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Single-step preparation of selected biological fluids for the high performance liquid chromatographic analysis of fat-soluble vitamins and antioxidants

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ABSTRACT

Fat-soluble vitamins and antioxidants are of relevance in health and disease. Current methods to extract these compounds from biological fluids mainly need use of multi-steps and multi organic solvents. They are time-consuming and difficult to apply to treat simultaneously large sample number.

We here describe a single-step, one solvent extraction of fat-soluble vitamins and antioxidants from biological fluids, and the chromatographic separation of *all-trans*-retinoic acid, 25-hydroxycholecalciferol, *all-trans*-retinol, astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, γ-tocopherol, β-cryptoxanthin, α-tocopherol, phylloquinone, lycopene, α-carotene, β-carotene and coenzyme Q_{10}. Extraction is obtained by adding one volume of biological fluid to two acetonitrile volumes, vortexing for 60 s and incubating for 60 min at 37 °C under agitation. HPLC separation occurs in 30 min using Hypersil C18, 100 × 4.6 mm, 5 μm particle size column, gradient from 70% methanol + 30% H_2O to 100% acetonitrile, flow rate of 1.0 ml/min and 37 °C column temperature. Compounds are revealed using highly sensitive UV-VIS diode array detector.

The HPLC method suitability was assessed in terms of sensitivity, reproducibility and recovery. Using the present extraction and chromatographic conditions we obtained values of the fat-soluble vitamins and antioxidants in serum from 50 healthy controls similar to those found in literature. Additionally, the profile of these compounds was also measured in seminal plasma from 20 healthy fertile donors.

Results indicate that this simple, rapid and low cost sample processing is suitable to extract fat-soluble vitamins and antioxidants from biological fluids and can be applied in clinical and nutritional studies.

1. Introduction

Fat-soluble vitamins are compounds with multiple biological roles and are essential to ensure correct cell functioning that strictly depends on their circulating levels. With the exception of vitamin D_3, humans are not able to synthesize fat-soluble vitamins (A, E and K) that are generally present in different amounts as pro-vitamins in various types of foods, mostly of vegetal origin. After their ingestion, pro-vitamins usually need one or more biotransformation step to generate the corresponding final active vitamin forms [1,2]. To ensure adequate levels within the body, a constant daily ingestion of pro-vitamin/vitamin-reach foods with diet is necessary. In this, the so called Mediterranean diet is considered one of the best dietary regimen, ensuring the consumption of a large variety of fruits, vegetables and plant-derived products (such as extra-virgin olive oil) containing relevant amounts of most fat-soluble pro-vitamins/vitamins [3].

It is important to stress that the Mediterranean diet also allows the intake of fat-soluble antioxidants, among which carotenoids, such as lutein, zeaxanthin, astaxanthin, α-carotene, β-carotene, lycopene, etc., are certainly one of the most well represented groups in foods of vegetal origin [4]. Several studies indicated that these compounds, due to their capacity to scavenge reactive oxygen species (ROS) [5], may exert beneficial effects in different pathological states characterized

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by increased ROS production, such as neurodegenerations [6], myocardial ischemia [7], diabetic retinopathy [8].

In the last decades, a renovated scientific interest has been developed not only towards fat-soluble antioxidants, but also towards fat-soluble vitamins. It was discovered that specific fat-soluble vitamins are either useful adjuvant in the pharmacological therapies of several pathologies [9,10] or possibly implicated in some chronic neurodegenerations [11–13]. These facts strongly increased the importance in the clinic setting of a correct identification and quantification of fat-soluble vitamins and antioxidants, often present in very low concentrations in body fluids [14].

Several methods have been proposed for the separation of hydrophobic vitamins and antioxidants in different biological matrices, mainly based on reversed-phase HPLC and using various highly sensitive detectors to quantify their concentrations at the nanomolar levels (fluorometric, elettrochemical, MS detectors) [15–19]. Due to the chemical nature (moderately to highly hydrophobic) of fat-soluble vitamins and antioxidants, and to the needs of their quantification in highly polar and complex biological samples (serum, plasma, seminal plasma), specific and efficient sample processing are required. To date, sample treatment for the extraction of fat-soluble vitamins are mainly based on complex multi-steps procedures [20]. Besides using large amounts of highly toxic organic solvents (chloroform, *n*-hexane), these methods have the disadvantage to be time-consuming and expensive, rendering problematic their application for the routine preparation of high number of samples in one shot.

In this work, we describe a novel procedure for the efficient fat-soluble vitamin and antioxidant extraction from biological fluids based on a single-step, single-organic solvent treatment of samples, coupled to a simple and fast reversed phase HPLC method for the simultaneous separation of *all-trans*-retinoic acid, 25(OH)-D_3, *all-trans*-retinol, astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, γ-tocopherol, β-cryptoxanthin, α-tocopherol, lycopene, α-carotene, β-carotene, phylloquinone and coenzyme Q_{10}. The method was validated in terms of sensitivity, reproducibility and recovery and was successfully applied to measure the aforementioned compounds in two biological matrices, serum and seminal plasma, obtained from two groups of healthy donors.

2. Materials and methods

2.1. Chemicals

Standards of *all-trans*-retinoic acid, 25(OH)-D_3, *all-trans*-retinol, astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, γ-tocopherol, β-cryptoxanthin, α-tocopherol, phylloquinone, lycopene, α-carotene, β-carotene and coenzyme Q_{10} were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) at the highest purity available. HPLC-grade solvents (methanol, acetonitrile, chloroform and ethanol) were supplied from Carlo Erba Reagenti (Milano, Italy). Ultrapure water was obtained from a Milli-Q system (Millipore, Millford, MA, USA).

2.2. Sampling of serum and seminal plasma

Peripheral venous blood samples were obtained from 50 healthy volunteers (33 males, 27 females; 48.6 ± 19.3 years of age) recruited among the personnel of the Catholic University of Rome. Assumption of dietary supplements, rich in fat-soluble vitamins or antioxidants, was used as the only exclusion criterion. Whole blood was collected from the antecubital vein into a VACUETTE® polypropylene tube containing serum separator and clot activator (Greiner-Bio One GmbH, Kremsmunster, Austria) and immediately protected from light. After 30 min at room temperature in the dark, samples were centrifuged at 1890*g* for 10 min at 10 °C and 250 μl of the separated sera were immediately withdrawn and processed for the extraction of fat-soluble vitamins and antioxidants.

Seminal plasma was prepared from ejaculates obtained from 20 fertile healthy volunteers (44.3 ± 12.6 years of age) recruited among the personnel of the University of Catania. The ascertained fertility (presence of offspring) was used as the only inclusion criterion. Within 60 min from ejaculation, the light-protected liquefied semen samples were centrifuged at 1480*g* for 10 min at 10 °C and the upper seminal plasma was immediately withdrawn and processed for fat-soluble vitamin and antioxidant extraction.

2.3. Single-step extraction of fat-soluble vitamins and antioxidants

An aliquot of each serum or seminal plasma sample (250 μl) was added to 500 μl of HPLC-grade CH_3CN. After vigorous vortexing for 60 s, these mixtures were incubated at 37 °C for 1 h in a water bath under agitation (to allow full extraction of lipid soluble compounds) and then centrifuged at 20,690*g* for 15 min at 4 °C to precipitate proteins. Clear supernatants were directly used for the reversed phase HPLC analysis of fat-soluble vitamins. All the aforementioned procedures were carried out by protecting samples from light, in order to avoid degradation of photo-sensitive molecules.

To assess recovery, aliquots of 5 randomly selected serum samples were either extracted with no addition or spiked with low (five samples) or high (five samples) concentrations of standard mixtures of fat-soluble vitamins and antioxidants, extracted and deproteinized according to what afore described and then analysed by HPLC.

Additionally, in order to compare efficiency of this new sample processing with a previously well established method (using a multi-step, multi-organic solvent treatment of samples), five randomly selected serum samples were treated for fat-soluble vitamin and antioxidant extraction according to Granado-Lorencio (Granado-Lorencio [15]).

2.4. Reversed phase HPLC assay of fat-soluble vitamins and antioxidants

Stock solutions of *all*-retinoic-*trans*-retinoic acid, *all*-retinol-*trans*-retinol, 25(OH)-D_3, α–tocopherol, γ-tocopherol and phylloquinone were prepared in ethanol to get a 1 mM final concentration. Lutein, zeaxanthin, astaxanthin, β-cryptoxanthin, α-carotene, β-carotene, *trans*-β-apo-8′-carotenal, lycopene and coenzyme Q_{10} were dissolved as 1 mM stock solutions in chloroform. All standards were carefully protected from light.

Stock solutions were stored at −80 °C for up to one month with a loss ≤ 1%. Proper standard mixtures with known concentrations used to validate the HPLC method (linearity, sensitivity, reproducibility) were prepared daily by diluting stock solutions with HPLC-grade CH_3CN. Before HPLC analysis, mixtures were vortexed for 60 s and then incubated for 1 h at 37 °C centigrade, using the same conditions adopted for biological fluid extraction.

2.5. HPLC apparatus and chromatographic conditions for fat-soluble vitamin and antioxidant separation

The HPLC apparatus consisted of a Spectra System P4000 pump system (Thermo Fisher Scientific, Rodano, Milan, Italy) equipped with a highly-sensitive, 5 cm light path flow cell, UV6000LP diode array detector (Thermo Fisher Scientific, Rodano, Milan, Italy), setup

for acquisition between 200 and 550 nm wavelengths. Data acquisition and analysis were performed using the ChromQuest software package provided by the HPLC manufacturer. Chromatographic separation was performed using a 100 × 4.6 mm, 5 μm particle size Hypersil Gold RP C18 column provided with its own guard column (Thermo Fisher Scientific, Milan, Italy). A gradient from solvent A (70% methanol 30% H_2O) to solvent B (100% acetonitrile) was formed as follows: 0.5 min at 100% A; 8 min at up to 100% B (hold for additional 27 min). A flow rate of 1.0 ml/min and a column temperature of 37 °C were maintained constant throughout the analysis, while a flow rate of 2 ml/min was used during column washing with 100% B (5 min) and the re-equilibrating time with 100% A before the next run (5 min). A thermostated autosampler AS3000 (SpectraSystem, Thermo Fisher Scientific, Rodano, Milan, Italy) was connected to the chromatographic system and programmed to inject a volume of 200 μl onto the column. Identification and quantification of fat-soluble vitamins and antioxidants in biological samples were obtained by comparing retention times and absorption spectra of different peaks to those of standard mixtures containing true compounds with known concentrations. The concentration of the different compounds in both serum and seminal plasma extracts were calculated from the standard run data at the following wavelengths: *all-trans*-retinoic acid = 345 nm; 25(OH)-D_3 = 265 nm; *all*-retinol-*trans*-retinol = 327 nm; astaxanthin = 479 nm; lutein = 450 nm; zeaxanthin = 445 nm; *trans*-β-apo-8′-carotenal = 458 nm; γ-tocopherol = 300 nm; β-cryptoxanthin = 480 nm; α–tocopherol = 295 nm; phylloquinone = 245 nm; lycopene = 475 nm; α-carotene = 477 nm; β-carotene = 481 nm; coenzyme Q_{10} = 274 nm.

2.6. Statistics

Differences across groups were estimated by the two-way ANOVA for repeated measures. Fisher's protected least square was used as the post hoc test. Only two-tailed *p*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. HPLC separation of standard mixtures of fat-soluble vitamins

In Fig. 1 a representative chromatogram of a standard mixture containing 0.1–5 μM of *all-trans*-retinoic acid, 25(OH)-D_3, *all-trans*-retinol, astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, γ-tocopherol, β-cryptoxanthin, α-tocopherol, phylloquinone, lycopene, α-carotene, β-carotene and coenzyme Q_{10} is shown at three wavelengths (265 nm, panel A; 327 nm, panel B, 450 nm, panel C). The different λ_{max} of the absorption spectra of each substance are summarized in Table 1. By properly combining the differences in λ_{max} and retention times of the various compounds, it is possible to observe from Fig. 1 that these HPLC conditions allow the separation and subsequent quantification of all the fat-soluble vitamins and antioxidants forming the standard mixture, even though the peaks of the isomeric carotenoids lutein and zeaxanthin (peaks 5 and 6 of panel C) were only partially resolved. It is worth underlining that standard mixtures underwent the same period of incubation at 37 °C occurring to selected biological fluids, prior to the HPLC analysis. Comparing areas of the peaks of chromatographic runs of mixtures analysed with no incubation with those of runs of incubated mixtures, it was possible to establish that one hour at 37 °C did not cause any appreciable degradation in any of the compounds of interest (values were comprised within the inter-assay coefficient of variation for peak areas reported in Table 3 data not shown).

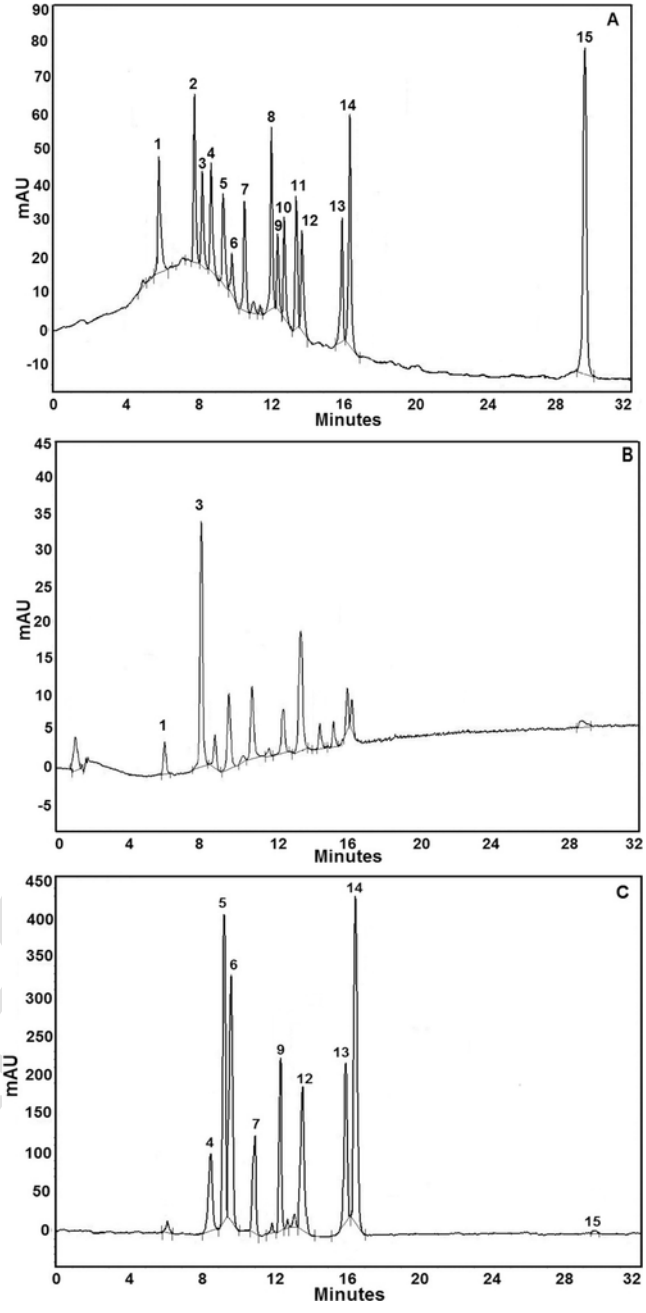

Fig. 1. Representative chromatogram of a standard mixture containing 0.1–5 μM of fat-soluble vitamins and antioxidants, shown at three different wavelengths. 1 = *all-trans*-retinoic acid; 2 = 25(OH)-D_3; 3 = *all-trans*-retinol; 4 = astaxanthin; 5 = lutein; 6 = zeaxanthin; 7 = *trans*-β-apo-8′-carotenal; 8 = γ-tocopherol; 9 = β-cryptoxanthin; 10 = α-tocopherol; 11 = phylloquinone; 12 = lycopene; 13 = α-carotene; 14 = β-carotene; 15 = coenzyme Q_{10}. Panel A = 265 nm; panel B = 327 nm; panel C = 450 nm. Chromatographic conditions are fully described under Materials and Methods.

The parameters to characterize the HPLC separation of these 15 fat-soluble vitamins and antioxidants, i.e. lower limit of detection (LLOD, evaluated with a signal to noise ratio > 3), lower limit of quantification (LLOQ, evaluated with a signal to noise ratio > 10) and linearity are summarized in Table 2. Minimal and maximal LLOQ values were observed for zeaxanthin (0.3 nM), and α-to-

Table 1
Maxima of absorption recorded between 200 and 500 nm wavelengths for 15 fat soluble vitamins and antioxidants.

	Wavelengths of maxima of adsorption (nm)
all *trans*-retinoic acid	345
25-hydroxycholecalciferol	265
all-retinol *trans*-retinol	327
Astaxanthin	301–479
Lutein	270–423–450–475
Zeaxanthin	335–445
trans-β-apo-8′-carotenal	218–270–458
γ-tocopherol	216–300
β-cryptoxanthin	275–456–480
α-tocopherol	295
Phylloquinone	245–266–330
Lycopene	296–448–475–503
α-carotene	265–450–477
β-carotene	454–481
Coenzyme Q_{10}	274

Absorption spectra were acquired at the maximal heights of the chromatographic peaks of each compound, after baseline subtraction.

Table 2
Lower limit of detection, lower limit of quantification and linearity of the reversed phase HPLC method for the detection of fat soluble vitamins and antioxidants in biological fluids.

	[a]LLOD (nM)	[b]LLOQ (nM)	3000× LLOQ (μM)	[c]Correlation coefficients of linearity straight lines
all-retinoic-*trans*-retinoic acid	1.2	2	6	0.996
25-hydroxycholecalciferol	2	3	9	0.993
all-retinol-*trans*-retinol	2	3	9	0.999
Astaxanthin	0.5	0.8	2.4	0.999
Lutein	0.5	0.8	2.4	0.999
Zeaxanthin	0.2	0.3	0.9	0.999
trans-β-apo-8′-carotenal	2	3	9	0.996
γ-tocopherol	10	16	48	0.998
β-cryptoxanthin	0.4	0.6	1.8	0.999
α-tocopherol	20	30	90	0.998
Phylloquinone	1	1.6	4.8	0.997
Lycopene	0.4	0.6	1.8	0.999
α-carotene	2	3	9	0.999
β-carotene	0.3	0.5	1.5	0.999
Coenzyme Q_{10}	2	3	9	0.999

[a] LLOD = Lower limit of detection, evaluated with a signal to noise ratio > 3.

[b] LOQ = limit of quantification, evaluated with a signal to noise ratio > 10.

[c] Linearity was determined by assaying standard mixtures with the following concentrations: LLOQ, 10× LLOQ, 20× LLOQ, 50× LLOQ, 500× LLOQ, 1500× LLOQ and 3000× LLOQ.

copherol (30 nM), respectively. Linearity was observed for all of the compounds in the concentration range between LLOQ and 3000× LLOQ (five mixtures with the intermediate concentrations of 10, 20, 50, 500 and 1500× LLOQ for each analyte were assayed), with high values of the respective correlation coefficients.

Reproducibility of the analysis, determined by calculating intra-assay (five consecutive chromatographic runs of the same mixture) and inter-assay (five chromatographic runs of five mixtures, freshly prepared on five consecutive days) coefficients of variations (CV) of peak areas and retention times, are reported in Table 3. Low values for the CVs (both intra- and inter-assay) of peak areas and retention times were recorded for these chromatographic conditions. It is worth recalling that to perform these tests all standard mixtures underwent the same extraction process used for biological samples, prior to the HPLC analysis.

Table 3
Reproducibility of the HPLC method for the separation of fat soluble vitamins and antioxidants.

	[a]Intra-assay coefficient of variation of retention times	[a]Intra-assay coefficient of variation of peak areas	[b]Inter-assay coefficient of variation of retention times	[b]Inter-assay coefficient of variation of peak areas
all-*trans*-retinoic acid	0.12 ± 0.07	1.65 ± 0.12	0.52 ± 0.12	2.12 ± 0.50
25-hydroxycholecalciferol	0.18 ± 0.02	1.10 ± 0.10	0.24 ± 0.09	2.24 ± 0.41
all-retinol-*trans*-retinol	0.14 ± 0.02	0.67 ± 0.06	0.39 ± 0.08	1.23 ± 0.48
Astaxanthin	0.15 ± 0.03	1.01 ± 0.08	0.59 ± 0.07	1.99 ± 0.86
Lutein	0.17 ± 0.05	0.84 ± 0.09	0.22 ± 0.01	1.13 ± 0.21
Zeaxanthin	0.13 ± 0.02	0.99 ± 0.10	0.77 ± 0.08	1.83 ± 0.34
trans-β-apo-8′-carotenal	0.12 ± 0.01	0.47 ± 0.02	0.28 ± 0.02	1.67 ± 0.23
γ-tocopherol	0.16 ± 0.04	1.25 ± 0.11	0.51 ± 0.03	1.05 ± 0.17
β-cryptoxanthin	0.15 ± 0.02	0.88 ± 0.09	0.32 ± 0.09	1.34 ± 0.25
α-tocopherol	0.11 ± 0.03	0.56 ± 0.05	0.44 ± 0.07	1.11 ± 0.10
Phylloquinone	0.19 ± 0.03	1.40 ± 0.20	0.58 ± 0.08	2.01 ± 0.42
Lycopene	0.12 ± 0.03	0.53 ± 0.04	0.34 ± 0.02	1.09 ± 0.21
Alfa-carotene	0.13 ± 0.01	0.87 ± 0.02	0.25 ± 0.01	1.03 ± 0.26
Beta-carotene	0.18 ± 0.02	0.77 ± 0.03	0.37 ± 0.04	1.02 ± 0.12
Coenzyme Q_{10}	0.21 ± 0.01	0.37 ± 0.02	0.55 ± 0.06	1.46 ± 0.39

[a] Each point is the mean ± SD of five consecutive chromatographic runs of the same standard mixture.

[b] Each point is the mean ± SD of five chromatographic runs of five different standard mixtures assayed in five consecutive days.

3.2. Evaluation of the extraction procedure of biological fluids for the HPLC analysis of fat-soluble vitamins and antioxidants

The suitability of the new extraction procedure of fat-soluble vitamins and antioxidants from biological fluids, as well as that of the HPLC conditions applied for their determination, was preliminarily studied by spiking five randomly selected serum samples with a mixture containing either low (10× LLOQ) or high vitamin and antioxidant concentrations (500× LLOQ). The non-enriched and enriched serum samples were then processed and assayed as previously described. Recovery, reported in Tables 4a and 4b, demonstrates a high efficiency of the new single-step method to extract fat-soluble vitamins and antioxidants, when these compounds are added to biological fluids at either low or high concentrations. The minimal recovery of 80% was recorded when zeaxanthin was added at low concentration (Table 4a). It is worth underlining that since β-tocopherol could not be separated from γ-tocopherol using these chromatographic conditions, and since this position isomer is present in cereals [21] and might therefore be detectable in biological samples, hereinafter these isomers are cumulatively indicated in sample extracts as β+γ-tocopherols.

To further corroborate the suitability of the present biological fluid processing finalized to measure fat-soluble vitamins and antioxidants by HPLC with UV-VIS-DAD detector, the same five serum samples were also processed using one of the conventional multi-steps method reported in literature [15]. Results summarized in Table 5, strongly demonstrate that a single-step extraction of serum with one organic solvent only (acetonitrile for 60 min at 37 °C) has the same efficiency than that of a multi-steps, multi-organic solvents, time consuming extraction process [15,14]. The statistical comparison of the results, performed according to the analysis of variance for repeated measures, showed no differences in the concentrations of

Table 4a
Recovery of the method for the detection of lipid soluble vitamins in biological fluids.

	Mean values in control serum samples (μmol/l serum)	Concentration added = 10× LLOQ (μmol/l serum)	Expected mean values (μmol/l serum)	Mean measured values (μmol/l serum)	Mean recovery (%)	%RSD
all-*trans*-retinoic acid	0.005 ± 0.001	0.02	0.025	0.022 ± 0.003	88.0	13.6
25-hydroxycholecalciferol	0.067 ± 0.022	0.03	0.097	0.087 ± 0.007	89.6	8.0
all-retinol-*trans*-retinol	5.24 ± 1.09	0.03	5.27	4.99 ± 0.09	94.7	1.8
Astaxanthin	0.005 ± 0.001	0.008	0.013	0.014 ± 0.001	107.7	7.1
Lutein	0.670 ± 0.200	0.008	0.678	0.66 ± 0.01	97.3	1.5
Zeaxanthin	0.002 ± 0.001	0.003	0.005	0.003 ± 0.0004	80	13.3
trans-β-apo-8′-carotenal	0.114 ± 0.031	0.03	0.117	0.127 ± 0.011	108.5	8.7
β + γ-tocopherol	1.24 ± 0.54	0.16	1.40	1.30 ± 0.08	92.8	6.2
β-cryptoxanthin	0.006 ± 0,004	0.006	0.012	0.010 ± 0.001	83.3	10.0
α-tocopherol	22.44 ± 3.13	0.3	22.74	21.67 ± 0.51	95.3	2.3
Phylloquinone	N.D.	0.016	0.016	0.017 ± 0.001	106.3	5.9
Lycopene	0.178 ± 0.046	0.006	0.184	0.168 ± 0.014	91.3	8.3
α-carotene	0.011 ± 0.005	0.03	0.041	0.047 ± 0.006	114.6	12.8
β-carotene	0.258 ± 0.043	0.005	0.263	0.249 ± 0.019	94.7	7.6
Coenzyme Q_{10}	0.055 ± 0.023	0.03	0.085	0.086 ± 0.004	101.2	4.7

Each value is the mean ± SD of five serum samples. Five control serum samples were extracted and analysed with no addition. Aliquots of each serum were spiked, before extraction, with a standard mixture at low concentration (10× LLOQ) of fat-soluble vitamins and antioxidants. N.D. = Not DetecTable Sample processing and chromatographic conditions are fully described in the Materials and Methods section.

Table 4b
Recovery of the method for the detection of lipid soluble vitamins in biological fluids.

	Mean values in control serum samples (μmol/l serum)	Concentration added = 500 x LLOQ (μmol/l serum)	Expected values (μmol/l serum)	Mean measured values (μmol/l serum)	Mean recovery (%)	%RSD
all-*trans*-retinoic acid	0.005 ± 0.001	1	1.005	1.022 ± 0.095	101.7	9.3
25-hydroxycholecalciferol	0.067 ± 0.022	1.5	1.567	1.499 ± 0.110	95.7	7.3
all-retinol-*trans*-retinol	5.24 ± 1.09	1.5	6.74	6.66 ± 0.54	98.9	8.1
Astaxanthin	0.005 ± 0.001	0.4	0.405	0.398 ± 0.041	98.3	10.3
Lutein	0.670 ± 0.200	0.4	1.07	1.06 ± 0.09	99.1	8.5
Zeaxanthin	0.002 ± 0.001	0.15	0.152	0.151 ± 0.018	99.3	11.9
trans-β-apo-8′-carotenal	0.114 ± 0.031	1.5	1.614	1.508 ± 0.14	93.4	9.3
β + γ-tocopherol	1.24 ± 0.54	8	9.24	9.18 ± 0.15	99.4	1.6
β-cryptoxanthin	0.006 ± 0,004	0.3	0.306	0.323 ± 0.018	105.6	5.6
α-tocopherol	22.44 ± 3.13	15	37.44	37.04 ± 2.42	98.9	6.5
Phylloquinone	N.D.	0.8	0.8	0.79 ± 0.07	98.8	8.9
Lycopene	0.178 ± 0.046	0.3	0.478	0.459 ± 0.32	96.0	7.0
α-carotene	0.011 ± 0.005	1.5	1.511	1.459 ± 0.088	96.6	6.0
β-carotene	0.258 ± 0.043	0.25	0.508	0.518 ± 0.013	102.0	2.5
Coenzyme Q_{10}	0.055 ± 0.023	1.5	1.555	1.571 ± 0.048	101.2	3.1

Each value is the mean ± SD of five serum samples. Five control serum samples were extracted and analysed with no addition. Aliquots of each serum were spiked, before extraction, with a standard mixture at high concentration (500 x LLOQ) of fat-soluble vitamins and antioxidants. N.D. = Not DetecTable Sample processing and chromatographic conditions are fully described in the Materials and Methods section.

the various compounds determined in the same serum samples extracted with the two methods.

3.3. Fat-soluble vitamins and antioxidants in serum and seminal plasma

After having demonstrated that the single-step procedure efficiently extracts lipid soluble vitamins and antioxidants from serum and that the reversed phase HPLC conditions we set up had the correct characteristics of sensitivity, reproducibility and linearity, we extracted and analysed by HPLC serum samples of 50 healthy controls to determine their circulating concentrations in lipid soluble vitamins and antioxidants.

A representative chromatogram of the separation of fat-soluble vitamins and antioxidants in serum of a control healthy subject and in seminal plasma of a fertile donor, subsequent to the single-step CH_3CN deproteinization/extraction, are shown, respectively, in Figs. 2 and 3 at three different wavelengths (265 nm, panel A; 327 nm, panel B; 450 nm, panel C). At 265 nm wavelength, 25(OH)-D_3 is clearly identified in serum but not in seminal plasma. Either β + γ- and α-tocopherol are visible at this wavelength in both extracts, as well as coenzyme Q_{10}, the peak of which is shown at 274 nm wavelength in the inserts of panel A of Figs. 2 and 3. A very small peak of phylloquinone (lower than its LLOQ) was also found in serum extract (not shown). When observing the same chromatograms at 327 nm wavelength, a clearly visible peak of vitamin A (peak number 3) and a small peak of *all-trans*-retinoic acid (peak number 1) are identifiable in both fluids. Observing these two chromatographic runs at a wavelength typical of carotenoids (450 nm), it is possible to note the incomplete separation of lutein and zeaxanthin, and that astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, β-cryptoxanthin, lycopene, α-carotene, β-carotene are all easily measurable in both serum and seminal plasma extracts.

The mean serum concentrations of the 15 compounds of interest measured in this group of healthy donors are summarized in Table 6. It is possible to note that we could not determine phylloquinone (vi-

Table 5
Comparison in the efficiency of the new single-step, single-organic solvent extraction of fat-soluble vitamins from control sera with that obtained using a well-established multi-step, multi-organic solvent protocol.

	Lipid-soluble vitamins and antioxidants in serum samples after a new single-step extraction (μmol/l serum)	Lipid-soluble vitamins and antioxidants in serum samples after a conventional multi-step extraction (μmol/l serum)
all-*trans*-retinoic acid	0.005 ± 0.001	0.006 ± 0.002
25-hydroxycholecalciferol	0.067 ± 0.022	0.049 ± 0.014
all-retinol-*trans*-retinol	5.24 ± 1.09	5.81 ± 1.34
Astaxanthin	0.005 ± 0.001	0.007 ± 0.002
Lutein	0.670 ± 0.200	0.598 ± 0.176
Zeaxanthin	0.002 ± 0.001	0.004 ± 0.002
trans-β-apo-8′-carotenal	0.114 ± 0.031	0.142 ± 0.060
β + γ-tocopherol	1.24 ± 0.54	1.09 ± 0.48
β-cryptoxanthin	0.006 ± 0.004	0.009 ± 0.004
α-tocopherol	22.44 ± 3.13	20.39 ± 5.01
Phylloquinone	N.D.	N.D.
Lycopene	0.178 ± 0.046	0.237 ± 0.079
α-carotene	0.011 ± 0.005	0.017 ± 0.006
β-carotene	0.258 ± 0.043	0.236 ± 0.031
Coenzyme Q_{10}	0.055 ± 0.023	0.058 ± 0.016

Each value is the mean ± S.D. of five control serum samples processed using either the new protocol for single-step, single solvent extraction of fat-soluble vitamins and antioxidants from biological fluids, or according to a conventional multi-step, multi solvent method as described by (Granado-Lorencio [15]). No statistical differences in any of the compounds assayed were observed between the two extraction methods. N.D. = Not Detectable. Extraction procedures and HPLC conditions are fully described under Materials and Methods.

tamin K_1) concentration. As also evident form the chromatogram shown in Fig. 2 (Panel A), the values of the peak area of phylloquinone in all serum samples assayed were below its LLOQ (6 nM), confirming that the physiological values of this substance in serum (0.5–1 nM) [22,23] are below the sensitivity of our highly sensitive diode array detector. That is, the single-step process effectively extracts even phylloquinone (see Tables 4a and 4b reporting the efficiency of the extraction in serum samples spiked with standard mixture containing measurable levels of phylloquinone) but a more sensitive detector (mass spectrometer) for its quantification in serum is required.

The other 14 fat-soluble compounds were always measurable in all serum samples and showed a moderate range of variability. The lowest and highest concentrations of lipid soluble vitamins and antioxidants in serum were found, respectively, for astaxanthin and zeaxanthin (0.004 ± 0.002 μmol/l serum for both compounds) and α-tocopherol (28.51 ± 7.08 μmol/l serum).

The applicability of the present single-step method was also tested to extract fat-soluble vitamins and antioxidants from seminal plasma obtained from 20 healthy, fertile control donors and subsequently analyzing these extracts using the above described HPLC conditions. Data reported in Table 7 indicate that, under the present HPLC conditions, seminal plasma does not contain measurable levels of 25(OH)-D_3, astaxanthin and phylloquinone. Quantifiable concentrations of the remaining 12 compounds (*all-trans*-retinoic acid, *all-trans*-retinol, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, β+γ-tocopherol, β-cryptoxanthin, α-tocopherol, lycopene, α-carotene, β-carotene and coenzyme Q_{10}) were found in all seminal plasma extracts, indicating that the present extraction method and HPLC separation can successfully be applied to monitor the status of lipid soluble antioxidants in human ejaculate. As it occurs in serum, α-to-

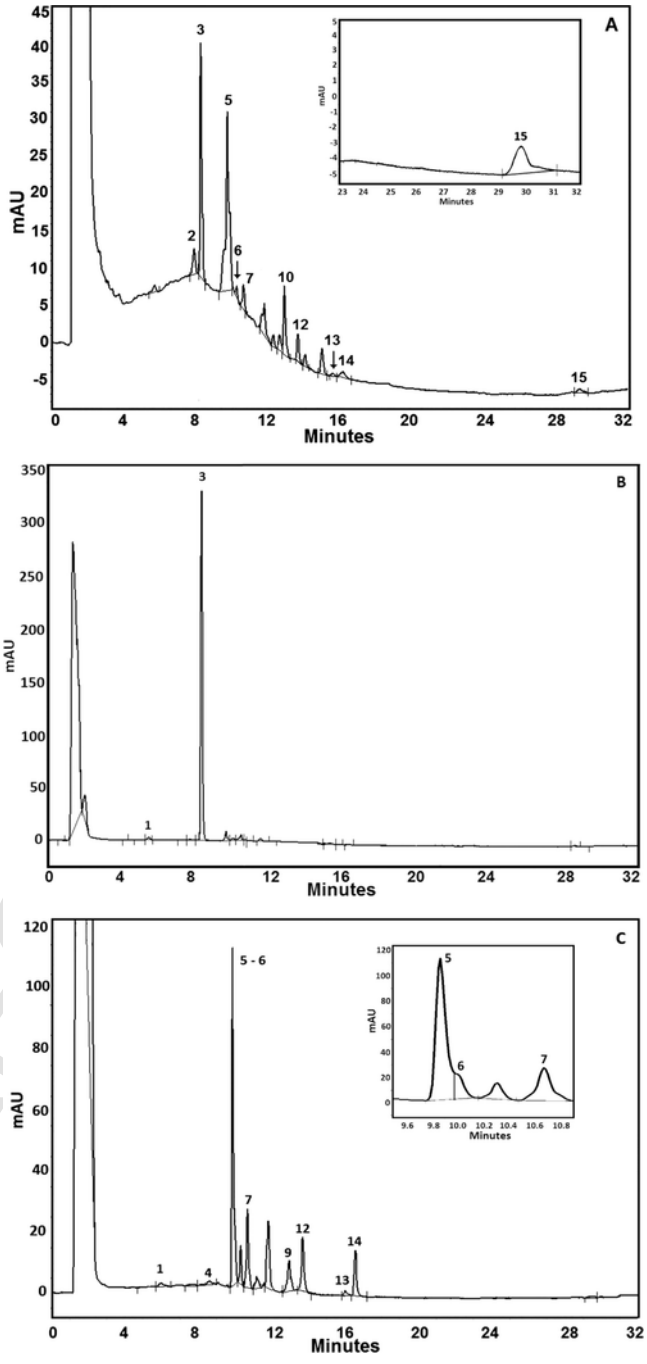

Fig. 2. Representative chromatogram of the separation of fat-soluble vitamins and antioxidants in serum of a control healthy subject, extracted according to the new single-step extraction, is illustrated at three different wavelengths (265 nm, panel A; 327 nm, panel B; 450 nm, panel C). Peak numbers correspond to the different compounds as indicated in the legend to Fig. 1, with the exception of peak 8 that in biological fluid extracts is = β+γ-tocopherol. In the insert of panel A, the chromatogram was magnified and replotted at 274 nm wavelength (the maximum of coenzyme Q_{10} absorption) to allow a better identification of the peak referring to coenzyme Q_{10}. In the insert of panel C, the chromatogram was magnified to better appreciate that the incomplete separation of lutein and zeaxanthin did not prevent correct calculation of both compounds. Sample processing and chromatographic conditions are fully described under Materials and Methods.

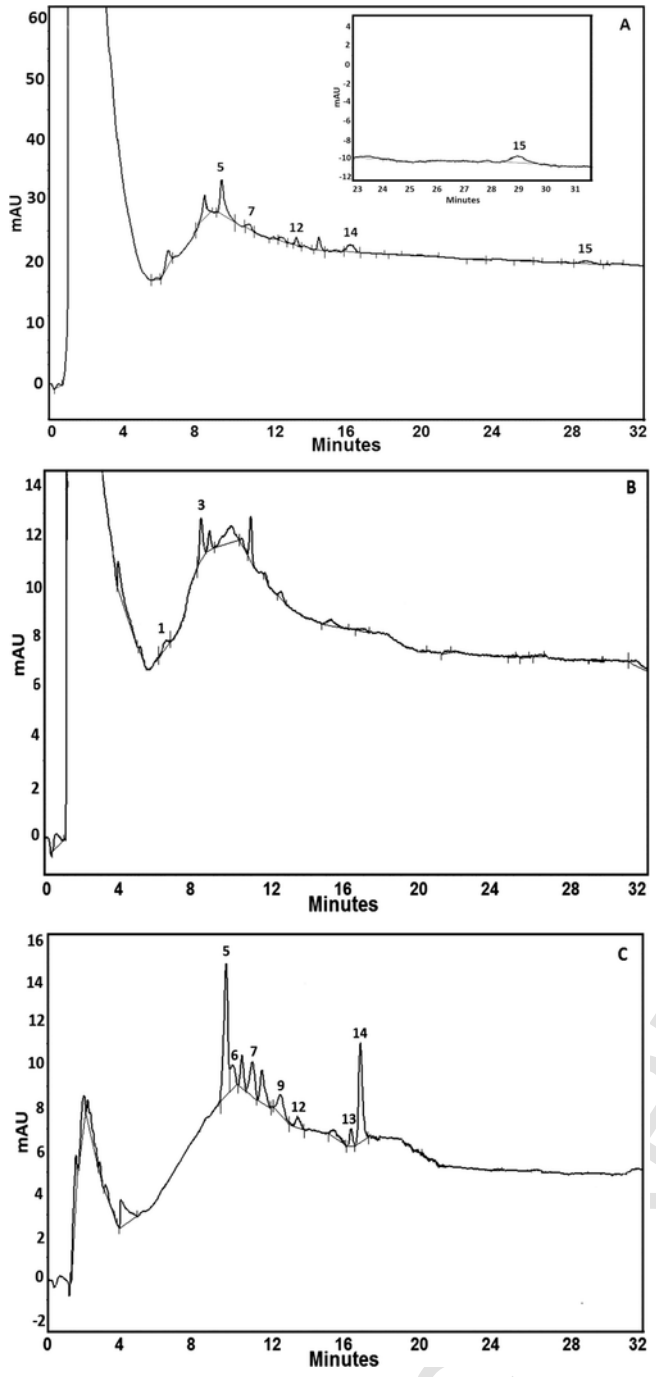

Fig. 3. Representative chromatogram of the separation of fat-soluble vitamins and antioxidants in seminal plasma of a control fertile subject, extracted according to the new single-step extraction, is illustrated at three different wavelengths (265 nm, panel A; 327 nm, panel B; 450 nm, panel C). Peak numbers correspond to the different compounds as indicated in the legend to Fig. 1, with the exception of peak 8 that in biological fluid extracts is = β+γ-tocopherol. In the insert of panel A, the chromatogram was magnified and replotted at 274 nm wavelength to allow a better identification of the peak referring to coenzyme Q_{10}. Sample processing and chromatographic conditions are fully described under Materials and Methods.

Table 6
Circulating values of fat soluble vitamins and antioxidants found in serum of 50 healthy controls.

	Concentration (μmol/l serum)
all-*trans*-retinoic acid	0.006 ± 0.003
25-hydroxycholecalciferol	0.074 ± 0.029
all-retinol-*trans*-retinol	5.69 ± 1.89
Astaxanthin	0.004 ± 0.002
Lutein	0.593 ± 0.211
Zeaxanthin	0.004 ± 0.002
trans-β-apo-8′-carotenal	0.152 ± 0.054
β + γ-tocopherol	1.68 ± 0.76
β-cryptoxanthin	0.008 ± 0.003
α-tocopherol	28.51 ± 7.08
Phylloquinone	N.D.
Lycopene	0.211 ± 0.096
α-carotene	0.010 ± 0.006
β-carotene	0.274 ± 0.099
Coenzyme Q_{10}	0.038 ± 0.028

Values are the mean ± S.D. of 50 serum samples obtained from healthy controls and processed using the new protocol for single-step, single solvent extraction of fat-soluble vitamins and antioxidants. N.D. = Not Detectable. Extraction procedures and HPLC conditions are fully described under Materials and Methods.

Table 7
Concentrations of fat soluble vitamins and antioxidants in seminal plasma obtained from a group of 20 fertile controls and detected by HPLC.

	Concentration (μmol/l seminal plasma)
all trans retinoic acid	0.001 ± 0.001
25-hydroxycholecalciferol	N.D.
all trans retinol	0.068 ± 0.028
Astaxanthin	N.D.
Lutein	0.067 ± 0.032
Zeaxanthin	0.001 ± 0.001
trans-β-apo-8′-carotenal	0.022 ± 0.005
β + γ-tocopherol	0.066 ± 0.024
β-cryptoxanthin	0.003 ± 0.001
α-tocopherol	3.06 ± 0.85
Phylloquinone	N.D.
Lycopene	0.003 ± 0.001
α-carotene	0.004 ± 0.002
β-carotene	0.008 ± 0.005
Coenzyme Q_{10}	0.006 ± 0.003

Values are the mean ± S.D. of 20 seminal plasma samples processed using the new protocol for single-step, single solvent extraction of fat-soluble vitamins and antioxidants. N.D. = Not Detectable. Extraction procedures and HPLC conditions are fully described under Materials and Methods.

copherol is the lipid soluble compound with the maximal concentration in seminal plasma (3.06 ± 0.85 μmol/l).

4. Discussion

Results reported in the present study allowed to validate a single-step, efficient, simple, rapid and low cost method to extract fat-soluble vitamins and antioxidants from biological fluids which, coupled to a proper reversed phase HPLC analysis, is suitable to determine the concentrations of *all-trans*-retinoic acid, 25(OH)-D_3, *all-trans*-retinol, astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, β+γ-tocopherol, β-cryptoxanthin, α-tocopherol, phylloquinone, lycopene, α-carotene, β-carotene and coenzyme Q_{10} in human serum and seminal plasma. Values of these fat-soluble compounds we found in serum of control healthy subjects (Table 6) are in accordance with previous studies in which different extraction and analytical methods were adopted [14,19,24–28], thereby strongly

demonstrating the suitability of both extraction method and HPLC conditions.

The classical multi-steps, multi-solvents procedures to prepare biological fluids for subsequent analysis of fat-soluble vitamins and antioxidants [14,15] are based on the initial deproteinization of samples (first step) with mildly polar organic solvents (methanol, acetonitrile, *iso*-propanol), followed by the extraction of the protein-free biological sample (second step) with a highly hydrophobic organic solvent (prevalently *n*-hexane). This highly hydrophobic phase, containing all fat-soluble compounds including vitamins and antioxidants, is recovered and evaporated to dryness (third step) and resuspended with a proper amount of mildly polar organic solvent (fourth step). This extract is lastly ready to be injected onto an HPLC column for the analysis of fat-soluble vitamins and antioxidants [14,25]. Alternatively, solid-phase extraction (SPE) with proper cartridges is the most utilized procedure to process biological fluids for fat-soluble vitamin and antioxidant determination [29,30]. However, even in this case, various steps (equilibration of the SPE cartridge and elution of samples) and solvents are necessary. It is therefore evident that any of these multi-steps, multi-solvents extraction procedures, as well as the SPE procedure, can hardly be applied to process simultaneously a large number of biological fluid samples. Hence, these processing protocols are relatively easy to be applied in research laboratories, but very difficult to be applied in the routine of clinical-biochemistry laboratories, where hundreds of samples/day have to be treated.

All these limitations are avoided by applying the single-step, one organic solvent treatment of biological fluids to efficiently extract fat-soluble vitamins and antioxidants described in the present study. Differently from the traditional extraction methods, the use of which renders difficult the simultaneous preparation of several samples, the present single-step deproteinization/extraction protocol with acetonitrile only, can concomitantly be applied to tenths of samples with nearly the same working time. In our personal experience during this study, we received in one morning 34 serum samples from healthy controls: in about 2.5 h, the whole process was completed for all 34 sera, and the first sample was placed in the autosampler and started to be analysed by HPLC to measure its fat-soluble vitamin and antioxidant levels.

Additional great advantages of this method are the minimal manipulation of samples, the reduction of costs-per-sample thanks to the use of moderately expensive solvent (acetonitrile), the elimination in the use of highly toxic organic solvents (*n*-hexane), the use of common laboratory equipments only (thermostated water bath, thermostated top bench centrifuge). It is worth recalling that the use of acetonitrile to extract serum samples for the analysis of fat-soluble vitamins has been proposed in the past [30]. According to that sample treatment, acetonitrile was supplemented with SDS, presumably to optimize extraction of lipid soluble vitamins potentially associated with albumin, and extracted samples in acetonitrile-SDS were passed through proper SPE cartridges before the HPLC assay [31]. Our results indicate that either SDS or SPE cartridges for sample cleaning are unnecessary, since a single-step extraction at 37 °C for one hour with acetonitrile only, efficiently extracts nearly the 100% of the serum fat-soluble vitamin and antioxidant content. This is clearly demonstrated by data summarized in Tables 4a and 4b reporting the percent recovery of serum samples spiked with low or high concentrations of standard mixtures of fat-soluble vitamins and antioxidants, and by data reported in Table 5 showing no statistical differences in the extraction efficiency of this single-step and the established multi-steps processing of samples. It is also worth underlining that the present extraction method allows to obtain clean samples directly injectable onto the HPLC column. The present study has been realized using the same chromatographic column and guard column for about 400 runs with no significant change in its separation efficiency.

Using this HPLC conditions, the separation of the compounds of interest is obtained in about 30 min, with a run-to-run time interval of about 45 min, i.e. a maximum of 32 samples/day can be assayed. As demonstrated by data summarized in Tables 2, 3, 4a, and 4b this HPLC method is characterized by high sensitivity, reproducibility and linearity in a wide range of concentrations. Referring to sensitivity, the use of a highly sensitive diode array detector (equipped with a 5 cm light path flow cell) consent to detect and to quantify the various compounds at the nanomolar level. This characteristic contributes to decrease the analytical costs since a moderately expensive HPLC apparatus is required, particularly when compared to HPLC methods based on the use of MS detectors [16,19,20,29]. It is important to recall that, notwithstanding the high sensitivity of the method, we could not quantify phylloquinone in serum since the area of the peak in all samples was always below its LLOQ, that is, MS detectors are needed for the quantification of phylloquinone in serum [22,23,32,33].

Since a renovated interest for the determination of fat-soluble vitamins and antioxidants has been developed in recent years, due to their possible involvement in different pathological states [9,34–36], we verified the suitability of the single-step extraction method, coupled with the presently characterized HPLC separation, to measure these fat-soluble compounds not only in serum samples of controls but also in seminal plasma of healthy fertile subjects. To our knowledge, this is the first time that a comprehensive profile of fat-soluble vitamins and antioxidants has been measured in extracts of seminal plasma. A number of previous studies correlated various plasma antioxidants with infertility [37,38]); very few measured antioxidants directly in seminal plasma [27,39]; none measured the pattern we assayed in our seminal plasma samples [40]. Our results indicate that fat-soluble vitamins and antioxidants are present in relatively low amounts in ejaculates of healthy fertile controls (Table 7), also indicating that 25(OH)-D_3, astaxanthin and phylloquinone are below their respective LLOQ and LLOD (no peaks corresponding to these compounds could be observed in any seminal plasma extract). However, the mean cumulative concentration of carotenoids in our seminal plasma (lutein + zeaxanthin + *trans*-β-apo-8′-carotenal + β-cryptoxanthin + lycopene + α-carotene + β-carotene) was 0.180 ± 0.014 μmol/l, representing about the 6% of the α-tocopherol concentration, i.e. of the fat-soluble antioxidant found in the highest concentration in this biological fluid. That is, this group of lipid soluble compounds certainly exerts a significant protective action in human seminal plasma (under physiological conditions) towards biochemical damages induced by oxidative/nitrosative stress. Since most of these carotenoids are present only in certain types of food, it is unquestionably possible that both their concentrations and pattern may have significant differences in populations with different nutritional habits. However, having established values of normality in this group of fertile controls, it will now be possible to verify whether a decrease in the concentration of one or more of these lipid soluble vitamins and antioxidants occurs in pathological seminal plasma, i.e. in seminal plasma from infertile subjects.

4.1. Conclusions

In conclusion, the single-step, single-organic solvent extraction of fat-soluble vitamins and antioxidants from biological fluids reported in the present study is a valid innovation over the existing methods [14,15], particularly in terms of time-for-sample processing and costs of reagents. Coupled with the HPLC conditions purposely set up,

which do not however allow separation of β- and γ-tocopherol and do not permit a complete separation of the lutein and zeaxanthin peaks, this procedure is suitable to extract and measure fat-soluble vitamins and antioxidants in a high number of human biological fluids (serum, plasma, seminal plasma, seminal fluid, urine, cerebrospinal fluid, saliva, microdialysis, synovial fluid). Although further confirmations are certainly needed, this method might be applied also in the routine of clinical-biochemistry laboratories, where a potentially high number of samples/day has to be processed and assayed. Therefore, the extraction and HPLC methods described in the present study might successfully be applied in clinical and nutritional studies, when supplementation of vitamins A, D, E, and/or carotenoids and/or antioxidant (coenzyme Q_{10}) is a powerful strategy for several pathologies. Monitoring of the aforementioned compounds in selected biological fluids is obviously necessary [9,10,14,36,41] and require a suitable, low-cost, time saving method to process large number of samples such as the new single-step, single-solvent procedure described in the present study.

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