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Methodology for simultaneous estimation of vitamins A and E in animal feeds using high performance liquid chromatography

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Evaluation of vitamin A in feeds is highly required because of its important roles in vision, maintenance of epithelial lining and immunity (McDonald *et al.* 1995) in animals. High performance liquid chromatography is one of the most powerful tools for the estimation of vitamins. This technique has replaced the earlier colorimetric and flouorimetric methods because of its high sensitivity, accuracy and speed. As such feed industry does not make use of this sensitive technique of vitamin analysis because of cost involvement. BIS has suggested to adapt the HPLC method 14565 issued by International Organisation for standardisation (ISO) for vitamin A analysis in feeds. This method requires validation before adoption by the industry. Vitamin E (α-tocopherol), another non enzymatic antioxidant, is involved in maintenance of immunity status of animals and prevents mastitis in dairy cows (Weiss *et al.* 1997, Kaur *et al.* 2002). Due to the critical role of these vitamins in animal nutrition, their quantitative analysis is very important to know their content in the feeds as well as to know the changes in their concentration under different storage and processing conditions. Hence, there is a need to revalidate ISO method for vitamin A estimation by HPLC, as well as to develop another HPLC method which can simultaneously estimate both vitamins A and E for adoption by the feed industry on routine basis.

Standards of α-tocopherol and all trans-retinol were procured commercially. HPLC grade acetonitrile, tetraphydrofuran, water and methanol were used for preparing mobile phases. Analytical grade ethanol, chloroform, petroleum ether and ascorbic acid were used. Stock solutions of retinol (20 μg/ml) and α-tocopherol (200 μg/ml) were prepared in 100% ethanol. Requisite aliquots of individual stock solutions were taken in brown colour volumetric flasks and dried under nitrogen at room temperature. The dried standards were reconstituted in mobile phase. A working standard solution containing 2.0 and 20.0 μg/ml of retinol and α-tocopherol was prepared at the time of use. All vitamin standards were stored at–20°C.

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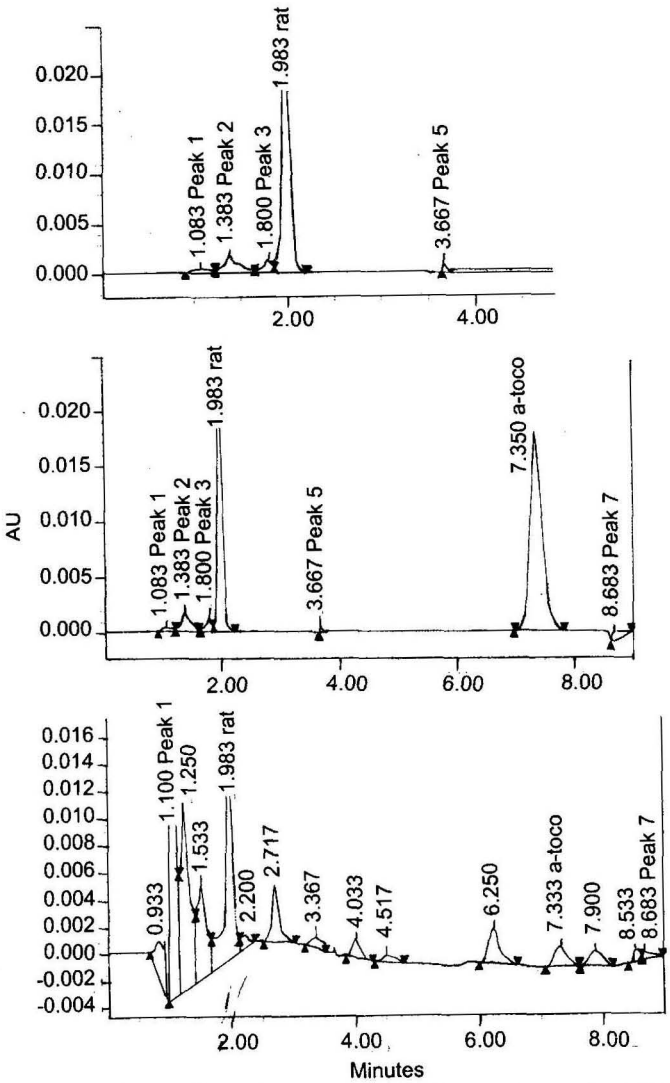
Concentrate mixture was fortified with vitamin A acetate and α-tocopherol acetate separately. Sample (1g) was subjected to saponification by addition of 10 ml of 95% ethanol and 2.0 ml of 60% potassium hydroxide (KOH). To prevent any significant loss in retinol and α-tocopherol during sample treatment, 3% ascorbic acid was added to ethanol. The extraction was done as per ISO method. Combined ether extract was washed with 10 ml of 0.5 N KOH and subsequently with distilled water to remove excess alkali. The ether extract was passed through Whatman phase separator filter paper to remove moisture and was dried under nitrogen in a water-bath maintained at 37°C. The dried residue was reconstituted in the mobile phase and was filtered through 0.22μ membrane filter for injection on HPLC. 20 μl of sample was injected on HPLC column. The samples were analyzed on HPLC system consisting of a model 510 pump, rheodyne injector with 20μl loop, model 486 tunable absorbance detector using multiwavelength detector and column (15 cm × 4.6 mm) packed with 5 μm silica particles.

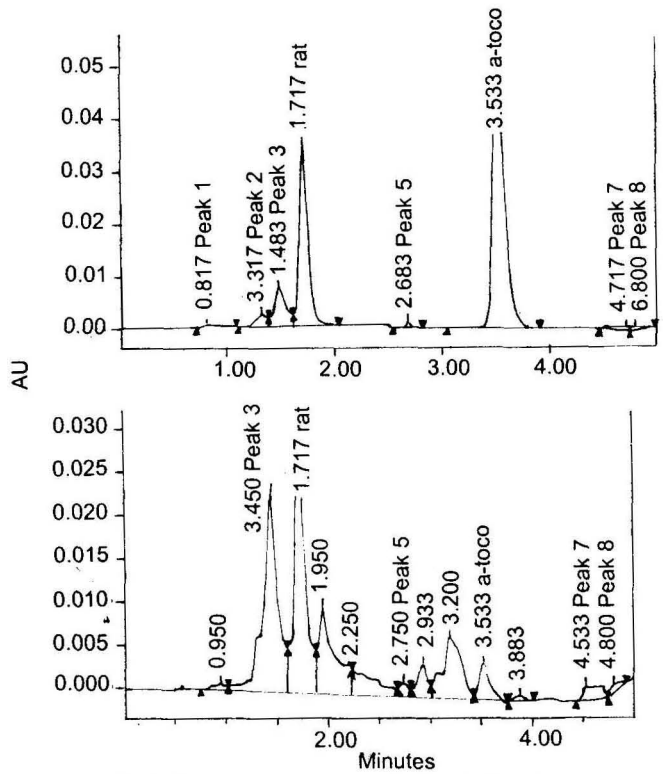
Solvent system of ISO method was used for the separation of vitamin A which consisted of methanol and HPLC water in the ratio of 95:5. ISO solvent was also used for the simultaneous analysis of vitamins A and E in feed samples. Various combinations of solvent system were tried to develop a method for simultaneous estimation of vitamins A and E in feed samples in minimum possible time. Solvent system consisting of acetonitrile, tetrahydrofuran and HPLC water in the ratio of 47:42:11 was selected as it could separate both the vitamins in 4 min time at a flow rate of 1.5ml/min. ISO method for the analysis of vitamin A alone was standardized using mobile phase comprising methanol: water (95:5, v/v) at a flow rate of 2ml/min. Retinol was separated at 1.95 min at 325 nm wavelengths. Fig.1 shows the chromatograph of standard retinol using ISO method.

Mobile phase of ISO method was used for the simultaneous analysis of retinol and α-tocopherol. The main difficulty in the simultaneous determination of retinol and α-tocopherol is their different spectral characteristics (absorption maxima). Therefore a specific programme was adopted for the



Figs 1-3. **1.** Chromatogram of standard vitamin A using ISO method (*top*) **2.** Chromatogram of standard retinol and α-tocopherol on a single run using ISO method (*middle*). **3.** Chromatogram of a feed sample using ISO method (*bottom*).

separation of retinol and α-tocopherol at 325 and 290 nm wavelengths respectively. Retinol and α-tocopherol-were separated at 1.95 and 6.95 min, respectively, and the wavelength from 325 was changed to 290 after 4 min of run (Fig. 2). The separation of vitamins A and E in the feed sample is shown in Fig. 3. Siong and Choo (1999) utilized HPLC method for simultaneous determination of retinol and α-tocopherol in 20 µl serum samples using methanol and water (95:5, v/v). The separation of retinol and α-tocopherol was achieved at 2.8 min and 8.1 min, whereas in the present experiment, the separation was achieved faster. The response factor for α-tocopherol was 8–9 times less than the response factor of retinol which might be due to the reason that α-tocopherol is better detected in fluorescence detector. Hewavitharana *et al.* (1996) estimated both vitamins A and E simultaneously using fluorescence detector in dairy foods and



Figs 4-5. **4.** Chromatogram of standard retinol and α-tocopherol on a single run usng developed method (*top*). **5.** Chromatogram of a feed sample using developed method (*bottom*).

separated the 2 vitamins in 20 min. Retinol also exhibits fluorescence but quantum efficiency is low (Lawn *et al.* 1983) than vitamin E and UV detection is used in most HPLC systems for determining vitamin A.

In the method used for simultaneous analysis of both vitamins A and E using methanol and water (ISO solvent), the time required for the separation of 2 vitamins was 8 min. To reduce the time of analysis, different combinations of solvent systems were tried so that the method can be easily adopted by feed industry for large scale analysis of feed samples. Various proportions of tetrahydrofuran with acetonitrile and water were tried at different flow rates to maximize the separation of 2 vitamins in shortest possible time without losing efficiency. Solvent system comprising acetonitrile, tetrahydrofuran and HPLC grade water in the ratio of 47: 42:11 (v/v/v) was the best mobile phase for the separation of retinol and α-tocopherol at flow rate of 1.5ml/min. The separation of standard retinol and α-tocopherol was achieved at retention time of 1.72 and 3.53 min at 325 and 290 nm wavelengths, respectively, and the wavelength was changed after 2.5 min (Fig. 4). Fig. 5 showed the separation of both the vitamins in a feed sample. Chawla and Kaur (2001) earlier developed isocratic HPLC method for simultaneous estimation of retinol, α-tocopherol and ß-carotene in plasma samples and separated the 3 vitamins at 2.17, 3.25 and 4.63 min, respectively, using tetrahydrofuran, acetonitrile and

HPLC water as solvent system. The analytical recoveries of vitamin A ranged from 94.60-95.72% for standard retinol concentrations in the range of 20–100ng and 98.15–103.50 for 200–400ng α-tocopherol.

Siong and Choo (1999) utilized HPLC method for simultaneous determination of retinol and α-tocopherol in 20 µl serum samples using acetonitrile, methanol and ethylacetate in the ratio of 88:10:2 (v/v/v) developed by Tee *et al.* (1994) and Tee and Khor (1995). They could not achieve separation of retinol and α-tocopherol along with added internal standards using acetonitrile, methanol and ethyl acetate as eluent. Gueguen *et al.* (2002) developed HPLC method using diode-array detector for separation of α-tocopherol, retinol and 5 carotenoids in human serum using methanol, acetonitrile and tetrahydrofuran (75: 20: 5, v/v/v) and full elution was obtained isocratically within 20 min. Woollard and Indyk (1986) achieved complete separation of α-tocopherol from vitamin A esters using mobile phase composition of hexane containing 0.08% propanol using both fluorescence and absorbance detectors. They could conveniently separate α-tocopherol from retinol in fluorescence detector, whereas it was not separated using UV detector.

An HPLC method utilizing only one detector for the separation of vitamins A and E is the most convenient and feasible as it will reduce the cost of analysis. The developed method utilizes only UV detector for the analysis of retinol and α-tocopherol at different wavelengths to attain maximum recovery. The developed method was faster than the ISO method. It could analyze both vitamins A and E in 4 min as compared to 8 min using ISO solvent system for vitamin A. Therefore it can be used on routine basis for simultaneous analysis of retinol and α-tocopherol in feed samples.

SUMMARY

Validation of International Organisation for Standardisation (ISO) method for vitamin A analysis in feeds was carried out. The method was extended for simultaneous estimation of vitamins A and E in animal feeds. The separation of vitamins A and E was achieved at 1.95 and 6.95 min, respectively, with solvent system consisting of methanol: water in the ratio of 95:5, as used in ISO method for the analysis of vitamin A alone. Various solvent systems comprising tetrahydrofuran, acetonitrile and water were tried at different flow rates to maximize the separation of 2 vitamins in shortest time so that the method can be easily adopted by feed industry for large scale analysis of feed samples. Solvent system consisting of acetonitrile, tetrahydrofuran and HPLC grade water in the ratio of 47:42:11(v/v/v) resulted in the separation of retinol and α-tocopherol at retention time of 1.72 and 3.53 min at 325 and 290 nm wavelengths, respectively, at flow rate of 1.5 ml/min. The developed method can be routinely used for the analysis of vitamins A and E in feed samples.

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