Application of high-performance liquid chromatography to determine the concentration of α-tocopherol in the blood of cattle

I.V. Artamonov[1], A.V. Platonov[1,2]*

[1]Vologda Research Center of the Russian Academy of Sciences, 56A, Gorky Street, Vologda, 160014; [2]Vologda Institute of Law and Economics of the Federal Penitentiary Service of Russia, 2, Shchetinin Street, Vologda, 160002, Russian Federation
* Corresponding author e-mail: platonov70@yandex.ru; Tel.: +7-817-259-78-10

Journal of Livestock Science (ISSN online 2277-6214) 13: 261-266
Received on 19/9/22; Accepted on 16/10/22; Published on 1/11/22
doi. 10.33259/JLivestSci.2022.261-266

Abstract

The purpose of the work was to develop within the laboratory method of analysis of α-tocopherol in the blood of animals corresponding to the laboratory equipment and the practical needs of the laboratory. The main objective of the study is to develop a method for the extraction and analysis of α-tocopherol in the blood serum of cattle, which could be used in studies related to the assessment of animal health and their productivity. Since most of the techniques are somehow related to the use of mass spectrometry (due to the low concentration of α-tocopherol in the blood), and the content of α-tocopherol is an important indicator in the system of evaluation criteria, an attempt was made to adapt the existing methods of extraction and analysis to the conditions and instrument base of the laboratory. In the process of work, the extraction of α-tocopherol in various variations was performed, the methods already described in scientific publications were tested. The result was an adapted extraction technique, as well as chromatographic analysis using fluorimetric detection, the separation conditions were selected. In particular, the selection of the composition of the rolling phase for the most efficient separation was carried out, which made it possible to reduce the analysis time from 23 minutes to 18 minutes (relative to the previously used technique).

Keywords: HPLC; tocopherol; cattle; blood serum; separation; extraction

Introduction

Tocopherol (vitamin E) is a natural antioxidant which prevents fatty acid oxidation in cell membrane. Tocopherol also plays an important role in sustaining immune response reactions to infectious diseases. A normal level of tocopherol in the cattle diet allows avoiding various dystrophic diseases including the white-muscle disease, as well as improves the quality of milk and meat (fresh keeping time for these products extends which has economic value) (Secrist et al., 1997).

Vitamin E is also important for muscle activity which affects lung and heart functions in animals and their rumen. The studies show that vitamin E deficiency leads to the increase of garget incidents which affects cattle reproductive performance. In vitro tests show the impact of vitamin E on long-chain fatty acids and cellulose consumption (Hino et al., 1993). Hypovitaminosis E is also connected to the occurrence of gonads atrophy in male cattle and to the damage of the placenta in female cattle which leads to fetal death (Agalakova et al., 2009; Shabunin et al., 2014). There is a delayed effect of vitamin E deficiency on the development of calves; in such cases, calves at the age of 3–6 weeks usually have a ”white muscle desease” associated with striated muscles degeneration (McDowell et al., 1996).

The cattle’s needs in vitamin E are not clearly defined. Different authors estimate the range from 15 to 60 mg/kg of dry food or from 150 to 600 mg per day for cattle fattening[1] (Burken et al., 2012). For comparison, according to the main document setting the reference values for the needs of dairy cows, in the USA the recommended daily dosages of tocopherol are 400–500 mg per one lactating cow and 1000–1400 mg per one dry cow (Nutrient Requirements of Dairy Cattle, 2001).

At the same time, the bioavailability of various forms of tocopherol is different (Hidiroglou et al. 1992). The standard form for tocopherol is the racemic mixture of d,l-α-tocopherol acetate.

The most vitamin E-rich feeds are legumes, oilseeds, fresh green mass. Grains and fermented feeds have a significantly lower content of tocopherols due to their oxidation and destruction during fermentation. Other sources of tocopherol that are of interest and are ofted used include fat-and-oil industry waste as well as alternative types of feed previously used to a limited extend, for example, hydroponic green feeds (Merkulova & Zueva, 2015) and green feeds in general (Mary et al., 2021). However, in recent decades, in the practice of animal husbandry, there has been a tendency to reduce the share of fresh grass feeds with a simultaneous increase in the share of canned food (Dillon, 2018). For this reason, vitamin supplements play an important role both in the form of an individual vitamin or complex, and in the form of foods rich in them.

Given the importance of vitamin E and its impact on the health and productivity of cattle, the accurate establishment of its content in the blood is necessary for the timely correction of diets and diagnosis of animal conditions.

The status of vitamin E is determined by its level in plasma or serum, and since tocopherol is not deposited in the body in significant quantities, it is believed that the level of tocopherol in the blood reliably reflects its consumption by animals (Pehrson & Hakkarainen, 1986).

Currently, there are many chromatographic techniques (as well as ELISA techniques, for example, immune-chemiluminescence analysis with high sensitivity (Cinquanta et al., 2017), allowing to determine the content of vitamin E in the blood serum (both animals and humans). They differ, as a rule, in the process of extracting tocopherol from blood serum and the process of detection. The most modern and sensitive is the chromatographic method with mass detection. However, in most cases, it is possible to use cheaper and less sensitive detectors.

The purpose of the work: the development of an intra-laboratory method for analyzing α-tocopherol in the blood of animals using classical liquid chromatography in combination withfluorimetric detection.

Material and methods

Forthe moat of dairy cows, in the amount of forty samples, were selected on the livestock farm of the collective farm named after the “50th anniversary of the USSR” Gryazovetsky district of the Vologda region, Russian Federation (Yurovo 58°58'06.7"N 39°59'57.5"E)

All the manipulations with the experimental animals were performed according to the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Official Journal of the European Union L276/33, 2010). Blood analysis was carried out in the Laboratory of bioeconomy and sustainable development (Vologda, Molochnoye, Russian Federation, 59°17'33.2"N 39°40'21.0"E).

Animal blood collected in a test tube with a coagulant was centrifuged at 4000 revolutions to separate the serum which was moved into eppendorf test tubes and stored frozen at a temperature of -25-30 degrees until analysis.

The serum was thawed at room temperature. 1 mL of whey was treated with a similar volume of methanol and ethanol mixture in a ratio of 95: 5 (v: v) cooled to -30 degrees[2] for protein denaturation and precipitation and

[1] Recalculation made for acetate dl-α-tocopherol, initial data are given in international units.

[2] For removing proteins you can also use a 10% solution of sulfosalycylic acid in a volume of half the volume of whey.

kept at a temperature of 2–3 degrees for 5 minutes[3]. 2.5 ml of hexane was added to the mixture and subjected to intense shaking on the vortex for 5 minutes.

After shaking, the mixture was centrifuged at 1600g to the complete separations of the components. The 2 mL aliquot was transferred to test tubes and evaporated at 35 degrees until the hexane was completely removed in the air current. The resulting residue was dissolved in 500 μL of the mobile phase and filtered through a 0.45 μm filter, and then introduced into the chromatographic system using an autosampler. Input volume – 100 μl[4].

We tested various movable phase formulations, including mixtures with methanol-water, acetonitrile-water, acetonitrile-methanol-water, acetonitrile-methanol. The results of the test partitions showed that the most optimal phase was the mobile phase of acetonitrile-methanol in the ratio of 50 : 50 (v : v) and separation in the isocratic mode. The column is Agilent Zorbax RP-C18 250 mm with a grain of 5 μm and an internal diameter of 4.5 mm at a flow rate of 1.5 ml / min at a temperature of 30 degrees.

We used a chromatographic system as a part of the Shimadzu LC-20AD pump module, the Shimadzu SPD-20A diode-matrix detector and the Shimadzu RF-20Asx fluorimeter detector, connected in series. Detection of tocopherol by a diode-matrix detector was carried out at a wavelength of 280 nm. For the fluorimetric detector, an excitation wavelength of 285 nm and an emission wavelength of 320 nm were established. Wavelength data were selected on the basis of the analysis of literature sources and tested in various combinations (see Table 1).

It is shown that the use of a fluorimetric detector is preferred; in comparison with it the PDA detector gives a 15 times less intense response which is especially important at vitamin E concentrations determined in the blood serum. The ratio of signals of the two detectors at the same concentrations of tocopherol is clearly shown in Fig. 1.

Results and Discussion

The calibration curve is plotted for concentrations of 2.25-72 μg/ml at six calibration levels (fig. 2). The work used the standard d,l-α-tocopherol Sigma-Aldrich.

The separation was carried out in an isocratic[5] mode by the rolling phase of several trains including those reflected in scientific publications on this topic, for example (Urbánek et al., 2006). In most cases, a mixture of methanol and water, methanol, acetonitrile and water is recommended as the mobile phase. Considering the high flow rates at which the separation takes place (1.5 ml / min), the selection of conditions demanding less time for the separation will ultimately reduce the consumption of reagents.

An important condition for stabilizing the separation is a sufficient conditioning time of the chromatographic column for at least 15 minutes at operating flow and temperature. Otherwise, the divergence of peak retention times can be up to 1 minute, which is unacceptable in the conditions of real separation. A comparison of the separation on the column before and after conditioning is presented in Fig. 3.

Validation of the extraction was performed by analyzing ten samples of cattle serum with one concentration level (obtained from one blood sample) and ten standard samples of d,l-α-tocopherol. The standard deviation from the analysis was 0.068 and 0.0817 for the serum sample and the standard sample, respectively. The standard deviation of retention times on a normally conditioned column was not more than 0.3295. The results of validation are given in Table. 2.

Compared to other techniques described in scientific publications (e.g., Aaran & Nikkari, 1988; Cavina et al., 1988; Hammer-Plecas & Woollard, 2010; Khan et al., 2010) studied in this study, our methodology provides comparable accuracy.

The result of the research consists in developing a method of quantitative analysis of α-tocopherol in the blood serum of animals, which is currently used in the laboratory in practical analysis and shows a stable result with sufficient accuracy.

Conclusion

Despite the fact that mass spectrometry is usually used for the analysis of blood vitamins due to its higher sensitivity and less complex sample preparation, the use of a system of classical liquid chromatography in combination with traditional detection methods (UV, fluorimetry) is quite appropriate for solving a fairly wide range of laboratory tasks. The proposed extraction technique is very simple, and the separation is short in time. Moreover, when using shorter columns (for example, 150 mm columns), it is possible to further reduce the separation time. When the conditions for preparing a chromatographic system are met, the technique allows obtaining a stable and repeatable result.

It should be noted that in the process of sample preparation, retinols, metabolites of ergo- and cholecalciferol and isomers of tocopherol are also simultaneously extracted from the serum sample. For this reason,

[3] The volume of serum from which the extraction is made can be reduced to 500 μL if necessary, but in some cases, with a low tocopherol content in the blood, the detector signal will be at the lower limit of the LOQ.

[4] Together with tocopherol, other fat-soluble vitamins, such as retinol and its isomers, are also extracted from the serum, so the technique can be used to analyze these compounds.

[5] Two channels to supply individual components of the mobile phase may be used. This does not affect the result of the separation.

the extraction technique is universal and can be used in the analysis of these compounds. In addition, the presented separation parameters are suitable for their analysis, since they have shorter retention times than α-tocopherol.

Table 1: Tocopherol signal intensity depending on detection conditions

λ, nm	Peak square	Height
290/325	26968928	1122798
285/320	40980903	1695238
295/340	8525661	336036
265	1155863	51547
280	2648502	118558
325	37024	1434
328	34601	1312

Table 2: Results of validation of the extraction and separation methodology

Test, №	Serum tocopherol concentration, μg/ml	Retention time, sec	Concentration of tocopherol in a standard sample, μg/ml*	Retention time, sec
1	2.7596	16.315	17.8984	16.446
2	2.8354	16.943	18.0213	16.539
3	2.7955	16.316	17.9875	16.648
4	2.7231	16.872	17.8546	16.855
5	2.6935	16.404	18.0547	16.829
6	2.7564	16.270	17.9846	16.601
7	2.7496	16.858	17.8123	16.376
8	2.7155	16.242	18.0124	16.090
9	2.5843	16.253	17.8865	16.039
10	2.6945	16.267	18.0036	16.031
Std. dev.	*0.0678*	*0.2921*	*0.0817*	*0.3295*
* The estimated concentration of the standard sample is 18 μg/ml obtained by sequential dilution of the standard sample with a concentration of 72 μg/ml (considering the α-tocopherol content in the Sigma-Aldrich standard of 96%).				

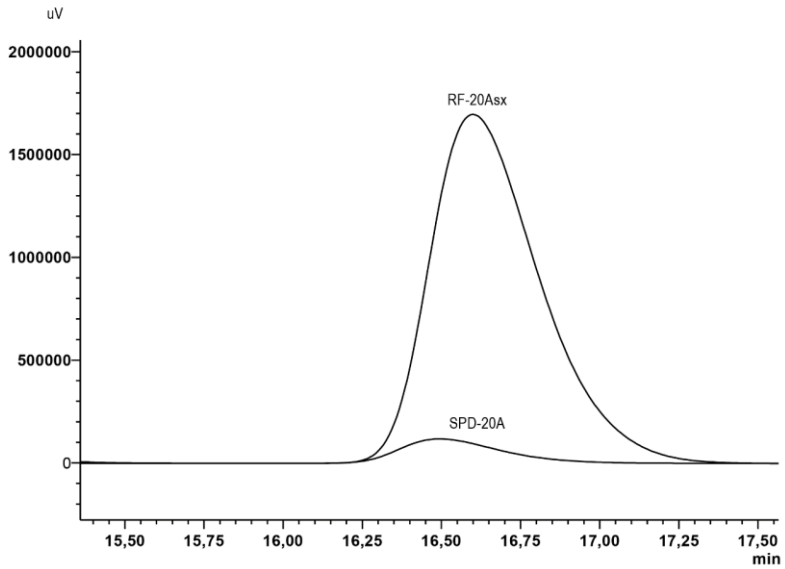


Fig. 1: The ratio of signals received from the PDA (SPD-20A) and fluorometric (RF-20Axs) detectors in response to one concentration of tocopherol (sample - tocopherol standard, 72 μg/ml).

#	Conc. µg/ml	Peak Square	SD
1	2.25	1148174	1.424
		1125285	
2	4.50	2432011	4.487
		2282442	
3	9.00	4658320	2.662
		4486191	
4	18.0	7979154	7.665
		8893716	
5	36.0	17632816	1.929
		17158358	
6	72.0	40980903	6.434
		37414421	

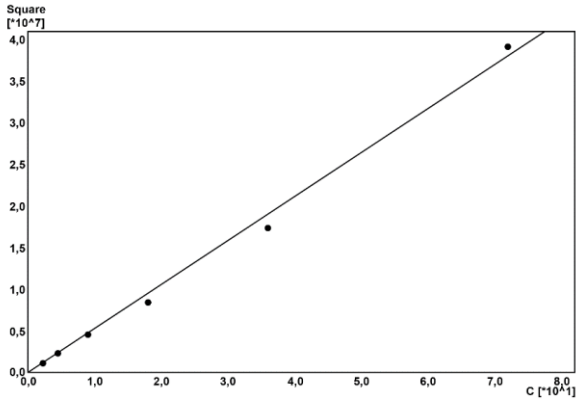
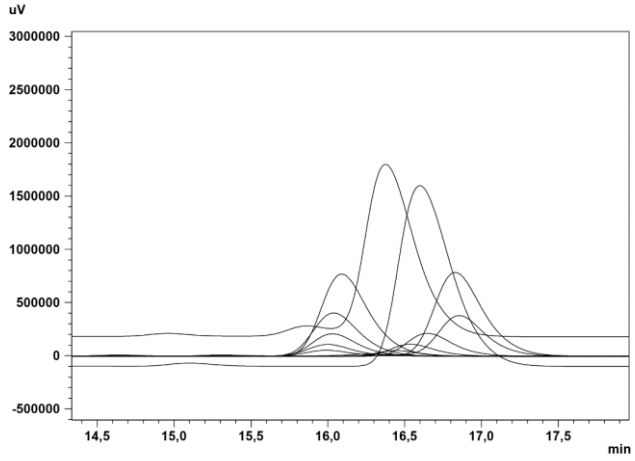
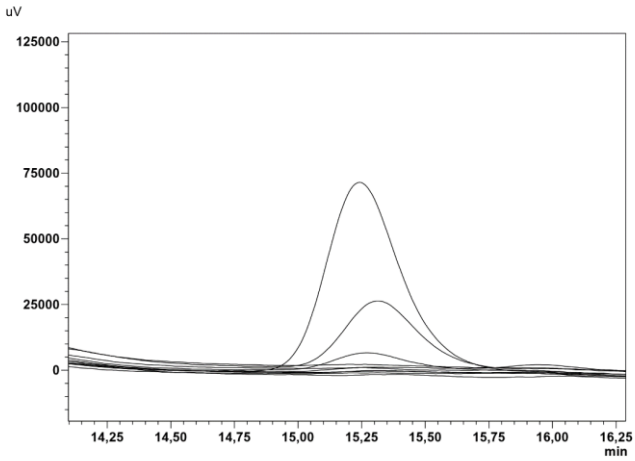


Fig. 2: Calibration curve for tocopherol. The concentrations used for the construction (considering the content of α-tocopherol in the standard sample), the area of the peaks obtained, and the deviation are indicated.



Separation before conditioning



Separation after conditioning

Fig. 3: Comparison of separation stability before and after conditioning of a chromatographic column for at least 15 minutes under separation conditions

References

1) Aaran, R.-K., & Nikkari, T. 1988. HPLC method for the simultaneous determination of beta-carotene, retinol and alpha-tocopherol in serum. Journal of Pharmaceutical and Biomedical Analysis, 6(6–8), 853–857. doi: 10.1016/0731-7085(88)80101-8
2) Agalakova, T.V., Netecha, V.I., & Shchepina, Yu.N. 2009. The effect of antioxidant drugs on the prevention of the death of early embryos in cows. Achievements of Science and Technology of AIC, 1, 46–48.
3) Burken, D.B., Hicks, R.B., VanOverbeke, D.L., Hilton, G.G., Wahrmund, J.L., Holland, B.P., Krehbiel, C.R., Camfield, P.K., & Richards, C.J. 2012. Vitamin E supplementation in beef finishing diets containing 35% wet distillers grains with solubles: Feedlot performance and carcass characteristics. Journal of Animal Science, 90(4), 1349–1355. doi: 10.2527/jas.2011-3833.
4) Cavina, G., Gallinella, B., Porrà, R., Pecora, P., & Suraci, C. 1988. Carotenoids, retinoids and alpha-tocopherol in human serum: Identification and determination by reversed-phase HPLC. Journal of Pharmaceutical and Biomedical Analysis, 6(3), 259–269. doi: 10.1016/0731-7085(88)80052-9.
5) Cinquanta, L., Fontana, D.E., & Bizzaro, N. 2017. Chemiluminescent immunoassay technology: what does it change in autoantibody detection? Autoimmunity Highlights 8(1). doi: 10.1007/s13317-017-0097-2.
6) Dillon, P.G. 2018. The evolution of grassland in the European Union in terms of utilisation, productivity, food security and the importance of adoption of technical innovations in increasing sustainability of pasture-based ruminant production systems. In Sustainable meat and milk production from grasslands : proceedings of the 27th General Meeting of the European Grassland Federation, Cork, Ireland, 17-21 June 2018 (Conference, pp. 3–16). Wageningen Wageningen Academic Publishers.
7) Hammer-Plecas, A., & Woollard, G.A. 2010. Simultaneous measurement of retinol, tocopherol and β-carotene in serum by reversed phase HPLC: Consideration of chromatographic conditions. Clinica Chimica Acta, 411(11–12), 912–913. doi: 10.1016/j.cca.2010.02.054.
8) Hidiroglou, M., Batra, T.R., Laflamme, L.F., & Markham, F. 1992. Possible roles of vitamin E in immune response of calves. International Journal for Vitamin and Nutrition Research, 62(4), 308–11.
9) Hino, T., Andoh, N., & Ohgi, H. 1993. Effects of β-carotene and α-tocopherol on rumen bacteria in the utilization of long-chain fatty acids and cellulose. Journal of Dairy Science, 76(2), 600–605. doi: 10.3168/jds.S0022-0302(93)77380-4.
10) Khan, A., Khan, M.I., Iqbal, Z., Shah, Y., Ahmad, L., & Watson, D.G. 2010. An optimized and validated RP-HPLC/UV detection method for simultaneous determination of all-trans-Retinol (Vitamin A) and α-Tocopherol (Vitamin E) in human serum: Comparison of different particulate reversed-phase HPLC columns. Journal of Chromatography B, 878(25), 2339–2347. doi: 10.1016/j.jchromb.2010.07.009.
11) Mary, A.E.P., Artavia Mora, J.I., Ronda Borzone, P.A., Richards, S.E., & Kies, A.K. 2021. Vitamin E and beta-carotene status of dairy cows: a survey of plasma levels and supplementation practices. Animal, 15(8), 100303. doi: 10.1016/j.animal.2021.100303.
12) McDowell, L.R., Williams, S.N., Hidiroglou, N., Njeru, C.A., Hill, G.M., Ochoa, L., & Wilkinson, N.S. 1996. Vitamin E supplementation for the ruminant. Animal Feed Science and Technology, 60(3–4), 273–296. doi: 10.1016/0377-8401(96)00982-0.
13) Merkulova, I.N., & Zueva, E.I. 2015. Prospects of development of milk cattle husbandry on the basis of innovative technologies in feed production. Agrarian Scientific Journal, 5, 90–94.
14) Nutrient Requirements of Dairy Cattle. 2001. National Academies Press. doi: 10.17226/9825.
15) Pehrson, B., & Hakkarainen, J. 1986. Vitamin E status of healthy Swedish cattle. Acta Veterinaria Scandinavica, 27(3), 351–360. doi: 10.1186/BF03548149.
16) Shabunin, S.V., Belyaev, V.I., & Papin, N.E. 2014. Gipovitaminozy cattle, prevention and treatment. Veterinary Medicine, Zootechnics and Biotechnology, 6, 19–27. https://www.elibrary.ru/item.asp?id=21974703.
17) Secrist, D.S., Owens, F.N., Gill, D.R., Boyd, L.J., & Oldfield, J.E. 1997. Effects of vitamin E on performance of feedlot cattle: A review. Professional Animal Scientist, 13(2), 47–54. doi: 10.15232/S1080-7446(15)31844-1.
18) Urbánek, L., Solichová, D., Melichar, B., Dvořák, J., Svobodová, I., & Solich, P. 2006. Optimization and validation of a high performance liquid chromatography method for the simultaneous determination of vitamins A and E in human serum using monolithic column and diode-array detection. Analytica Chimica Acta, 573–574,267–72. doi: 10.1016/j.aca.2006.02.032.