Journal of Chromatography, 176 (1979) 488–492

CHROM. 11,925

Note

Determination of α-tocopherol in animal feedstuffs using high-performance liquid chromatography with spectrofluorescence detection

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(Received April 17th, 1979)

The importance of vitamin E in animal nutrition has been well established. Of its various molecular forms, α-tocopherol has been recognised as the most biologically important. A comprehensive review has been published by Moustgaard and Hyldgaard-Jensen[1].

Many methods for the determination of the tocopherols in feedstuffs have been introduced, most of them based on separation of the tocopherols by column, paper, or thin-layer chromatography, followed by a colorimetric reaction.

The separation steps, however, are usually laborious and time consuming, and the colorimetric reactions such as that of Emmerie and Engel[2], are often subject to interference from other compounds[3].

Recently, Vatassery and Hagen[4] published a study on the determination of α-tocopherol in brain samples using high-performance liquid chromatography (HPLC) and Vatassery *et al.* have also described the separation of other tocopherols[5] using this technique. Furthermore De Leenheer *et al.*[6] has described the determination of serum α-tocopherol using HPLC.

In the present paper we describe the application of HPLC to the determination of α-tocopherol in animal feedstuffs, such as hay, meal, barley, silage, etc. The method described consists of three main steps *viz.* extraction, saponification, and chromatography.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph pump (Model 6000), injector (Model UK6) and column (μBondapak C_{18}, 300 × 3.9 mm) were purchased from Waters Assoc., Hartford, Great Britain. The fluorescence spectrophotometer (Model 204) and flow cell were purchased from Perkin-Elmer, Beaconsfield, Great Britain. The chart recorder (type CR160) was purchased from J. J. Lloyd Instruments, Southampton, Great Britain.

Reagents

AnalaR grade ascorbic acid, potassium hydroxide, methanol, ethanol, and

diethyl ether were used untreated, and were obtained from Hopkin and Williams, Ramford, Great Britain. *n*-Hexane obtained from BDH, Poole, Great Britain, was re-distilled before use to remove fluorescent impurities; D-α-tocopherol was obtained from Eastman-Kodak, Rochester, N.Y., U.S.A. and used without further treatment. Gelatin-coated beadlets of tocopherol acetate feed supplement (Rovimix E25) were obtained from Roche, Dunstable, Great Britain. Barley, hay, grass silage, pig meal etc. were samples submitted for routine α-tocopherol analyses.

Procedure

Extraction. Samples, *e.g.* barley, hay, and meal, were milled before use and wet samples such as silage were finely chopped and mixed. Samples of 5 g were weighed into 125-ml PTFE, wide-mouth bottles and 10 ml of 5% ascorbic acid in 0.1 *N* hydrochloric acid was added, followed by 10 ml ethanol. The bottles were shaken for 15 min in a reciprocating shaker. A 40-ml volume of water and 40 ml of diethyl ether were added, and the samples were again shaken for 1 h, followed by centrifugation at 2000 *g* for 10 min. The upper ether layer was transferred to a 150-ml round-bottomed flask and an additional 40 ml of ether was added to the bottles, which were then shaken for a further 30 min. The bottles were again centrifuged, and the ether layers were combined with those from the first extraction. The ether extracts were reduced in volume to about 5 ml using a rotary film evaporator, and transferred to 100 × 16 mm test tubes, fitted with ground-glass stoppers.

Saponification. The ether was evaporated from the extracts under the stream of nitrogen using a hot water bath (50°) and 3 ml 2% ascorbic acid in ethanol was added. The tubes were placed in a 70° water bath and allowed to equilibrate for a few minutes. A 0.7-ml volume of 60% potassium hydroxide in water was added and the tubes were heated for 15 min at 70°. They were then cooled and 4 ml *n*-hexane and 3 ml distilled water was added and the tubes were shaken vigorously for 1 min, followed by low-speed centrifugation (1000 *g*) for 5 min. The hexane layer was removed for chromatography.

Chromatography. The mobile phase used in the HPLC was methanol–water (95:5). The flow-rate was 3 ml/min. Fluorescence detection was used with an excitation wavelength of 296 nm and emission wavelength of 330 nm.

The standard consisted of 20 μg/ml α-tocopherol in *n*-hexane. 50 μl of this solution was injected, as was 20–100 μl of the unknown sample extracts, depending on the concentration of α-tocopherol. Concentration was related to the height of the fluorescent peaks. A typical trace using different samples is shown in Fig. 1.

RESULTS

Recovery experiments were carried out to check the extraction and saponification steps in the assay. Free α-tocopherol, α-tocopherol acetate, and gelatin-coated beadlets of α-tocopherol acetate were added to a meal sample and carried through the whole procedure. Results are shown in Table I. Recoveries range from 80–92%.

Table II shows a comparison between the wet extraction procedure described, and a standard Soxhlet extraction procedure, where the samples were extracted for 2 h in a Soxhlet apparatus, using diethyl ether. It can be seen that the Soxhlet extrac-

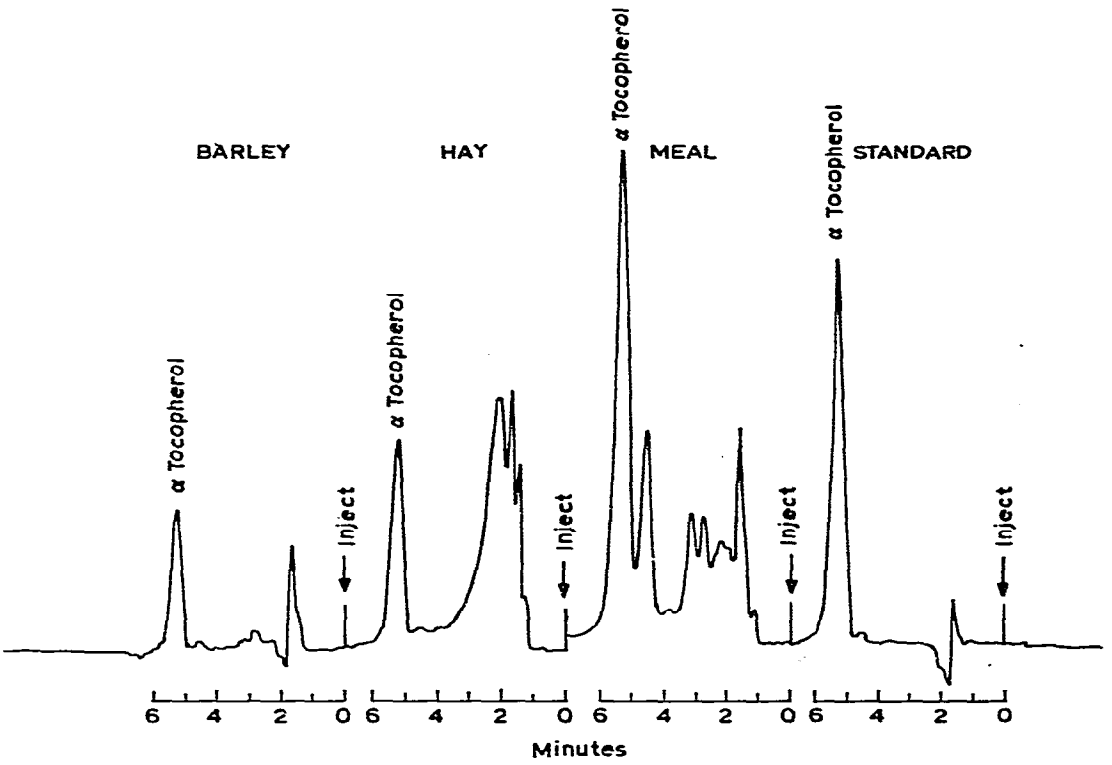

Fig. 1. Typical chromatograms of α-tocopherol standard (1 μg) and three feed extracts.

tion gave generally lower results, particularly for the gelatin beadlet supplemented sample, and also the silage sample.

Table III shows the results of replicate analyses of a meal sample containing natural tocopherol, and also with 20 ppm tocopherol acetate added in the form of gelatin coated beadlets.

TABLE I

RESULTS FROM A MEAL SAMPLE SUPPLEMENTED WITH VARIOUS FORMS OF α-TOCOPHEROL

Sample in duplicate	*α-Tocopherol found (ppm)*	*Recovery (%)*
Meal	5.0	—
	4.8	—
Meal + 100 ppm free α-tocopherol	85.0	80.1
	86.6	81.7
Meal + 100 ppm α-tocopherol acetate	90.2	85.3
	87.1	82.2
Meal + 100 ppm α-tocopherol acetate as gelatin-coated beadlets	88.2	83.3
	96.7	91.8

TABLE II

COMPARISON OF THE WET EXTRACTION PROCEDURE USED IN THE METHOD, WITH SOXHLET EXTRACTION

Sample	*Wet extraction α-tocopherol (ppm)*	*Soxhlet extraction α-tocopherol (ppm)*
Hay	3.5	3.5
	3.7	2.5
Meal	5.4	4.5
	5.4	4.3
Meal supplemented with 100 ppm tocopherol acetate as gelatin beadlets	88	18
	91	10
Silage	28.5	4.2
	24.2	8.7

TABLE III

RESULTS FROM REPLICATE ANALYSIS ($n = 5$) OF A MEAL SAMPLE WITH AND WITHOUT ADDED TOCOPHEROL

Sample	*Mean α-tocopherol (ppm)*	*Range*	*S.D.*	*Coefficient of variation (%)*
Meal	5.36	5.2–5.5	±0.11	2.0
Meal + 20 ppm tocopherol acetate as gelatin coated beadlets	23.3	22.5–24.3	±0.72	3.1

DISCUSSION

The method described has been developed to analyse a wide variety of animal feedstuffs which may contain only naturally occurring tocopherols, or may be supplemented with additional tocopherol in the form of free tocopherol, tocopherol acetate, or gelatin beadlets containing tocopherol acetate. The initial shaking with ethanol–hydrochloric acid prior to extraction was used to dissolve gelatin off any supplement beadlets present. It would also appear that this method of extraction improves the release of tocopherol from plant material, compared with the standard Soxhlet extraction. Ascorbic acid was added to prevent oxidation of the tocopherols. The wet extraction procedure was particularly suitable for supplemented samples and also for wet samples such as silage or grass. Further drying may destroy some of the tocopherols present and is therefore not used in the present procedure. Dry matter determinations were carried out separately. The standard Soxhlet extraction procedure was shown to be unsatisfactory for gelatin beadlet supplemented or wet samples (Table II).

The saponification step was necessary to convert any α-tocopherol acetate to the free form, and also to remove fats and other materials which may cause interference in the chromatographic stage.

Fluorescence rather than ultraviolet detection was used since we found that other compounds with similar retention values to the α-tocopherol caused considerable interference when absorbance was measured at 280 nm. The fluorescence trace

on the other hand, showed clean peaks free from interference of other compounds. The peak preceeding the α-tocopherol has been shown to consist of γ- and β-tocopherols[6], and these two forms cannot be separated using the μBondapak C_{18} column. If required it may be possible to resolve the tocopherols using the column system described by Vatassery *et al.*[5].

The method described has been used in this laboratory for several months and has shown itself to produce fast and accurate results with a minimum of steps. Emulsification of the extract was not found to be a problem compared to some other wet extraction procedures *e.g.* diethyl ether. Recoveries from a variety of samples has been in the region of 80–90%. We have only used the method for animal feedstuffs to date, but it should similarly enable the analysis of a wide range of food in the human nutritional field to be carried out.

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