Method for the Extraction and Cleanup of Animal Feed for the Determination of Liposoluble Vitamins D, A, and E by High-Pressure Liquid Chromatography

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A high-pressure liquid chromatography (LC) procedure and cleanup has been developed for the determination of vitamins A, D, and E in feeds. The method is quantitative for all three vitamins. Recovery studies on different feeds showed an average recovery of vitamin A (acetate), 96.4% ± 2.4; vitamin E acetate, 92.6% ± 2.92; vitamin D_3, 91.25% ± 5.9. Minimum detectable amounts are: vitamin A, 250 IU/kg; vitamin D_3, 10000 IU/kg; vitamin E, 5 IU/kg. The LC procedure was compared with an AOAC colorimetric procedure for the vitamins A and E.

Liposoluble vitamins (A, D, E) and their guaranteed presence in animal feeds are of prime importance both in terms of biological development and nutritional equilibrium (Bekes et al., 1977; Combs, 1976; Thompson, 1976; Edwards, 1976; Dorr and Balloun, 1976). Analysis of liposoluble vitamins by the high-pressure liquid chromatography (LC) technique is both rapid and precise, particularly for those in the form of concentrated solutes, or in multivitamin preparations (Abe et al., 1975; Tsukido et al., 1976; Conrad, 1975; Williams et al., 1972; Egberg et al., 1977). A determination of vitamin E in a feed, without cleanup, has also been accomplished by LC (Shaikh et al., 1977). However, the latter case is of limited applications since the extracts of almost all finished feeds must be purified before LC can be used, especially if vitamin D is to be determined.

The method described here has the advantage of extracting the vitamins and precipitating the chlorophyll and many other elements present in the sample without altering the vitamin concentration. This eliminates the need for an intermediate step such as in some of the presently used methods (Kobayashi et al., 1976; Wiggins, 1976; Hoffman-LaRoche, 1974; Johnson and Vicker, 1973).

Using this method it is possible to analyze and quantify (1) vitamin A (retinol) and the derivatives such as palmitate and acetate; (2) vitamin D_3 (cholecalciferol); (3) vitamin E, *dl* (alcohol and acetate).

MATERIALS AND METHODS

Materials. 1. Primary stock solution (solution A): (a) vitamin A alcohol (U.S. P.C. Inc., Rockville, Md.), 16.63 mg in 100 mL of methanol; (b) vitamin A acetate (INC, Pharmaceutical Incl, Montreal, P. Q.), 5.24 mg in 100 mL of methanol; (c) vitamin A palmitate (INC, Pharmaceutical Inc., Montreal, P. Q.), 6.51 mg in 100 mL of methanol; (d) vitamin D_3 (INC, Pharmaceutical Inc., Montreal, P. Q.), 50 mg in 100 mL of methanol; (e) vitamin E acetate (tocopherol acetate) (INC, Pharmaceutical Inc., Montreal, P. Q.), 200 mg in 100 mL of methanol; (f) vitamin E alcohol (INC, Pharmaceutical Inc., Montreal, P. Q.), 316 mg in 100 mL of methanol.

2. Solution B for LC is obtained from the primary stock solution A: (a) 5 mL of vitamin A alcohol (solution A) in 100-mL flask completed with methanol to give 8.315 ng/μL; (b) 5 mL of vitamin A acetate (solution A) in 100 mL of methanol to give 2.62 ng/μL; (c) 1 mL of the vitamin D_3 (solution A) in 100 mL of methanol to give 5 ng/μL; (d) 5 mL of the vitamin E acetate (solution A) in 100 mL of methanol to give 100 ng/μL; (e) 5 mL of the vitamin E alcohol (solution A) in 100 mL of methanol to give 158 ng/μL.

These values were chosen so that a 5-μL injection in the LC would give approximately 0.5 scale deflection.

3. Tetraethylenepentamine techn. (Aldrich Milwaukee, Wis.).

4. Sodium Phosphate Tribasic, 20% solution in water (Fisher Scientific Company).

5. Solvents: All solvents were residue free and glass distilled. Solvents were purchased from Caledon Laboratories Ltd., Georgetown, Ontario, Canada.

Equipment. Hewlett-Packard Integrator Model No. 3370A was used to quantitate peaks. Peak areas were expressed in millivolt/seconds.

A liquid chromatograph (Waters Associates Inc., Milford, Mass) Model 202 equipped with a Model 440 UV detector M6000 pump, U6K septumless injector. The Model 440 UV detector was used at sensitivities of 0.2 to 0.005 absorbance units full scale. Chromatograms were recorded on a Linear Instruments (Irvine, Calif.) Model 260 10-mV single-pen recorder with a chart speed of 20 cm/h. The reverse-phase μ Bondapak C/18 material used in the column was from Waters Associates, Inc., Milford, Mass.

A wrist-action shaker (Burrel Corporation) was used.

Procedure. Column packing: The column used was a 25 cm × 4.2 mm (i.d.) stainless steel tubing. The modified balance density slurry technique (Linder et al., 1976) was adopted by slurrying Microbondapak C/18 silanized reverse-phase packing in anhydrous 2-propanol. [Tetrachloroethane–dioxane mixture (3:1) was used with limited success.] The slurry was then charged into a precolumn and pressurized using the solvent delivery system of the liquid chromatograph between 5500 and 6000 psi at a flow rate from 2 to 9.9 mL with intermittent vibration of the column. When the column pressure was steady, the pressure was cut off, the column was allowed to attain atmospheric pressure, and the packing was then eluted with methanol.

Method of Extraction. All experiments were run under subdued lighting. Five grams of ground feed was transferred to a 500-mL Erlenmeyer (actinic glass) and shaken mechanically for 90 min in a solution of dioxane–isooctane (20:80) using a wrist-action shaker. The solution was decanted and the organic phase was filtered on a sintered glass filter (10 μm) with Celite. The residue was washed twice with the dioxane–isooctane solvent mixture (20:80) and filtered once more through Celite filter.

Tetraethylenepentamine (0.3 mL) was added. This mixture was shaken mechanically for 20 min and then evaporated to near dryness in a 500-mL round-bottom flask on a rotary evaporator. The residue was extracted three times with 100 mL of acetonitrile and filtered through Whatman filter paper No. 31 or 41. The filtrate

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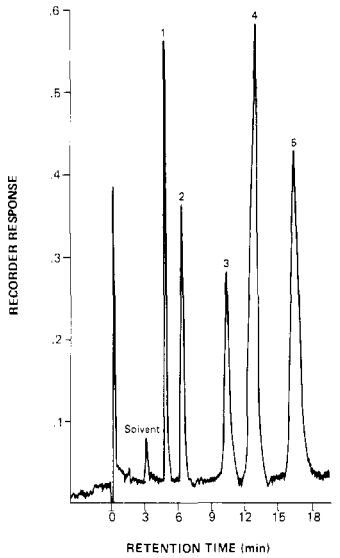

Figure 1. High-pressure liquid chromatography of a mixture of five vitamins: peak 1, 33 ng of vitamin A alcohol; peak 2, 13.1 ng of vitamin A acetate; peak 3, 25 ng of vitamin D_3; peak 4, 474 ng of vitamin E alcohol; peak 5, 500 ng of vitamin E acetate. UV detector sensitivity setting, 0.005 aufs.

was evaporated to approximately 60–80 mL.

Three separate extractions were performed, adding 100 mL of isooctane each time to the acetonitrile. The solutions (isooctane–acetonitrile) were shaken mechanically for 15, 10, and 5 min, respectively. The acetonitrile phase was then discarded.

Sodium phosphate tribasic solution (20%) (4 mL) was added to the isooctane solution. When the mixture is shaken an abundant white precipitate is formed. The solution was filtered on Celite and evaporated fully.

Five–ten milliliters of MeOH was added by a volumetric pipet. The resultant solution is suitable for injection into the LC. The solution is quantified against known reference standard (solution B) of vitamins A, D, and E.

Note: If the vitamin concentration in the sample is very low, then 10 or 15 g of feed may be extracted, but then up to 0.6 mL of tetraethylene pentamine is added at the appropriate step.

RESULTS AND DISCUSSION

The vitamin level in a feed in solution in methanol is stable for up to 3 weeks providing the solution is refrigerated. The addition of tetraethylenepentamine renders the chlorophyll partly insoluble and a limited part of it remains in the acetonitrile phase during the partition of the acetonitrile–isooctane phases. Other amines such as pyridine, 1,3-diaminopropane, and 3,3′-dipropylamine were used without success, due to larger amounts needed for precipitation of the chlorophyll. Concerning the three acetonitrile extractions, it was found using pure standards that approximately 75% of the vitamins went into the acetonitrile layer after one extraction. A second acetonitrile extraction probably would suffice, but due to the large difference in polarity of the vitamins, three extractions were chosen to be sure to achieve quantitative recovery. The addition of the sodium phosphate tribasic solution allows the removal of any traces of amine and precipitates traces of inorganic salts found in the organic phase. Other salts tried such as sodium borate, ammonium chloride, sodium bicarbonate, and sodium phosphate dibasic caused no precipitate and lowered the vitamin concentration.

The chromatogram shown in Figure 1 illustrates the resolution of a mixture of vitamins with the LC system.

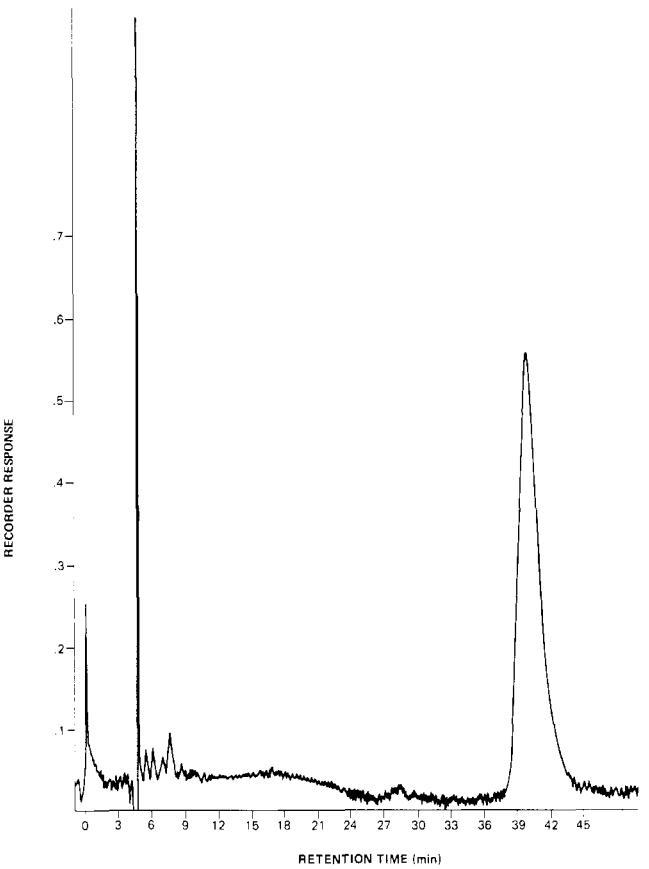

Figure 2. High-pressure liquid chromatography of vitamin A palmitate, 325.5 ng. UV detector sensitivity setting, 0.005 aufs.

Table I. Retention Time Reproducibility[a]

	mean[b]	standard deviation	coeff. of variation, %
vitamin A alcohol	4.99	±0.024	0.48
vitamin A acetate	6.39	±0.038	0.59
vitamin A palmitate	39.23	±0.10	0.25
vitamin D_3	10.84	±0.20	1.84
vitamin E alcohol	13.86	±0.18	1.29
vitamin E acetate	17.67	±0.17	0.96

[a] LC conditions: 1 mL/min of (95:5 v/v, CH_3OH–H_2O, using one μ Bondapak/C_{18} column (25 cm × 4.2 mm i.d.) and UV detector with a 280-nm filter at a sensitivity setting of 0.005 aufs and a chart speed of 20 cm/h. [b] Average of eight injections.

This system includes a μ Bondapak/C_{18} column, a methanol–water (95:5) solvent system, and a Model 440 UV detector. The chromatography of the five vitamins, A acetate, A alcohol, D_3, E alcohol, and E acetate, was completed in 18 min and the vitamin A palmitate in 39.2 min (Figure 2) at a flow rate of 1 mL/min. The retention time of the vitamins is summarized in Table I.

Figure 3 illustrates the linear dynamic range, i.e., the number of times the sample concentration can be increased from the minimum detectable level to the concentration at which the detector is no longer linearly proportional to sample concentration. The calibration curve does not go through zero. All of the work in the paper was carried out at 280 nm, which was not the ideal wavelength for all of the vitamins but was chosen as a compromise to achieve good sensitivity for all three vitamins A, D, and E simultaneously. Though the curves do not go through zero, they are linear over the range of concentration chosen.

At a wavelength of 280 nm, 5 ng of vitamin A acetate and alcohol, 5 ng of vitamin D_3, 50 ng of vitamin E acetate,

Table II. Recovery of Fat-Soluble Vitamins A, D_3, and E in Animal Feeds[a, b]

sample	unspiked			spiked			found			% recov.		
	A	D_3	E	A	D_3	E	A	D_3	E	A	D_3	E
dairy supplement	2739		39	3803	100000	50	6523	88000	91	99	88	97.5
mineral feed, special vitamins	3258		11	1153	40000	20	4529	40000	28.5	95	100	97.7
protein sow supplement	5919		50	761	40000	10	6524	36000	58.5	97	90	92.5
dairy supersweet mix	982			1523		20	2456		18.5	98		92.5
all mash layer (14%)	1184		13.5	3808	100000	50	4892	87000	57.5	93	87	90.5

[a] Vitamin A represents vitamin A acetate or palmitate or both of them. D_3 was not present. E represents E acetate or E alcohol or both of them. The overall percent recoveries of D_3 in feed are 91.25 ± 5.9; of A, 96.4 ± 2.4; of E, 92.6 ± 2.92. [b] The vitamins are expressed as IU/kg.

Table III. Comparison of AOAC and LC Method for Vitamin A, D, and E[a]

	guarantee			AOAC method[b]			LC method		
	A	D_3	E	A	D_3	E	A	D_3	E
pre-mix mineral	220		220	296		236	301		215
pre-mix for turkey and pig	35723	3527	88	36919		73.4	32983	2900	68
pre-mix for poultry	7709	1651	7700	7966		6600	7518	1693	7230
feed (cattle)	285		385	348		273	297		328
feed (rabbit)	18931		44	18000		38	17770		35
feed (horse)	11000		11	10000		10	9953		10

[a] The vitamin A is either vitamin A acetate, A alcohol, or A palmitate, or the sum of two or three of them; vitamin E is either E acetate or E alcohol or the sum of these two. Vitamins are expressed as IU/kg, except for pre-mix samples which are IU/g. [b] For vitamin D_3, the AOAC method was not available in our laboratories.

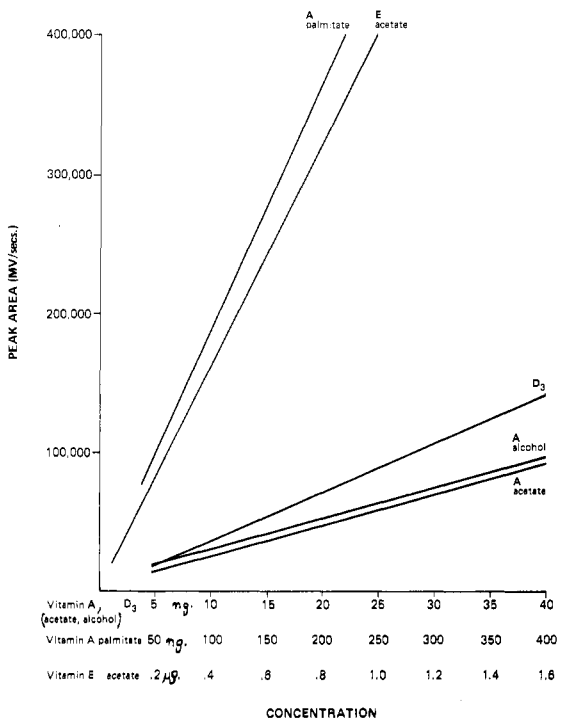

Figure 3. Linear dynamic range of the five vitamins.

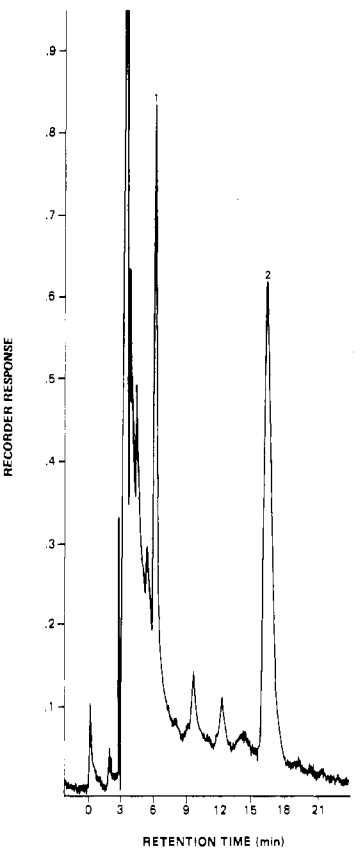

Figure 4. High-pressure liquid chromatography of vitamin A acetate (peak 1) and vitamin E acetate (peak 2) in a 10-g feed sample. UV detector sensitivity setting, 0.005 aufs.

and 60 ng of vitamin A palmitate can be detected. The discrepancy in half-scale response of the vitamins A and E is due to the much lower extinction coefficient of the latter. But the linear dynamic range is adequate for most problems of quantitative analysis of all three vitamins.

Recovery studies were performed on five different samples from various sources. Blanks were run in triplicate on all samples prior to spiking to determine levels of vitamins. No peaks were found to interfere with either vitamin A, D, or E. The quantities of vitamin spiked are summarized in Table II. Minimum detectable amounts are: vitamin A, 250 I U/kg; vitamin D_3, 10 000 IU/kg; vitamin E, 5 IU/kg.

The recovery percentages are the following: vitamin A acetate, 96.4 ± 2.4; vitamin D_3 81.25 ± 5.9; vitamin E acetate, 92.6 ± 2.92.

Figure 4 is the chromatogram of a feed sample (10 g) using the method previously described. Elution is from a μ Bondapak/C_{18} column with methanol–water (95:5) at

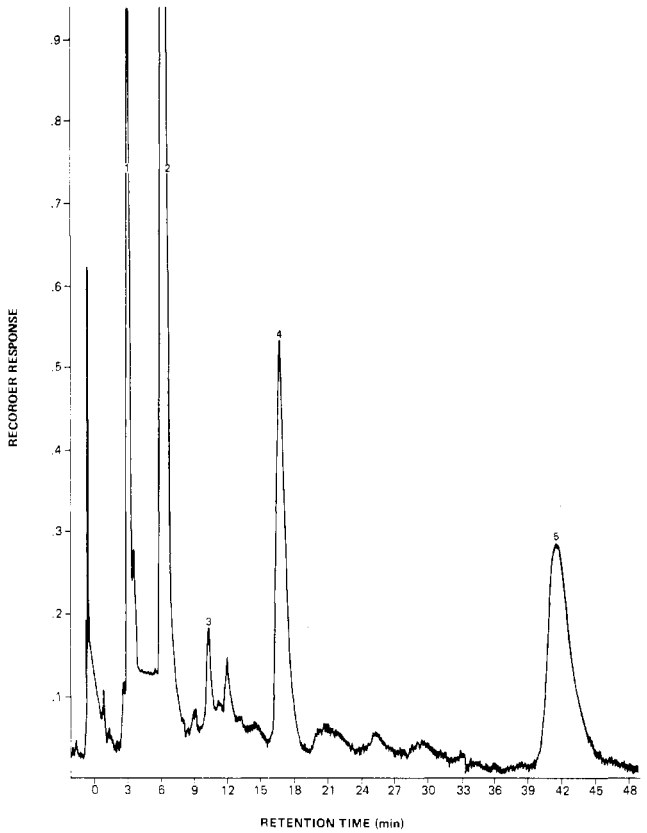

Figure 5. High-pressure liquid chromatography of vitamin A acetate, peak 2; vitamin D_3, peak 3; vitamin E acetate, peak 4; vitamin A palmitate, peak 5 in a 0.4-g sample of poultry vitamin pre-mix. UV detector sensitivity setting 0.005.

a flow rate of 1 mL/min with a 280-nm filter UV detector. This sample contains vitamin A acetate (peak 1, 1500 IU/kg) and vitamin E acetate (peak 2, 20 IU/kg). Figure 5 is the chromatogram of a sample of poultry pre-mix (0.4 g) eluted under the same conditions described for Figure 4. This sample contained vitamin A acetate (peak 2), vitamin D_3 (peak 3), vitamin E acetate (peak 4), and vitamin A palmitate (peak 5) which gives a broader peak. The first peak which appears is the solvent peak. The levels are found in Table III.

The identification of the vitamins was performed in reference to a standard. The identification of vitamins A and E was achieved by collecting each from the column and using the AOAC Colorimetric Method (Association of Official Analytical Chemists, 1970, 1974). Vitamin D_3 was collected and the UV spectra in mobile phase show a maxima at 264 nm (literature gives 264.5 nm).

A series of duplicate analyses was conducted on the same day by both the LC and the official AOAC procedure, and the HPLC method compared favorably with the colorimetric method. The comparative results are shown in Table III.

In conclusion, we have found the LC reverse-phase method, with preliminary cleanup as described, to be an efficient technique for the determination of vitamins A, D, and E and some of their common derivatives. The method is simple, sensitive, reproducible, and in addition very rapid in comparison with the standard methodology. The minimum quantity of vitamin D_3 is a function of detector sensitivity and not of the method described.

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