The Quantification of Menadione as Nutritional Supplement in Feedingstuffs

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Abstract

Menadione (2-Methyl-1, 4-naphthoquinone) also known as vitamin K3, is the synthetic form of vitamins K. Although it has a weak biological activity, menadione can be converted into a more active form in the presence of gut bacteria. It is widely used in medicine as an anti-hemorrhagic agent and also as nutritional supplement in feedingstuffs for animals.

The purpose of the present study is to quantify the menadione sodium bisulfite in feedingstuffs via volumetric and spectrophotometric analyses.

The analyses carried out on feedingstuff samples for chickens and dogs revealed concentrations of menadione sodium bisulfite of 0.5960 mg/kg and 1.424 mg/kg, respectively.

The difference between the results obtained using the two parallel methods did not exceed 6%.

Keywords: menadione sodium bisulfite, feedingstuffs, volumetric analysis, spectrophotometric method, premix vitamin-minerals

1. Introduction

Lately, the quality of feedingstuffs has generated an increased interest mainly due to their importance in providing the necessary supplements (vitamins and minerals) for animals. The absence of vitamins and minerals in animal foods causes adverse effects which affect the health of the animal. Therefore, the quality of feedingstuffs needs to be closely monitored and surveyed.

Vitamin K plays a physiological role being a cofactor in the synthesis of blood clotting. It has been reported to be involved in the process of osteocalcine and bone formation as well as in other biomedical functions (SARIN S. K., et al., 2006 [1]).

Vitamin K3, 2-methyl-1,4-naphthoquinone (menadione sodium bisulfite) is an effective clinical drug which can be used to cure vitamin K deficiency. Vitamin K3 has been the subject of many researches focused on demonstrating its anticancer potential (LAMSON, D.W., PLAZA, S.M. 2003, [2]).

Substances with vitamin K activity can be supplied to the animal from a combination of vitamins sources such as K1, K2 and K3. Vitamin K1, also known as phylloquinone, is found in green leafy vegetables and vegetable oils. Vitamin K2 is produced by gut bacteria while vitamin K3 is chemically synthesized. Vitamins K1 and K2 are "active" upon absorption.

Vitamin K3 must be "alkylated" by gut bacteria or tissue enzymes to become active (SHEARER, M.J. 2000, [3]). Animals can utilize either the active forms that are supplied in...
their diet, or they can make vitamin K2 from vitamin K3. Animals cannot produce their own Vitamin K3, therefore it must be provided through diet.

Menadione is important for certain species of animals - dogs, poultry, pigs and aquatic animals - which are not able to produce vitamin K3 during metabolism as a result of their short digestive tract and the fast rate of food passage.

Menadione can be used as a vitamin K supplement in pet foods since it is more stable and tolerates heating better than other forms of vitamin K. Experiments demonstrated that the menadione bisulfite is a nutritional supplement for the prevention of vitamin K deficiency if added up to a level which cannot exceed 2 g per ton of complete feed for chicken and turkey feeds and in the feed of growing and finishing swine up to a level which cannot exceed 10 g per ton of complete feed (PADMAKUMAR B. et al., 2008, [4]).

The menadione sodium bisulfite complex is the only form of vitamin K3 accepted by the Food and Drug Administration to be used as source of vitamin K in pet foods.

Many companies manufacturing pet foods make sure to provide the analysis report which accompanies the released product. Yet, in the analysis are often listed substances which are present in the product without further percentage information regarding the amount.

The feedingstuff for dogs analysed for the present study is the Purina Dog Chow Junior Large Breed product which contains menadione as menadione sodium bisulfate complex. The review bulletin of the product does not specify the quantity of vitamin K3. Experiments were also carried out on feedingstuffs for chickens (chicken feed SC Zoovet SRL Slobozia, Ro) such as chicken concentrate with 10% PVM.

This paper presents the results of quantitative determination of menadione sodium bisulfate complex in feedingstuffs using the volumetric and spectrophotometric methods.

2 Experimental part

Reagents, equipment and methods

Vitamin K3 was purchased from pharmacies and feedingstuffs from pet shops. All reagents used were analytically pure.

The reagents in colorimetric method were the following: 96 % (v/v) ethanol, 1,2-dichloroethane a.p., 10 % (w/v) solution of anhydrous sodium carbonate a.p., 37 % (w/v) hydrochloric acid (d = 1.19g/cm³), 25 % (w/v) ammonia (d = 0.91g/cm³), ammoniacal ethanol.

The preparation of 2,4-Dinitrophenylhydrazine reagent:

40 mg 2,4-dinitrophenylhydrazine a.p. are dissolved in about 40 mL boiling absolute ethanol, allowed to cool and then transferred into a 50 mL volumetric flask. 1 mL hydrochloric acid is added and absolute ethanol is used to fill to the mark. The reagent has to be prepared right before use.

Standard menadione solution preparation:

20 mg menadione (vitamin K3) are dissolved in 1,2-dichloroethane and to fill-to-line up to 200 mL volumetric flask. The aliquots of this stock solution are diluted with 1,2-dichloroethane to obtain a series of solutions with menadione concentrations varying between 5 and 30 µg per mL. These solutions have been freshly prepared.

The qualitative colorimetric reaction between 2-methyl 1,4-naphthoquinone and 2,4-dinitrophenylhydrazine described by (NOVELLI, A., 1941, [5]), was carried out by means of a quantitative procedure designed to measure menadione alone or when admixed in the preparation of feedingstuffs.
0.1 mL of 5% Na bisulphite and 0.5 mL of dinitrophenylhydrazine HCl 2N are added to an alcoholic solution, containing 10-900 µg menadione.

The solution is heated in boiling water for 20-30 minute then ethyl alcohol is added to replace the losses resulted from evaporation and then it is left to vaporize nearly to dryness. 1-2 mL 95% ethanol and 1.6 mL NH₄OH 2N are added to the residual red precipitate which is afterwards shaked and then heated in a water bath for 1 min.

The characteristic green colour will result attaining its maximum intensity in about 10 minutes after the removal of the tube from the water bath.

It follows a dilution of the product with alcohol 50% to 10 mL. The optical density of the blue-green coloured complex is measured at 635 nm.

This method is sensitive up to 10 µg menadione. The colour obtained is proportional with the menadione concentration in the 10 µg-2000 µg range.

**Extraction**

An important part of the experiment is the active substance extraction procedure from raw material 10 mL H₂SO₄ 1M solution are added to the test sample (1g) which is afterwards shaken for 10 minutes. Further on, exactly 50 mL diluted ethanol are added to the solution which is then shaken mechanically for 15 minutes at room temperature. The extract resulted is transferred into centrifuge tubes and centrifuged at 3000 - 5000 r.p.m.

2 mL extract, accurately measured, are placed in a 25 mL separator funnel to which are added 5 mL 1,2-dichloroethane mix and 2 mL sodium carbonate solution. The solution is vigorously shaken for 30 seconds and then the 1,2-dichloroethane phase is collected in a 10 mL separator funnel. 2 mL water are added, followed by 15 seconds shake. The dichloroethane phase is collected and the traces of water are removed with strips of filter paper. All dichlorethane phases have been placed into volumetric flasks of 10 mL and kept in dark. For the concentrate and premixed samples, an aliquot part of the extract was diluted with 1,2-dichloroethane to obtain a menadione concentration of 2 to 10 µg per mL. For feedingsuffs, an aliquot part of the extract was evaporated to dryness under reduced pressure in nitrogen atmosphere, on water bath at 40ºC. The residue was rapidly treated with 1,2-dichloroethane to obtain a solution containing 2 to 10 µg menadione per mL.

**Hydrazone formation**

2.0 mL dichloroethane extract are transferred in a 10 mL volumetric flask to which are added 3.0 mL 2,4-dinitrophenylhydrazine reagent. The flask is safely closed in order to prevent the evaporation since it will undergo heating for two hours at 70 ºC on water bath. The extract is allowed to cool, and then are added 3.0 mL ammoniacal ethanol, the solution is mixed, absolute ethanol is added to fill to the mark and then the solution is mixed again.

**Optical density measurement**

The optical density of the blue-green coloured complex was measured with a spectrophotometer at 635 nm and compared with a blank reagent. The quantity of menadione was determined through reference to calibration curves established for each series of analyses.

**Calibration curve**

2.0 mL menadione standard solution was treated with 2,4-dinitrophenylhydrazine as described above. The optical density was measured and the calibration curve was plotted with
the optical density values as ordinate and the corresponding quantities of menadione in mg/mL as abscissa (NAGARAJA, P., et al., 2002, [6], SASTRY, C.S.P, et al., 1987, [7]). (see Fig. 3)

The menadione content of the sample was calculated taking into account the weight of the test sample and of the dilutions carried out during analysis.

The final results were expressed in mg of menadione per kg of sample. Measurements of pH levels were carried out with the Consort C-862multiparameter. Spectra were recorded and processed with the Spectro UV-VIS Double Beam PC 8 Auto Scanning Cell UVD-3200 spectrophotometer, Lobomed, INC.

### Volumetric method

The second method used for the quantitative determination of menadione was the volumetric method which consists in the titration of the menadione sample and iodine solution mixture with sodium thiosulphate following the protocol described in FR VIII (FR VIII, 1965, [8]).

The reagents used for this method were methyl alcohol, Zn powder, H2SO4, iodine 0.1N, sodium hydrocarbonate, acetic acid, starch as indicator and sodium thiosulphate 0.1N. All reagents were analytically pure. A micro burette was used for the volumetric determination.

The quantity of 0.00861g menadione corresponds to 1 mL of iodine 0.1N.


### 3. Results and discussions

Simple and accurate quantitative analyses (spectrophotometric and volumetric methods) were applied in order to evaluate the amount of menadione sodium bisulfite in feedingstuffs.

### Spectrophotometric method

Following the scheme of reaction (figure 1), menadione reacts with 2,4-dinitrophenylhydrazine, in specific conditions, developing a green colour:

![Figure 1. The scheme of reaction between menadione and 2,4-dinitrophenylhydrazine](image)

The osazone formed between menadione and 2,4-dinitrophenylhydrazine has a maximum of absorption (A=1.316) at λ = 639 nm (figure 2).
Figure 2. Absorbtion VIS spectra of osazone formed between menadione and 2,4-dinitrophenylhydrazine

A calibration curve was realized (figure 3) taking into account the maximum absorption of menadione-osazone coloured compound at 639 nm.

![Graph of calibration curve with equation and R^2 value](image)

Figure 3. Calibration curve of osazone derivate from menadione

The results obtained using the spectrophotometric method intended to measure the concentration of menadione in feedingstuffs for chickens and dogs are presented in table 1.

<table>
<thead>
<tr>
<th>Feedstuff sample</th>
<th>Absorbance (λ=635 nm)</th>
<th>Average of absorbance (λ=635 nm)</th>
<th>Menadione concentration µg/mL</th>
<th>Menadione mg/kg sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purina Dog Chow feed</td>
<td>0.085</td>
<td>0.065</td>
<td>0.075±0.010</td>
<td>2.7898</td>
</tr>
<tr>
<td>Chickens feed with 10% PVM*</td>
<td>0.033</td>
<td>0.035</td>
<td>0.033±0.002</td>
<td>1.1780</td>
</tr>
</tbody>
</table>

*PVM-Premix Vitamin-Mineral

The determinations were made in triplicate in all experiments. The feedingstuffs for chickens (chicken feed SC Zoovet SRL Slobozia) were achieved from pet shops under the form of chicken concentrate with 10% PVM.

The premix contains menadione supplement (equivalent with menadione sodium bisulphate complex) but the label on the product does not specify the concentration value of menadione.
The FDA recommendation regarding the concentration of menadione sodium bisulphate in dry food for dogs ranges between 0.97mg/kg and 1.32mg/kg. Also, the amount of vitamin K3 recommended by NRC "Nutrient Requirements of Dogs" for dogs and cats is approximately 1 part per million of diet (BALDWIN, K., 2010, [12]).

The results regarding the menadione concentration in feedingstuffs for dogs (Purina Dog Chow Junior Large Breed) fit the range of recommended values. 1.3949mg menadione per kilogram Purina Dog Chow Junior Large Breed dry food represents a value close to the maximum FDA recommended value, thus being considered acceptable for a healthy metabolism of dogs.

The amount of menadione determined through experiments in chickens feed with 10% PVM is of 0.5890mg/kg, being under the recommended FDA limit value. Generally the concentration of menadione in feedingstuffs is related to the weight of the animal.

**Volumetric method**

The volumetric method consists in the titration of the menadione sample and iodine solution mixture with sodium thiosulfate according to FR VIII.

Quinone/hydroquinone represents a complex redox system which involves two electrons and two protons in its redox reaction (fig.4):

![Figure 4. The redox system of quinone/hydroquinone](image)

Menadione has a quinonic structure. In a reducing medium (Zn and H2SO4) menadione is immediately reduced to hydroquinone (figure 4).

Hydroquinone is weakly acidic so it may be a reducing factor in a reaction with oxidizing substances. The iodine solution is an oxidizing agent in a weak basic medium such as NaHCO3 solution.

This behaviour of menadione allowed applying the iodometric method in order to perform the quantitative analyse of menadione.

The menadione hydroquinone has a weak affinity for reduction therefore it can be easily oxidized by an iodine solution and transformed in a quinonique form.

Used in accurately measured quantities at the beginning of the reaction, iodine reacts with menadione-hydroquinone and the excess of iodine will react with the sodium thiosulfate as follows:

![sodium thiosulphate](image) → sodium tetrathionate
The quantity of iodine reacting with menadione represents the difference between the initially measured iodine and the iodine reacting with the thiosulfate. The stoichiometry of the reaction between menadione and iodine allows the calculation of the quantity of menadione occurring in the experimental sample. For 1 mL iodine solution 0.1N correspond 0.00861 g menadione.

The experiments were carried out on the same extracts obtained from feedingstuffs for dogs and chickens that were used for the spectrophotometric method.

The results obtained after applying the volumetric method for feedingstuffs are expressed in figure 5. The amounts of menadione obtained using both methods (volumetric and spectrophotometric methods) are compared (table 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quantity of menadione (mg/kg)</th>
<th>Average</th>
<th>Difference</th>
<th>STDEV</th>
<th>CV*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volumetric method</td>
<td>Spectrophotometric method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs feed</td>
<td>1.4530</td>
<td>1.3949</td>
<td>1.4240</td>
<td>0.0581</td>
<td>0.041</td>
</tr>
<tr>
<td>Chickens feed</td>
<td>0.6030</td>
<td>0.589</td>
<td>0.5960</td>
<td>0.0140</td>
<td>0.010</td>
</tr>
</tbody>
</table>

*CV=coefficient of variation

The variation coefficient of the two methods used to determine menadione is small, 2.89% in the case of dogs feed and 1.66% for chickens feed, meaning that both methods (spectrophotometric and volumetric methods) can be successfully applied in the laboratory.

**Conclusion**

The colorimetric and volumetric methods allowed the quantification of K₃ vitamin in feedingstuffs with different complexities.

The difference between the results of the two parallel determinations (colorimetric and volumetric methods carried out on the same sample) did not exceed 6%, in relative value, for menadione contents lower than 10 ppm.

This study provides variants of analysis easy to apply and also financially advantageous in order to quantify menadione in feedingstuffs. Once established the protocol for processing of analyzed samples for dosage menadione, volumetric or spectrophotometric methods of
analysis presented in this paper can be successfully applied in quality control laboratories accredited for feedingstuffs.

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