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FLUORIMETRIC DETERMINATION OF MENADIONE SODIUM BISULPHITE (VITAMIN K_3) IN ANIMAL FEED AND PREMIXES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN DERIVATIZATION

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for the determination of menadione sodium bisulphite (vitamin K_3) in animal feed and premixes is described. After aqueous extraction, the vitamin is converted into menadione, which is extracted and separated on a reversed-phase HPLC column. After menadione has been reduced in a post-column reaction coil, fluorimetric measurement of the reduced vitamin permits the determination of menadione sodium bisulphite in animal feed and premixes at concentrations as low as 0.02 μg/g.

Several types of animal feed have been analysed according to the method described and also with the European Community colorimetric method. From high concentration levels down to *ca.* 15 μg/g the results obtained with the two methods are in good agreement. For concentrations in the range 1–3 μg/g (complete diets) a tendency to lower results by the HPLC method is observed. The detection limit of the HPLC method is considerably lower than that of the European Community colorimetric method. The within-assay coefficient of variation of the method applied to feeds is 6.0%. The within-assay analytical recovery of menadione sodium bisulphite added to feeds is 94.4 ± 6.8% (mean ± S.D.).

INTRODUCTION

The general term vitamin K is used to indicate 2-methyl-1,4-naphthoquinone (menadione, vitamin K_3) and its derivatives. Vitamin K plays an important role in blood coagulation and bone mineralization[1]. The two naturally occurring and biologically most important forms are phylloquinone (vitamin K_1) from plant origin and the menaquinones (vitamin K_2 series) synthesized by bacteria. The significance of vitamin K has often been studied with animals, especially poultry, where deficiencies are likely to occur[2]. For this reason and because the biologically active menaquinone-4 can be formed *in vivo* from menadione[3,4], the latter compound is added to animal feed. To improve its stability and absorption efficiency the water-soluble derivative menadione sodium bisulphite is often used.

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Numerous methods for the determination of menadione sodium bisulphite in feeds have been published, *e.g.*, gas–liquid chromatographic (GLC)[5,6], colorimetric[7,8] and HPLC methods[9,10]. However, most of them have one or more drawbacks. GLC methods are not sufficiently sensitive to determine levels below *ca.* 4 μg/g. The official colorimetric method of the European Community (EC) for the determination of menadione sodium bisulphite in feed[7] includes a time-consuming colour reaction. Further, at low levels (1–3 μg/g) the colorimetric measurement is often subject to interference by sample components. The sensitivity of the HPLC method described by Ranfft and Rückemann[9] only permits determinations in premixes and supplements. The detection limit of the HPLC method with UV detection described by Manz and Maurer[10] is 0.5 μg/g. However, we observed that UV detection of the menadione peak of complete diet samples with concentrations below *ca.* 2 μg/g is often subject to interference by sample components.

Abe *et al.*[11] described an HPLC method with post-column reaction fluorimetry for the determination of phylloquinone and menaquinone-4 in biological materials. These non-fluorescent naphthoquinones were reduced to their fluorescent dihydroxynaphthalenes in a post-column reaction coil.

Lack of specificity of the UV detection of menadione led us to develop an HPLC method with post-column reaction fluorimetric detection. This detection system is a modification of that of Abe *et al.*[11], extended with debubbling of the reagent and air segmentation, which are actually improvements.

After aqueous extraction, menadione sodium bisulphite is converted into menadione. The latter compound is extracted with *n*-hexane and separated on a reversed-phase HPLC column. Reduction of menadione to 2-methyl-1,4-dihydroxynaphthalene by a post-column reaction followed by fluorimetric detection permits the determination of menadione sodium bisulphite in feeds and premixes at concentrations as low as 0.02 μg/g.

EXPERIMENTAL

Apparatus

HPLC was performed with a system incorporating a Gilson Model 302 constant-flow pump (Meyvis, Bergen op Zoom, The Netherlands), a Rheodyne Type 7010 HPLC injection valve equipped with a 100-μl sample loop and a Shimadzu Type RF-530 fluorescence spectrophotometer (Pleuger, Amstelveen, The Netherlands) equipped with a 100-μl Hellma Type 179.50-QS debubbler fluorescence flow-cell.

A Knauer stainless-steel column (250 × 4.6 mm I.D.) was home-packed with ODS-Hypersil 5 μm (Shandon Southern Products, Astmoor, U.K., Cat. No. 580 × 9) by the balanced-density slurry technique. A Gilson Minipuls 2 four-channel peristaltic pump (Meyvis) was used for reagent supply and air segmentation. Elution profiles were displayed on a Kipp BD-8 recorder (Kipp Analytica, Delft, The Netherlands).

Reagents

Analytical-reagent grade reagents were used. Tannin was obtained from Brocacef, Maarssen, The Netherlands (Cat. No. TA 680) and sodium borohydride from Riedel-de Haen, Hannover, F.R.G. (Cat. No. 62861). The derivatization reagent was

prepared by dissolving sodium borohydride in absolute ethanol at a concentration of 0.8 g/l and degassing by ultravibration for 5 min. This solution was stable at ambient temperature for 2 h. The HPLC mobile phase was water–ethanol (4:6) flushed with a stream of helium gas for 10 min before use. Menadione was obtained from Sigma, St. Louis, MO, U.S.A. (Cat. No. M-5625). A stock standard solution was prepared by dissolving menadione in absolute ethanol at a cencentration of 200 μg/ml. This solution was stored in the dark at 4°C and was stable for at least 1 month. A working standard solution was obtained by diluting the stock standard solution with HPLC mobile phase to a concentration in the range 0.2–2 μg/ml. This solution was stored in the dark at 4°C and was stable for at least 1 week. A solution containing 200 μg/ml of menadione sodium bisulphite (Sigma, Cat. No. M-5750) in water–ethanol (6:4) was used for recovery experiments. This solution was freshly prepared for each series of analyses.

Extraction procedure

A portion of 1–10 g of a ground feed or premix was shaken for 10 min with 96 ml of water–ethanol (6:4). After adding 4 ml of 10% (w/v) tannin solution and shaking for 1 min, the supernatant was centrifuged at 2000 *g* for 5 min. The supernatant was filtered under vacuum through a fine-pore glass filter. Subsequently, a 40-ml portion was transferred into a 250-ml brown glass separating funnel. After adding 50 ml of *n*-hexane and 20 ml of 10% (w/v) sodium carbonate solution, the funnel was shaken for 1 min. The two layers separated and the lower layer (water–ethanol) was discarded. The *n*-hexane layer was washed twice with *ca.* 100 ml of distilled water and, after the water layer had been discarded, dried with strips of blue-ribbon filter-paper. An aliquot of the *n*-hexane layer was evaporated to dryness under nitrogen with the aid of a rotary vacuum evaporator. The residue was dissolved in a known volume of mobile phase by ultravibration for 1 min in order to obtain a menadione concentration in the range 0.2–2 μg/ml. If turbid, this solution was filtered through a disposable 0.2-μm filter and stored in the dark at 4°C prior to analysis by HPLC within 1 week.

Recovery experiments

The recovery of menadione sodium bisulphite added to a sample was determined by adding an aliquot (*a* ml) of a solution containing 200 μg/ml of menadione sodium bisulphite in water–ethanol (6:4). Thereafter the sample was analysed as described but with extraction with $(100-a)$ ml of water–ethanol (6:4).

High-performance liquid chromatography

HPLC analysis of menadione was carried out by injecting 100 μl of the sample extract on to the ODS-Hypersil column. The column was eluted isocratically with the mobile phase at a flow-rate of 0.6 ml/min. The effluent from the column was fed directly into a post-column reaction system to effect reduction of menadione. Fig. 1 shows the arrangement of this system.

The reduced menadione was detected using the fluorescence spectrophotometer set at an excitation wavelength of 325 nm and an emission wavelength of 425 nm. The recorder was set at 10 mV full-scale. The duration of the chromatographic run was about 25 min. The menadione sodium bisulphite concentration of the original

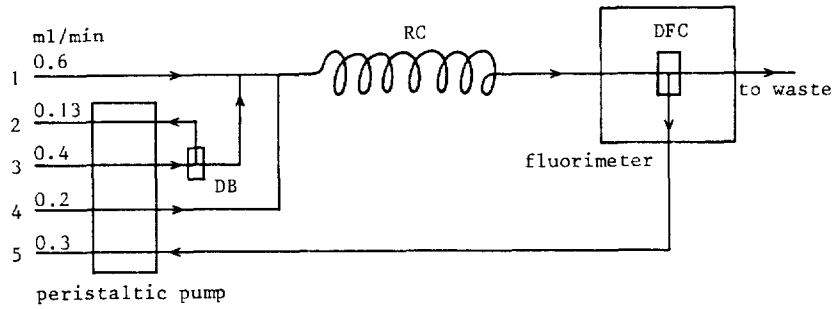

Fig. 1. Flow diagram of the post-column reaction system for reducing menadione to fluorescent 2-methyl-1,4-dihydroxynaphthalene. DB = debubbler to remove incidental hydrogen bubbles, Acculab No. 116-0202-03; RC = stainless-steel reaction coil; DFC = debubbler fluorescence flow cell; 1 = HPLC effluent; 2 = debubbled reagent, back to reagent reservoir; 3 = derivatization reagent; 4 = air; 5 = effluent with air to waste.

sample was calculated from peak heights with the working standard solution as a reference.

RESULTS

Characteristics of the method

Fig. 2 shows typical elution profiles of the working standard solution and of extracts of a cat food and a premix. In blank experiments only a solvent peak was recorded. After replacement of the sodium borohydride reagent by HPLC mobile phase no peaks with the same retention time as menadione were observed.

As far as has been investigated, the fluorescence response was linear for concentrations from 0.02 μg to 200 mg of menadione sodium bisulphite per g of sample. Assuming that the signal-to-noise ratio should be 5, the detection limit of the method corresponds to a concentration of 0.02 μg/g.

A number of animal foodstuffs and premixes were analysed for menadione sodium bisulphite by the HPLC method described and also by the EC colorimetric method[7] and the results are given in Table I. Similar results were obtained with both methods for samples with concentrations higher than *ca.* 15 μg/g. Below this level lower results were obtained by the HPLC method, probably because of its higher specificity. Further, the detection limit of the HPLC method is considerably lower than that of the EC colorimetric method.

After analysing a few sample extracts and the working standard solution, omiting air segmentation in the reaction coil, considerable peak broadening and spikes due to hydrogen bubbles were observed.

Optimization of post-column reaction fluorimetry

To optimize the post-column reaction conditions, 100-μl aliquots of a working standard solution containing 1 μg/ml of menadione were subsequently injected under the chromatographic conditions described. The length of the reaction coil was varied from 600 to 1800 mm in 200-mm steps. The concentration of sodium borohydride in ethanol was varied from 0.04 to 0.14% (w/v) in steps of 0.02%. Further, the net flow of the reagent was varied from 0.21 to 0.60 ml/min in *ca.* 0.12 ml/min steps.

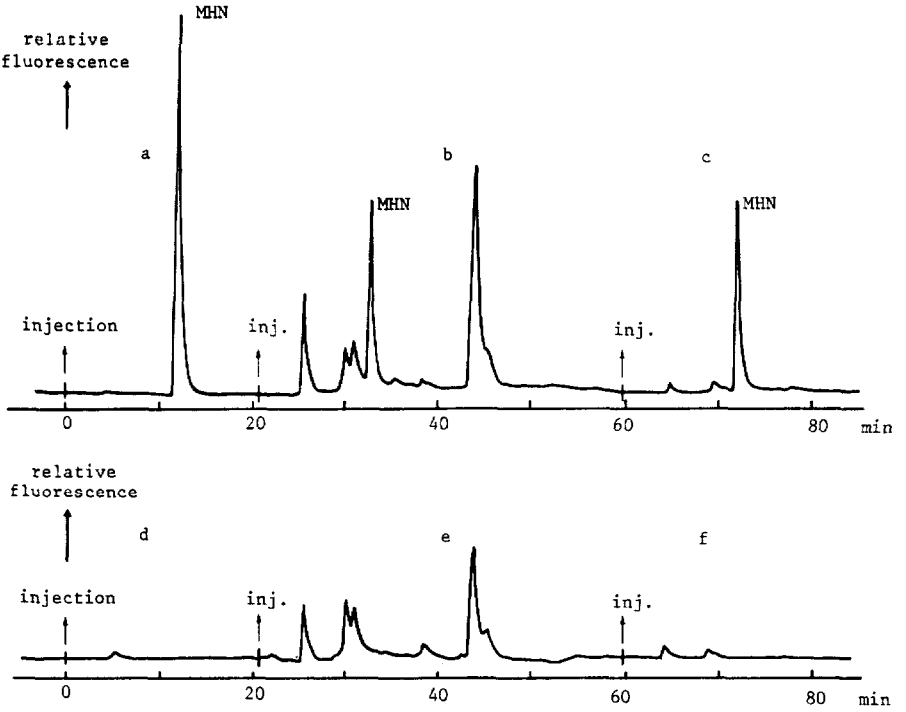

Fig. 2. Typical elution profiles of (a) the working standard solution (1 μg/ml), (b) an extract of a cat food (0.1 μg/g) and (c) a premix (180 μg/g); (d), (e) and (f), elution profiles of the samples (a), (b) and (c), respectively, with HPLC mobile phase instead of derivatization reagent. MHN = 2-methyl-1,4-dihydroxynaphthalene.

Maximum peak heights were obtained when using a reaction coil at least 1200 mm long and a sodium borohydride concentration of at least 0.06% (w/v). For the reagent flow-rate range mentioned, a slightly decreasing peak height was observed, corresponding to the dilution factors as a result of an increase in the total flow-rate.

Accordingly, a length of the reaction coil of 1400 mm, a concentration of the reagent solution of 0.08% (w/v) and a net reagent flow-rate of 0.27 ml/min were chosen for the assay.

The highest fluorescence yield was observed with the sodium borohydride dissolved in analytical-reagent grade absolute (99.98%) ethanol.

Stability of 2-methyl-1,4-dihydroxynaphthalene

To determine the rate of deterioration of this compound in the reaction coil, aliquots of HPLC mobile phase and reagent solution were mixed in the ratio used in the HPLC procedure in a standard-sized quartz fluorescence cuvette. After removing hydrogen bubbles by ultravibration for 5 min, the cuvette was placed in a Perkin-Elmer Model 204 A fluorescence spectrophotometer set at the wavelengths of the assay (excitation at 325 nm, emission at 425 nm). The relative fluorescence was set at zero. A 40-μl aliquot of a working standard solution containing 1 μg/ml of menadione was then added and mixed with the contents. The rate of deterioration

TABLE I

COMPARISON OF RESULTS OBTAINED WITH HPLC AND COLORIMETRIC METHODS

Type of sample	*Menadione sodium bisulphite**			
	HPLC		*Official EC method*	
	Sample (μg/g)	*Recovery** (%)*	*Sample (μg/g)*	*Recovery** (%)*
Premix, chalk-based	362	96	365	98
Premix, chalk-based	246	96	235	–
Premix, based on chalk and wheat grit	240	92	231	90
Premix, based on chalk and wheat grit	71	96	69	97
Premix, chalk-based	49	97	51	98
Premix, chalk-based	45	96	48	93
Premix, based on chalk, Calcium phosphate and wheat grit	12	98	15	94
Rat breeding diet	1.9	98	3.0	90
Dog breeding diet	2.4	91	2.9	97
Fish feed	0.3	101	< 1	–
Cat feed	0.1	104	Colour interfered	
Dog feed	2.2	94	Colour interfered	
Calf milk	0.4	91	Colour interfered	

* Average of duplicate analyses.
** Recovery experiments were performed as described under Experimental.

of 2-methyl-1,4-dihydroxynaphthalene was determined by recording the decrease in its relative fluorescence, by placing the cuvette in the excitation light beam for 4 sec every minute for 15 min.

A fairly constant rate of deterioration was observed during the period mentioned. After 15 min the relative fluorescence showed a decrease of 30%. As in the method described the period between the origin and the measurement of 2-methyl-1,4-dihydroxynaphthalene does not exceed *ca.* 1 min, the reliability of the method is not affected by detorioration of the compound.

TABLE II

PRECISION OF THE HPLC METHOD IN DETERMINING MENADIONE SODIUM BISULPHITE

Parameter	*Within-assay precision*	
	Rat breeding diet	*Recovery test**
n	6	6
Mean	2.48 μg/g	94.4%
S.D.	0.15 μg/g	6.8%
C.V.	6.0%	–

* 3.8 μg of menadione sodium bisulphite per gram of sample were added.

Precision and recovery

To determine the within-assay precision of the method and the recovery, several 10-g portions of a rat breeding diet with and without the addition of menadione sodium bisulphite were analysed within 1 day. The results in Table II show that the within-assay coefficient of variation is sufficiently low. The recovery of menadione sodium bisulphite added to the rat food is good. As menadione sodium bisulphite in feed is not stable during storage[12], the between-assay precision of the method was not investigated.

CONCLUSION

A specific and sensitive HPLC method for the determination of menadione sodium bisulphite in animal feed and premixes has been described. The linearity range and sensitivity permit determinations at concentrations as low as 0.02 μg/g, which is hardly possible with other methods. Debubbling of the derivatization reagent and segmentation significantly improved the HPLC elution profiles.

The results obtained with this method and with the official EC method are in good agreement for premixes with concentrations down to *ca.* 15 μg/g. However, for the analysis of animal feeds containing less than *ca.* 15 μg/g, the HPLC method is to be preferred because of its higher specificity and sensitivity.

REFERENCES

1 M. L. Scott, *Vitam. Horm.*, 24 (1966) 633.
2 J. Marks, *The Vitamins in Health and Disease*, Churchill, London, 1968, p. 68.
3 W. V. Taggart and J. T. Matschiner, *Biochemistry*, 8 (1969) 1141.
4 V. T. Burt, E. Bee and J. F. Pennock, *Biochem. J.*, 162 (1977) 297.
5 V. W. Winkler and J. M. Yoder, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 1219.
6 D. A. Libby and A. J. Sheppard, *J. Ass. Offic. Anal. Chem.*, 48 (1965) 973.
7 *Off. J. Eur. Commun.*, 17 (1979) L 108, 23.
8 S. S. M. Hassan, *J. Ass. Offic. Anal. Chem.*, 64 (1981) 611.
9 K. Ranfft and H. Rückemann, *Z. Lebensm.-Unters.-Forsch.*, 167 (1978) 150.
10 U. Manz and R. Maurer, *Int. J. Vitam. Nutr. Res.*, 52 (1982) 248.
11 K. Abe, O. Hiroshima, K. Ishibashi, M. Ohmae, K. Kawabe and G. Katsui, *Yakugaki Zasshi*, 99 (1979) 192.
12 E. Leerbeck and W. Hjarde, *Acta Agr. Scand.*, 25 (1975) 313.