Simultaneous Determination of Five Fat-Soluble Vitamins in Feed by High-Performance Liquid Chromatography Following Solid-Phase Extraction

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Abstract

A high-performance liquid chromatography method is developed for the simultaneous determination of menadione, retinyl acetate, cholecalciferol, α-tocopherol, and α-tocopherol acetate in feed. The present study uses an enzyme to destroy the coating film, ethanol to extract free vitamins, and Oasis HLB cartridges to purify. Vitamins are separated using an Atlantis dC18 column. The mobile phase is methanol–water (98:2 v/v). Detection is performed with a UV–vis detector at 230 and 265 nm. The linearity, accuracy, and repeatability of this method are all satisfactory. Application of the method is suitable for the determination of the fat-soluble vitamins in general feed.

Introduction

Biologically active vitamins are a class of low molecular weight organic compounds which maintain normal physiological functions for animal organisms, containing both the characteristics of water- and fat-solubility. Fat-soluble vitamins play an important role in the stimulation of synthesis and degradation of nutrients, enhancing immune function and growth performance. In vitamin deficiency, animals tend to develop disease (1). However, an overdose of vitamins results in poisoning, evident in animals' legs (2). Although some ruminants and grazing monogastric animals can obtain autonomically vitamins B and K from bacteria, most animals passively acquire vitamins from feed intake. Currently, feed supplemented with vitamin additive is growing in popularity, which requires a parallel determination method for vitamin detection in animal feed.

In the past two decades, various analytical methods have been developed for this purpose, for example, capillary electrophoresis (3,4), spectrophotometry (5), fluorimetry (6), colorimetry (7), and chromatography (8). Among them, determination by high-performance liquid chromatography methods (HPLC), due to its rapid separation, high sensitivity, and accurate quantitation, has become popular in the detection of vitamins in various matrices (9,10). Recently, a single vitamin analysis (11–14) has been developed. Rusing et al. (15) reported a simultaneous analytical method for vitamins A and E in rodent feed by HPLC. In 1993, a simultaneous HPLC method to determine vitamins K_1, K_2, and K_3 in animal feed was developed (16). In addition, HPLC has been used for the simultaneous determination of fat-soluble vitamins A, D, and E in animal feed and foodstuff (17). However, the simultaneous determination of five vitamins (A, D_3, E, E-ac, and K_3) in feed is rarely reported.

The previously mentioned methods mainly consist of the process of sample saponification, liquid–liquid extraction, and HPLC analysis. In them, the regular alkaline saponification procedure readily causes the oxidation of vitamins because vitamins are unstable under the experimental conditions. Moreover, it has the risk of large variation, low recovery and reproducibility, and is also a time-consuming and complicated operation. Therefore, Qian et al. (18) reported a new method of one-step extraction instead of the traditional saponification for the simultaneous determination of vitamins A, D, E, and pro-vitamin D_2 in animal feeds. However, concerning the sample preparation, the method recommended that the whole determination process should be accomplished in a short period and under gentle operating conditions. Recently, enzymolysis was developed as an alternative to saponification; for example, a lipase-catalyzed reaction was used to determine the fat-soluble vitamins in milk power and infant formula (19). This method with hydrolytic enzymes is rarely reported for vitamin detection in animal feed.

Another challenge for the detection of vitamins in feed is multiple interference from the components in feed, which leads to inaccurate determination. The traditional purification method is to extract the fat-soluble vitamins with an organic solution, such as hexane, heptane, ether, or chloroform, which is solvent-consuming and causes incomplete extraction (16,20,21). Solid-phase extraction is efficient enough to conquer this in terms of its rapidity, high efficiency, and use of less solvent, which reasonably makes it a qualified pretreatment of feed samples for HPLC determination, to exclude the interferents (22).

In this study, we developed an HPLC method with a new pretreatment for the simultaneous determination of menadione

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(K_3), retinyl acetate (A-ac), cholecalciferol (D_3), α-tocopherol (E), and α-tocopherol acetate (E-ac) in animal feeds. Their chemical structures are shown in Figure 1. The fat-soluble vitamins were extracted with basic proteinase and ethanol, and purified with Oasis HLB cartridges. The five vitamins were separated using reversed-phase HPLC combined with a UV–vis detector. Application of the method is suitable for determination of all fat-soluble vitamins in broad animal feed. As we know, few studies on the determination of vitamins in feed with proteinase extraction and SPE purification have been reported.

Experimental

Reagents and materials

Vitamin A-ac, E, K_3, and D_3 standards were purchased from Sigma-Aldrich (St. Louis, MO), and vitamin E-ac from Supelco (Bellefonte, MA). These standards were used without further purification. Basic proteinase named "Savinase" was obtained from Roche (Shanghai, China). HPLC-grade methanol was supplied by Fisher Scientific International (Hampton, NH). All other chemicals were analytical grade and water used in the study was double-deionized water (Milli-Q, Millipore Corp., Milford, MA) of 18.2 MΩ/cm resistivity.

Apparatus and procedures

HPLC analysis was performed on an LC-10A HPLC system (Shimadzu, Kyoto, Japan) with an Atlantis dC_{18} column (4.6 mm × 150 mm; 5 µm) (Waters Corp., Dublin, Ireland). OASIS HLB cartridges (1 cc), used for purification of samples, were purchased from Waters Corp. The solid-phase extraction system was a vacuum manifold processing station obtained from Agilent Technologies (Palo Alto, CA). Ultrasonic cleaner (Kunshang, China) was used for promoting the sample dissolution.

Standard solutions

Stock standard solution (1 mg/mL) was prepared separately by dissolving 10 mg of individual vitamin in 10 mL of methanol and stored in darkness at 4°C. Working standard solutions were prepared daily by methanol dilution of the stock standard solutions in appropriate propotions into the concentrations of 5 µg/mL for K_3, 20 µg/mL for A-ac, 10 µg/mL for D_3, 40 µg/mL for E, and 40 µg/mL for E-ac.

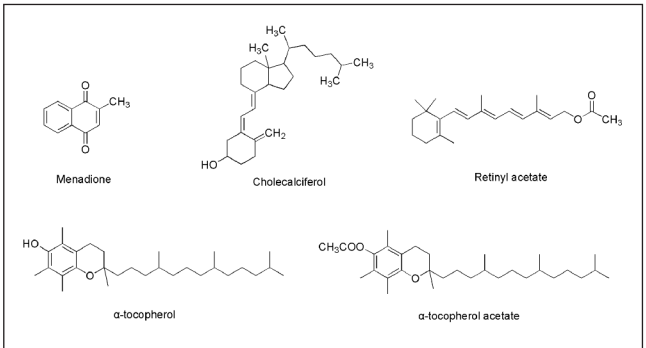

Figure 1. Chemical structures of the five fat-soluble vitamins.

Chromatographic conditions

The chromatographic conditions were chosen in terms of peak shape, column efficiency, retention time, resolution, and sensitivity. For the separation of the fat-soluble vitamins, a Waters Atlantis dC18 column (particle diameter 5 µm, 150 × 4.6 mm i.d.) with a matching guard cartridge was used. The mobile phase was methanol–water (98:2, v/v). The mobile phase flow rate was 1.0 mL/min, and the injection volume was 10 µL. The column oven temperature was 35°C. Detection with a UV–vis detector was carried out at 230 nm for E and E-ac, and 265 nm for K_3, A, and D_3.

Extraction of feed sample

Firstly, the feed sample (0.1 g vitamin additive, 1–2 g multivitamin, 5 g premix, and 10 g complete feed), spiked with five fat-soluble vitamins, was weighed in a 100-mL volumetric amber flask, and the Savinase proteinases (approximately 100 mg, 200 mg, 500 mg, and 1 g corresponding to the different samples) were added. In order to release vitamins easily, 10 mL of freshly prepared 0.2% ammonia solution was added to the flask. The mixture was shaken in an ultrasonic bath at 40–50°C for 20–30 min. Then approximately 65 mL of ethanol was added to extract the free vitamins. The solution was allowed to cool to room temperature in the dark, and the solution volume was made up to 100 mL by the addition of ethanol. The mixture was shaken vigorously for 1 min and was centrifuged at 5000 rpm for 10 min. Finally, the supernatant was cleaned with SPE as follows.

Solid-phase extraction

A 1 mL aliquot of the supernatant was decanted into a 5 mL tube and evaporated to near dryness in a water bath at 55°C, under a stream of nitrogen. The extract was reconstituted in 1 mL of 65% ethanol–water solution (ethanol–water, v/v), then the solution was purified by the Oasis HLB cartridge according to the optimal process of SPE. Firstly, the OASIS HLB column was preconditioned by passing through 1 mL of methanol, followed by 1 mL of double-deonized water. Secondly, 1 mL of the extract solution was slowly passed through the OASIS HLB column at a flow rate of 1 mL/min. After washing with 1 mL of 5% methanol, the analyte was eluted with 1 mL of ethanol.

Determination of inter- and intra-assay variation

The mixed standards of five fat-soluble vitamins were spiked in feed samples. Intra-assay variation was measured for five replicates of mixed vitamins, while for inter-assay variation, the solution was determined for seven consecutive days. Linear analysis revealed calibration equations with the expected correlation coefficient.

Results and Discussion

Chromatographic parameters

To obtain the best peak shape, resolution, and retention time, the chromatographic conditions of standard solutions, such as analytical column, mobile phase composition, flow rate, column temperature, and detector wavelength, were optimized. For example, several analytical columns, such as Separon C_{18} (150 × 3

mm, 7 µm) (23), Nova-Pack C_{18} (150 × 3.9 mm, 4 µm) (24), and MetaChem Polaris C_{18}-A (150 × 4.6 mm, 3 µm) (25) were used to separate the water- and fat-soluble vitamins with HPLC. Here Atlantis dC_{18} was chosen as the analytical column, which seemed a better fit to separate the vitamins. Methanol and water were chosen as the mobile phase. Nine ratios of methanol–water (50:50, 60:40, 80:20, 85:15, 90:10, 92:8, 95:5, 98:2, and 99:1, v/v) were tested. Considering the optimal resolution, the ratio of 98:2 was selected. Because the peak of vitamins K_3 was earlier, three flow rates (1.0, 0.8, and 0.6 mL/min) were tested in order to adjust the retention time. The results showed that the lower flow rates did not delay the retention time of vitamins K_3, but increased the retention time of vitamins E and E-ac. Thus 1 mL/min was considered sufficient. For the detector wavelength, photodiode array detector was used to scan the wavelength from 200 to 400 nm, and a larger absorbance peak was observed at 230 nm for vitamins E and E-ac, and at 265 nm for vitamin K_3, D_3, and A-ac; then both wavelengths 230 and 265 nm were monitored.

A typical chromatogram of the mixture of five fat-soluble vitamins under the optimal HPLC conditions is presented in Figure 2.

Extraction step

Vitamins used for animal feed are either contained in the initial raw plant material or introduced as special additives with solid preparations. Because vitamins are unstable and readily oxidized, the synthetic vitamin supplements are often coated with collagen or gelatin beadlets to enhance their stability. The beadlet must be penetrated to dissolve the vitamins. Previous studies suggested that organic solvents alone did not extract vitamins completely from vitamin-fortified feeds and supplements. Hung (26) and Qian et al. (18) added 1% and 5%, respectively, of 20% sodium phosphate tribasic solution to the organic solvent to dissolve the protective covering film. In the present work, the basic proteinase named Savinase was chosen to disrupt the gelatin beadlets. Three enzymolysis times (10, 30, and 60 min) were assayed. Considering the higher recoveries for most vitamins and faster extaction time, 30 min enzymolysis was chosen. In accordance with the optimal bio-action of Savinase proteinase, 40°C bath temperature was set. During the ultrasonic process, a variation of less than 5–10°C was allowed.

Three ammonia concentrations (0.02%, 0.1%, and 0.2%) and three ammonia volumes (10 mL, 15 mL, and 20 mL) were assayed. The results suggested that the ammonia concentration did not play an important role in the method, and the volume was chosen according to the sample weight. The sample must be kept in a liquid status during the whole ultrasonic process. If ammonia volume was insufficient to wet the whole sample, the extraction would be incomplete. Ethanol was then compared with methanol to extract the free vitamins, and the results showed that higher recoveries of vitamins were obtained with ethanol. Moreover, ethanol has a relatively low toxicity exposure for operators. Therefore, ethanol was selected as the extractant. In addition, according to previous studies, most investigators used butylated hydroxytoluene (BHT) (27) or ascorbic acid (13) as the antioxidant to avoid vitamin oxidation. However, in our study, similar results were acquired with or without BHT.

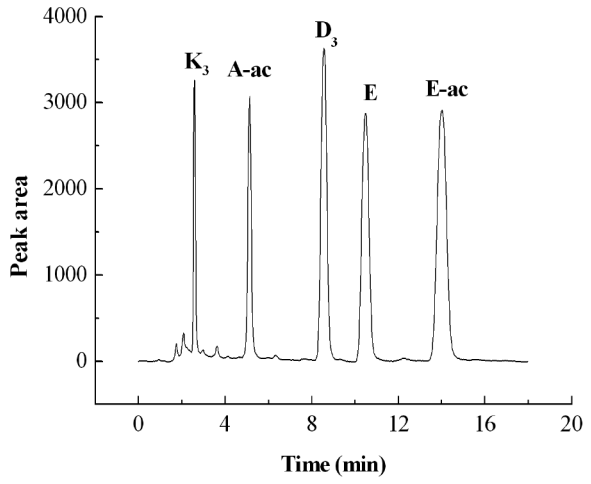

Figure 2. High-performance liquid chromatograms of standard mixture of five fat-soluble vitamins. Peaks: K_3, t_R = 2.59 min; A-ac, t_R = 5.14 min; D_3, t_R = 8.57 min; E, t_R = 10.50 min; E-ac, t_R = 14.04 min. Chromatographic conditions: mobile phase, methanol–water (98:2, v/v); flow rate, 1 mL/min; detection wavelength, 230 nm.

Purification of samples by SPE

An Oasis HLB cartridge containing poly divinylbenzene-co-*N*-vinylpyrrolidone sorbents was chosen as the SPE column, because the copolymer which exhibits both hydrophilic and lipophilic retention characteristics plays a valid role in the extraction of medium-polar and non-polar organic compounds from mixtures of water and organic solvent (28). Thus, this cartridge is suitable for the clean-up of feed, including fat- or water-soluble vitamins.

If the extraction solution containing 90% ethanol as loading solution was directly transferred into the cartridge, the vitamins would almost run down. To optimize the SPE loading conditions, various proportions of ethanol and water as the loading solution were assessed. Ten concentrations of ethanol ranging from 15% to 95% were assayed. The retention abilities of

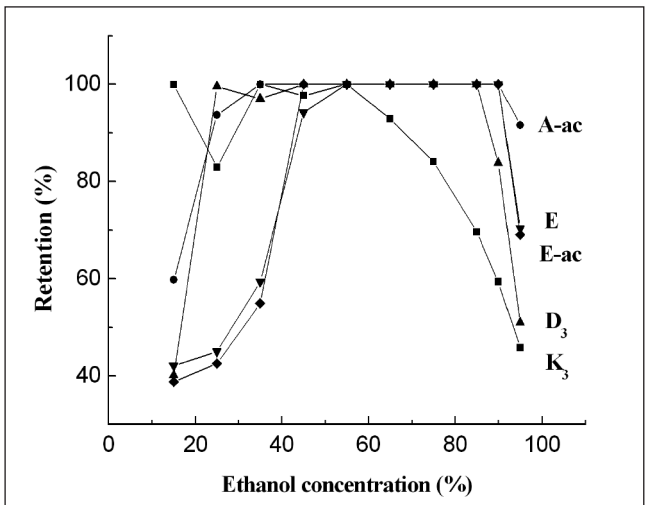

Figure 3. Dependence of the retention abilities of five vitamins on the Oasis HLB cartridge on different concentrations of ethanol as the loading solvent.

vitamins on the HLB column under different concentrations of ethanol solution are presented in Figure 3. The results indicate that all vitamins can be held on the cartridge under 45%, 55%, and 65% ethanol solvent. Finally, in view of better solubility for the five vitamins, 65% was selected. In addition, water and 5% methanol–water were assayed as the detergent. The results showed no difference. The 5% methanol solution was chosen as a common detergent. Several solvents with different polarities, such as tetrahydrofuran, acetonitrile, ethyl acetate, acetone, cyclohexane, ethanol, and methanol, were chosen as the eluent. Three volumes (1, 2, and 3 mL) were assayed. Taking into consideration the volatility, highest recovery, and smallest volume, 1 mL of ethanol was set as eluent. Summarily, the optimal clean-up steps are presented in Table I. The chromatograms were compared before and after an SPE-treated concentrate feed spiked vitamins (Figure 4), and clearly showed the importance of the utilization of the Oasis HLB cartridge, especially for vitamin K_3.

Table I. The Step and Purpose of Solid-Phase Extraction in Feed Clean-up

Step	Operating	Purpose
Conditioning	1 mL MeOH + 1mL water	Preparation of sorbent
Loading	1 mL of 65% ethanol–water extraction solution	Retention of analytes on cartridge
Washing	1 mL of 5% MeOH	Exclusion of salts and other organic interference
Elution	1 mL ethanol	Elution of fat-soluble vitamins

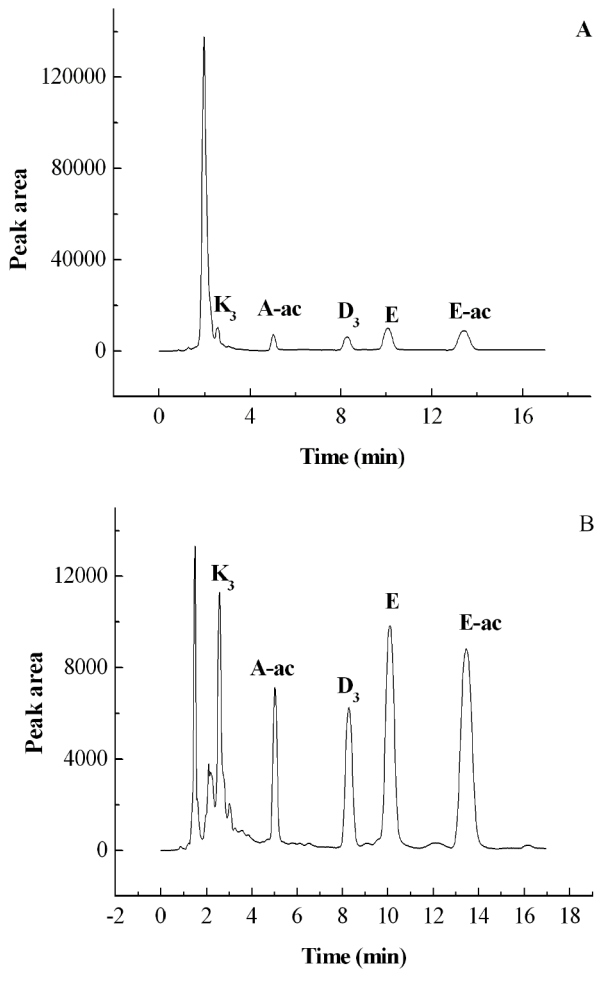

Figure 4. High-performance liquid chromatograms of vitamins K_3, A-ac, D_3, E, and E-ac spiked in concentrate feed without (A) and with (B) prepurification with Oasis HLB cartridge.

Method validation

The method was validated with respect to linearity and sensitivity, and also precision and accuracy. The linearity was studied

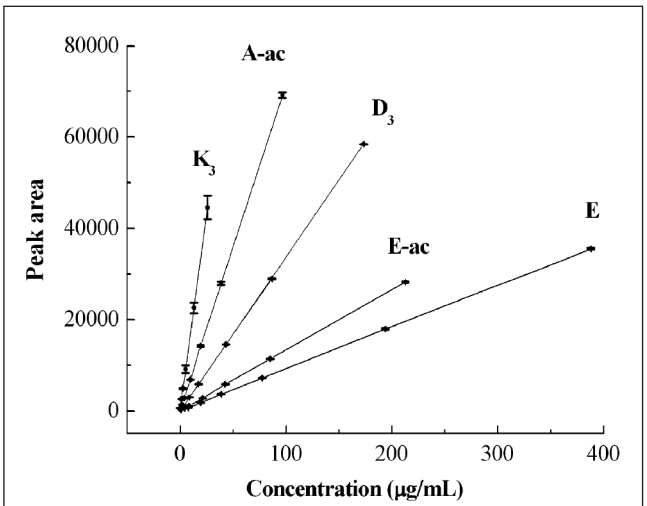

Figure 5. Calibration curves of standard solutions containing five fat-soluble vitamins.

Table II. Recovery of Fat-Soluble Vitamins Spiked in Feed Samples

Vitamin	Added (mg/g)	Measured (mg/g)	Recovery (%)	C.V. (%)
K_3	0.01	0.013 ± 0.001	129.6	8.8
	0.1	0.095 ± 0.005	94.6	4.8
	1	0.876 ± 0.076	87.64	8.6
A-ac	0.1	0.092 ± 0.007	91.66	7.8
	0.4	0.427 ± 0.004	106.66	1.0
	2.0	1.788 ± 0.149	89.41	8.4
D_3	0.02	0.021 ± 0.002	104.64	7.5
	0.24	0.234 ± 0.005	97.66	2.0
	1.8	1.756 ± 0.065	97.53	3.7
E	0.08	0.079 ± 0.004	99.33	4.9
	0.4	0.403 ± 0.013	100.74	3.3
	1.6	1.662 ± 0.048	103.88	2.9
E-ac	0.08	0.079 ± 0.006	99.31	7.9
	0.8	0.809 ± 0.035	101.1	4.3
	2.4	2.329 ± 0.068	97.05	2.9

Table III. Declared and Determined Concentrations of Vitamins in Feed Samples (*n* = 3)

	Premix feed			Ferment feed		
Vitamin	Declared (mg/kg)	Measured (mg/kg)	C.V. (%)	Declared (mg/kg)	Measured (mg/kg)	C.V. (%)
K_3	35	31.9 ± 1.6	4.9	8	9.84 ± 0.8	8.1
A-ac	258	251.0 ± 10.2	4.1	5.16	4.62 ± 0.25	5.4
D_3	5	4.7 ± 0.08	1.8	0.05	ND†	–
E	nd*	ND†	–	nd*	ND†	–
E-ac	5900	5906.8 ± 12.8	0.2	20	19.28 ± 0.51	2.6

* Not declared.
† Not found.

by a series of mixed standard solutions of each vitamin at six levels. The method was linear in the ranges as follows: from 0.26 to 26.0 µg/mL for menadione, from 2.0 to 100.0 µg/mL for retinyl acetate, from 0.8 to 86.8 µg/mL for cholecalciferol, from 3.5 to 390.0 µg/mL for α-tocopherol, and from 4.5 to 213.0 µg/mL for α-tocopherol acetate. A satisfactory linearity was observed (Figure 5), and indicated the correlation coefficients (r^2) ranging from 0.99 to 1. The limit of detection (LOD), defined as the compound concentrations which produced a signal-to-noise ratio generally greater than three, ranged from 0.075 µg/mL to 1.26 µg/mL. The limit of quantitation (LOQ) of the assay was evaluated as the concentration greater than 10 times the value of the signal-to-noise ratio. LOQ values ranged from 0.25 µg/mL to 4.2 µg/mL.

To evaluate the precision and accuracy of the method, inter- and intra-assay variation repeatability was estimated. The recovery of vitamins spiked in concentrated feed was determined by five replications at the medium concentration: 5.56 µg/mL for menadione, 20.34 µg/mL for retinyl acetate, 15.36 µg/mL for cholecalciferol, 41.2 µg/mL for α-tocopherol, and 45.6 µg/mL for α-tocopherol acetate. The results indicated that recoveries of all vitamins ranged from 91% to 103% and coefficients of variation were less than 6% for both intra-assay and inter-assay determinations.

Analysis of spiked feed samples

Recoveries from spiked feed samples were determined at three different concentrations by comparing the peak area ratios for extracted vitamins from the feed samples and the respective values derived from the vitamins' calibration curves (Table II). The statistical results show satisfied recoveries that ranged from 87% to 130% and coefficients of variation were less than 10%. Therefore, combined with the extraction and clean-up procedures, this HPLC method was ideal to accurately determine vitamins in feed samples.

Identification and determination of vitamins in real samples

This method was suitable for the simultaneous determination of vitamins K_3, A-ac, D_3, E, and E-ac in vitamin additives, multivitamin, premix, and some complete feed. Two feed samples were prepared and analyzed in order to check the applicability of the method in real feed samples. Concentrations measured after triplicate analyses are summarized in Table III. Because the amount of vitamin D_3 was trace in those samples, the samples were pre-concentrated 10 times. The results suggest that vitamin K_3 had a great deviation in the present method and the other vitamins were well determined.

Conclusion

This work successfully developed a new method including the procedure of emzymolysis, extraction, and purification for the simultaneous determination of five fat-soluble vitamins (K3, A-ac, D3, E, and E-ac) using HPLC combined with UV detector under the double wavelength conditions. Instead of saponification, emzymolysis made the operation easy and saved time. Solid-phase extraction proved to be a useful way for purifying the five fat-soluble vitamins from interferences in complex feed samples, especially for vitamin K3, which was well-separated from the feed matrix. Moreover, HPLC separation was excellent with a retention time less than 16 min. The accuracy of this method was tested and obtained average recoveries ranging from 87% to 130%, and coefficients of variation were less than 10%. Thus, the developed method is proficient in the determination of fat-soluble vitamins in feed samples, such as multi-vitamin supplements, premixed feed, and formula feed.

Acknowledgments

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