

The influence of haemolysis on the determination of vitamin E in cattle

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Abstract

The study deals with the influence of haemolysis on the results of vitamin E determination in plasma (serum) in cattle. Although nowadays specific High Performance Liquid Chromatography (HPLC) techniques are used almost exclusively for the determination of vitamin E, this indicator is also influenced by haemolysis. This occurs in the pre-analytical phase due to the fact that iron contained in haemoglobin is able to catalyze peroxidation reactions. Subsequently, changes occur mainly in polyunsaturated fatty acids in the lipoprotein components of serum/plasma. The vitamin E present inhibits this process, as a result of which its concentration is reduced. The experiment was performed by preparing model samples with a defined degree of haemolysis by adding haemolysate to the centrifuged plasma in the range of ca 0–12 g/l, i.e. mild to severe haemolysis. After 4 h of incubation at room temperature, the vitamin E concentration was determined by the HPLC method. Haemolysis was found to reduce the test result; mild one (approximately up to 2 g/l) non-significantly, medium and severe haemolysis by up to tens of percent, which warrants repeated sampling. False reductions in results will not endanger the patient's health, but economic losses may occur due to unnecessary check-ups and increased vitamin E supplementation.

Tocopherol, HPLC, lipid peroxidation

Vitamin E (alpha-tocopherol) is one of the substances of considerable physiological importance in animals and therefore its determination is carried out as one of the frequent laboratory analyses. One of the factors that may affect the result of this assay is haemolysis, despite the fact that vitamin E is now almost exclusively analyzed by specific High Performance Liquid Chromatography (HPLC) techniques.

Haemolysis is a phenomenon that always occurs in a certain percentage of cases. It can be caused by the breakdown of erythrocytes *in vivo* or more often by an inappropriate method of sampling or due to incorrect or long-term storage of the collected blood samples before centrifugation. Depending on the degree of haemolysis, a number of indicators are subsequently affected. In human laboratory medicine, the approximate percentage of haemolytic samples is reported to be about 3% (Lippi et al. 2008), although this value is highly variable. There are probably no similar statistics available in veterinary medicine for the time being, but it can be assumed that it will be significantly higher. In this case, the patient is often non-cooperating; the sampling is usually performed in the field, frequently under adverse climatic conditions, often followed by several hours of transport to the laboratory. These circumstances significantly increase the likelihood of haemolysis.

The effect of haemolysis on the routine laboratory analysis has been relatively well studied. In principle, several mechanisms are applied – an increase in the indicator results due to significantly higher analyte concentration in erythrocytes, such as potassium, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), etc., or the intervention of some substances in the reaction mechanism of the analytical method (usually decreasing the result). The colour of haemoglobin also modifies the absorbance of the measured sample (usually increasing the result). In practice, these mechanisms are often applied

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together, creating a complex combination of influences on the final result. However, in the case of influencing the result in the determination of vitamin E, a different mechanism applies. After releasing intracellular haemoglobin into plasma, free radicals are formed and peroxidation reactions are initiated (e.g. Winterbourn 1990). In particular, the double bonds of unsaturated fatty acids contained in plasma lipoprotein complexes are attacked. Vitamin E as an antioxidant interferes with this process and chemical changes occur in its molecule. A number of intermediates of a radical nature (e.g. tocopheroxyl radical) and oxygen products of lipoperoxidation (e.g. Yamauchi 1997) are formed. As a result, the concentration of vitamin E decreases. These phenomena have already been studied, for example, in the storage of pig sera and liver analysis (Hooser et al. 2000).

The aim of our work was to assess the effect of different degrees of haemolysis on the results of vitamin E determination in cattle.

Materials and Methods

The haemolysate was prepared in the standard manner: blood collected in heparin was centrifuged and then plasma was collected. Erythrocytes were gently washed three times in saline and the same amount of distilled water was added to the washed erythrocyte suspension. The sample was shaken and frozen at -20°C . Haemoglobin concentration was determined on a BC-2800Vet haematology analyser (Mindray, China). Model samples were prepared by adding the haemolysate to non-haemolytic plasma at the amounts of 2, 5, 10 and 20 $\mu\text{l}/200 \mu\text{l}$ of plasma, and the current haemoglobin concentration was calculated from the dilution.

Pyrogallol (Sigma-Aldrich, Darmstadt, Germany) was dissolved in water and added to the analysed sample to a final concentration of about 50 mmol/l.

Lipoperoxidation was performed after the addition of haemoglobin to plasma for 4 h at room temperature with occasional shaking.

Vitamin E was determined by HPLC system Ultimate 3000 (Dionex, Sunnyvale, USA) according to Sowell et al. (1994) with minor modifications. Briefly, after deproteination with ethanol and extraction into hexane, the organic layer was collected, dried and dissolved in the mobile phase. The Ultimate 3000 HPLC system was used. Column: Acclaim 120, C18 3 μm , $4.6 \times 100 \text{ mm}$, mobile phase – methanol, flow – 1.0 ml/min, isocratic mode, temperature – 30°C , injection – 20 μl , fluorimetric detection $\lambda_{\text{ex}} = 292 \text{ nm}$, $\lambda_{\text{emis}} = 325 \text{ nm}$. Lyophilized serum for vitamin A and E determination was used as a calibrator: ClinCal-Calibrator (RECIPE, Munich, Germany).

The concentration of malondialdehyde (MDA) in the samples was determined according to Mateos et al. (2005) with minor modifications.

The used statistical parameters - mean, standard deviation, line equation, coefficient of determination and value of statistical significance ($P < 0.05$ was considered as significant) were calculated in the usual way using the statistical program Excel 2010.

The analysed plasma was obtained at the Ruminant and Swine Clinic, University of Veterinary Sciences Brno, or on nearby farms as part of diagnostic (5 cows with an acute inflammation) and preventive (28 healthy cows) examinations during the year 2020.

Results

The main results of our experiment are presented in Fig. 1. It is apparent that an increase in haemoglobin in the sample resulted in a decrease in vitamin E concentration. We chose the linear dependence, mainly for the reasons of simplicity. It is possible that if using a different mathematical model, the statistical parameters would be more favourable, on the other hand, a more complicated model would be thus created, which was not our intention. The not very high value of the coefficient of determination ($R^2 = 0.7719$) indicates that this dependence is also influenced by other factors. However, the result is highly significant ($P < 0.001$) and this is an important conclusion of our experiment.

Fig. 2 shows the time course of the decrease in vitamin E concentration in the described system for two different haemoglobin concentrations. It is obvious that a significant decrease in vitamin E occurs mainly within the first hours, whereas further decrease more or less stagnates.

Fig. 3 shows the increase in the MDA concentration in the system, which can be considered as a confirmation of the ongoing lipoperoxidation reactions.

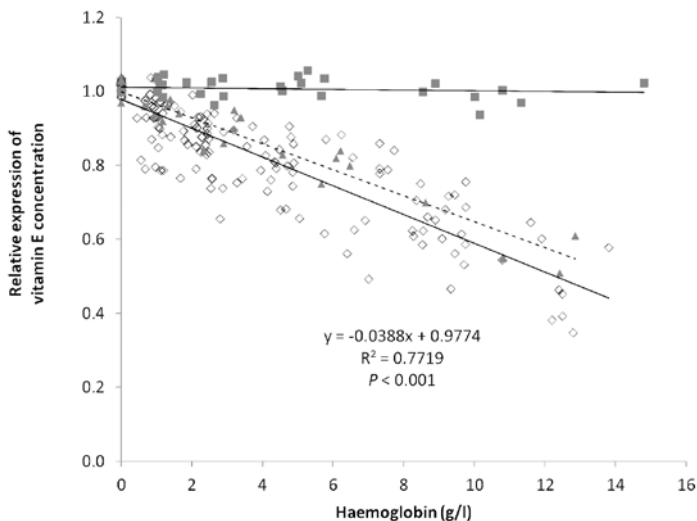


Fig. 1. Relative decrease of vitamin E concentration depending on the degree of haemolysis of the sample. Line equation, coefficient of determination (R^2) and statistical significance of correlation (P) belong to healthy animals (—), dashed line belongs to animals with an acute inflammation (-----).
 ◇ Samples of healthy animals; ■ Samples with the addition of pyrogallol; ▲ Samples of animals with acute inflammation

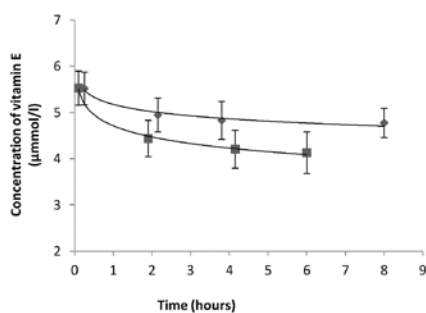


Fig. 2. Time dependence of the decrease in vitamin E concentration for sample with different haemoglobin concentration (mean \pm standard deviation, $n = 3$ for each point).

◆ Haemoglobin = 3.65 g/l (mild haemolysis); ■ Haemoglobin = 8.23 g/l (strong haemolysis)

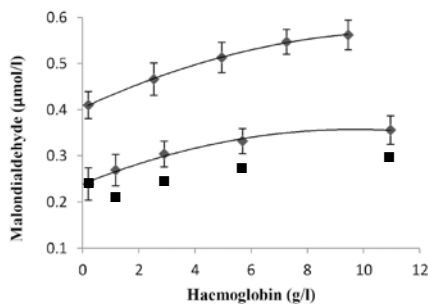


Fig. 3. Dependence of the increase in malondialdehyde concentration on the haemoglobin content for two different samples (measured after 72 hours, mean \pm standard deviation, $n = 3$ for each point).

◆ Sample 1; ■ Sample 2

Discussion

Determining the exact level of vitamin E in cattle is important because this vitamin contributes, in addition to non-specific antioxidant effects (Yamauchi 1997; Píšťková et al. 2019; Mikulková et al. 2020), also to immune functions (Eicher-Pruiett et al. 1992; Eicher et al. 1994; Otomaru et al. 2015) and their deficiency can manifest itself in the form of impaired health condition (Kume and Toharmat 2001; Torsein et al. 2011).

It is clear from Fig. 1 that a concentration of haemoglobin of up to about 2–3 g/l, i.e. mild haemolysis, has in most cases only a small effect on the result. In the case of moderate and severe haemolysis, the modification of the result is significant and such finding warrants a new sampling. We also performed the experiment on samples of patients with ongoing acute inflammation, which were delivered to our laboratory by field veterinarians. The equation of the regression line in this case is $y = -0.0351x + 0.9987$ ($R^2 = 0.9182$), i.e. very similar to that obtained from healthy animals. Therefore, this condition does not significantly affect the result. Perhaps prolonged or chronic inflammation, when the antioxidant stores have already decreased or been depleted, could perhaps have a greater effect on vitamin E loss.

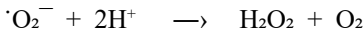
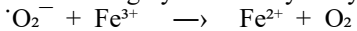
The addition of pyrogallol (a substance with antioxidant properties) to the reaction mixture confirms that the decrease in vitamin E is related to peroxidation reactions, as in this case increasing haemoglobin concentration had no effect on the decrease in vitamin E (in a square of Fig. 1). Nowadays, pyrogallol is often used in the industry to protect a variety of substances from harmful peroxidation (e.g., Cynthia et al. 2018).

The coefficient of determination has the value $R^2 = 0.7719$, i.e. not too high. One of the reasons for the variability of the results may be the relatively low homogeneity of the analyzed set. Samples were taken throughout the year on various farms and at the Clinic of Ruminant Diseases. This may explain the lower homogeneity of the examined file. On the other hand, this inhomogeneity represents a certain advantage, namely that the results may have a more universal practical application.

Another reason is the complexity of the lipid peroxidation process itself. It is a process that can be compared to a classic fire – it depends on (1) the flammability of the material, (2) the number of fires and (3) the effectiveness of extinguishing agents.

(1) It is a known fact that most polyunsaturated fatty acids (e.g. arachidonic or eicosapentaenoic) forming biological membranes and lipoprotein components of blood plasma are subject to peroxidation. The percentage of these acids in membranes and lipoproteins is variable and depends to a large extent on the composition of the feed mixture. For instance Monticelli et al. (1992) state how diets with different compositions affect the presence of fatty acids in erythrocyte membranes and subsequent changes in the physico-chemical behaviour of these membranes. Similarly, plasma membranes and lipoproteins with a higher proportion of unsaturated fatty acids will be more prone to lipoperoxidation and thus a greater “consumption” of vitamin E will occur.

(2) In aerobic organisms in the process of respiration there is always the formation of a certain amount of reactive oxygen species (1–2%) (Bergendi 1988). Those in the presence of transition valence metals (mainly Fe and Cu) can react by a series of reactions to form the highly reactive hydroxy radical $\cdot\text{OH}$.



It is able to react with methylene carbon of the double bonds of unsaturated fatty acids and start the lipoperoxidation chain. The last reaction (Fenton's reaction) was studied in great detail and it was found that the presence of iron can be free, in the low molecular weight chelate but also, which is important, as a haemoprotein (Winterbourn 1990). Sadrzadeh et al. (1984) have shown that haemoglobin can catalyse the formation of

a hydroxy radical and the mechanism of its formation is similar to the Fenton's reaction. In experiments *in vitro*, they showed that free haemoglobin accelerates the peroxidation of arachidonic acid, which is an important component of biological membranes. Therefore, the presence of transition metals (especially Fe and Cu), which are present in high (but in individual cases different) concentrations in erythrocytes, is another variable factor that can affect the intensity of peroxidation.

(3) The organism has a number of mechanisms to defend against the effects of free radicals. They can be divided into high molecular weight (superoxide dismutase, glutathione peroxidase, catalase, etc.) and low molecular weight (vitamin C, glutathione, carotenoids, Se, etc.). The second category also includes vitamin E. Due to its structure, its hydrophobic part is "immersed" in the membrane (or other hydrophobic structures) and the hydrophilic chromate ring is on the surface where it can interact with other antioxidants (mainly vitamin C) and thus contribute to its regeneration. Sufficient antioxidant capacity of the organism significantly suppresses the effects of free radicals and protects the organism from a number of pathological conditions (e.g. Konvičná et al. 2015) and we believe that this is the parameter that probably influenced the most the results of our experiment.

Lipoperoxidation is also affected by cholesterol levels in the system (Balgavý et al. 2001). With its flat hydrophobic molecule, it is inserted into the membrane and thus acts as an effective barrier in propagating the radical chain reaction. In addition, it increases the order of the lipid bilayer, which results in lower oxygen solubility in the lipid bilayer and thus the formation of fewer reactive oxygen radicals (Filipek et al. 2001). However, the role of cholesterol in the process of lipoperoxidation is much more complex; it can affect the activity of enzyme mechanisms, under certain conditions it can also have a prooxidative effect, however, this issue is still being studied intensively (Girotti and Korytowski 2019). In any case, this is another factor that can affect the lipoperoxidation process.

The result is also influenced by the fact that the plasma concentration of vitamin E is not identical with the concentration inside the erythrocytes. And mixing these two components together will change the total concentration. Since the concentration of vitamin E in erythrocytes is lower than in plasma, haemolysis essentially has the effect of diluting the sample. Considerable attention is paid to this issue, especially in human medicine (e.g. Simon et al. 1997; Sotomayor et al. 2019). The experiment we performed was designed so that the volume changes were up to 10%, so it is a factor that can be taken into account, but it certainly does not have a significant effect in this case.

All the mentioned factors (and undoubtedly others too) interfere with the described mechanism. And since the level of all these factors is variable, their mutual combination can create conditions that contribute to a relatively large variance of the measured results. It should be added that none of the discussed indices (feed ration composition, fatty acid composition, antioxidant capacity indicators, transition metal concentration, cholesterol, etc.) was monitored in our work. We are of the opinion that a single indicator cannot explain the complexity of the whole problem, otherwise a huge amount of data would be created, the interpretation of which would be complex and the whole experiment would be disproportionately complicated and expensive. Our goal was to evaluate the effect of haemolysis on the concentration of vitamin E for practical laboratory purposes and, in our opinion, the experiments performed and the conclusions based on them fulfill this purpose.

The experiment shown in Fig. 2 supports the fact that our chosen peroxidation time of 4 h is suitable. A significant decrease in vitamin E occurs mainly during the first 2 h or so. Although with the increasing time there is a further decrease, but less significant, and it is clear that the decisive factor in this case is not the time but the concentration of haemoglobin in the sample. It represents a kind of initial ignition and with the increasing time of peroxidation in the given system it more or less stagnates. By supplying another impulse to the system (an increase in temperature, an increase in the amount of oxygen, the

concentration of the transition metal etc.), the peroxidation reaction would probably start again, but this would already take place outside normal laboratory conditions. There is a number of other interesting experiments that could certainly be performed on this topic.

Figure 3 confirms the idea that the loss of vitamin E causes peroxidation reactions – in the system the concentration of MDA as the final product of lipoperoxidation increases over time. Of course, other oxygenated products are formed (e.g. 4-hydroxynonenal, 6-hexanal), which are more reactive and the determination of which is sometimes preferred today, however, for our purposes the determination of MDA is fully sufficient. Even in this field, it would certainly be possible to carry out further experiments. As in the previous case (Fig. 2), we did not perform a more detailed statistical evaluation, partly because the conclusions are obvious and these experiments were rather supportive.

In conclusion, haemolysis causes a false reduction in the results of the vitamin E determination. However, in this case there is no risk of neglecting the deficiency of this important substance, but economic losses can occur. The reason is an unjustified increase in the dosage of vitamin supplements (dietary or parenteral) or unnecessary repeated sampling.

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