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Simultaneous determination of fat-soluble vitamins A, D and E and pro-vitamin D_2 in animal feeds by one-step extraction and high-performance liquid chromatography analysis

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Abstract

Traditionally, alkaline saponification for the extraction of vitamins and step-wise HPLC analyses have been widely used for analysis of fat-soluble vitamins in animal feeds. The objective of present study was to develop an analytical method using one-step extraction and simultaneous determination of the vitamins A, D and E and pro-vitamin D_2 in animal feeds by HPLC. Various analytical conditions were tested which included sample particle size, extraction solvents, the ratio of solvent to sample, extraction approach, extraction time, N_2 protection, prepurification with Sep-Pak cartridge and detection wavelength. The accuracy of the developed method has been proven by comparison with the AOAC method. The reproducibility of the developed method has been substantiated by repeated recovery experiments. The detection limit for the four vitamins was 10 ng/g of feed sample. One of the important properties of the present method is rapidity and ease of use.

Keywords: Feeds; Vitamins

1. Introduction

Reliable, sensitive and rapid methods of determination of vitamins in animal feeds are essential for nutrition research as well as foods and animal industries. Consequently, it is very important to be able to determine simultaneously vitamins A, D and E in a single assay. The complexity of the simultaneous determination of fat-soluble vitamins in animal feeds is due to their instability during extraction as well as different spectral characteristics for analysis. Currently, the official methods of the Association of Official Analytical Chemists (AOAC) [1] for vitamins A, D and E are separate assays with detection based on colorimetry. The complexity and time-consuming assays make the AOAC methods difficult to use for routine analysis.

Although new procedures using high-performance liquid chromatography (HPLC) have been utilized in the past years, the reliability, simplicity and rapidity need to be substantiated or improved. Cohen and LaPointe [2] and McMurray et al. [3] developed HPLC methods for the determination of vitamin E in animal feeds. Rushing et al. [4] reported a method

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for a simultaneous analysis of vitamins A and E in rodent feed by HPLC. White [5] also developed a HPLC method to determine vitamins K_1, K_2 and K_3 in animal feeds. In addition, HPLC has been used for analysis of vitamins A, E, D and K in vitamin premix supplements [6], in foods [7], and in serum [8]. Most of these published methods consist of sample saponification, vitamin extraction and HPLC analysis. Saponification or alkaline digestion readily causes oxidation of fat-soluble vitamins although some investigators have tried to overcome this obstacle by adding antioxidants such as butylated hydroxytoluene [9] and ascorbic acid [4]. Nevertheless, the saponification procedure is time-consuming and of complexity of the process. Hexane has been used as extraction reagent in most published methods, but its effectiveness may not be as high as that of other organic reagents. In addition, there are numerous other conditional factors such as sample particle size, ratio of sample to reagent, extraction time, extraction equipment and prepurification that can influence extraction efficiency. Furthermore, reliability for reported methods [2–5] was based on their recovery experiments and no data are available for comparison of results achieved by their methods with those by AOAC methods.

The objective of the present work was to develop a method for simultaneous determination of fat-soluble vitamins A, D and E and pro-vitamin D_2 in animal feeds by an one-step extraction and HPLC analysis.

2. Experimental

2.1. Materials

The all-*trans* vitamin A (retinol acetate), vitamin D (cholecalciferol), vitamin E (DL-α-tocopherol) and pro-vitamin D_2 (ergosterol) were purchased from Sigma (St. Louis, MO, USA), and were used as standards. These vitamins were prepared in *n*-butanol. All other reagents were also from Sigma.

2.2. Design of the initial extraction procedure

The present study aimed to develop a one-step extraction procedure to substitute two-step sample preparation procedure (saponification and extraction) that has been used in most reported methods, and then simultaneous analysis of fat-soluble vitamins in a single run. An animal feed, or sample A (Cat. No.: 5056, Ralston Purina, St. Louis, MO, USA) was used for the conditional experiments for extraction procedure. An initial extraction procedure was developed as follows. Feed sample A was ground in a Waring blender and screened through a 80 mesh sieve. One g of the sample was precisely weighed and transferred to a 10-ml screw-capped extraction tube. Four ml of *n*-hexane was added to the tube and the tube was flushed with a steam of N_2 to protect vitamins from air exposure before capping. The mixture was shaken on a vortex mixer for 0.5 min, rested for 5 min, and shaken another half minute. After centrifugation at 4000 rpm for 5 min, 1 ml of supernatant was transferred to a 1.5-ml vial and evaporated under nitrogen to remove the solvent. The residue was re-dissolved in 0.3 ml *n*-butanol before being injected into the HPLC system.

2.3. Study of conditional factors

Each of conditional factors was studied respectively when other conditions stayed unchanged as the designed initial extraction procedure as described above. These series of extraction conditions were determined as following (also see Table 1):

1. Sample particle size (mesh): 40, 60 80, 100 and 120
2. Extraction solvent: *n*-hexane, *n*-hexane–benzene (70:30), *n*-hexane–ethyl acetate (70:30), chloroform, acetone–chloroform (30:70)
3. Ratio of sample to solvent (g:ml): 1:2, 1:4, 1:6
4. Extraction with and without N_2 protection
5. Extraction time (s): 30, 60 and 90
6. Extraction with: supersonic vibrant mixer (Sonicor, SC-50T, Sonicor Instrument, Copiague, NY, USA), rotary mixer (Shaking Water Bath, YB-521, American Scientific Products, USA), or vortex mixer (Genie 2, Fisher Scientific, Atlanta, GA, USA)
7. With and without Sep-Pak cartridge prepurification (Waters, Milford, MA, USA)

To evaluate these conditional factors, the AOAC (1990) procedures were conducted in parallel to determine vitamins A, D and E (no AOAC method is available for analysis of pro-vitamin D_2) in feed sample A, and these values were used as standards to

Table 1
The values of vitamins A, D and E and pro-vitamin D_2 in animal feeds determined under various analytical conditions as compared with those obtained by AOAC analysis (μg/g)[a]

	Vitamin A		Vitamin D		Vitamin E		Pro-vitamin D_2	
	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)
AOAC analysis	3.71	10	5.09	3	5.58	6	–	–
Sample size, mesh								
40	0.61	18	5.28	6	2.49	14	14.2	5
60	0.54	32	4.38	11	3.21	16	15.5	5
80	0.58	6	4.65	12	3.21	25	17.3	6
100	–	–	5.10	5	1.58	15	12.8	1
120	–	–	5.37	7	1.67	27	14.9	3
Extraction solvent								
n-Hexane	0.32	17	6.13	4	2.40	5	16.1	5
n-Hexane–benzene (70:30)	0.67	58	6.22	2	2.42	10	16.0	2
n-Hexane–ethyl acetate (70:30)	0.97	20	7.97	6	2.60	11	18.1	3
Chloroform	1.70	28	7.28	3	2.50	19	21.3	6
Acetone–chloroform (30:70)	3.04	7	7.30	4	2.52	6	17.5	3
Ratio of sample:solvent (g:ml)								
1:2	0.20	29	5.58	3	2.08	10	13.2	3
1:4	0.58	6	4.65	11	3.21	25	17.3	6
1:6	0.39	20	5.83	10	2.10	10	15.1	3
N_2 protection								
$+N_2$	0.35	12	5.38	8	3.52	12	16.8	4
$-N_2$	0.37	24	5.51	14	4.49	44	16.6	11
Extraction time								
30 s	0.18	21	5.39	7	2.03	12	14.3	3
60 s	0.58	6	4.65	11	3.21	25	17.3	6
90 s	0.42	31	6.31	4	2.16	8	15.1	4
Extraction								
Supersonic vibrant mixer	–	–	1.67	12	0.78	34	5.2	15
Vortex mixer	0.58	6	4.65	12	3.21	25	17.3	6
Rotary mixer	–	–	2.52	15	0.74	5	6.2	9
Sep-Pak cartridge prepurification								
+Sep-Pak cartridge	2.08	14	4.60	13	3.11	18	2.6	13
−Sep-Pak cartridge	3.51	7	4.96	2	5.50	6	12.1	2

[a] Each of conditional factors were studied respectively when other conditions stayed unchanged as the designed initial extraction procedure as described in Section 2.2 for design of initial extraction procedure. The HPLC analytical conditions are described in Section 2.4.

compare with values determined in series of conditional experiments.

2.4. Chromatography

Chromatographic separations were performed on a 150×3.9 mm Novapak C_{18} column (Waters). Methanol was used as mobile phase at a flow-rate of 1.5 ml/min and a pressure of 1000 p.s.i. (1 p.s.i.= 6894.76 Pa) All injections were 50-μl loop injections on a M710B autosampler (Waters). A Model M510 Waters pump and a Model M490 Waters variable-wavelength UV–visible detector set at 290 nm were used. All quantitation was by peak area using a Waters M740 integrator. Based on the established chromatographic conditions, repeated injections of

0.1, 0.5, 1, 5 and 10 μg of the standard vitamins A, D and E and pro-vitamin D_2 were made 10 times onto the HPLC system. The regression equations relating peak area (y, $\cdot 10^5$) to injected amounts (x, μg) of vitamin A, D and E and pro-vitamin D_2 were generated as $y=1.7x$, $y=0.55x$, $y=0.65x$ and $y=0.7x$ with r^2 values of 0.997, 0.999, 0.999 and 0.999, respectively. The linear dynamic ranges can be extended down to 10 ng or up to 50 μg of each vitamin. The retention time for vitamins A, D and E and pro-vitamin D_2 were 1.9, 3.6, 4.1 and 4.9 min, respectively. A Shimadzu MPS-2000 universal spectrophotometric scanner was used to determine the spectrograms of these four vitamins in *n*-butanol. A 290 nm wavelength, where all four vitamins presented a relatively high absorbance, was used for the analysis.

2.5. Assay procedure development and recovery experiments

The results of determined vitamins A, D and E values in series of experiments for conditional factors as described above were compared to those measured by the AOAC methods, and best conditions were selected to develop a new assay procedure. To evaluate the reliability of the new procedure, feed samples A and B (Cat. No.: 5014, Ralston Purina) were used to determine their vitamin contents using the new procedure and the AOAC methods. Data of the determined vitamins A, D and E mean values and coefficients of variation (C.V.s) were compared. In addition, recovery experiments were also conducted using both new procedure and the AOAC methods. Known amounts of vitamins A (5 μg), D (5 μg) and E (10 μg) and pro-vitamin D_2 (5 μg) in butanol were added to feed samples A and B prior to extraction in the new procedure or to saponification in the AOAC methods. The recovery was determined using the formula: recovery$=(A_1-A_2)/A_3\times 100\%$; where A_1 represents the area of a peak obtained from feed samples with the added standard vitamins, A_2 represents the area of the peak obtained the same feed sample without standard vitamins, and A_3 represents the area of the peak obtained from the same amount of added standard vitamins.

3. Results and discussion

3.1. Development of assay procedure

Saponification or alkaline digestion readily causes oxidation of fat-soluble vitamins although some investigators have tried to overcome this obstacle by adding antioxidants such as butylated hydroxytoluene [9] and ascorbic acid [4]. Saponification procedures also have the potential to introduce large variation and have low recovery and reproducibility, in addition to being time-consuming. The objective of present study was to develop a one-step extraction procedure to directly extract fat-soluble vitamins from animal feeds without saponification. Fat-soluble vitamins in feeds share some similar properties, one of which is that they are easily resolved in organic solvents such as chloroform. Based on the mechanism of liquid–solid extraction [10], the factors that influence the efficiency of extraction include sample particle size, extraction solvent, the ratio of sample to solvent, air exposure, extraction, extraction time and Sep-Pak cartridge prepurification.

In a series of experiments (Table 1), by comparing with AOAC, it was observed that 80 mesh particle size, acetone–chloroform (30:70) extraction solvent, 1:4 of ratio of sample:solvent, N_2 protection, 60 s for extraction, vortex mixing and without Sep-Pak cartridge prepurification were critical for direct extraction of fat-soluble vitamins from animal feeds. Sample particle size that may influence the efficiency of extraction, however, no data were available for comparison. We observed that large sample particle size (40 and 60 mesh) caused incomplete extraction of vitamin A with large variation (Table 1), but that small size led to under-measurement of vitamin A, possibly due to its instability [4].

n-Hexane has been used as solvent for extraction of fat-soluble vitamins in most available methods [4,8]. Chloroform and methylene chloride are also used as extraction solvents in some protocols [5,10]. In the present study, hexane had low resolvability for fat-soluble vitamins, and chloroform led to overestimation of vitamin D with large variation (Table 1). Chloroform, with its high specific gravity, resulted in incomplete centrifugation and cloudy extraction mixtures. By comparing with data obtained by the AOAC method, acetone–chloroform (30:70)

extraction was found to be the best for extraction of fat-soluble vitamins in feeds. Also, the present study indicated that, of the conditional factors under investigation, extraction solvent was most crucial to effective extraction of vitamins from feed samples, especially for vitamin A (Table 1). Vitamin A yield was much lower when hexane was used as solvent for extraction (less than 1 μg/g under any conditions compared to the AOAC value of 3.71 μg/g) than when extraction was performed using acetone–chloroform (30:70) mixture (3.04 vs. 3.71 μg/g of AOAC value) (Table 1).

Nitrogen has been used in some protocols for protection of extracted vitamins from oxidation [11]. However, others have suggested that it was not necessary to use N_2 because the solvent vapor that replaces air over the surface of extraction mixture has a protective antioxidant effect [11]. Our data indicated that N_2 protection did not influence the mean values of determined vitamins A, D and E and pro-vitamin D_2 but decreased the variation, especially for vitamin A (Table 1). Hung [10] used rotary mixer for extraction of vitamins for 1 h. In our experiments, samples were strongly mixed using vortex mixer for 1 min, rested for 5 min then vortexed another minute (Table 1). Rotary mixer and supersonic mixer were not as effective as vortex for extraction of fat-soluble vitamins from animal feeds.

Before injection of samples into the HPLC system, a Sep-Pak cartridge was used to purify the extracted vitamins and clarify the sample [5,10]. In our series of conditional studies, pre-treatment of samples with Sep-Pak cartridge resulted in low analysis values with large variation (Table 1). The extracted vitamins were readily retained in the cartridge, and were difficult to wash out. In addition, acetone–chloroform solvent mixture was easily evaporated during pre-column clean-up, which led to large variation.

In summary, the assay procedure was developed and described as the following: 1 g of 80-mesh sample was precisely weighed and added to a 10-ml screw-capped extraction tube. Four ml of acetone–chloroform solvent (30:70) mixture, was added to the tube and the tube was flushed with N_2 to protect vitamins from air exposure before sealing with the cap. The mixture was shaken on a vortex mixer for 1 min, rested for 5 min, and mixed another minute. After centrifugation at 4000 rpm for 5 min, 1 ml of supernatant was transferred to a 1.5-ml vial and evaporated under nitrogen to remove the solvent. The residue was redissolved in 0.3 ml *n*-butanol before injection onto the HPLC system. Conditions for HPLC analysis are described in Section 2.4. Fig. 1 presents typical chromatograms of vitamins A, D and E and pro-vitamin D_2 extracted from animal feeds and standard samples. The present method can not separate vitamin D_2 from D_3 therefore the value obtained represents total vitamin D content.

3.2. Recovery and reproducibility

To substantiate the developed method, recovery

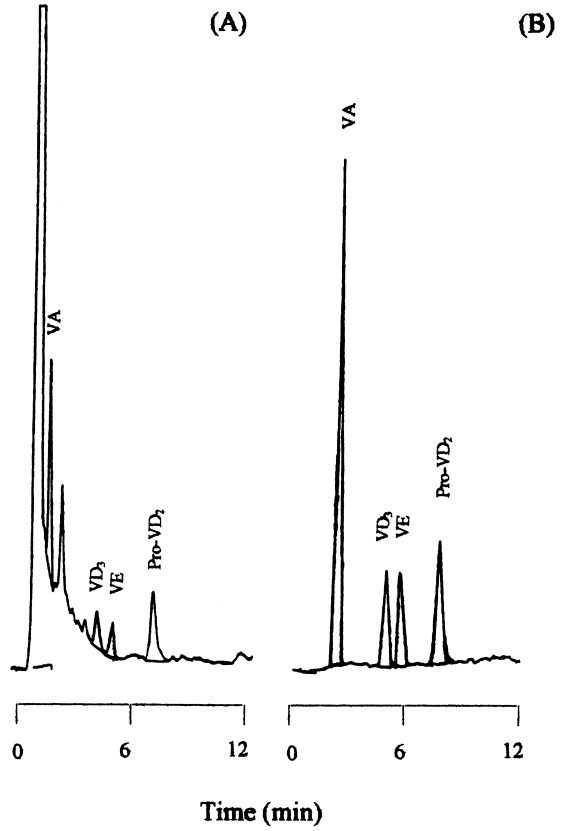

Fig. 1. Typical chromatograms of vitamins A, D and E and pro-vitamin D_2 extracted from animal feed (containing 3.2, 5, 4.8 and 10 μg/g of vitamins A, D and E and pro-vitamin D_2, respectively) (A) and standard samples (containing 5 μg of each vitamin) (B) determined using the present method. Column: 150× 3.9 mm I.D. Novapak C_{18}; mobile phase: methanol at a flow-rate of 1.5 ml/min; detection wavelength: 290 nm.

Table 2
Comparison of the present method[a] and AOAC method in analysis of fat-soluble vitamins in animal feeds

	Analysis				Recovery			
	Present method		AOAC method		Present method		AOAC method	
	Mean (μg/g)	C.V. (%)	Mean (μg/g)	C.V. (%)	Mean (%)	C.V. (%)	Mean (%)	C.V. (%)
Sample A								
Vitamin A	3.51	7	3.71	10	87.2	5	90.5	8
Vitamin D	4.96	2	5.09	3	93.1	3	97.0	9
Vitamin E	5.49	6	5.57	6	102	3	89.6	3
Pro-vitamin D_2	12.1	2	–	–	95.5	3	–	–
Sample B								
Vitamin A	5.36	8	5.24	7	84.1	6	83.1	5
Vitamin D	12.6	8	12.4	4	107	5	90.6	5
Vitamin E	10.8	6	10.3	5	107	7	102	11
Pro-vitamin D_2	1.73	10	–	–	103	6	–	–

[a] The assay procedure developed is described in Section 3.1 for development of assay procedure.

experiments were conducted using the developed method compared with the AOAC method (Table 2). Two animal feed samples were used in the recovery experiments and each assay was performed in six replicates. The C.V. values were all below 8% in analysis of vitamins A, D and E and pro- vitamin D_2 and the recoveries are all close to 100% except for vitamin A that was 84 to 87% using the present protocol. Vitamin A is an unstable vitamin and is readily destroyed [4]. The results obtained by the present method are consistent with those measured by the AOAC methods. In addition, the present method can also be used for analysis of pro-vitamin D_2, for which there is no AOAC method.

Some studies suggest that organic solvents alone do not extract vitamins quantitatively from vitamin-fortified feeds and supplements. This is due to gelatin beadlets that are often added in vitamin supplements as a means to enhance their stability. This thus requires penetration by water or alkali for dissolution [12]. Hung [10] added 1% of 20% sodium phosphate tribasic solution to the organic solvent to dissolve the protective coating of gelatin. In our preliminary study, five volumes of 20% sodium phosphate tribasic solution was added to the mixture of 30 volumes of acetone and 65 volumes of chloroform and the obtained data were not different with those obtained using the organic solvent mixture alone during extraction (data not shown). It is believed that the gelatin beadlets can be destroyed under strong vibration during extraction because normal feed samples contain 7–8% of moisture that is enough to dissolve the coating.

The repeatability of retention time and peak area is shown in Table 3. In addition, we also applied the developed method to determine the fat-soluble vitamins in other five animal feeds. The results indicated that the C.V. values were below 8% and the re-

Table 3
The repeatability of retention time and peak area[a]

	Retention time		Peak area (arbitary units)	
	Mean (min)	C.V. (%)	Mean	C.V. (%)
Vitamin A	1.908	0.1	1590	3.0
Vitamin D	3.552	0.2	482	4.6
Vitamin E	4.096	0.1	528	2.0
Pro-vitamin D_2	4.925	0.1	590	4.0

[a] The repeatability for the present established method was determined by repeatedly injecting 10 μl of the standard vitamin mixture (100 μg/ml for each vitamin standard) into the HPLC system.

coveries were 95±6, 94±10, 91±7 and 99±4%, respectively for vitamins A, D and E and pro-vitamin D_2 (original data not shown).

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References

[1] AOAC, Official Methods of Analysis, Washington, DC, 15th ed., 1990.
[2] H. Cohen, M.R. LaPointe, J. Assoc. Off. Anal. Chem. 63 (1980) 1254–1257.
[3] C.H. McMurray, W.J. Blanchflower, D.A. Rice, J. Assoc. Off. Anal. Chem. 63 (1980) 1258–1261.
[4] L.G. Rushing, W.M. Cooper, H.C. Thompson, J. Agric. Food Chem. 39 (1991) 296–299.
[5] S. White, Anal. Proc. 30 (1993) 266–267.
[6] S. Scalia, G. Ruberto, F. Bonina, J. Pharm. Sci. 84 (1995) 433–436.
[7] P.C.H. Hollman, J.H. Slangen, P.J. Wagstaffe, U. Faure, D.A.T. Southgate, P.M. Finglas, Analyst 118 (1993) 475–480.
[8] L. Yakushina, A. Taranova, J. Pharm. Biomed. Anal. 13 (1995) 715–718.
[9] R.G. Pozo, E.S. Saitua, I. Uncilla, J.A. Montoya, J. Food Sci. 55 (1990) 77–78.
[10] G.W.C. Hung, J. Liq. Chromatogr. 11 (1988) 953–969.
[11] G.F.M. Ball, J. Micronutr. Anal. 4 (1988) 255–283.
[12] J.N. Thompson, J. Assoc. Off. Anal. Chem. 69 (1986) 727–738.