Simultaneous Analysis of Vitamins A and E in Rodent Feed by High-Pressure Liquid Chromatography

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A method is presented for the coextraction and simultaneous analysis of vitamins A and E from three rodent feeds (NIH-31, Masoro, and Emory Morse) formulations. The method consists of extraction of the vitamins for 16 h with ethanolic KOH containing ascorbic acid followed by partitioning into hexane and subsequent analysis by normal phase HPLC using hexane/2-propanol/glacial acetic acid (990/10/0.2) mobile phase, a 5-μm LC-CN column, and detection at 296 nm. Recoveries of vitamin A from NIH-31, Masoro, and Emory Morse feeds spiked at 50 μg/g were 95.5, 74.6, and 78.2%, respectively, while those for vitamin E spiked at 250 μg/g were 103, 103, and 85.2%, respectively. Ancillary data are also presented which demonstrate the stability of vitamin A alcohol and vitamin E in ethanolic KOH for as long as 21 h. Additional data are presented detailing results of analyses of five lots of NIH-31 and Masoro feeds and seven lots of Emory Morse feed as received from the supplier.

INTRODUCTION

Currently the official methods of the Association of Official Analytical Chemists (AOAC) for vitamins A and E in animal feeds are separate assays with detection based on colorimetry. New procedures utilizing modern analytical techniques such as high-pressure liquid chromatography (HPLC) are needed. A few HPLC procedures for the determination of vitamin E alone in animal feed have been published.

In the past few years, HPLC has been utilized for the determination of vitamins A and E. De Leenheer et al. (1979) Driskell et al. (1982), and Nierenberg and Lester (1985) have published procedures for the simultaneous analyses of vitamins A and E in serum and plasma. Cohen et al. (1980) and McMurray et al. (1980) have developed methods for the determination of vitamin E in animal feeds. The *Official Methods of Analysis* of the AOAC (1990) lists separate analyses for vitamin A and vitamin E in feeds; however, both have sample saponification and extraction steps that are similar. Therefore, the possibility of a combined procedure with coextraction of vitamin A and vitamin E appeared feasible. This line of reasoning was investigated which resulted in development of an analytical procedure for the coextraction of vitamins A and E from three rodent feeds with subsequent simultaneous analysis of these vitamins by HPLC with detection at 296 nm.

MATERIALS AND METHODS

Chemicals. The *all-trans*-vitamin A (Aldrich Chemical Co., Milwaukee, WI), the *all-trans*-vitamin A acetate (Sigma Chemical Co., St. Louis, MO), and the vitamin E, *dl*-α-tocopherol (Fluka Chemical Corp., Ronkonkoma, NY), were used as received. The vitamin E standard (5 mg/mL) was prepared in methanol, and the vitamin A acetate standard (1 mg/mL) was prepared in 95/5 hexane/2-propanol. All standards and samples were prepared under subdued yellow lighting. The concentration of the vitamin A acetate standard prepared daily was determined by diluting 200-fold with methanol and reading its absorbance using a 1-cm cuvette and a UV spectrophotometer set at 324 nm. The *Merck Index* lists the $E^{1\%}_{1cm}$ for vitamin A acetate as 1550. All solvents were distilled in glass HPLC quality from J. T. Baker (Phillipsburg, NJ).

Sample Preparation. Pelletized feeds (2 kg) (NIH-31 and Masoro, Ralston Purina Co., St. Louis, MO; Emory Morse, Emory Morse Co., Guilford, CT) were ground in a Waring blendor and sieved through a no. 8 sieve. This material (2 kg) was further blended for 20 min in a Model LV twin shell blender (Patterson-Kelley, East Stroudsburg, PA).

Extraction Procedure. Duplicate 10-g samples of each feed type were placed in 250-mL screw-cap Erlenmeyer flasks. Extraction solvent consisting of 50 mL of ethanol, 0.5 g of ascorbic acid, and 50 mL of aqueous potassium hydroxide (500 g/L) was added to the 250-mL Erlenmeyer flasks. Additionally, two samples of each feed type were weighed, 99 mL of extraction solvent was added, and then the mixture was spiked with 0.5 mL of each vitamin standard. Standards of vitamin A acetate and E were carried through the procedure by spiking 0.5 mL of each into 99 mL of extraction solvent. After the flasks were sealed with Teflon-lined screw caps, they were shaken overnight (16 h) on an Eberbach reciprocating shaker at 200 excursions/min. Five milliliters of sample or standard was removed and added to separate Teflon-lined screw-capped tubes. Five milliliters of ice-cold deionized water and 5 mL of hexane were added, and the mixture was shaken for 3 min by hand. The layers were allowed to separate. The hexane layer was transferred with a Pasteur pipet to a second tube containing 0.5 mL of 1 M KH_2PO_4 (pH 7). This was shaken by hand for 30 s and then centrifuged to separate the layers. Fifty microliters of the hexane layer was injected into the HPLC system.

HPLC. Chromatographic separations were performed on a 4.6 × 250 mm Supelco (Supelco Inc., Bellefonte, PA) 5-μm LC-CN column. The mobile phase was hexane/2-propanol/glacial acetic acid (990/10/0.2) flowing at 2 mL/min at approximately 700 psi. All injections were 50-μL loop injections on a Rheodyne (Rheodyne Inc., Cotati, CA) injector. A Model SP8700 pump (Spectra Physics, San Jose, CA) and a Model SP8440XR variable-wavelength UV-visible detector set at 296 or 440 nm were used. The retention times (t_R) for vitamins A and E were 6.4 and 2.5 min, respectively. A Hewlett-Packard (Palo Alto, CA) Model 1040A photodiode array UV-visible detector was used to record the UV spectra of vitamins A and E. All quantitation was by peak area using a Shimadzu (Shimadzu Scientific Instruments, Inc., Columbia, MD) CR3A integrator. The concentration of the spiked minus the concentration of the unspiked samples divided by the concentration of the standard times 100 equals percent recovery.

Stability. The stability of vitamins A and E was determined by spiking 100 μL of vitamin A alcohol (1 mg/mL) and 400 μL of vitamin E (1 mg/mL) into each of five flasks containing 1 g of ascorbic acid and 99.5 mL of ethanolic/KOH. Also, 100 μL of the vitamin A acetate (1.15 mg/mL) and 400 μL of vitamin E (1 mg/mL) were spiked into flasks containing 1 g of ascorbic acid and 99.5 mL of ethanolic/KOH. These were spiked at such

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times (e.g., -21, -4, -2, -1, and -0.25 h) that would allow all samples to be simultaneously removed from the Eberbach shaker, extracted with hexane, and assayed by HPLC as previously described.

Recovery Experiments. Duplicate 10-g samples of animal feed of each of the three diet types were spiked by adding 99 mL of extraction solvent, 0.5 mL of a 5 mg/mL vitamin E solution in MeOH, and 0.5 mL of a 1 mg/mL vitamin A acetate solution in 95/5 hexane/2-propanol. Duplicate 10-g samples of unspiked feed of each diet type were also prepared for analysis. All samples were then taken through the extraction procedure and analyzed by HPLC as previously described to determine the accuracy and precision of the procedures. This process was repeated on three separate days.

RESULTS AND DISCUSSION

Stability of vitamins can be a problem area for the researcher and must be evaluated by using conditions that will be used in the course of methodologies employed. The vitamin E primary standard (5 mg/mL) used in our study was stable for over a month in methanol; however, the vitamin A alcohol standard (1 mg/mL) in hexane deteriorated rapidly. Therefore, the more stable vitamin A acetate was selected for use as a standard. A fresh vitamin A acetate standard was prepared daily and the concentration determined spectrophotometrically at 324 nm in a manner similar to the determination of retinol reported by Thompson (1986).

Most reverse-phase HPLC procedures for the analysis of vitamin E involve evaporating the sample extract to dryness and reconstituting in a reverse-phase compatible solvent. However, thin films of vitamin E are reported by the AOAC *Official Methods of Analysis* (1990) to oxidize rapidly on exposure to air. Therefore, a normal phase HPLC system was selected to eliminate this problem and also reduce sample handling. A normal phase solvent system consisting of hexane/methylene chloride/2-propanol similar to that used by Cohen et al. (1980) for determination of vitamin E in animal feeds was tested for the chromatography of vitamins A and E in our application. Although good separation of vitamins A and E was achieved, it was subsequently found that methylene chloride reduced the concentration of vitamin A at microgram per milliliter concentrations. This effect was also noted with chloroform. Percolating these solvents through alumina prior to use diminished this effect but did not eliminate it. Therefore, these solvents are not recommended for vitamin A determinations.

Organic solvents alone do not extract vitamin A quantitatively from vitamin-fortified feeds and supplements. This is because the vitamin supplements are often added as gelatin beadlets by the manufacturer to enhance stability and thus require penetration by water or alkali for dissolution (Thompson, 1986). Furthermore, McMurray et al. (1980) have determined that saponification before extraction rather than after, as recommended by the AOAC (1990), procedure most efficiently extracts natural vitamin E from the food matrix as well as saponifies all vitamin E esters which may have been added in the form of vitamin supplements. Alkaline hydrolysis also functions to convert all of the vitamin A esters, commonly the palmitate and/or acetate, into retinol (vitamin A). This hydrolysis also acts as a cleanup step by partitioning the hydrolyzed fats, a large number of pigments, and other material into the water layer while partitioning the vitamins into the hexane layer. Table I illustrates the stability of vitamins A and E under alkaline extraction conditions. Both vitamins A and E are stable in ethanolic KOH containing the antioxidant ascorbic acid for at least 21 h. Additional information suggests that vitamin A is not destroyed during alkaline hydrolysis and will survive for at least a week in ethanolic KOH containing the antioxidant pyrogallol according to Thompson (1986). Additional experiments were performed in our laboratories to compare efficiency of alkaline hydrolysis at ambient temperature and at 70 °C as recommended by the AOAC procedure. Triplicate NIH-31 feed samples, as received from the supplier, were taken through our procedure utilizing the previously stated hydrolysis conditions at ambient temperature and the AOAC procedure. Samples hydrolyzed under ambient conditions were analyzed for vitamin A after 1, 2, 4.5, 6.5, and 20.5 h. There was no statistically significant difference between vitamin A data derived from ambient versus 70 °C hydrolysis conditions.

Table I. Stability of Vitamins A and E in Ethanolic KOH[a]

time, h	vitamin A acetate[b]	vitamin E[b]	vitamin A alcohol[c]	vitamin E[c]
0.25	100	100	100	100
1	101	104	98	96
2	100	101	101	97
4	102	102	103	97
21	102	103	100	96

[a] 0.25-h values normalized to 100%. [b] Vitamin A acetate, 1.15 μg/mL; vitamin E, 4 μg/mL. [c] Vitamin A alcohol, 1 μg/mL; vitamin E, 4 μg/mL.

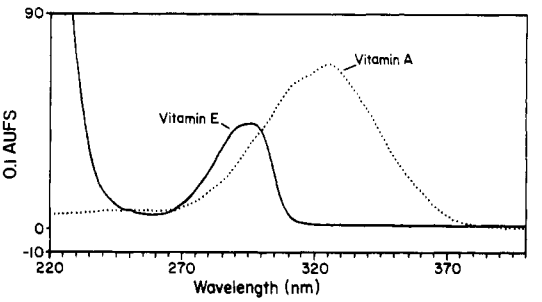

Figure 1. UV spectra of vitamins A and E; maxima at 324 and 296 nm, respectively.

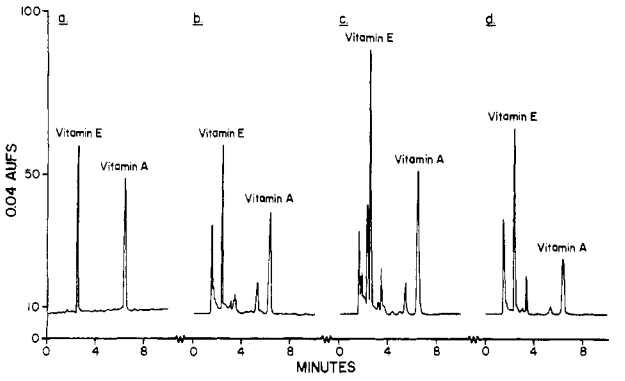

Figure 2. (a) Normal phase HPLC chromatogram of standards with the detector set at 296 nm: 250 ng of vitamin E and 44 ng of vitamin A (alcohol); (b–d) 5-mg equivalents of NIH-31, Emory Morse, and Masoro feeds, respectively; all at 0.04 AUFS.

Figure 1 illustrates the UV spectra of vitamins A and E recorded with a Hewlett-Packard photodiode array HPLC detector using a mobile phase of hexane/2-propanol/glacial acetic acid. Vitamins A and E have UV maxima at 324 and 296 nm, respectively. As determined from its spectrum, vitamin A also absorbs at 296 nm. Figure 2a is the normal phase HPLC chromatogram of a 50-μL injection of a standard of vitamins A and E with detection at 296 nm. This wavelength was selected to maximize the response of vitamin E, which has a relatively weak molar absorptivity, and still detect the vitamin A. Parts b–d of Figure 2 are HPLC chromatograms of extracts of NIH-31,

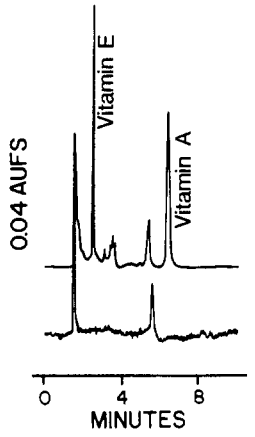

Figure 3. Normal phase HPLC chromatogram of extract of NIH-31 feed at 296 (upper trace) and 440 nm (lower trace).

Table II. Recovery Data for Vitamins A and E in Three Types of Rodent Feed[a]

	% recovery[b]				
	day 1	day 2	day 3	$\bar{x} \pm$ SD	$\bar{x} \pm$ SE
NIH-31					
vitamin A	99.0	98.2	89.4	95.5 ± 5.3	95.5 ± 3.1
vitamin E	104	101	103	103 ± 2	103 ± 1
Masoro					
vitamin A	71.8	72.2	79.9	74.6 ± 4.6	74.6 ± 2.6
vitamin E	118	97.8	94.4	103 ± 13	103 ± 7
Emory Morse					
vitamin A	79.5	80.1	75.1	78.2 ± 2.7	78.2 ± 1.6
vitamin E	86.3	84.4	84.9	85.2 ± 1.0	85.2 ± 0.6

[a] Vitamin A spiked at 50 μg/g of feed; vitamin E spiked at 250 μg/g of feed. [b] Data from days 1–3 are means of duplicate determinations on each day.

Emory Morse, and Masoro feeds, respectively, illustrating their vitamin A and vitamin E content. Figure 3 is a HPLC chromatogram of the extract of NIH-31 feed at 296 and 440 nm. The higher wavelength was examined to determine if carotenoids in the yellowish extract from the NIH-31 feed were coeluting with these vitamins. No coelution was observed at the retention times of these vitamins as shown in Figure 3.

Due to the literature-reported susceptibility of vitamins A and E to air oxidation (Windholz, 1983; AOAC, 1990), recovery experiments from feed were carried out by spiking these vitamins into the extraction solvent containing the antioxidant ascorbic acid and allowing them to equilibrate/partition between the feed and solvent by shaking 16 h. This approach was adopted rather than spiking directly onto the feed, which leaves the vitamins exposed to air and feed components after evaporation of the solvent. Spiking directly onto the feed gave low and erratic recoveries. Manufacturers enhance the stability of their supplemented vitamins in feed by encapsulating them in a gelatin beadlet. This not only reduces vitamin exposure to air but also prevents direct contact between the vitamin and feed components. Recovery of vitamin E (spiked at 250 μg/g) was typically 100% from NIH-31 and Masoro feeds and 85% from Emory Morse feed. The recovery of vitamin A (spiked at 50 μg/g) was generally 75% from Masoro and Emory Morse feeds and 95% from NIH-31 feed. Table II details the results of the recovery experiments performed in duplicate on three separate days.

The utility of the method is demonstrated by the data in Table III which details the results of duplicate assays of several lots of three feed formulations as received from the supplier (NIH-31, Emory Morse, and Masoro feeds)

Table III. Vitamins A and E in Three Rodent Diet Formulations As Received from Supplier[a]

lot no.	vitamin E	SE[b]	CV[c]	vitamin A	SE[b]	CV[c]
			NIH-31			
1	73.5	1.0	1.4	16.5	0.8	5.1
2	48.2	0.4	0.8	10.8	1.0	9.1
3	70.9	0.7	0.7	15.3	0.5	3.5
4	70.0	2.4	2.4	16.0	0.4	2.7
5	72.6	0.5	0.5	10.7	0.3	2.7
			Emory Morse			
1	89.0	0.7	0.8	7.8	0.5	6.5
2	116	1.2	1.0	13.0	0.4	3.4
3	116	0.9	0.8	14.0	0.6	3.9
4	115	4.4	3.8	13.2	0.4	2.9
5	116	1.5	1.2	13.4	0.7	4.9
6	119	0.9	0.7	12.9	0.3	2.7
7	119	1.0	0.8	13.7	0.7	5.3
			Masoro			
1	64.1	0.1	0.2	7.7	0.5	6.8
2	93.7	1.1	1.1	13.7	1.7	12.
3	57.5	0.3	0.5	6.6	0.2	2.8
4	59.6	0.5	0.8	7.0	0.4	5.8
5	90.3	1.8	1.8	14.8	0.4	2.6

[a] Vitamins A and E in micrograms per gram of feed, corrected for recovery; average of duplicate assays performed on three separate days. [b] SE, standard error, $N = 3$. [c] CV, coefficient of variance.

for vitamins A and E performed on three separate days. All results are reported in micrograms per gram of feed. Formerly, vitamin A values were reported in international units (IU); however, this unit as originally defined ceased to exist in 1956. Even the newer retinol equivalents (RE) unit should not be employed indiscriminately and is not always applicable to animal nutrition. Thompson (1986) suggests that the analyst is advised to express results in micrograms or milligrams and refrain from calculating units or equivalents. The coefficient of variance (CV) for the assay of vitamin E in feed (Table III) averaged 1.1% for 17 lots of feed. The CV for vitamin A averaged 4.9% for the same 17 lots.

In conclusion, a HPLC procedure is described for the simultaneous analysis of vitamins A and E after saponification and coextraction from three rodent feed formulations. This procedure provides a simple and time efficient alternative analysis for these vitamins that formerly required two separate assays.

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