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GAS CHROMATOGRAPHY OF RETINOL AND α-TOCOPHEROL WITHOUT DERIVATIZATION

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SUMMARY

An improved gas chromatographic method for the analysis of retinol and α-tocopherol in biological samples is described. The use of cold on-column injection in combination with wall coated open tubular column gas chromatography eliminates thermal decomposition of vitamin A and yields efficient separations of fat-soluble vitamins (A, D_2, D_3, and E) without derivatization. Peak tailing was judged to be minimal. Vitamins were quantified by flame ionization detection responses down to 3.5 ng injected, and their identities were confirmed using gas chromatography–mass spectrometry. Extracts of biological samples were saponified, and sterols were removed using digitonin-impregnated celite chromatography before analysis by gas chromatography and gas chromatography–mass spectrometry. Recoveries of vitamins from a test diet ranged from 89 to 103%.

INTRODUCTION

Inertness due to deactivation of reactive sites in gas chromatography (GC) columns and cold (room temperature) on-column injection make it possible to successfully conduct GC on thermally labile analytes without the need for derivatization [1,2].

Observation of decomposition of retinol (vitamin A) in hot injectors or at active sites on column surfaces have hindered development of GC methods for this vitamin [3]. Several methods have been proposed to convert retinol into derivatives that can withstand normal injector temperatures [3].

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GC analysis of α-tocopherol (vitamin E) offers the advantage of more sensitivity and selectivity than classical spectrophotometric methods [4]. GC separations of α-tocopherol have been improved by silylation [5]. However, these derivatizations are often neither quantitative nor reproducible. The inertness of modern GC systems made it attractive to attempt simultaneous analyses of underivatized retinol and α-tocopherol. The major objective of this study was to develop a simultaneous GC analysis method for underivatized retinol and α-tocopherol as prerequisite for gas chromatographic–mass spectrometric (GC–MS) analyses. A general preparation scheme for biological samples is also described.

EXPERIMENTAL

Biological samples

Rat liver and blood plasma were harvested from an anesthetized 18-month old male Sprague–Dawley rat that had been fed a commercially available cereal-based rat diet (Ralston Purina, St. Louis, MO, U.S.A.) supplemented with fat-soluble vitamins (ten times the requirement for fourteen days). Wholemeal wheat flour was purchased in a local supermarket. Fresh tuna liver was a gift from California Home Brand Foods (Terminal Island, CA, U.S.A.).

For the purpose of recovery determinations a test diet was formulated as follows (per 100 g): 65 g cornstarch, 20 g casein (90% purity), 5 g triolein, 5 g mineral mix, 5 g cellulose, 10 mg retinol (85% purity), 10 mg cholecalciferol and 10 mg α-tocopherol. Vitamin standards were obtained from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

The sample clean-up procedure consisted of a lipid extraction, followed by saponification, extraction of the non-saponifiable lipids and final isolation of the vitamins on digitonin-impregnated celite columns. The lipids were extracted according to the method of Bligh and Dyer [6], modified for smaller samples (1–2 g) and labile analytes. Dichloromethane was used instead of chloroform, and centrifugation at 1250 *g* was employed instead of filtration. The final solvent composition was: 6.4 and 5.5 ml (11.9 ml) water, including sample water, 2×8 ml (16 ml) dichloromethane, 16 ml methanol and 2.5 ml saturated aqueous sodium chloride solution. To avoid decomposition of retinol and α-tocopherol the following precautions were taken: samples were kept under argon; they were protected from light; 1 mg butylated hydroxytoluene (BHT) was added before extraction; and the water contained 0.05% EDTA.

The saponification procedure and the extraction of the non-saponifiable material was derived from the method of Slover et al. [7] and is appropriate for up to about 75 mg of lipid. The harvested lower layer plus washing was dried under argon at 40°C and 1.6 ml of 3% pyrogallol in absolute ethanol was added. A 200-μl volume of saturated, aqueous potassium hydroxide was added 30 s later while argon was still flowing through the tube. Then the tube was sealed under argon and vigorously shaken for 15 min at 65°C. Saponification was terminated by cooling the tube on ice.

Because of the limited solubility of retinol in non-polar organic solvents, cyclohexane–isopropanol (99:1, v/v) was used in the following extraction and clean-up steps. To extract non-saponifiable lipids, 2 ml of cyclohexane–isopropanol was added under argon and mixed (vortex mixer) for 5 s. Then 1.2 ml of distilled water was added under argon, vigorously mixed for 3 min and centrifuged for 3 min at 800 *g*. The upper layer was transferred to a clean 15-ml screw-cap tube wrapped in aluminum foil. The lower layer was then washed once with 2 ml cyclohexane–isopropanol, centrifuged and the upper layer transferred to the same 15-ml screw-cap tube. This fraction containing the non-saponifiable lipids was dried under argon at 40°C and dissolved in 1 ml cyclohexane-isopropanol.

Sterols were removed from the non-saponifiable lipid extract by chromatography on digitonin-impregnated celite columns [8]. The digitonin-celite column was prepared as follows: 180 mg digitonin and 1.5 ml water were mixed in a 50-ml screw-cap tube until a homogenous foam was obtained. Then, 3 g of dried celite were added and thoroughly mixed. The mix (digitonin-impregnated celite) was suspended in cyclohexane and loaded into a 1 cm×30 cm column. The loaded column was washed with 5 ml of cyclohexane and wrapped in aluminum foil.

The non-saponifiable lipids (in 1 ml cyclohexane–isopropanol) were loaded on the digitonin-impregnated celite column and eluted with 30 ml cyclohexane–isopropanol at a flow-rate of 1–2 ml/min. The solvent was evaporated under argon at 40°C and the residue redissolved in 1 ml cyclohexane–isopropanol containing 35 μg/ml squalane (Sigma) as an internal standard. The purified sample was stored in a 1-ml amber vial with a PTFE-lined screw cap.

Wall coated open tubular (WCOT) column gas chromatography

A Hewlett-Packard gas chromatograph (Model 5830A) fitted with a flame ionization detector, an on-column injector (J&W Scientific, Folsom, CA, U.S.A.) and a fused-silica capillary column, 15 m×0.25 mm I.D. coated with a 0.25-μm film of methylsilicone (J&W Durabond-1™) was used. Chromatography conditions included: an initial temperature of 220°C followed by a gradient of 3°C/min to 270°C and a 20-min hold at 270°C. The flame ionization detector temperature was 290°C, and ultrapure hydrogen was used as the carrier gas, flow-rate 50 cm/s. Volumes of 1 μl were injected on the GC column.

Mass spectrometry

To confirm the identities of eluted GC peaks the gas chromatograph was coupled to a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, U.K.) that was operated at a source temperature of 180°C and 70 eV electron ionization voltage. Total ion current (TIC) chromatograms and full mass spectra were obtained for individual and combined vitamins. The selected-ion monitoring (SIM) mode was used to analyze rat blood plasma. Retinol was detected by monitoring its molecular ion at *m/z* 286 and a fragment ion at *m/z* 268 (anhydroretinol) and α-tocopherol was monitored with its molecular ion at *m/z* 430.

Quantitative analysis

Standard solutions were prepared containing 1, 3.5, 10, 35, 100, 350 and 1000 ng of retinol and α-tocopherol, dissolved in 1 μl of a 35 ng/μl solution of squalane

standard. Each standard was analyzed in triplicate to establish the standard calibration curves used to quantify the vitamins in biological extracts. To correct for variations in injection volume, the integrated flame ionization detection (FID) peak-area responses of the vitamins were expressed relative to the area of the internal squalane standard. Linear regression analysis on the calibration plots yielded regression equations that were used for subsequent quantification of retinol and α-tocopherol in biological samples.

The integrated peak areas of retinol and α-tocopherol from biological samples were determined in triplicate and average values were calculated. Known amounts of standards of retinol and α-tocopherol were added to another set of three samples. These samples were extracted and analyzed in the same manner as their non-spiked counterparts. The increase in the integrated peak areas due to added standard was used to calculate recoveries during the entire analytical procedure. The amounts of retinol and α-tocopherol present in the sample were calculated from the appropriate regression equations.

RESULTS AND DISCUSSION

Retention times and integrated responses

Chromatograms of the 10-ng standards are shown in Fig. 1. The temperature program was chosen to shorten retention times as much as possible to minimize exposure of labile analytes to elevated column temperatures without significantly compromising chromatographic resolution. Mean retention times and standard deviations of 29 individually prepared standard solutions were 4.15 ± 0.07 and 12.65 ± 0.14 min for retinol and α-tocopherol, respectively. The integrated FID peak-area responses obtained from three individually prepared standard solutions (35 ng/μl) of retinol and α-tocopherol were 8594 ± 638 and 14754 ± 957, respectively. α-Tocopherol gave a significantly stronger FID response than retinol and thus the sensitivity of the method is accordingly higher for α-tocopherol than for retinol. The differences in the FID responses can be explained in terms

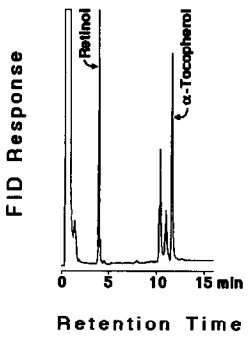

Fig. 1. GC of fat-soluble vitamin standards: 10 μg/ml each in cyclohexane–isopropanol (99:1, v/v); injection volume, 1 μl. Temperature program: 220°C, 0 min; 3°C/min to 270°C, 5 min. The two peaks preceding α-tocopherol are the pyro and isopyro isomers of cholecalciferol (vitamin D_3).

of the reducing state of the analytes. α-Tocopherol is a more reduced compound than retinol and thus gives a stronger FID response.

Dynamic range

Regression lines and equations relating FID responses to varying quantities of retinol and α-tocopherol were constructed from the mean values of three individually prepared standards of each concentration ranging from 1 ng to 1 μg per μl injected. The regression equations obtained for retinol and α-tocopherol were as follows: $y=2.30+1.06x$ ($r=1.00$) and $y=2.58+1.03x$ ($r=1.00$), respectively. The FID responses of both vitamins were linear down to 1 ng which was the smallest amount injected. This shows that the vitamins were not adsorbed significantly to active sites in the column or the injector. Thus the derived regression equations were reliable for quantifying retinol and α-tocopherol over the full range from 1 to 1000 ng.

Sample clean-up

Recoveries of fat-soluble vitamins from biological extracts are often low because of their instability [9–11]. This is especially true for retinol [12,13] and its metabolites which have been shown to readily undergo *cis–trans* isomerization on exposure to normal fluorescent light [14]. Effective stabilization of the analytes was considered important throughout extraction and isolation to maximize recovery. Samples were stored and treated under argon whenever possible and protected from light with aluminum foil, amber glassware and reduced light. Moreover, BHT was added at the beginning of the clean-up. It remained with the fat-soluble vitamins throughout the procedure and was observed in the final gas chromatograms. EDTA was added to chelate metals and minimize metal ion-catalyzed decomposition. Finally pyrogallol was added during saponification to trap free radicals. Without these precautions as much as 50% of the vitamins were found to decompose. Satisfactory recoveries have been obtained for retinol and α-tocopherol, which are shown in Table I. In the rat liver samples retinol recoveries were only 73% but this value is in question because of coeluting interferences.

TABLE I

RECOVERIES AND CONTENTS OF RETINOL AND α-TOCOPHEROL IN BIOLOGICAL SAMPLES

In all cases, $n=3$. C.V. = coefficient of variation.

Sample	Compound	Recovery (%)	C.V. (%)	Vitamin content (μg/g)	C.V. (%)
Rat liver	Retinol	73	16.3	$2.3 \cdot 10^3$	21.4
	α-Tocopherol	100	14.6	17.4	15.6
Tuna liver	Retinol	92	14.3	649	20.8
	α-Tocopherol	101	10.1	45	15.0
Wheat flour	α-Tocopherol	97	14.8	10	17.1

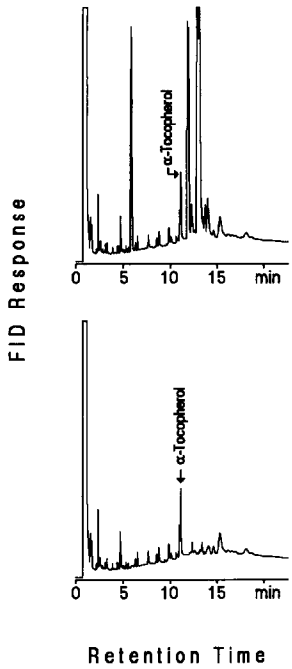

Fig. 2. Sterol removal by digitonin-celite column chromatography. Upper chromatogram: before treatment; lower chromatogram, after treatment.

The initial total lipid extraction procedure was necessary to allow an effective subsequent saponification under mild conditions with minimum impact on the analytes. The Bligh and Dyer [6] total lipid extraction procedure has been shown to be highly effective, probably due to the formation of a transient monophasic sample dispersion [15].

The digitonin column chromatography is an important clean-up step to remove interfering sterols, such as cholesterol, β-sitosterol and stigmasterol. Precipitation of interfering sterols with digitonin using methanol–water systems and subsequent extraction with cyclohexane was incomplete and tedious. Therefore digitonin column chromatography was employed. The method of Sheppard et al. [8] was chosen and modified for smaller samples sizes. The effectiveness of this additional clean-up step is illustrated in Fig. 2 with a wholemeal wheat flour sample. Digitonin-celite chromatography removed interfering plant sterols from the non-saponifiable lipid fraction of wheat flour, so that α-tocopherol was well resolved, identified and quantified. Since recoveries from the column exceeded 95% for retinol and α-tocopherol, digitonin-celite chromatography was also employed to remove the large quantities of cholesterol present in the liver samples.

Recovery determination

The purified test diet was designed for recovery determinations and contained 100 μg/g each of retinol and α-tocopherol. Recoveries were 89 ± 12 and $98 \pm 12\%$ ($n=3$), respectively. A chromatogram of the test diet sample is shown in Fig. 3.

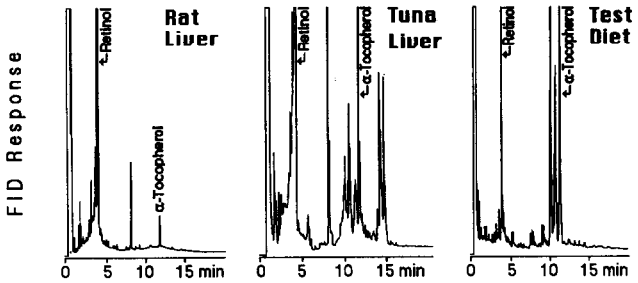

Fig. 3. GC of biological samples in cyclohexane–isopropanol (99:1, v/v); injection volume, 1 μl. Temperature program: 220°C, 0 min; 3°C/min to 270°C, 20 min.

Biological samples

Fig. 3 shows the gas chromatograms of the tuna liver, rat liver and test diet samples. Relevant peaks were identified by coelution with known standards as well as from mass spectra obtained by GC–MS. Table I shows recoveries and

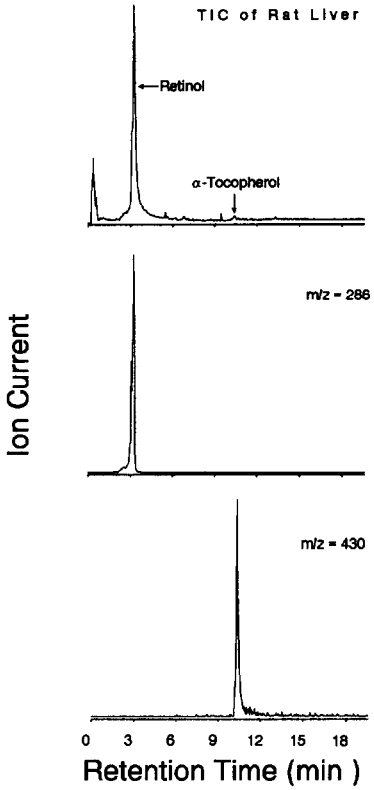

Fig. 4. GC–MS ion chromatograms of a rat liver sample. Top: total ion current (TIC). Middle and bottom: reconstructed ion chromatograms for *m/z* 286 and *m/z* 430, respectively.

amounts of retinol and α-tocopherol as determined by the present method in tuna liver, rat liver and wholemeal wheat flour.

The total retinol content of tuna liver was determined to be 649 μg/g with a recovery of 92% and peak identity was verified by GC–MS. The mass spectrum of the retinol peak showed an intense molecular ion at *m/z* 286 and characteristic fragment ions at *m/z* 268 and 255. MS of the smaller peaks preceding the large retinol peak indicated the presence of several isomers of retinol, identified from the prominent molecular ion at *m/z* 286. It is likely that these are *cis* isomers of retinol since they are expected to have slightly shorter retention times than the all-*trans* isomer. Because this isomerization was not observed in other biological samples and since adding authentic retinol standard to the sample did not increase the areas of these isomers, it was concluded that the isomers were naturally present in the tuna liver.

The α-tocopherol content of tuna liver was estimated at 45 μg/g with a recovery of 101%. The peak was identified by GC–MS with the molecular ion of α-tocopherol at *m/z* 430.

The rat liver sample shows a large retinol peak on the chromatogram. The retinol content was estimated at about 2.3 mg/g rat liver. This is about three to four times higher than literature values [16] for adult rats fed normal retinol levels. However, this value is reasonable because the liver was taken from an 18-month-old animal given a diet supplemented with fat-soluble vitamins. The α-tocopherol content in this rat liver was estimated to be 17.4 μg/g, which is in agreement with published values [17]. Fig. 4 shows the ion current chromatograms from a GC–MS analysis of a rat liver sample. The chromatogram on the top shows the total ion current (TIC) and below are reconstructed ion chromatograms for *m/z* 286 (retinol) and *m/z* 430 (α-tocopherol).

The recovery of α-tocopherol from wholemeal wheat flour was 97% and the content of this vitamin was estimated to be 10 μg/g, which agrees with values reported by others [18, 19].

CONCLUSION

Until recently adsorption and degradation of retinol or α-tocopherol during GC could only be prevented by derivatization procedures [3,5]. Inert fused-silica WCOT columns with a covalently bonded methylsilicone stationary phase, an appropriate temperature program, ultrapure hydrogen as a carrier gas and cold on-column injection are key elements in the successful chromatography of underivatized retinol and α-tocopherol in the present study.

The mass spectra obtained from GC–MS show molecular ions in high yields for retinol and α-tocopherol, demonstrating a high degree of inertness of the chromatographic system as a prerequisite for successful identification and quantification by MS detectors. Analyses requiring high selectivity, sensitivity and accuracy are best performed using GC–MS in the SIM mode, which also allows for the use of internal standards labelled with stable isotopes.

Using FID alone, the method is adequate for a variety of biological samples containing at least 5 μg of the analyte per gram of sample. Liver, fish liver and

body oils as well as certain foods like cereals and dairy products seem to be appropriate samples for GC–FID. However, for routine analyses of fat-soluble vitamins in foods and tissues the reader is referred to more sensitive and less labor-intensive high-performance liquid chromatographic methods [3, 5, 15, 18, 20, 21].

Both retinol and α-tocopherol are labile compounds and their effective protection during the sample preparation significantly improved their recoveries.

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