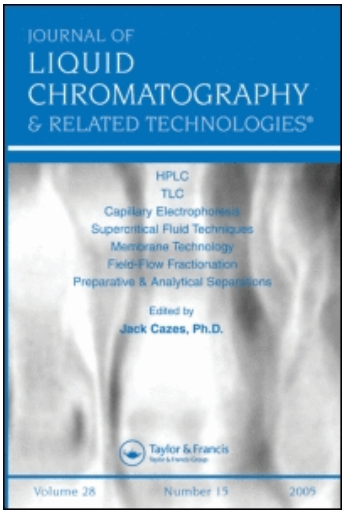

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DETERMINATION OF ALPHA-TOCOPHEROL IN BIOLOGICAL SPECIMENS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method is described for determining α-tocopherol in liver and other biological specimens. Hexane extracts of saponified samples are acid-washed and then injected onto a reversed phase column using hexane:propan-2-ol (99:1 v/v) for elution, and fluorimetry at 210 nm excitation for detection.

INTRODUCTION

Continuing interest in the role played by vitamin E in animal and human health necessitates provision of a diagnostic service for α-tocopherol, the most biologically active form of vitamin E. Many methods for determining α-tocopherol have been published but most are limited for routine use by their comparatively low sensitivity or precision, doubtful specificity, or complexity. The advent of high performance liquid chromatography (HPLC) brought the potential for overcoming these limitations. The HPLC method reported here was developed to realize this potential and

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is suitable for the relatively rapid determination of α-tocopherol in biological specimens.

MATERIALS

Chemicals

α-Tocopherol was obtained from Sigma (St. Louis, Mo.,U.S.A.). Other tocopherols were prepared from the unsaponifiable matter (1) of wheatgerm oil and soybean oil by separation with chloroform on Sephadex LH 20 (Hoogenboom, unpublished). Hexane was distilled over potassium hydroxide from technical hexane (Mobil Pegasol 1516). L-Ascorbic acid was reagent grade and ethanol, potassium hydroxide, sulphuric acid, and propan-2-ol were Analar grade, British Drug Houses (Poole, U.K.).

Standard Tocopherol solution

A stock solution of α-tocopherol (500 mg/l) was prepared in ethanol and stored in an amber bottle at 4 °C. The concentration of α-tocopherol in this solution was calculated from the maximum absorbance of a 10^{-1} dilution at 292 nm, using a molar absorbtivity of 32600 (2).

HPLC system

The HPLC system comprised a Tracor (Austin, U.S.A.) 995 pump; Rheodyne (Berkeley,U.S.A.) 7120 injector with 40 µl loop; Whatman Partisil PXS 10/25 ODS column; and a Varian (Palo Alto, U.S.A.) Fluorichrom fluorescence detector with deuterium lamp that was fitted with forced air convection cooling. A Corion (Holliston, U.S.A.) 210 nm interference filter (25 nm ½ band width, 15% minimum transmission) was used for excitation; and a Corion Schott 295 nm cut-off filter and Varian 325 nm band filter for emission. Detector responses were monitored as peak areas on a Perkin Elmer (Norwalk, U.S.A.) Sigma 10 Data Station. The HPLC eluant, de-gassed hexane:propan-2-ol (99:1, v/v) was pumped at a flow rate of 1.6 ml/min (3 MPa).

METHOD

To 0.8 g of liver or other tissue, 1 ml of serum, or 0.2 g of feedstuff in a Quickfit (Stone, Staffs., U.K.) C24/C24R tube were added 2 ml of ascorbic acid soln, 100 g/l, followed by 2 ml of 7.5 M potassium hydroxide soln, followed by 4 ml of ethanol. The tubes were then fitted with air condensers and placed in a water bath (80 °C) contained in a fume cupboard. After 10 min, samples were vortex mixed for 5 sec and then returned to the water bath for a further 20 min. Saponified samples were cooled in a cold water bath for 10 min before mechanically shaking with 10 ml of hexane for 5 min. The hexane extract (5 ml) was shaken with 0.5 ml of sulphuric acid soln, 600 ml/l, for 2 min and then allowed to stand for 10 min before injecting 40 µl onto the HPLC column.

RESULTS AND DISCUSSION

Saponification and Extraction

Saponification is necessary to convert any tocopherol esters to the free form and to remove fats and other interfering substances before chromatography. Many experimental conditions have been used for this saponification step to limit the oxidation of tocopherol that readily occurs under alkaline conditions.

Difficulties were initially encountered here in achieving saponification of sample, maintaining tocopherol stability, separation of the hexane extract, and extraction of the tocopherol. These factors are inter-related. Unless suitable ratios of reagents are used, emulsions form that require long periods of settling or centrifugation. Also the tocopherol, being soluble in both ethanol and hexane, distributes itself between the phases to varying extents depending upon the experimental conditions chosen.

The finally developed saponification and extraction procedure overcame most of these problems. Saponification at 80 °C, with air condensers, enabled samples to be easily processed in batches

without the difficulties experienced in refluxing at 100 °C as recommended in earlier methods (3,4). Oxidation of α-tocopherol was not detected over a 60 min period at 80 °C but 30 min, with a short period of vortex mixing was sufficient to ensure tissue breakdown and release of the free tocopherol. Emulsions did not form during shaking: the hexane extract, containing 1.5% ethanol, rapidly separated out on standing.

Washing the hexane extract with 60% (v/v) sulphuric acid facilitated a short HPLC analysis time by removing interfering compounds with longer retention times than α-tocopherol. The sulphuric acid wash also removed the ethanol extracted into the hexane. If the sulphuric acid wash is omitted from the sample extracts and the working α-tocopherol standards then the ethanol present in the HPLC injections shortens the retention times slightly.

Chromatography

A typical chromatogram obtained from a bovine liver extract is shown in Fig. 1. With the described procedure, the retention time of α-tocopherol was short (2.6 min), with no significant detectable interferences. This resulted in a rapid sample throughput. The comparative cleanliness of the acid-washed hexane extract ensured little deterioration of column performance over extended usage.

The present procedure does not appear to have been reported previously for tocopherol. Until the advent of chemically bonded stationary phases in 1969, most liquid chromatography was performed with a polar stationary phase, commonly silica or alumina, and a non-polar mobile phase. Tocopherol was initially chromatographed here and elsewhere using this so-called normal phase system. As adapted to HPLC, this usually comprised a silica column with hexane:diisopropyl ether (95:5) at a flow rate of 1.5 ml/min to give a retention time of about 10 min for α-tocopherol. The normal phase system has the advantage that hexane sample extracts, or even the (oil) sample itself (5), can be injected directly onto

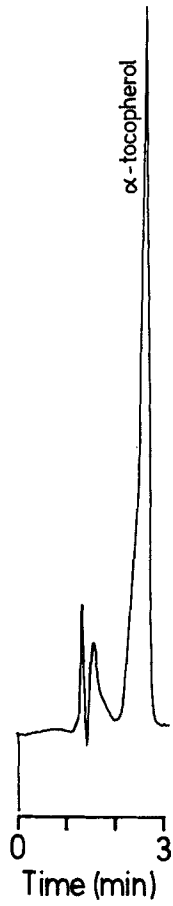

FIGURE 1. HPLC chromatogram of extracted bovine liver.

the HPLC column. Most workers, however, now use the more stable reversed phase system with a non-polar stationary phase, typically octadecylsilane (ODS) bonded to silica, and a polar mobile phase, typically methanol:water (90-97:10-3) at 2-3 ml/min, to give a retention time of 5-20 min for α-tocopherol. This reversed phase system necessitates taking the hexane extract to dryness, which may result in low and variable recoveries, before re-dissolving in methanol for injection onto the HPLC column.

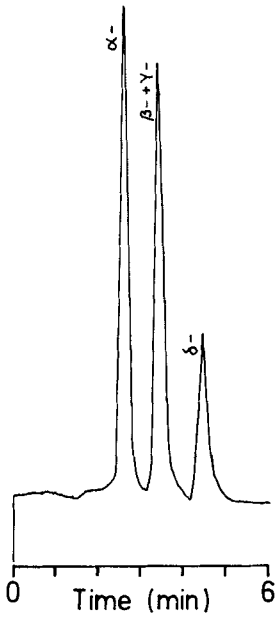

FIGURE 2. HPLC chromatogram showing separation of α-, β- + γ-, and δ-tocopherols on ODS column using hexane: propan-2-ol (99:1) as mobile phase.

The system developed here has the advantage that hexane extracts can be injected directly onto a reversed phase column, using propan-2-ol as a polar modifier in the hexane mobile phase to resolve the α-tocopherol in a short time. Separation of the α-tocopherol is presumably by both partition and adsorption processes as with the more commonly used reversed phase systems (6). As α-tocopherol elutes only slowly through a reversed phase column unless the polarity of the hexane eluant is increased, 40 µl or more of hexane sample extract can readily be injected without undue peak broadening.

Detection

Fluorimetry provides a sensitive and relatively specific method for detecting α-tocopherol. Excitation at 292 nm, the

absorption maximum for α-tocopherol, was initially used but this was later replaced with excitation at 210 nm (7) to increase the sensitivity.

Where the identity of a peak may be in doubt, it can be confirmed as α-tocopherol by checking the response with different excitation/emission filters. In our experience, this has always confirmed the identity of a peak as α-tocopherol whenever the retention time coincided with that of the standard.

The stability of the fluorescence detection system was improved by fitting a cooling fan to the deuterium lamp housing. Without this lamp cooling, baseline fluorescence decreased markedly for 2-3 hours after start-up. With the cooling fan operating, acceptable baseline stability was achieved within 15 min.

Separation of other tocopherols

Although this method was developed primarily for α-tocopherol it may also be used for the separation and determination of some other tocopherols (Fig. 2). The relative retention times for α-, β- + γ-, and δ-tocopherol were 1.00:1.46:2.12, respectively. The behaviour of tocopherol with this and other HPLC column/eluant systems will be described in more detail elsewhere.

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