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Note

Analysis of the fat-soluble vitamins using narrow-bore high-performance liquid chromatography with multichannel UV–VIS detection*

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Vitamins are biologically active compounds which are essential for normal health and growth. A deficiency of vitamins in the diet can cause debilitating symptoms however, excessive amounts can prove toxic. Regulations exist which demand quantitative and specific measurement of the individual vitamins in foods. Vitamins generally lack stability when exposed to heat, light or oxygen and their levels in food can be as low as a few micrograms per 100 g. They are often accompanied by an excess of compounds with similar physical and chemical properties which can interfere with their analysis. During the past decade high-performance liquid chromatography (HPLC) has been developed as a technique for vitamin analysis with many advantages over previously used crude biological and chemical assays which had poor reproducibility and method robustness[1]. Normal-phase HPLC is generally preferred for vitamin A[2] and vitamin E[3] in foodstuffs. The inherent ability of HPLC to separate the vitamins from interfering substances, coupled with the availability of selective detectors provides a fast and reliable method of vitamin analysis which is both quantitative and specific. Recently the techniques of microcolumn HPLC and multichannel UV detection have been developed, both of which can offer significant advantages over previous HPLC methods for vitamin analysis.

Miniaturisation of the HPLC column and, consequently, sampling systems, detectors and ancillary equipment have been studied extensively. Recently this work was summarised by Kucera[4]. Narrow-bore HPLC employs columns of internal diameter between 1 mm and 2.5 mm, and equipment is now available which is compatible with this column geometry. The instrumentation used for the study described in this paper consisted of a pump and detector which are compatible with conventional columns (4.6 mm I.D.) but have been designed with reduced dispersion effects and have available a specially designed 1-μl flow cell. This system has been evaluated for external variance and system compatibility with narrow-bore column technology[5]. The evaluation technique employed uses a comparison of the column peak standard deviation (σ_{col}) with the measured external peak standard deviation (σ_{ext}) to calculate the expected resolution loss[6,7]. Table I shows the results obtained for

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TABLE I

CALCULATED RESOLUTION LOSSES WITH DISPERSION

k	σ_{col} (μl)	σ_{ext} with 1-μl flow cell (μl)	Resolution loss (%)
1	7.1	4.7	16
2	10.7	4.7	< 10

capacity factors (k) of 1 and 2. The columns, external tubing, injection system and flow cell used for these calculations are of the same dimension as those employed for this application. Previous applications of microcolumns with multichannel UV detection optimised to give low dispersion, have involved the use of specialised equipment dedicated to this column geometry[8].

The ability of multichannel UV detection to identify relevant chromatographic peaks and to monitor at multiple UV wavelengths simultaneously is particularly pertinent to the detection of vitamins which have differing UV absorbance maxima and are assayed in complex mixtures[9]. The detector employed provided a three-dimensional plot of wavelength *vs.* absorbance *vs.* time (a chromascan) which could be extensively manipulated post run, providing qualitative and quantitative information. This avoided any re-injection of vitamin solutions which could quickly deteriorate. The extra facilities of derivative spectra for identity confirmation and spectral subtraction for background suppression proved extremely useful in this application.

EXPERIMENTAL

Materials

Vitamins for use as reference materials were obtained from Sigma (St. Louis, MO, U.S.A.). Solvents were HPLC grade (Fisons, Loughborough, U.K.).

Instrumentation

The HPLC system consisted of a PU4015 pump with a PU4021 multichannel UV/VIS detector equipped with a 1-μl flow cell and a Rheodyne 7520 injection valve with a 1-μl rotor. The detector output was connected to a PU4850 data station having two additional 128K memory boards. (All components were from Philips Analytical, Cambridge, U.K.).

Chromatographic conditions

Sample manipulations were performed in the absence of oxygen, direct sunlight, or the light of fluorescent tubes to avoid degradation of the vitamins.

All data were obtained as chromascans from which spectra and chromatograms could be taken using the PU4850 data station.

A 3-cm guard and a 10 cm × 2.1 mm I.D. Spheri-5 Silica cartridge (Philips Analytical) were used with a mobile phase of hexane–isopropanol (99.5:0.5, v/v) and a flow-rate of 0.4 ml/min. All reference materials were dissolved in hexane. Retinyl palmitate (vitamin A palmitate) was extracted from 50 g of dried milk by ultrasonication in hexane, evaporation to dryness and redissolution in 1 ml of hexane. Retinol

(vitamin A) and α-tocopherol (vitamin E) were extracted from 0.5 g of animal feed after saponification followed by extraction with hexane, evaporation to dryness and redissolution in 10 ml of hexane.

RESULTS AND DISCUSSION

Standard solutions containing 0.5 mg/ml of each of retinol and α-tocopherol were chromatographed and retention times, spectra and area response factors were established. Examination of the reference spectra revealed the absorbance maximum of retinol A as 330 nm and that of α-tocopherol as 290 nm.

Animal feed was assayed for retinol and α-tocopherol; chromatograms and spectra are shown in Fig. 1. Recoveries were calculated and results are shown in Table II. The identity of α-tocopherol was confirmed by comparison of first- and second-order derivative spectra with those obtained from the reference spectra. These spectra are reproduced in Fig. 2.

Fig. 3 shows the chromascan and the chromatogram and spectrum of retinyl palmitate in dried milk; this spectrum reveals the presence of a coeluting compound. A spectrum of this compound, obtained as a baseline spectrum taken a few seconds before retinyl A palmitate began to elute, was normalised and subtracted from the spectrum of the vitamin. The resulting spectrum was identified as that for retinyl A palmitate. The spectrum of the co-eluting compound showed no UV absorbance at

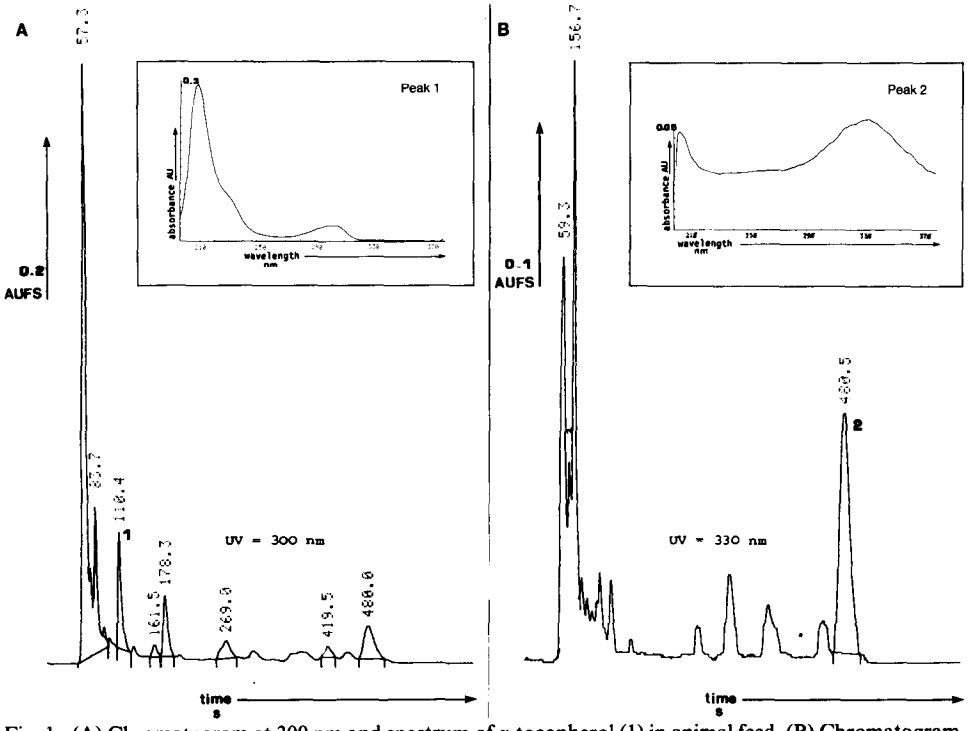

Fig. 1. (A) Chromatogram at 300 nm and spectrum of α-tocopherol (1) in animal feed. (B) Chromatogram at 330 nm and spectrum of retinol (2) in animal feed.

TABLE II

RECOVERY OF VITAMINS FROM THEIR SAMPLE MATRICES

Sample	*Vitamin*	*Concentration recovered (μg/μl)*	*Concentration specified (μg/μl)*	*Recovery (%)*
Animal feed	Retinol	0.046	0.050	92
		0.045	0.050	90
Animal feed	α-Tocopherol	0.0223	0.025	89.2
		0.0217	0.025	86.8
Dried milk	Retinyl palmitate	0.1812	0.19	95.4
		0.1840	0.19	96.8

330 nm, hence this compound will not interfere with the quantitative determination of the vitamin at this wavelength. Recoveries of the vitamin from dried milk were calculated and results are shown in Table II.

Fig. 4 shows spectra obtained from an on-column loading of 10 ng and 500 pg of retinyl palmitate and 10 ng on column of α-tocopherol, showing the exceptionally high mass sensitivity achieved.

Throughout this work no column deterioration was observed and retention behaviour was reproducible.

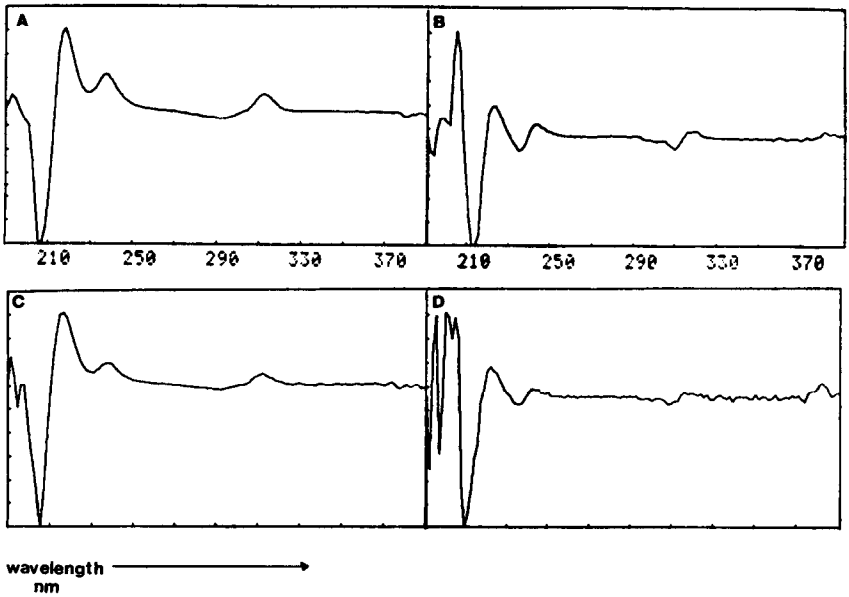

Fig. 2. Comparison of first- and second-order derivative spectra for α-tocopherol in animal feed with those for reference material. (A) First order derivative spectrum of α-tocopherol reference material; (B) second order derivative spectrum of α-tocopherol reference material; (C) first-order derivative spectrum of α-tocopherol in animal feed; (D) second-order derivative spectrum of α-tocopherol in animal feed.

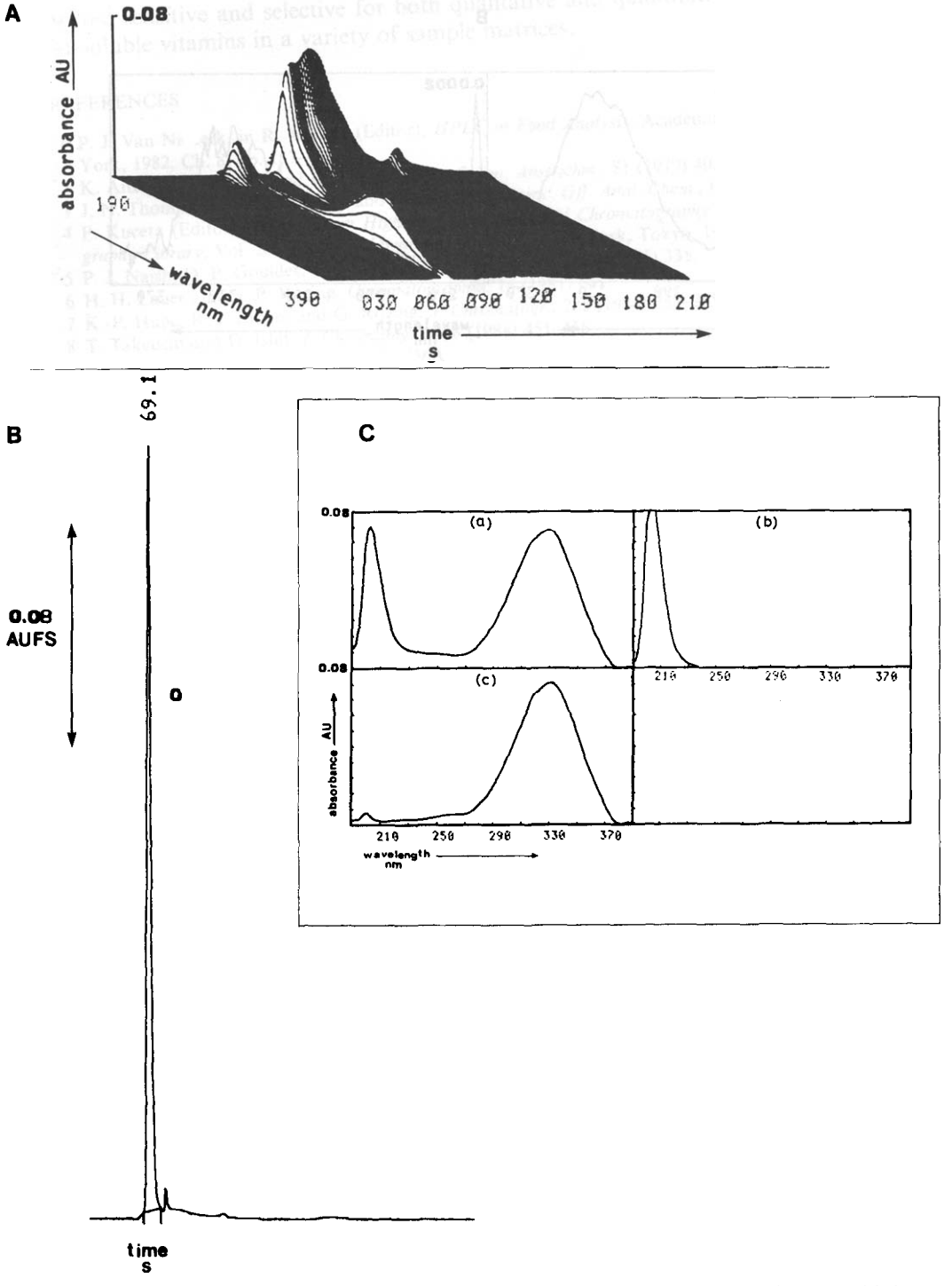

Fig. 3. (A) Chromascan of retinyl palmitate in dried milk. (B) Chromatogram of retinyl palmitate in dried milk at 330 nm. (C) Subtraction of spectra. (a) Spectrum of retinyl palmitate in dried milk. (b) Spectrum of coeluting compound. (c) Resultant spectrum, a − b.

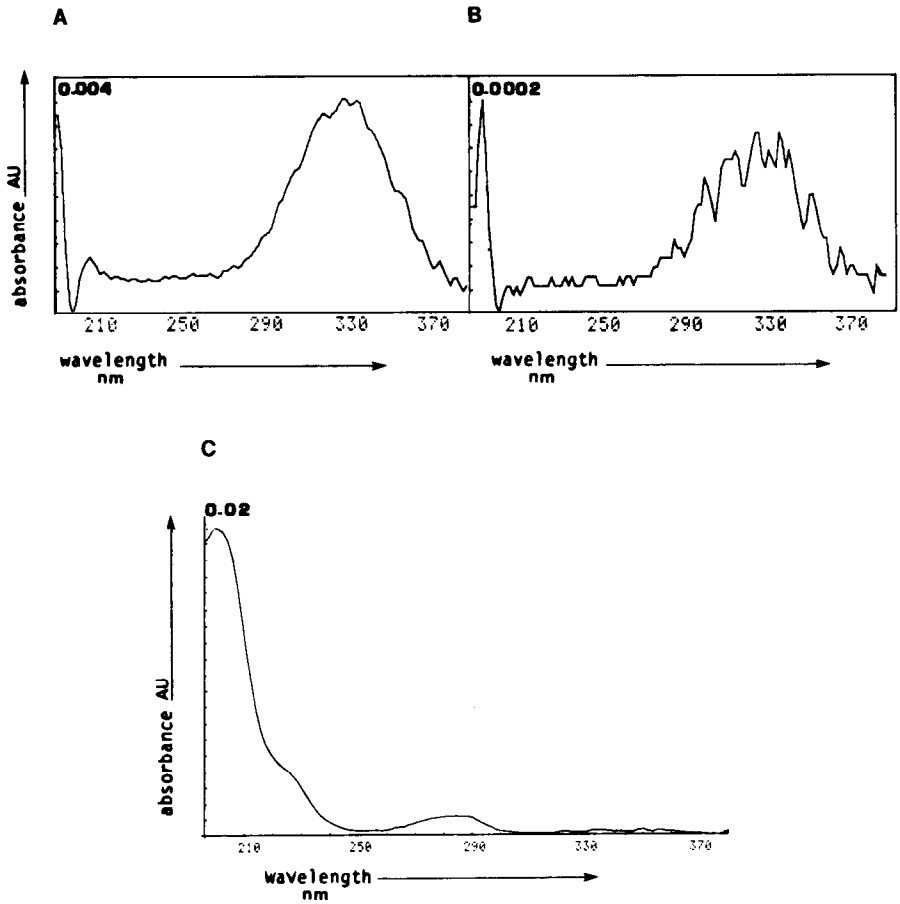

Fig. 4. (A) Spectrum of retinyl palmitate, 10 ng on-column loading. (B) Spectrum of retinyl palmitate, 500 pg on-column loading. (C) Spectrum of α-tocopherol, 10 ng on-column loading.

CONCLUSIONS

The work described in this paper has shown how the high mass sensitivity of narrow-bore columns can be utilised for vitamin analysis. This application demonstrates a reduction by a factor of five in the sample consumption. The columns used performed well, showing good efficiency and thus good resolving power.

Multichannel UV detection proved to be extremely versatile, the vitamins could be clearly identified in the presence of many co-extracted compounds. Data obtained from a single injection could be quickly and easily manipulated to give a large amount of information. This method of detection minimises the possibility of erroneous interpretation and quantitation of data, as chromatographic peaks are clearly identified and spectral purity of these peaks ascertained.

The combination of narrow-bore HPLC and multichannel UV detection has been shown to provide a robust and reliable method of analysis. The technique

proved sensitive and selective for both qualitative and quantitative analysis of the fat-soluble vitamins in a variety of sample matrices.

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