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Simultaneous determination of retinol, β-carotene and α-tocopherol in adipose tissue by high-performance liquid chromatography

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Abstract

A method is described for evaluation of fat-soluble vitamin in human adipose tissue with the aim to obtain, accurately and within the shortest analysis time, a time-integrated measure of exposure to vitamins from the diet. Fat tissue was deproteinized with ethanol and extracted with *n*-hexane. Normal-phase HPLC was performed in a Lichrosorb Si60 column with a gradient of *n*-hexane–2-propanol at 1 ml/min. Detection was accomplished using a diode-array system (for retinol and β-carotene) in series with a fluorescence detector (α-tocopherol). The method was validated and applied to human adipose tissue in a total of 140 subjects. The mean contents found were 0.43, 0.84, 240.3 μg/g for retinol, β-carotene and α-tocopherol, respectively. The method is sensitive enough for detecting the compounds in 1.6 mg of adipose tissue considering the lowest concentration found.

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

1. Introduction

There has been a rapidly growing clinical interest over the past years in the relationship between antioxidant micronutrients and several degenerative human health conditions, in particular for vitamins E, C, β-carotene and selenium [1]. Epidemiological and clinical assays suggest that the fat-soluble vitamin E, vitamin A and β-carotene protect against coronary heart disease and they may be important in the prevention of some cancers [2–5]. This association is less clear for vitamin C [4].

Food frequency questionnaires are commonly used in epidemiological studies. This approach has several limitations, namely the considerable individual day-to-day variation [2,6]. Moreover, the dietary assessment methods may also not be accurate because of limitations in the nutrient databases [6,7]. An attractive and more objective alternative is to compare results of questionnaires with biochemical measurements. Blood concentrations are frequently used in these measurements but, for antioxidant fat-soluble vitamins, they provide information on the recent vitamin intake, instead of being a measure of long-term dietary intake. Although suitable biomarkers are not available for many nutrients, studies have shown that a large fraction of body tocopherol (almost 90% of the body pool) is concentrated in adipose tissue, and since there is a low turnover of tocopherol in adipose tissue, this may be a better indicator of a long-term intake than blood concentrations [8]. The same has been observed for carotenoids [2] being the

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adipose tissue and liver the most important sites of deposition.

HPLC has been widely applied to the study and analysis of fat-soluble vitamins in several matrices. The stationary phase used are both normal and reversed-phase HPLC, the elution being either isocratic or with gradient and the quantification with fluorimetric, ultraviolet or electrochemical detectors [9,10].

The complexity of the simultaneous determination of α-tocopherol, retinol and β-carotene is caused by differences in their spectral characteristics (absorption maxima vary in the range 295–452 nm). Several methods have been developed, mainly in serum, but usually they use a programmable UV detector [11–13]. This approach may require double injection in order to quantify the three compounds and, moreover, the detection of α-tocopherol by UV causes loss of sensitivity towards this compound being more adequate to use a fluorescence detector simultaneously [14–16]. The multiple injection problem can be easily resolved using a diode-array detector (DAD) [17–19] but not the low sensitivity towards the tocopherols. By using simultaneously a DAD and a fluorescence detector it is possible to quantify the three compounds with sensitivity and also to identify coeluting impurities.

The aim of this work was to develop a rapid, sensitive and specific method for simultaneous determination of α-tocopherol (the most active and most abundant form of vitamin E), retinol (vitamin A), and β-carotene (provitamin A) in human adipose tissue by HPLC, with simultaneous detection by DAD and fluorescence. The method was validated and applied to a total of 140 adipose tissue samples. The results are to be compared with the concentrations found by food intake questionnaires and also with the risk for acute myocardial infraction, as part of a Portuguese health program.

2. Experimental

2.1. Chemicals

All-*trans*-retinol and all-*trans*-β-carotene were obtained from Fluka (Madrid, Spain) and D,L-α-tocopherol was obtained from Merck (Darmstadt, Germany) and used without further purification. The internal standard *rac*-tocopherol (tocol) was obtained from Universal Biologicals Ltd. (Gloucestershire, UK). *n*-Hexane and 2-propanol were HPLC gradient grade from Merck (Darmstadt, Germany). TBHQ (*t*-butylhydroquinone) was from Aldrich (Madrid, Spain).

All other chemicals were of analytical reagent grade.

2.2. Standard solutions

Stock solutions of retinol, β-carotene and α-tocopherol were prepared in *n*-hexane, and kept under nitrogen atmosphere at −20°C. The actual concentration of the standards was determined daily by measuring the absorvence of diluted stock solutions on a Jenway 6105 spectrophotometer (Essex, UK) and using published absorption coefficients (A1%/cm): retinol 1835 at 327 nm; β-carotene 2592 at 452 nm; and α-tocopherol 72 at 295 nm [13,19].

The working standards of each compound were prepared daily and appropriate dilutions made ranging from 0.03 to 0.25 μg/ml for retinol and β-carotene and ranging from 2.0 to 10.0 μg/ml for α-tocopherol. The internal standard solution was prepared at 10.0 μg/ml.

In addition, all solvents used in the preparation of stock or standard solutions were saturated with nitrogen before use and all the operations were performed under red light.

2.3. Chromatography

HPLC was performed using a system incorporating two PU-980 pumps, a MD-910 diode-array detector, a FP-920 fluorescence detector and a AS-950 auto-sampler (Jasco, Japan).

The chromatographic separation was achieved with a normal-phase Lichrosorb Si60 column (4.6 mm×25 cm, 5 μm) from Merck. The solvent system used was a gradient of *n*-hexane (A) and *n*-hexane/2-propanol(90:10) (B). The gradient was as follows: 7% B from 0 to 4 min; linear gradient to 30% B within 3 min; 30% B from 7 to 11 min; linear gradient to 100% B from 11 to 12 min; 100% B from 12 to 14 min; and again 7% B at 14.1 min, with a total run time of 23 min. The flow-rate was 1.0 ml/min and the injection volume was 20 μl.

Detection was accomplished simultaneously with a

DAD (452 nm for β-carotene, 327 nm for retinol and 295 nm for the internal standard) connected in series with a fluorescence detector programmed for excitation at 295 nm and emission at 330 nm, gain 10, for detection of both α-tocopherol and internal standard. Signals from both detectors were recorded simultaneously by the data handling system (Jasco-Borwin).

Peak identification and purity were based on photodiode array spectroscopic data, retention time and peak spiking with authentic standards.

2.4. Sample pre-treatment and extraction

The subcutaneous adipose tissue biopsy specimen was taken from the buttock by needle aspiration according to Beynen and Katan [6]. The samples were kept in the original plastic adapter, and stored at −70°C until analysis.

Adipose tissue samples were removed from the connector, weighed (32.5 mg on average), and transferred into brown-coloured microcentrifuge tubes together with the internal standard solution. Absolute ethanol containing ascorbic acid (10 mg/ml) and saturated NaCl solution (200 μl each) were added and the mixture was homogenised. The extracting solvent was added (500 μl of *n*-hexane with 2% 2-propanol and 0.01% TBHQ) and the mixture was homogenised, vortexed and centrifuged (5 min, 12 000 *g*) (Biofuge pico, Heraeus, Germany). The supernatant was removed to a second tube on ice. The pellet was resuspended with the extraction solvent and further extracted twice with equal amounts, as described.

The pooled extracts were evaporated at ambient temperature under a stream of nitrogen on a Reacti-Therm module (Pierce, Rockford, IL, USA) and reconstituted with 400 μl of the initial chromatographic eluent (*n*-hexane with 0.7% of 2-propanol). The extracts were transferred to glass inserts and placed in brown HPLC injection vials.

3. Results and discussion

3.1. Extraction method

The fat-soluble vitamins under study are very unstable pigments, being specially sensitive to light, heat, oxygen and peroxide [9,20]. Tocopherols are also rapidly oxidised in alkaline medium [20]. Therefore, special precautions have to be taken when handling them. All the operations were performed in subdued lighting with a red globe lamp (25 W) providing the only illumination.

In general, an initial comprehensive saponification-extraction scheme is used, both to hydrolyse any ester linkage and remove the overwhelming proportions of triglyceride lipid, prior to exhaustive multi-step solvent extraction, washing and concentration of target analytes. We have adapted several published extraction methods with and without saponification [8,16,21–23] in order to find the best extraction procedure. Due to the impossibility to use human fat in the development of the extraction method we have used control samples. The selection of control samples is quite conflictive, not only due to differences in texture but also on the concentration of the compounds under study. We have started with both homogeneous pig and chicken adipose tissue as control samples but the pig fat was rejected after the first extraction attempts due to the absence of β-carotene. Therefore we have proceed our studies with chicken adipose tissue. The results for the various extraction methods tested are presented in Table 1 and compared with our final method. Al-

Table 1
Comparison of different extraction procedures (relative %)

Ref.	8	16	22 (met. c)	23	21	22 (met.a)	22 (met.b)	Final
Saponification	No	No	No	No	Yes	Yes	Yes	No
β-carotene	82	100	77	82	64	60	67	95
α-tocopherol	85	97	95	100	46	41	63	100
retinol	42	53	40	45	84	90	100	55

though retinol was higher in the methods with prior saponification, due to retinol esters hydrolysis, there were losses for both β-carotene and α-tocopherol. Rey et al. [22] have already observed this for α-tocopherol in high fat pig samples. Based on this observation we have proceed our studies without saponification.

As recommended, we tested the usefulness of different antioxidants for stabilising fat-soluble vitamins during the extraction and drying steps [20]. Driskell et al. [24] described that the loss of vitamin A can be eliminated completely by the addition of ascorbic acid (0.1% w/v) to the ethanol before it is used in the pre-assay extraction. Since it proved to give no interferences in the chromatography separation we have used this water-soluble antioxidant in the first extraction step. More important was the choice for a fat-soluble antioxidant due to the characteristics of the compounds under study. BHT (butylated hydroxytoluene) is often described for this purpose but, under the conditions of our assay, it is eluted near the β-carotene peak and thus interferes

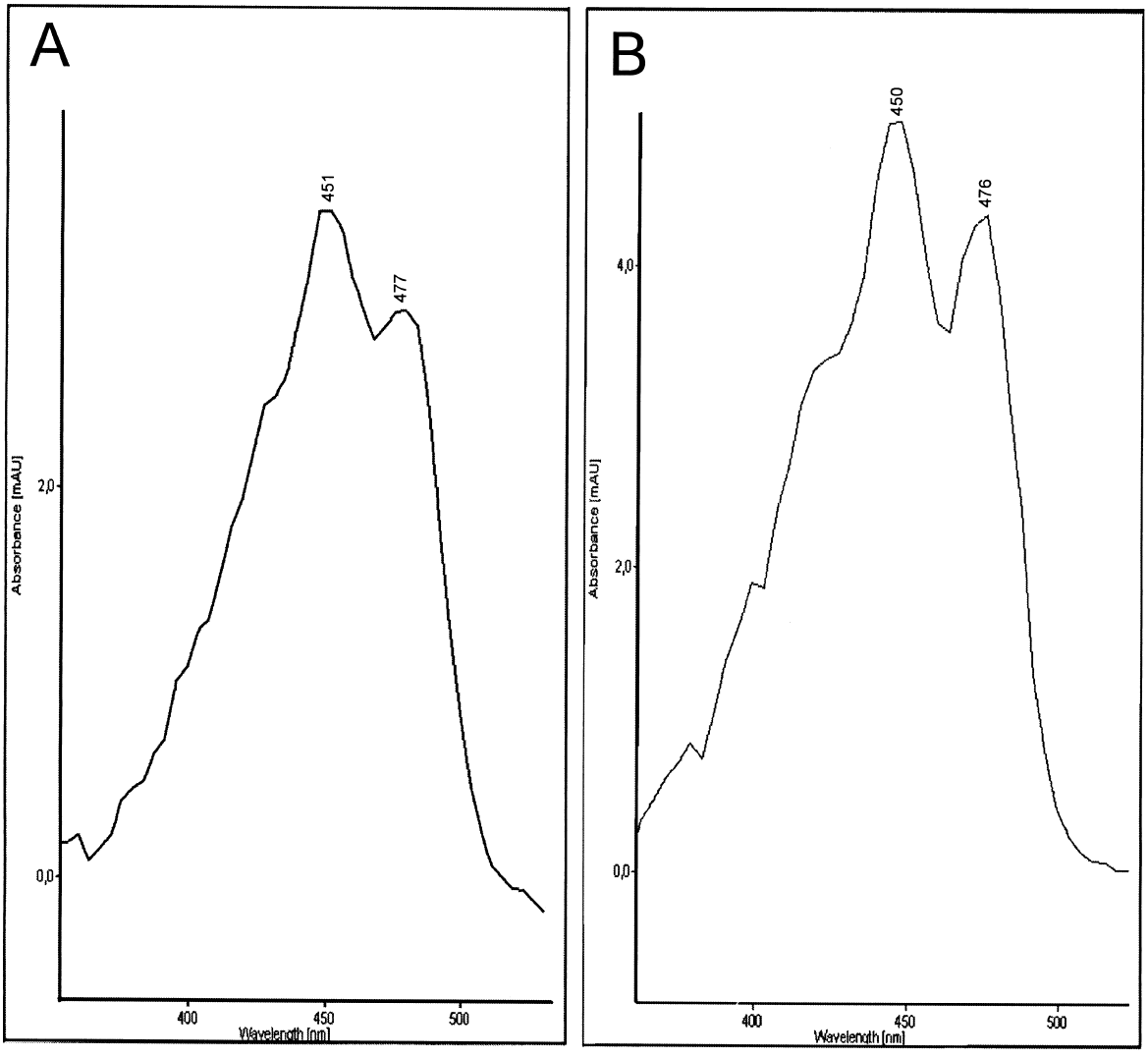

Fig. 1. A DAD β-carotene spectra for a low concentration standard (A) and chicken adipose tissue (B).

Table 2
Analytical characteristics

Compound	k' (C.V.%) ($n=20$)	LOD (ng/ml)	LOQ (ng/ml)	Range[a] (μg/ml)	Slope
β-carotene	0.14 (3.9)	0.1	0.3	LOQ-1.0	1.3145
α-tocopherol	1.60 (1.0)	0.1	0.4	LOQ-10.0	0.5300
retinol	6.13 (0.2)	0.2	0.4	LOQ-1.0	0.9291

[a] Studied range.

with its determination. Also BHA was tested but eluted near the peak of the internal standard. The use of TBHQ was already described by Madhavi [25] and in our conditions eluted after retinol, the last compound to leave the chromatographic column. With some chromatographic adjustments this antioxidant eluted at 22 min without interfering with any compound.

Dissolution of the final extract is a critical step in the quantitative analysis of β-carotene and retinol, since their concentration is frequently near the detection limit. When necessary we have use ultrasound to ensure complete solubilization [15].

The DAD proved its usefulness not only in the simultaneous detection of the compounds but also by providing spectra of the eluted compounds avoiding misinterpretations with impurity peaks. A DAD β-carotene spectra is represented in Fig. 1 both for a low concentration standard (A) and chicken adipose tissue (B).

3.2. Method validation

Several analytical performance parameters were validated [26]. The total procedure was examined for the linearity of the calibration plots. For each batch of samples, a new set of standards, usually five, was simultaneously prepared and treated in parallel with the samples. Injections in duplicate were made and the peak areas of the compounds subjected to the overall extraction procedure were measured and the peak-area ratios against the I.S. were calculated. A linear relationship was obtained with the correlation coefficients being above 0.99 for all three compounds (Table 2).

Assuming that the signal-to-noise ratio should be at least 3, and using an injection volume of 20 μl, the detection limits of the method (LOD), assessed in the presence of the internal standard, were calculated. The results are represented in Table 2. The quantification limits were determined by sample dilutions. Assuming the lowest concentrations found in the human adipose tissue for retinol, the minimum sample amount necessary for the determination can be set at around 1.6 mg.

Method precision was evaluated in terms of within-day and day-to-day reproducibility studies with homogeneous chicken sample and the results are represented in Table 3.

To calculate recoveries, chicken fat samples to which known amounts had been added were analysed together with untreated samples. The results are represented in Table 3.

Fig. 2 represents the chromatograms for DAD and fluorescence detector of a standard solution. The

Table 3
Analytical precision and recovery (chicken adipose-tissue)

Compound	Precision C.V.(%)		Recovery		
	Within-day ($n=6$)	Inter-day ($n=6$)	Real (μg/g)	Added (μg/g)	Mean recovery ($n=6$)[a]
β-carotene	3.87	5.01	0.46	0.2–0.4	99.6±4.8
α-tocopherol	2.55	3.82	6.59	1.0–2.0	100.2±1.9
Retinol	1.25	5.26	0.54	0.3–0.6	99.1±3.5

[a] Mean±SD.

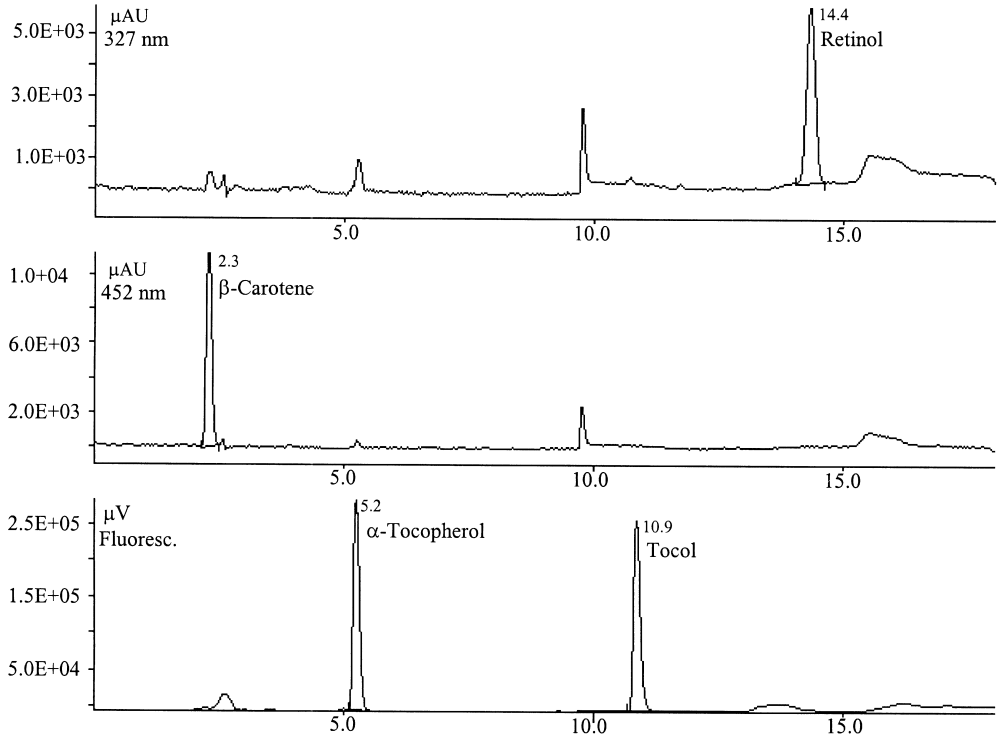

Fig. 2. Chromatogram obtained with a standard sample. HPLC conditions as given in experimental.

tubing between the DAD and the fluorescence detector causes a delay in retention time of tocol.

The use of NP-HPLC has been described as causing over-estimation of the β-carotene concentration due to its elution very close to the dead volume of the column [23]. The results prove that β-carotene can be determined under these conditions. Moreover, the use of NP-HPLC is more adequate for fat samples since the high triglyceride content does not interfere with the chromatographic determination and extra sample manipulation is avoided. Also, the absence of chlorinated solvents or acetonitrile in the eluent, as necessary in RP-HPLC, is a further guarantee of stability in the analysis of these easily oxidizable molecules.

3.3. Analyses of human adipose-tissue

The overall procedure was developed for analysis of human adipose-tissue samples as part of a Portuguese health program (''EPIcardis''), intended to find correlations between vitamin concentrations and the risk for acute myocardial infraction, as referred, and also to validate food questionnaires.

A typical HPLC pattern of a human adipose tissue sample is represented in Fig. 3.

In total we have analysed 140 samples, including cases and controls. Vitamin results were unavailable for three subjects (no adipose tissue found). The yield of adipose tissue per aspiration was 32.5±13.5 mg (mean±SD; n=140) with individual values ranging from 2.3 to 63.5 mg. The mean adipose tissue concentrations found in adipose-tissue represented as mean (min–max) were 0.43 μg/g (0.10–0.99) for retinol, 0.84 μg/g (0.15–2.22) for β-carotene, and 240.3 μg/g (36.8–669.4) for α-tocopherol.

The results achieved were in agreement with a European multicentre case-control study for α-tocopherol and β-carotene in the same tissue [2] and

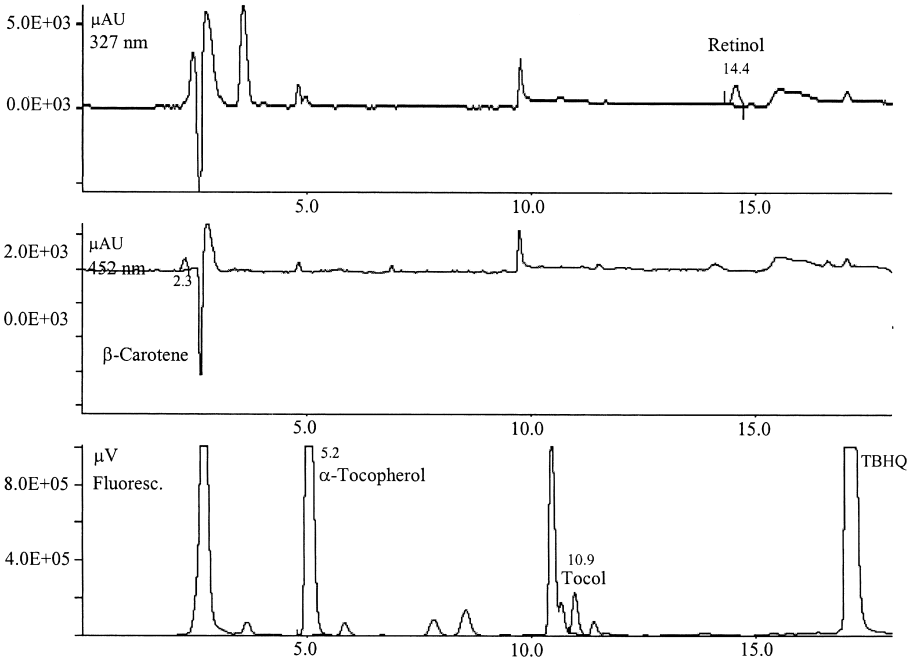

Fig. 3. Chromatogram from a human adipose-tissue. HPLC conditions as given in experimental.

with the results of a similar Norwegian study [8] achieved with different methodologies. These results will be statistically analysed elsewhere by the institution that collected the samples and requested the analysis.

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