrugat, M.C. De la Torre-Boronat, Clin. Chem. 45 (10) (1999) 1870.
[21] L.A. Kaplan, J.A. Miller, E.A. Stein, M.J. Stampfer, Methods Enzymol. 189 (1990) 155.
[22] E. Gimeno, E. Calero, A.I. Castellote, R.M. Lamuela-Raventós, M.C. de la Torre, M.C. López-Sabater, J. Chromatogr. A 881 (2000) 255.
[23] K.S. Epler, L.C. Sanders, J. Chromatogr. 595 (1992) 89.
[24] The United States Pharmacopeia (U.S.P. XXIII), Mack Printing, Easton, PA, 1989, p. 1711.
[25] W. Horwitz, Anal. Chem. 54 (1986) 671.