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Evaluation of HPLC method for the rapid and simple determination of α-tocopherol acetate in feed premixes*

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ABSTRACT

Alpha-tocopherol was determined in feeds for chickens and pigs. Samples for chromatographic (HPLC) analysis were prepared using a method encompassing one-step extraction with a 30:70 (v/v) mixture of acetone:chloroform. The samples were eluted isocratically from a LiChroCART™ 250-4 column with UV detection using a UV-VIS detector at a wavelength of 290 nm. The eluent was methanol at a flow rate of 1 ml/min. 20 µl samples were injected into the column using an autosampler. The retention time of the α-tocopherol acetate peak was 13.41 min with a coefficient of variation of 2.78%. The repeatability of the area under the curve, expressed as the coefficient of variation, was 22.7% for α-tocopherol concentrations ranging from 0.5 to 1 µg/ml, 8.3% for a range from 1-11 µg/ml, and 1.8% for a range from 11 to 3000 µg/ml. The coefficient of variation for the full assay of α-tocopherol acetate in samples encompassing all of its stages, starting from the weighing of a sample to its chromatographic analysis, did not exceed 7.3%. The recovery of tocopherol measured against an external standard was 92.4%, and against a standard processed through the sample preparation steps 93.2%. Alpha-tocopherol acetate standard stored at 4°C and protected from the light was found to be very stable. Two of the advantages of the developed method are its speed and ease of performance.

KEY WORDS: vitamin E, α-tocopherol acetate, premix

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INTRODUCTION

The basic methods of assaying vitamins are given in AOAC (1990). These methods include gas chromatography (procedures: 988.14, 989.09, 969.40; AOAC, 1990) and colorimetry (procedure 973.30). Because these techniques are complicated, most laboratories develop their own methods based, however, on the above procedures and adapted to determining the vitamin E content in feeds, animal tissues, foods, and blood using HPLC. Determining vitamin E and other fat-soluble vitamins in feeds is associated with difficulties in taking samples and processing them for analysis (Sanz and Santa-Cruz, 1986; Stary et al., 1989; Barbas et al., 1997; Qian and Sheng, 1998; Abidi, 2000; Ye et al., 2001).

Sample processing (purification, extraction) is one of the most important elements determining assay precision, i.e. its sensitivity, recovery and repeatability.

In general, there are two ways of processing a sample:

1. Extraction preceded by alkaline digestion in conjunction with saponification,
2. Digestion (or not) and direct extraction with an organic solvent without saponification.

Digestion at elevated temperatures (70°C) is usually done with ethanol or methanol, with the former being preferred due to its higher boiling temperature and probably because of this, a somewhat greater efficiency of extracting vitamins from samples (Buttriss and Dipplock, 1984). During processing, antioxidants and substances inhibiting the degradation of the assayed compounds are added before saponification. These substances include ascorbic acid, pyrogallol, EDTA or butylated hydroxytoluene (BHT). Kramer et al. (1997) believe that the fat contained in most tissues and feeds does not interfere with subsequent chromatographic analysis (by HPLC) and therefore saponification before extraction can be omitted. Processing samples without saponification before assaying fat-soluble vitamins has been described, among others, by Eriksson et al. (1978) (multivitamin tablets), Pozo et al. (1990) (fat tissue), Barbas et al. (1997) (muscle tissue),Qian and Sheng (1998) (feeds), Ihara et al. (2000) (blood). The main factor affecting the efficiency of extracting vitamin E and its derivatives is the type of solvent used. Most available methods (Rushing et al., 1991) employ *n*-hexane to extract fat-soluble vitamins. Chloroform and methyl chloride are also used for this purpose (White, 1993). Nonetheless, using a 30:70 mixture of acetone and chloroform seems to give the best results of extracting vitamins from feeds (Qian and Sheng, 1998).

A one-step method of extraction with acetone-chloroform (30:70) was applied in the assay of vitamin E in the form of α-tocopherol acetate (Qian and Sheng, 1998), after which the solvent was evaporated and the residue dissolved in *n*-butanol.

MATERIAL AND METHODS

Reagents

The following solvents were used: HPLC-grade methanol, *n*-butanol, chloroform (MERCK, Darmstadt, Germany) and analytical grade acetone (MERCK, Darmstadt, Germany). Deionized water prepared with a MILLI-Qplus deionizer (Millipore, Vienna, Austria) was used in the analyses. The α-tocopherol acetate standard was obtained from Sigma-Aldrich (St. Louis, USA).

Chromatographic analysis

HPLC was performed on Schimadzu (Kyoto, Japan) HPLC equipment equipped with an LC-6A pump, SIL-10A$_{XL}$ autosampler, and UV-VIS SPD-6AV detector. Data was integrated using HSM-D7000 LaChrom software from Merck-Hitachi (Darmstadt, Germany). Chromatographic separation was carried out using a LiChroCART™ 250-4 Superspher™ 100 RP-18-4 μ column (Merck, Darmstadt, Germany). Methanol was the mobile phase; elution was carried out at a rate of 1 ml/min. 20 μl aliquots of the studied samples were used for analysis, detection was at a wavelength of 290 nm.

External standard in n-butanol

Solutions of α-tocopherol acetate in concentrations from 187.5 to 3000 μg/ml (from W_{z1} to W_{z5}) were prepared directly in *n*-butanol.

Standard processed through the sample preparation

A standard of α-tocopherol acetate (W_{st}) in a mixture of acetone:choloroform (30:70) was prepared at a concentration of 50000 μg/ml. To glass Schott vials (12 ml) containing 4 ml of a mixture of acetone:chloroform (30:70) 100 μl of the above standard were added, the vials filled with nitrogen and hermetically sealed. They were shaken for 1 min using a Vortex shaker (Reax control Heidolph, Schwabach, Germany). Next, 1 ml was transferred to a chromatographic vial (1.5 ml), evaporated to dryness under nitrogen in a water bath (at 40°C). Before HPLC analysis, the residue was dissolved in 1 ml *n*-butanol.

Preparation of the blank sample

In order to determine the purity of the reagents, the following blank sample was prepared: 4100 μl of a 30:70 mixture of acetone and chloroform were added to a

Schott vial then filled with nitrogen and hermetically closed. The vial was shaken for 1 min (Vortex, Reax control Heidolph, Schwabach, Germany), left still for 5 min and once again shaken for 1 min. After centrifugation at 4000 rpm for 5 min, 1 ml of the supernatant was transferred to a 1.5 ml chromatographic vial. The supernatant was evaporated to dryness under nitrogen in a water bath (at 40°C) and the residue was dissolved in 1 ml *n*-butanol. This sample was used for HPLC analysis.

Processing of samples with and without added standard

α-Tocopherol was determined in six premixes used in feeding broiler chickens, laying hens and pigs. Samples of the premix were ground in a Fritsch pulocrisette® (Switzerland) with a sieve diameter of ø 0.80 mm (Qian and Sheng, 1998). Samples without added standard were processed as follows: a 1 g sample was weighed with a precision of 0.001 g, transferred to the above-mentioned extraction vials and 4100 µl of a 30:70 mixture of acetone and chloroform were added. In order to remove the air, the vials were filled with nitrogen and closed. The mixture was shaken for 1 min (Vortex, Reax control Heidolph, Schwabach, Germany), left still for 5 min and then shaken again for 1 min. After centrifugation at 4000 rpm for 5 min, 1 ml of supernatant was transferred to 1.5 ml a chromatographic vial. The supernatant was evaporated to dryness under nitrogen in a water bath (at 40°C), and the residue dissolved in 1 ml *n*-butanol. The vials were closed and before injecting into the HPLC column, shaken on the Vortex for 10 sec. Samples with added standard were prepared as above, except that instead of 4100 µl of 30:70 mixture of acetone and chloroform, 4000 µl of this mixture and 100 µl of the standard were added (W_{st}).

RESULTS AND DISCUSSION

Retention and blank sample

The retention time of α-tocopherol acetate was 13.41 min with a coefficient of variation of 2.78%. These data are for a series of analyses conducted on the same day. As the column aged, the retention time of α-tocopherol acetate gradually lengthened. A typical chromatogram from the analysis of a sample for α-tocopherol acetate is given in Figure 1. Chromatograms of blanks prepared in the same way as samples had low background noise without peaks in the region of the assayed vitamin.

Standard stability

Fat-soluble vitamins are sensitive to long-term exposition to light. This applies mainly to vitamins A and D, less to vitamin E and its derivatives. In order to deter-

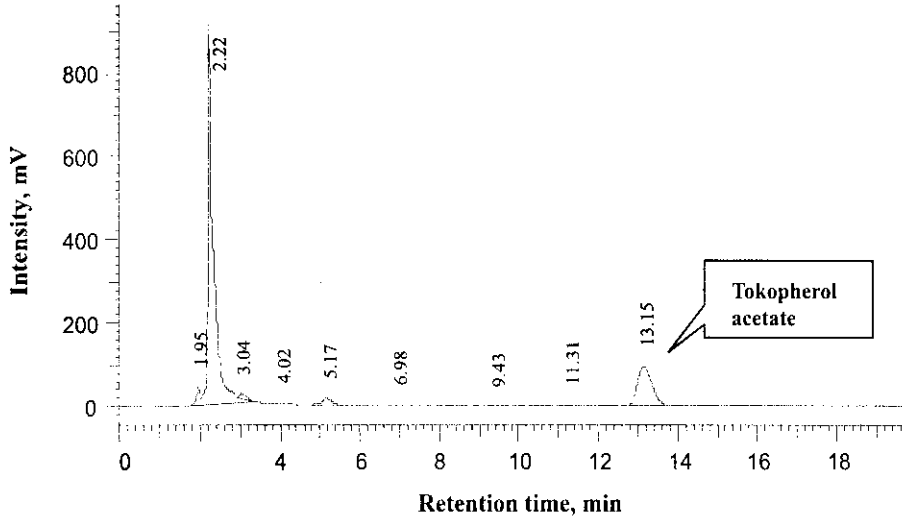

Figure 1. Typical chromatogram for analysis of α-tocopherol. Isocratic elution system with UV-Vis detector at 250 nm and LiChroCART™ 250-4 column was used. Eluent (metanol) rate flow was 1 l/min. 20 μL analyzed samples were injected onto the column

mine the stability of α-tocopherol acetate, five standard solutions ranging in concentration from 188.812 to 3021 μg/ml were prepared in *n*-butanol. The standards were stored at a temperature of about 4°C and protected from the light. The standards obtained in this way were assayed by HPLC on three dates: on the first, third and eighth days. In all of the samples the standard was found to be very stable. The coefficient of variation of the area under the curve (AUC) for α-tocopherol acetate did not exceed 1%. The small variability in the AUC points not only to the high stability of the compounds in *n*-butanol, but also to the high stability of the entire chromatographic system, including the column, during the eight-day period.

Calibration and limit of quantitative assay

The determinations of α-tocopherol acetate performed in replicated using the method of serial dilutions were linear in a wide range of concentrations. The best linear regression parameters of the dependence of the AUC for a given peak on the concentration of the compound in this peak were obtained for one series of concentrations (i.e. from the baseline standard to its 10-fold dilution). For α-tocopherol acetate the linear regression equations for the concentration range from 0.545 to 1.09 μg/ml had the form $y=3398(\pm 687)\,x$; in the range from 1.09 to 10.9 μg/ml, $y=3481(\pm 135)\,x$; and in the range from 2 to 32 μg/ml $y= 2901(\pm 36)\,x$. The coefficients of correlation r^2 equaled 0.9243, 0.9940 and 0.9987. F equaled 24.4, 663.8,

and 6304, and the residual coefficient of variation, 19.3, 11.8, and 5.8%, respectively. The limit of quantitative assay of α-tocopherol acetate was 0.5 μg/ml.

Repeatability of chromatographic determination

The repeatability of the AUC for α-tocopherol acetate was determined on standards in a wide range of concentrations of this compound in *n*-butanol. In most studies, the repeatability of chromatographic determinations is given as standard deviation (SD) or the coefficient of variation (relative standard deviation - RSD) , without specifying the range of concentrations for which this parameter was determined, whereas the AUC of a peak depends on the concentration of the analyzed compound. In our study, this coefficient was highest at concentrations from 0.5 to 1 μg/ml α-tocopherol acetate. Much better repeatability of the AUC for α-tocopherol acetate was obtained when its concentration was higher. Chromatographic analysis of the studied standards was conducted in numerous n-element k-series (n replicates n each series, n=2). The following equation was used to calculate the coefficient of variation (Dobecki, 1998):

$$srV = \sqrt{\frac{\sum_{k} V_i^2}{k}}$$

where:

srV – the pooled coefficient of variation for k-series of determinations
Vi – the coefficient of variation for the i-th series of determinations
k – the number of series.

The repeatability of chromatographic determination of α-tocopherol acetate is presented in Table 1.

TABLE 1

Repeatability of chromatographic determination of u α-tokopherol (n=2)

Range of concentration of α-tocopherol acetate μg/ml	Number of series (n=2) k	Pooled coefficient of variation, srv %
0.5-1	3	22.7
1-11	8	8.3
11-3000	46	1.8

Repeatability of sample preparation and chromatographic analysis

Six premixes were analyzed in two replicates. Sample processing and its HPLC analysis were as follows: a 1-g sample was weighed, vitamins extracted with a mi-

xture of solvents, the extract sampled, the solvent evaporated under nitrogen, the residue dissolved in *n*-butanol and HPLC performed. The coefficient of variation of the determination of α-tocopherol acetate in samples encompassing all of the stages starting from weighing the sample and ending in chromatographic analysis depends mainly on the reproducible and appropriately short time of processing the sample, the amount of the sample and its homogeneity. In our study all of these conditions were fulfilled (short analysis time, a sufficient sample size (1g) careful grinding of the sample on a ø 0.80 mm sieve), which was expressed as a low coefficient of variation of the methods, which did not exceed 7.3%. Data on the content of α-tocopherol acetate in the analyzed premixes and value of the coefficients of variation are given in Table 2.

TABELA 2

Content of α-tocopherol acetate in premixes and coefficients of variation

	Means content mg/kg	Coefficient of variation %	Expected content of α-tocopherol acetate* mg/kg
Premiks I	1546.4	7.3	1500
Premiks II	1196.3	0.1	1000
Premiks III	4342.4	1.7	4000
Premiks IV	1391.4	5.9	1500
Premiks V	4175.9	1.2	4000
Premiks VI	1873.7	2.3	2000

* values declared by firm offering premixes

Recovery of α-tocopherol

Commonly available publications usually do not contain details about how recovery is determined. In this study we determined recovery of α-tocopherol acetate by assaying blank samples, standard processed through the sample preparation, samples, samples with added standard and external standard. The determinations were conducted on six premixes in two replicates, which made it possible to determine the size of the losses of the analyzed acetate form of vitamin E during the preparation of samples and determination the matrix effect of the sample.

Losses during sample processing

On the basis of comparing the area under the curve of α-tocopherol the acetate peak in the external standard with that of the compound present in the standard processed through the sample preparation, the recovery of α-tocopherol acetate was determined, but without determining the matrix effect of the sample. Re-

covery was computed as $R=100 \times (A_W/A_Z)$, where A_W is the area under the curve of α-tocopherol acetate peak in the standard processed through the sample preparation, A_Z the area under the curve of the α-tocopherol acetate peak in the external standard, prepared directly in *n*-butanol.

The matrix effect

On the basis of comparing the area under the curve of the peak of α-tocopherol acetate added to the sample with that of α-tocopherol acetate from the standard processed through the sample preparation, the recovery of this compound as the so-called matrix effect was determined. It was calculated as $R_M = 100 \times (A_D-A_P) /A_W)$, where A_D is the AUC representing α-tocopherol acetate in the sample with a known amount of standard added, A_P the AUC of α-tocopherol acetate in the sample (without added standard), A_W the AUC of this vitamin in the standard processed through the sample preparation. R_M is used in calculating the content of the vitamin in the sample when the standard is processed in the same way as the analyzed sample.

Full recovery

Full recovery takes into account the losses arising during sample processing (extraction, pipetting, evaporation) and the matrix effect (sorption on the surface of the sample, interference of the assayed vitamin with other sample constituents). It is calculated as follows: $R_C = (R \times R_M)/100 = 100 \times [(A_D-A_P)/A_Z]$. The value expressing full recovery (R_C) is taken into account in calculating the vitamin content in the sample determined against an external standard. The value of the discussed parameters (R, R_M, R_C) and the standard deviations of their arithmetic averages (SE) are presented in Table 3.

TABELA 3

Recovery of α-tocopherol acetate

Index	Recovery of α-tocopherol acetate, %
R	99.1
SE	0.60
R_M	93.2
SE	1.44
R_C	92.4
SE	1.56

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STRESZCZENIE

Ocena przydatności metody HPLC do szybkiego i prostego oznaczania zawartości octanu α-tokoferolu w premiksach paszowych

W paszach stosowanych w żywieniu kur oraz trzody chlewnej oznaczono α-tokoferol. Próbki do analiz chromatograficznych (HPLC) przygotowywano według metody polegającej na jednostopniowej ekstrakcji za pomocą mieszaniny acetonu z chloroformem (30:70, v/v). Zastosowano izokratyczny system elucji z detekcją przy pomocy detektora UV-VIS przy długości fali 290 nm, używając kolumny chromatograficznej LiChroCART™ 250-4. Jako eluenta użyto metanolu o prędkości przepływu 1 ml/min. Badane roztwory w ilości 20 µl podawano na kolumnę za pomocą autosamplera. Czas retencji piku octanu α-tokoferolu wynosił 13,41 minuty ze współczynnikiem zmienności 2,78%. Powtarzalność pola piku, wyrażona przez współczynnik zmienności, wyniosła dla zakresu stężeń octanu α-tokoferolu od 0,5 do 1 µg/ml 22,7%, dla zakresu stężeń od 1-11 µg/ml 8,3%, a dla zakresu stężeń od 11 do 3000 µg/ml 1,8%. Współczynnik zmienności pełnego oznaczenia octanu α-tokoferolu w próbkach obejmujący wszystkie jego etapy, poczynając od naważenia próbki a kończąc na jej oznaczeniu chromatograficznym, nie przekroczył 7,3%. Odzysk tokoferolu mierzony w stosunku do wzorca zewnętrznego bezpośrednio przygotowanego w *n*-butanolu wynosił 92,4%, a w stosunku do wzorca wewnętrznego, przeprowadzonego przez procedurę przygotowania próbki, wynosił 93,2%. Stwierdzono wysoką trwałość standardu octanu α-tokoferolu przechowywanego w temperaturze około 4°C i chronionego przed światłem. Jedną z ważniejszych cech opracowanej metody jest jej szybkość i łatwość wykonania.