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Quantitative analysis of vitamin E, cholesterol and phospholipid fatty acids in a single aliquot of human platelets and cultured endothelial cells

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Abstract

A reliable procedure is described for the joint analysis of vitamin E (tocopherols), cholesterol and phospholipids in the same minute sample of human platelets and on human cultured endothelial cells. The whole procedure is based on the extraction of total lipids, thin-layer chromatography of all compounds of interest and microcolumn purification of tocopherols and cholesterol. The combined use of butyl hydroxytoluene and ascorbic acid in the purification steps allowed a complete recovery of the tocopherols analyzed, as well as of cholesterol by high-performance liquid chromatography. The detection of these lipids was performed with fluorometric, spectrophotometric and evaporative light-scattering detectors whose respective sensitivities were compared. The fatty acid composition of phospholipid classes from the same sample, separated on the same silica gel plate, was determined by gas–liquid chromatography. The whole procedure is rapid since it requires about 4 h to analyse tocopherols and cholesterol and to prepare methylated fatty acids, 28 samples being easily completed within one working day. The evaluation of the whole membrane antioxidant status requires as little as one 25 cm^2 confluent culture flask (about 0.75×10^6 cells) for endothelial cells or two ml of blood (3×10^8 platelets). © 1997 Elsevier Science B.V.

Keywords: Vitamins; Cholesterol; Phospholipids

1. Introduction

Vitamin E (tocopherols) deficiency was shown to induce a variety of hematological problems in rats including higher platelet counts, increased platelet adhesion [1] and aggregation in vitro [2,3] as well as in vivo [4]. Consequently, investigations on these cells have been frequently proposed to assess the nutritional status in humans and animals [5,6]. Similarly, vitamin E deficiency produced marked changes in vascular function associated with lipid peroxidation and endothelial cell (EC) structural alterations [7]. Vitamin E was also shown to have important effects on several EC functions involved in normal hemostasis. It was reported that vitamin E is able to stimulate EC proliferation [8] and to protect these cells against age-related injury [9]. The best known function of vitamin E is to prevent peroxidation of unsaturated fatty acids by scavenging lipophilic radicals within membranes. Furthermore, a

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role as membrane stabilizer [10] has also been proposed.

Several alterations of the lipid components included in the diet of fed animals or in the growth medium of cultured cells lead to changes in membrane lipid composition that are frequently related to membrane physiological processes. In general, these changes concern the membrane cholesterol to phospholipid ratio, the unsaturation degree of phospholipid acyl chains and tocopherol status. Several reports indicate how these compounds can be altered in platelets [11,12] or in EC [13–16]. Recent observations on cultured cells emphasized the importance of the joint examination of the tocopherol levels and fatty acid unsaturation [17]. Thus, the determination of tocopherols, cholesterol and phospholipid composition in the same sample appears desirable to appreciate the oxidative and lipid status of cellular membranes. Previously proposed methodologies for the determination of tocopherols and cholesterol in biological samples are based on saponification [18–22] or protein precipitation [23,24], both combined with non-polar solvent extractions. As these processes are not compatible with glycerolipid analysis, all compounds cannot be determined on the same minute sample. When abundant, tocopherols can be determined by high-performance liquid chromatography (HPLC) with ultraviolet detection [22] but fluorescence [18,19,22,25] or electrochemical [20,23,24,26] detections are needed for small biological samples. Cholesterol can be determined by gas–liquid chromatography (GLC) after derivatization [27] but HPLC coupled with ultraviolet detection [22,26] offers a convenient alternative. The evaporative light-scattering detection was not previously applied to the HPLC determination of these molecules after purification.

Our specific interest was to find an efficient and rapid method that would allow precise measurement of the three types of membrane compounds extracted simultaneously from the same microsample of platelets or cultured EC. This would permit comparisons between the vitamin E status and the biochemical structure of the membrane lipids after any experimental treatment. These determinations required one extraction, one thin-layer chromatography (TLC) separation, GLC analysis of the fatty acid composition of phospholipids and a determination of tocopherols and cholesterol with the same HPLC method after a microcolumn purification step.

2. Experimental

2.1. Reagents

HPLC-grade solvents, cholesterol, ascorbic acid, butylated hydroxytoluene (BHT), and silica gel G60 (230–400 mesh) were from Merck (Darmstadt, Germany). Thin-layer silica gel plates LK5 were from Whatman (Clifton, NJ, USA). Racemic α-, γ-, δ-tocopherol, tocopherol acetate, standard fatty acids and phospholipids were from Sigma (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France). BF_3-methanol reagent was from Alltech France (Templeuve, France). Deionized water was obtained from a Milli-Q station (Millipore, Saint-Quentin-Yvelines, France).

2.2. Sample preparation

Washed platelets were prepared from blood of healthy donors according to Cazenave et al. [28], suspended in a known volume of Tyrode's buffer containing 0.35% (w/v) fatty acid-free albumin (Sigma) and 2 m*M* Ca^{2+} (pH 7.3) and counted with a Sysmex K-1000 counter. After low speed centrifugation (7 min at 1100 *g*), a known amount of cells was suspended in 0.8 ml of 25 m*M* EDTA, pH 7.0. Total lipids were extracted by vortexing during 20 min after addition of 1 ml chloroform and 2 ml methanol, and during 5 min after further additions of 1 ml chloroform and 1 ml of 1 *M* NaCl. After a low speed centrifugation, the lower phase was washed with 2 ml of 1 *M* NaCl and evaporated under nitrogen.

Human EC were obtained from fragments of mammary arteries or saphenous veins and cultivated in 25 cm^2 tissue culture flasks (Falcon Plastics) in the presence of 30% human pooled serum as previously described [29,30]. Cells were photographed with a Nikon (Diaphot-TMD) inverted microscope equipped with an automatic camera, then cell densities were calculated from cell counts in calibrated fields. When the cultures became confluent (about 0.75×10^6 cells), each flask was drained, rinsed with

physiological saline and the cells scraped in 4 ml methanol. After addition of 4 ml chloroform and 1.6 ml water, the suspension was vortexed for 20 min and centrifuged. The lower phase was washed with 2 ml of 9 g/l NaCl and evaporated under nitrogen. BHT was added (0.15 g/l) to all solvents used for extractions and dissolution.

2.3. TLC separation of phospholipids, cholesterol and tocopherols

Individual phospholipids, cholesterol and tocopherols in the total lipid extract were separated by one-dimensional TLC on Whatman LK5 silica gel plates [31]. TLC plates were previously washed by migration up to the top in chloroform–methanol (1:1, v/v) and impregnated with boric acid. Six samples were applied as streaks in the preconcentration zone along the full width of the plate which was developed up to 1 cm below the top in a dark chamber in a solvent system of chloroform–ethanol–water–triethylamine (35:30:7:35, v/v). To prevent tocopherol oxidation, ascorbic acid previously dissolved in water and BHT in ethanol were added to the solvent system to give a final concentration of 10 g/l and 0.15 g/l, respectively. Lipid spots were located under UV light after a primuline spray with the help of commercial standards. The mobilities of the various lipids of interest, expressed as R_F values were: sphingomyelin: 0.08, phosphatidylcholine: 0.13, phosphatidylinositol: 0.22, phosphatidylserine: 0.35, phosphatidylethanolamine: 0.42, cardiolipin: 0.67, cholesterol+tocopherols: 0.86–0.89.

2.4. Analysis of phospholipid fatty acid composition

Each spot on the silica gel plate corresponding to individual phospholipid was scraped off and transferred to a screw-capped glass tube and a known amount of heptadecanoic acid was added as an internal standard before direct transmethylation with 1 ml of 14% BF_3 methanol as previously described [31]. GLC analysis of fatty acids was performed with an instrument (Model 5890A, Hewlett-Packard France, Les Ulis, France) equipped with a carbowax fused silica 30 m×0.25 mm I.D. capillary column, 0.25 m film thickness (Alltech). Output signal was analyzed with an electronic integrator (Datajet, Thermo Separation Products) monitored by the WOW software (Thermo Separation Products). Mass data for each fatty acid were transformed into molar percentage using individual molecular mass. The molar distribution of phospholipid classes was calculated using data for the internal standard in each class. The peroxidizability index was calculated from the global fatty acid composition of the whole phospholipid pool as the number of bis-allylic positions per 100 mol fatty acids [17]. The antioxidant index was calculated as the ratio of the total polyunsaturated fatty acids to α-tocopherol (mol/mol).

2.5. Analysis of tocopherols and cholesterol

After the TLC separation, the spot corresponding to the mixture of unseparated cholesterol and tocopherols (average R_F=0.87) was scraped off and transferred with a little funnel to a small silica gel column. This column consisted in a pasteur pipette (15 cm length) containing a small plug of glass wool which retained the proper amount of silica gel G60 (2 cm height). The column was washed with 3 ml hexane before use. After the scraped powder was loaded, the column was washed with 2 ml hexane. Then, purified fractions containing tocopherols and cholesterol were eluted with 3 ml hexane-*tert.*-butyl methyl ether (92.5:7.5, v/v) and with 3 ml *tert.*-butyl methyl ether, respectively. The solvent phases were evaporated under nitrogen and the residues redissolved in 0.2 ml or less of HPLC mobile phase. Samples and standards were kept in inactinic glass ware at −20°C up to one week until HPLC analysis.

Tocopherols and cholesterol were separated on a 120×4.6 mm I.D. HPLC column packed with 5 μm LiChrosorb RP18 (Merck) with methanol–water (98:2, v/v) as mobile phase [18]. The flow-rate was 1.5 ml/min using an HPLC pump (Model PU-980, Jasco, Prolabo, Fontenay-sous-Bois, France) equipped with a degaser (GT-103, Gastorr, Prolabo). Detection was carried out either with a spectrofluorimeter (Model SFM 23/B, Kontron Instruments, St-Quentin-Yvelines, France), an ultraviolet detector (Model Spectrachrom 100, Thermo Separation Products, Les Ulis, France) or a light-scattering detector (DDL21, Eurosep Instruments, Cergy Pontoise, France). The output signal integration and data

analysis were performed on integrators (Datajet, Thermo Separation Products) monitored by the WOW software (Thermo Separation Products). Tocopherols were measured by spectrofluorimetry with an excitation wavelength of 298 nm and an emission wavelength of 327 nm. Spectrophotometry was used to measure tocopherols, tocopherol acetate and cholesterol at 292 nm, 284 nm and 206 nm, respectively. All these lipids could also be quantified with the evaporative light-scattering detector. The signal-to-noise ratio of the instrument was maximized by measuring the integrator responses given by α-tocopherol and cholesterol amounts as a function of air-inlet pressure, evaporation temperature and high voltage setting. On the basis of these assays, the air pressure was set at 1 bar, the evaporation temperature at 60°C and the high voltage at 500 V or 700 V for injected amounts higher or lower than 500 ng, respectively. With the isocratic system used in this study, the representative retention times were: δ-tocopherol, 4.0 min; γ-tocopherol, 4.7 min; α-tocopherol, 5.6 min; tocopherol acetate, 7.5 min and cholesterol, 9.4 min (Fig. 1).

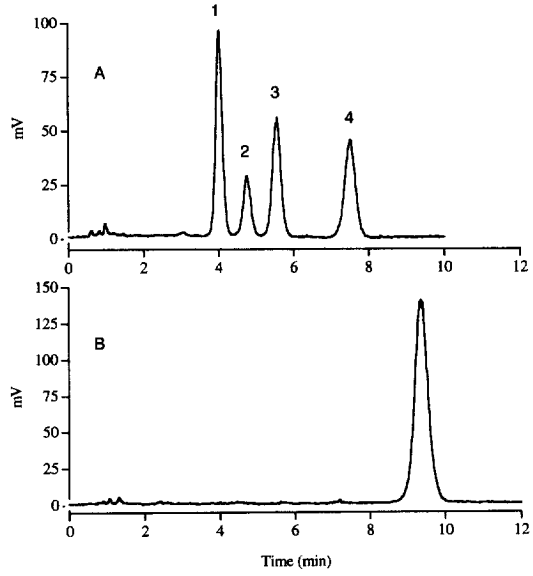

Fig. 1. (A) Typical chromatogram of a mixture of tocopherols (1 μg each) eluted from the silica gel microcolumn by 3 ml of *tert.*-butyl methyl ether (92.5:7.5, v/v). Peaks 1=δ-tocopherol, 2=γ-tocopherol, 3=α-tocopherol, 4=tocopherol acetate. Column C_{18} RP (125 mm×4.6 mm I.D., 5 μm; mobile phase, methanol–water (98:2, v/v); flow-rate, 1.5 ml/min; detection with the light scattering detector. (B) Chromatogram of the fraction eluted from the silica gel microcolumn by 3 ml pure *tert.*-butyl methyl ether containing 3 μg cholesterol.

2.6. Statistics

All data are reported as means standard error (SEM). The significance of the difference between groups of cultured EC was determined by the use of Student *t*-test. Values of $p \leq 0.05$ were considered to be significant.

3. Results

3.1. Lipid methodology

The TLC system used in this study separated very efficiently the phospholipid classes, including phospatidylinositol and phosphatidylserine, while tocopherols and cholesterol migrated together near the solvent front. Seven samples could be easily chromatographed on one plate for a 2-h run.

In contrast to phospholipids or cholesterol, attempts to measure tocopherol amounts after combined separations on thin-layer plate and solid-phase column in the absence of antioxidants were not successful. Thus, poor recoveries (47±1.7%, n=4) were obtained for δ-tocopherol added to cell extracts. Whereas the quantification problem could be partially resolved by using an internal standard, efforts were made to improve tocopherol recoveries. We determined that the addition of 0.15 g/l BHT to the solvent system improved the recovery up to 86.5±2.2% (n=4) and that a further addition of 10 g/l ascorbic acid increased the recovery up to 98.7±0.2% (n=4). Thus, the internal standardization for the determination of tocopherols appeared useless in our combined TLC/HPLC method. Nevertheless, δ-tocopherol could be used as an internal standard since it was absent in our samples. If δ-tocopherol is present or if a fluorimetric equipment is not available, tocopherol acetate can be used with light scattering or spectrophotometric detection. The recovery for cholesterol and phospholipids was always higher than 98%.

While tocopherols and cholesterol could be separated in the same HPLC run, a solid-phase separation

step was introduced to remove contaminants (fatty acids are retained on the gel) and to allow the use of different sample volumes for the two lipid classes. This supplemental clean-up prior analysis improved the background noise and the detection sensitivity through a reduction of sample size. The fluorometric detector could be used only for the determination of unsubstituted tocopherols (Table 1). The range of response factors measured at the medium sensitivity of our instrument (between 10 and 200 ng) was $18–59\times10^{-6}$. At the highest sensitivity setting, the response factors in the range 1 to 10 ng were 10 times lower. The minimum detection limit was about 0.2–0.5 ng (0.5–1.2 pmol) while the quantification limit was about 0.5–1 ng (2.4–4.8 nmol). This detection limit compares well with that reported with similar methodology [19] or with electrochemical detection [20–23] while a 60 pg detection limit was claimed possible with this last equipment [24]. With the UV spectrophotometer as detector, all tocopherols and cholesterol could be quantified, the range of response factors for the different compounds (between 0.1 and 10 μg) being $4.8–15.5\times10^{-6}$. The correlation coefficients were always higher than 0.995. The minimum detection limit for these compounds was about 15–30 ng (35–70 pmol) per injection and the quantification limit was about 50–100 ng (110–150 nmol) per injection (Table 1). With the evaporative light-scattering detector, the responses were found linear for tocopherols in the range 0.1–0.5 μg and for cholesterol in the range 0.5–3 μg, however the slope varied markedly with the compound analyzed (response factors from 1.4 to 5.4×10^{-6} with a high voltage setting of 500 V). The correlation coefficients were always higher than 0.996. The minimum detection limit was about 10–70 ng (25–180 pmol) and the quantification limit was about 40–200 ng (100–500 pmol) per injection (high voltage: 700 V) (Table 1). It is noteworthy that detection and quantification limits were similar for unsubstituted tocopherols when using either a light-scattering or a UV detector. As the UV detector had a lower background noise than the light-scattering detector, its quantification limits for cholesterol were twice lower.

We evaluated the within-run precision of α-tocopherol and cholesterol estimations by using the three detectors proposed in this work. Platelet aliquots were prepared so that final lipid amounts injected were about ten times higher than their quantification limits. The precision was estimated as the coefficient of variation (in percentage deviation from the mean) by performing the whole protocol 6 times. The results are shown in Table 2.

Table 1
Sensitivity, detection and quantification limits for determination of tocopherols and cholesterol by different detection systems

	α-Tocopherol	γ-Tocopherol	δ-Tocopherol	Tocopherol acetate	Cholesterol
UV detection					
response factor (μg/area)[a]	6.6×10^{-6} (292 nm)	15.5×10^{-6} (292 nm)	4.8×10^{-6} (292 nm)	10.5×10^{-6} (284 nm)	6.95×10^{-6} (206 nm)
detection limit (ng)[b]	15	30	15	20	30
quantification limit (ng)[c]	50	100	50	60	100
Light-scattering detection					
response factor (μg/area)	1.4×10^{-6}	5.4×10^{-6}	1.4×10^{-6}	1.9×10^{-6}	1.77×10^{-6}
detection limit (ng)	10	20	10	70	70
quantification limit (ng)	40	100	40	200	200
Fluorescence detection					
response factor (ng/area) (medium sensitivity)	59×10^{-6}	59×10^{-6}	18×10^{-6}	-	-
detection limit (ng) (high sensitivity)	0.5	0.5	0.2	-	-
quantification limit (ng)	1	1	0.5	-	-

[a] The response factor linking the area counts and the injected amount was calculated as the slope of an average calibration curve.
[b] The detection limit was estimated as the amount injected giving a signal-to-noise ratio of 3.
[c] The quantification limit was estimated as the smallest amount giving a signal intensity distinguished from the diluent blank with a 95% confidence (calculated by interpolating the mean ±2 SD signal intensity value of the zero calibration on an averaged calibration curve).

Table 2
Within-run precision determination for α-tocopherol and cholesterol with the three detectors (C.V. in % deviation from the mean)

	Fluorescence detector	UV detector	Light-scattering detector
α-Tocopherol	1.2	3.6	6.6
Cholesterol	-	3.9	4.2

3.2. Analysis of cellular lipids

Lipid determinations on platelets were made after processing about 6×10^8 cells isolated from 2 ml of blood and concentration results are given on a basis of 10^6 cells (Table 3). Thus, our determinations revealed that the amounts of cholesterol (about 100 nmol or 38 μg) and α-tocopherol (670 pmol or 300 ng) present in 6×10^8 platelets could be easily determined with a light-scattering detector or even with a spectrophotometric detector, whereas the determination of γ-tocopherol required a spectrofluorimetric equipment. With respect to phospholipids, 6×10^8 platelets allowed precise fatty acid analysis even of the poorly represented classes (phosphatidylserine or phosphatidylinositol). Only the composition of the most unsaturated platelet phospholipids are given in Table 4. Phosphatidylinositol was characterized by a high arachidonic acid content (47%), while phosphatidylethanolamine, rich in (n-3) fatty acids, was the most unsaturated phospholipid.

Lipid determinations on endothelial cells were performed on about 0.75×10^6 cells contained in only one 25 cm^2 culture flask and, as platelets, concentration results are given on a basis of 10^6 cells (Table 3). The amount of cholesterol per flask of arterial (37 nmol or 14 μg) or venous (57 nmol or 22 μg) EC could be determined with a light-scattering detector or a spectrophotometric detector. The corresponding amount of α-tocopherol (about 34 pmol or 15 ng in a flask of arterial EC and 22 pmol or 10 ng in a flask of venous EC) could be determined only by spectrofluorimetry. Arterial and venous EC cultivated in one 25 cm^2 flask yielded at confluence similar amounts of phospholipids as that found in platelets contained in 2 ml of blood and thus similar analyses could be processed. When comparing the lipid composition of the two types of cultured EC, it appeared that the phospholipid contents were similar in arterial and venous EC while the cholesterol content was 55% higher in venous than in arterial EC ($p<0.05$), leading to significantly different cholesterol/phospholipid ratios ($p<0.05$). The tocopherol content was 38% lower in venous than in arterial EC ($p<0.05$), while the α-tocopherol/γ-tocopherol ratio was similar in both cell types. The number of polyunsaturated fatty acid

Table 3
Lipid composition of human platelets and cultured endothelial cells

	Platelets	Arterial endothelial cells	Veinous endothelial cells
Cholesterol (nmol/10^6 cells)	0.16±0.02[a]	49.4±5.4[b]	76.6±4.2[b]
α-Tocopherol (pmol/10^6 cells)	1.12±0.16	45.7±16.6	28.8±13.2
γ-Tocopherol (pmol/10^6 cells)	0.21±0.04	4.5±1.2	2.8±1.4
Phospholipids (nmol/10^6 cells)	0.25±0.03	147±4	160±5
Cholesterol/phospholipid ratio (mol/mol)	0.52±0.03	0.34±0.03	0.48±0.03
α-Tocopherol/phospholipid ratio (pmol/nmol)	4.22±0.37	0.31±0.11	0.18±0.07
Antioxidant index (PUFA/α-tocopherol ratio, mol/mol)	160±4	2584±95	4431±611
PUFA	33.8±0.9	40.1±1.5	39.9±5.5
DBI	146±4	156±8	156±19
PEROX. INDEX	94.9±2.6	93.1±11.9	96.8±5.6
UNSAT/SAT	1.27±0.12	1.48±0.14	1.46±0.09

[a] Values are means±SEM of samples from 5 subjects.
[b] Values are means±SEM of triplicates.
PUFA: polyunsaturated fatty acids (mol%); DBI: double bond index (mean number of double bonds per 100 mol fatty acids); PEROX. INDEX: peroxidizability index (number of bis-allylic positions per 100 mol fatty acids); UNSAT/SAT: unsaturated to saturated fatty acids ratio (mol%/mol%). Mole percents of fatty acids were calculated on the basis of amounts of fatty acids in all phospholipid fractions determined from integrated peak areas on chromatograms and the respective molecular masses of fatty acids.

Table 4
Fatty acid composition of the main glycerophospholipids in human platelets[a]

	PE	PS	PI	PC
14:0	-	0.7±0.2	0.6±0.2	0.5±0.1
16:0	4.4±0.4	2.1±0.3	2.7±0.3	31.8±1.0
18:0	16.5±0.2	39.6±2.0	39.4±1.6	14.4±0.5
20:0	0.5±0.0	1.3±0.1	-	0.9±0.1
16:1n-9	0.6±0.1	0.7±0.1	-	0.5±0.0
18:1n-9	5.2±0.1	20.1±0.9	4.6±0.8	20.5±0.5
20:1n-9	-	-	-	0.8±0.1
18:1n-7	0.9±0.0	0.9±0.1	1.2±0.1	2.0±0.1
18:2n-6	2.3±0.2	1.0±0.0	0.9±0.0	8.2±0.6
20:3n-6	0.7±0.1	1.7±0.1	0.7±0.0	1.8±0.1
20:4n-6	36.7±0.5	27.0±2.5	47.4±0.5	14.6±0.6
22:4n-6	4.9±0.4	1.3±0.2	0.5±0.1	0.9±0.1
18:3n-3	1.0±0.2	-	-	-
20:5n-3	0.7±0.3	-	-	-
22:5n-3	3.1±0.2	0.9±0.1	0.4±0.0	0.6±0.1
22:6n-3	3.4±0.3	1.9±0.2	0.4±0.1	0.9±0.1
PUFA	53.5±1.0	34.2±2.7	50.5±0.6	28.0±0.6
DBI	229±5	160±10	207±3	120±2
UNSAT/SAT	1.87±0.27	1.30±0.10	1.33±0.08	1.10±0.02
Phospholipid (mol%)	30.4±1.1	10.2±0.4	6±0.2	40.4±0.9

[a] Values are means (mol%)±SEM of samples from 5 subjects. Fatty acids less than 0.5% of the total were not included in the table. PE: phosphatidylethanolamine; PS: phosphatidylserine; PI: phosphatidylinositol; PC: phosphatidylcholine; PUFA: polyunsaturated fatty acids; DBI: double bond index, mean number of double bonds per 100 mol fatty acids; UNSAT/SAT: unsaturated to saturated fatty acids ratio (mol%/mol%). The composition of cardiolipin (1.1±0.3 mol%) and sphingomyelin (11.8±1.0 mol%) was determined but not given in the table.

molecules per α-tocopherol (antioxidant index) was significantly lower (42%) in arterial than in venous EC ($p<0.05$).

In our culture conditions, the most important phospholipid classes of arterial (Table 5) and venous EC (Table 6) had very similar fatty acid compositions, the only significant difference being a phosphatidylethanolamine unsaturation lower in arterial than in venous EC ($p<0.05$).

4. Discussion

The development of HPLC has increased the sensitivity of analytical procedures for many membrane lipid components. Several laboratories have already published reports on tocopherol analysis in several cell types [19–21,24,25,32]. The present method was developed to analyze accurately several important membrane lipids in the smallest number of cells. As tocopherols, cholesterol and phospholipids must be determined in the same microsample, we omitted the saponification step, classically used for the determination of tocopherols and cholesterol [20–22]. Tocopherol decomposition was minimized due to the fast processing of samples and the use of antioxidants along the extraction and TLC separation, nearly 100% recoveries for low amounts being routinely obtained. The preparation of 28 samples ready to be analyzed for their content in cholesterol, tocopherols and phospholipid fatty acids can be easily processed without special training within one working day. While the analysis of cholesterol and tocopherols in the 28 samples takes no longer than 8 h, the analysis time of fatty acids by GLC is obviously longer (about 45 min per sample). Our results for α-tocopherol content and for the ratio of γ- to α-tocopherol in human platelets are in agreement with previously published values [25,32–34]. Thus, these results together with our validation study indicate the usefulness of the proposed analytical procedure in the determination of labile

Table 5
Fatty acid composition of the main glycerophospholipids in arterial endothelial cells in culture[a]

	PE	PS	PI	PC
14:0	1.4±0.3	1.2±0.5	0.5±0.2	0.6±0.3
16:0	9.2±0.6	9.7±1.3	5.3±1.8	29.8±4.3
18:0	15.0±1.2	35.0±2.5	36.9±0.5	10.1±0.8
16:1n-9	1.3±0.2	1.9±0.6	0.6±0.2	0.8±0.2
18:1n-9	8.4±0.0	14.5±0.5	7.2±0.4	17.4±1.0
16:1n-7	-	-	-	1.0±0.2
18:1n-7	1.2±0.3	2.2±0.3	0.8±0.2	2.9±0.3
18:2n-6	6.2±0.7	9.1±0.9	5.9±0.7	17.7±0.5
20:2n-6	-	0.7±0.0	-	1.0±0.2
20:3n-6	1.4±0.1	3.2±0.2	2.3±0.3	2.5±0.4
20:4n-6	20.3±1.0	4.8±0.6	35.1±3.1	10.3±1.6
22:4n-6	7.7±0.6	6.8±0.9	2.5±0.3	2.0±0.4
20:5n-3	0.6±0.2	-	-	-
22:5n-3	3.7±0.3	3.6±0.3	1.1±0.1	0.9±0.1
22:6n-3	6.1±0.8	5.2±0.6	1.0±0.0	1.8±0.3
PUFA	47.1±1.6	34.4±1.8	48.6±3.3	37.1±2.6
DBI	203±6	148±7	191±13	136±13
UNSAT/SAT	1.51±0.09	1.16±0.10	1.36±0.14	1.51±0.25
Phospholipid (mol%)	23.1±0.7	4.3±0.2	8.5±1.3	55.9±0.8

[a] See legend of Table 4. The composition of cardiolipin (1.6±0.1 mol%) and sphingomyelin (6.6±0.7 mol%) was determined but not given in the table.

Table 6
Fatty acid composition of the main glycerophospholipids in veinous endothelial cells in culture[a]

	PE	PS	PI	PC
14:0	-	2.0±1.6	1.2±0.5	1.1±0.5
16:0	7.2±0.6	9.3±5.0	5.3±2.0	32.5±3.3
18:0	17.5±0.4	32.2±4.3	34.0±1.2	8.4±0.8
16:1n-9	0.6±0.2	1.8±1.7	1.1±0.6	0.9±0.3
18:1n-9	11.7±0.2	16.0±1.0	8.8±1.5	18.7±0.6
16:1n-7	-	-	-	1.3±0.3
18:1n-7	2.2±0.2	2.6±0.7	1.8±0.4	2.8±0.2
18:2n-6	7.1±0.4	9.8±3.2	6.0±0.7	17.7±1.5
20:2n-6	0.6±0.0	0.6±0.2	-	0.9±0.1
20:3n-6	1.7±0.1	3.1±1.2	2.3±0.2	2.2±0.4
20:4n-6	17.9±1.2	3.9±1.6	33.5±3.4	7.9±0.9
22:4n-6	9.4±0.9	6.2±1.8	2.7±0.7	1.9±0.5
20:5n-3	0.6±0.1	-	-	-
22:5n-3	4.6±0.3	4.7±2.7	1.3±0.3	0.9±0.1
22:6n-3	6.9±0.2	5.7±2.1	1.0±0.0	1.6±0.1
PUFA	49.6±2.2	34.8±7.2	47.5±4.2	33.9±3.2
DBI	217±7	152±34	190±15	124±10
UNSAT/SAT	1.97±0.13	1.28±0.23	1.48±0.11	1.40±0.19
Phospholipid (mol%)	24.1±0.6	5.8±0.5	8.5±1.2	56.1±1.6

[a] See legend of Table 4. The composition of cardiolipin (1.7±0.1 mol%) and sphingomyelin (3.8±0.9 mol%) was determined but not given in the table.

antioxidants such as tocopherols present in minute amounts of tissue and cells. In cultured EC, we were able to measure very low α-tocopherol concentrations, in agreement with previous values determined on cells from human umbilical cord veins cultured in similar conditions [35]. In contrast, a four-fold concentration of α-tocopherol was reported for the same type of cultured EC [36]. These discrepancies could result from differences in cellular specificity or culture conditions. As far as we know, no values for γ-tocopherol content in cultured EC were previously reported. When compared with platelets, endothelial cells have elevated antioxidant index but similar to that recently reported for other cultured cells [17]. These results confirm an apparent tocopherol deficiency in EC cultivated in medium unsupplemented with vitamin E, as it was previously suggested [17]. This relative deficiency is likely related to the limited amount of tocopherols contained in the tissue culture growth medium, the addition of 30% human serum leading to final α- and γ-tocopherol concentrations of about 10 and 1.4 μM, respectively. These observations may provide a basis to investigate the relationships between vitamin E and membrane unsaturation. The results for the platelet cholesterol to phospholipid molar ratio are in agreement with values previously reported for healthy subjects [37,38]. The cholesterol content of human arterial EC given in the present work is similar to that reported for EC cultivated from bovine carotid arteries [39]. In the absence of other comparative studies, no conclusion can be drawn from the observed higher cholesterol to phospholipid ratio in venous than in arterial EC. The results for the fatty acid profiles are in agreement with those previously published for platelets [31] and cultured EC [40,41]. The slight differences observed between our results and the previous reports are probably caused either by the influence of exogenous lipids on the membrane composition or by cell processing. The elevated proportion of polyunsaturated fatty acids, and mainly of arachidonic acid, in phospholipids of our cultured cells is likely related to the use of human serum in the cultivation medium [41]. It should be emphasized that the presence of antioxidants in the chromatographic eluent probably reduced a spontaneous oxidation allowing a maximal recovery of lipid double bonds.

In summary, we have described a reliable tocopherol assay in parallel with efficient cholesterol and phospholipid determinations in platelets and cultured endothelial cells. The simple procedure presented here has proved to be very efficient and fast, enabling the routine processing of large numbers of samples. Our methodology allows precise lipid determinations in microsamples and may have particular value in the management of experimental or pathological situations where oxidative vulnerability is expected.

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