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Liquid chromatographic determination of fat-soluble vitamins in paprika and paprika oleoresin

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Fat-soluble vitamins, A, D, E, K_1, and vitamin esters, A acetate and E acetate, have been resolved and quantitated in paprika and paprika oleoresin. The analysis was carried out by reversed-phase gradient elution high performance liquid chromatography with spectrophotometric detection. Paprika samples were extracted with ethyl acetate and the extract directly injected at room temperature without prior hydrolysis or isolation steps. Thus, the method can be useful for the quality control analysis or the routine determination of fat-soluble vitamins in commercial samples of paprika or paprika oleoresin.

INTRODUCTION

Fat-soluble vitamins are present in foods in variable concentrations, those of vitamins D and K being very low when compared with vitamins A and E (Macrae, 1988). Classical methods for the determination of vitamins A, D, E and K in foods, lack specificity, display poor internal precision, are time consuming, and are not suitable for simultaneous determinations. Vitamin A is currently analyzed by the Carr–Price colorimetric method (AOAC, 1975). Similarly, a classical vitamin E determination uses the Emmerie–Engel reaction (AOAC, 1975). Vitamins D and K_1 are commonly determined by bioassays (AOAC, 1975).

The application of high performance liquid chromatography (HPLC) to the determination of vitamins normally requires previous steps to separate the vitamins from the complex food matrices. On the other hand, ester derivatives of fat-soluble vitamins are usually present and hydrolysis steps might not be adequate to free the alcohol form of the vitamin. In addition, vitamin E is extremely sensitive to degradation by alkaline hydrolysis, vitamin D suffers a variety of isomerization reactions and vitamin K_1 is destroyed (Barnett *et al.*, 1980).

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Liquid chromatographic separations of the fat-soluble vitamins in different foodstuffs have been reported (Carpenter, 1979; Parrish, 1979, 1980; Zonta *et al.*, 1982; Cort *et al.*, 1983; Stancher & Zonta, 1983; Reynolds & Judd, 1984; Wickroski & McLean, 1984; Hogarty *et al.*, 1989). Vitamin E (total tocopherol) in paprika and paprika oleoresin has also been determined by several methods (Fuente *et al.*, 1976; Domenech *et al.*, 1977; Kanner *et al.*, 1979; Hiroyasu & Murota, 1985; Viñas *et al.*, 1992). However, methodology for the simultaneous rapid determination of vitamins A, D, E and K_1 in red pepper, paprika and paprika oleoresin has not been reported.

In the present study, a procedure for the simultaneous determination of the fat-soluble vitamins and fat-soluble vitamins ester derivatives in paprika and paprika oleoresin is reported. The proposed method avoids previous purification and hydrolysis steps. A gradient elution technique allows the determination of the vitamins in the presence of paprika carotenoids. Therefore, the procedure is fast and simple and may be useful for routine analysis when a large number of samples are involved.

MATERIALS AND METHODS

Apparatus

The HPLC system consisted of a Perkin-Elmer Series 4 liquid chromatograph operated at room temperature,

with a flow-rate of 1·0 ml min^{-1}; a Perkin-Elmer LC-85B variable wavelength scanning spectrophotometric detector with an 8 μl flow-cell and programmable detection wavelength to improve the sensitivity of the determination; a 7125-075 Rheodyne injector valve with a 20 μl sample loop; and a Perkin-Elmer Sigma 15 chromatography data station. The column was a 12·5 cm × 0·46 cm I. D. stainless-steel tube packed with Spherisorb ODS-2 with a particle size of 5 μm. A Supelco guard column packed with the same stationary phase was also used.

Reagents

Methanol, acetonitrile, tetrahydrofuran and ethyl acetate (Romil Chemicals, Loughborough, UK) were liquid chromatography grade. Doubly distilled water was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). The solvents were degassed by sparging with helium gas.

Vitamins A (*trans* retinol), A acetate (*trans* retinol acetate), D_2, E, E acetate and K_1 were obtained from Sigma (St Louis, MO, USA). Solutions were prepared by dissolving 25 mg of the commercial products, without further purification, in 25 ml of methanol, and stored in dark bottles at 4°C. Working solutions were obtained by dilution with ethyl acetate immediately before the measurements. The exact concentrations of these solutions were determined spectrophotometrically by using the following extinction coefficients ($E^{1\%}_{1\,cm}$): vitamin A, 1832 at 325 nm (Wickroski & McLean, 1984); vitamin A acetate, 1560 at 325 nm (Beaulieu *et al.*, 1989); vitamin E, 75·8 at 292 nm (Nierenberg & Lester, 1985); vitamin E acetate, 43·6 at 285 nm (Nierenberg & Lester, 1985); vitamin D_2, 458 at 264·5 nm (Beaulieu *et al.*, 1989); vitamin K_1, 419 at 248 nm (Windholz *et al.*, 1983).

Calibration graphs

The chromatographic conditions were as follows: mobile phase, methanol/water (98:2, v/v); flow-rate, 1·0 ml min^{-1}; room temperature; sample-loop, 20 μl; detection wavelength, 325 nm for vitamins A and A acetate, 280 nm for vitamins D_2, E and E acetate, and 248 nm for vitamin K_1. Calibration graphs were prepared by plotting peak area against concentration.

Determination of fat-soluble vitamins in paprika and paprika oleoresin

Samples of commercial paprika were analyzed as received. A paprika sample of 1 g was accurately weighed and extracted with 20 ml of ethyl acetate in a covered beaker. The mixture was stirred for 10 min, filtered through paper and the solution was diluted to volume with ethyl acetate in a 25 ml standard flask. For paprika oleoresin, samples of 5–40 mg were weighed and diluted to volume with ethyl acetate in a 10 ml standard flask. These solutions were kept in the dark when not in use. Aliquots of these solutions were filtered through a 0·45 μm Nylon filter before injection into the chromatograph and the gradient elution programme given in Table 3 was applied. The flow-rate was 1·0 ml min^{-1} throughout. Other chromatographic conditions were the same as those for the calibration graphs. The esters were quantified as the equivalent amount of vitamin A acetate or vitamin E acetate.

RESULTS AND DISCUSSION

HPLC optimization

Adjustment of the capacity factors (k') was the initial step to optimize the mobile phase composition. Methanol, acetonitrile and tetrahydrofuran were the selectivity-adjusting solvents and water the strength-adjusting solvent (Glajch *et al.*, 1980). The model adopted for prediction of k' values was based on the relationship between k' of a solute and the composition of the binary mobile phase (Schoenmakers *et al.*, 1978):

$$\ln k' = A_2x^2 + A_1x + A_0$$

where x is the volume fraction of one of the two solvents and A_2, A_1 and A_0 are the coefficients obtained from the quadratic form for $k'(x)$. Table 1 summarizes the measured capacity factors of the vitamins with three methanol/water mobile phase compositions, 0%, 2% and 5% water. The k' values were used for determination of the A coefficients in the equation; the coefficients for each vitamin are also shown in Table 1. The predicted capacity factors (lines) and the measured values (symbols) are shown in Fig. 1. The solid lines drawn through the data points represent best fitting quadratic curves, from which the coefficients were derived. Therefore, the model was satisfactory for prediction of the separation.

Table 1. Capacity factors of the fat-soluble vitamins for three methanol/water mobile phases and the calculated coefficients

	A	AA	D_2	E	EA	K_1
			Capacity factors			
0% water	0·64	1·27	2·68	3·00	4·49	2·53
2% water	0·99	1·66	4·11	5·01	7·50	9·27
5% water	1·66	2·93	7·91	9·03	15·01	23·77
			Coefficients			
A_2	0·0030	0·0117	0·0070	−0·0079	−0·0026	−0·0588
A_1	0·1795	0·1084	0·1789	0·2587	0·2538	0·7351
A_0	−0·4642	0·2384	0·9984	1·1053	1·5053	0·9413

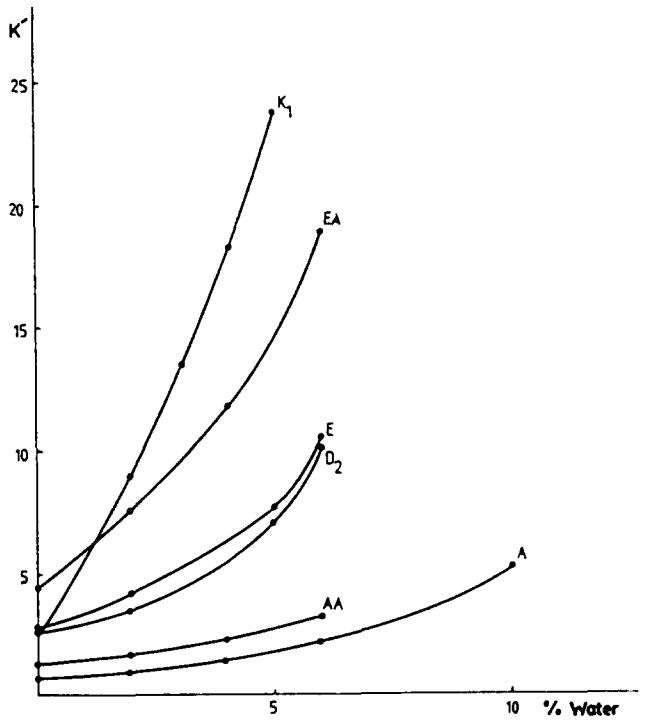

Fig. 1. Predicted (lines) and measured (symbols) capacity factors of vitamins with the methanol/water mobile phase. Flow-rate, 1 ml min $^{-1}$; sample loop, 20 μl; UV detection, 325 nm for A and AA, 280 nm for D_2, E and EA, and 248 nm for K_1.

The capacity factors for all vitamins markedly increased with higher water concentrations. Thus k' ranges were 1·66–23·77 for the methanol/water (95 : 5) mixture and 0·99–9·27 for the (98 : 2, v/v) mixture. Thus, a (98 : 2, v/v) methanol/water mobile phase was selected for the first apex of the selectivity triangle.

Total solvent strength for the methanol/water (98 : 2, v/v) composition was 2·55. Binary mixtures giving the same solvent strength would be acetonitrile/water (80 : 20, v/v) and tetrahydrofuran/water (57 : 43, v/v). A comparison of the chromatograms showed that all vitamins were well resolved with methanol/water; however, some vitamins co-eluted when acetonitrile/water or tetrahydrofuran/water mixtures were tried. Separation with acetonitrile/water (80 : 20, v/v) caused a delay in the elution of all vitamins and no resolution between peaks was obtained. In contrast to what might be expected on the basis of polarity, acetonitrile was a weaker eluent than methanol. This was in close agreement with the results obtained for the determination of fat-soluble vitamins in human serum (Lefevere *et al.*, 1979). Separation with tetrahydrofuran/water (57 : 43, v/v) caused the co-elution of vitamins D_2, E and E acetate and the pair A, A acetate was poorly resolved. Thus, since methanol/water mobile phase satisfied the requirements of good resolution for all peak pairs and appropriate analysis time, ternary mobile phases were not investigated.

The substitution of water by a less polar organic solvent, such as acetonitrile or ethyl acetate, produced a strong decrease in the k' values of the vitamins. Figure 2 shows the variation of capacity factors of the vitamins with stronger solvents than water to optimize the total solvent strength. These binary mixtures were not selected since some vitamins co-eluted at the void volume as non-retained compounds.

The values of separation factors (α) and resolution (R_s) are shown in Table 2. For the methanol/water mixtures, both separation parameters increased at the

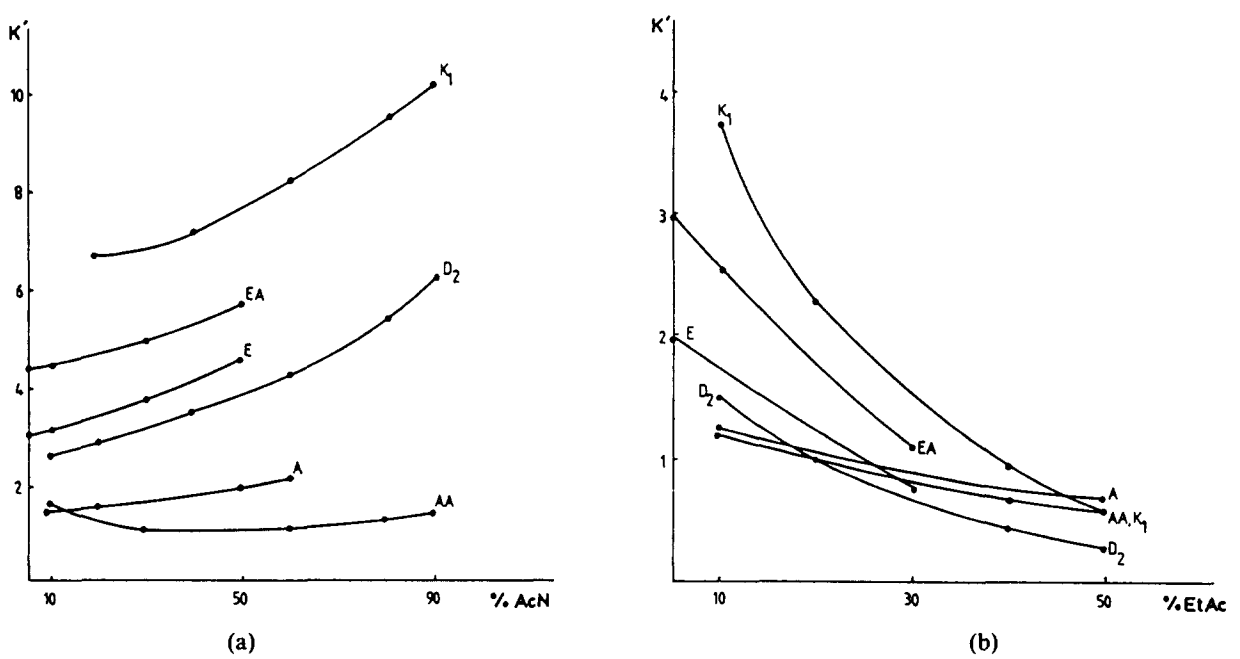

Fig. 2. Predicted (lines) and measured (symbols) capacity factors of vitamins with the methanol/acetonitrile (a) and methanol/ethyl acetate (b) mobile phases. Flow-rate, 1 ml min^{-1}; sample loop, 20 μl; UV detection, 325 nm for A and AA, 280 nm for D_2, E and EA, and 248 nm for K_1.

Table 2. Selectivity (α) and resolution (R_s) of the fat-soluble vitamins

Mobile phase	A/AA	AA/D_2	D_2/E	E/EA	EA/K_1
		Selectivity (α)			
MeOH/H_2O					
100 : 0	1·99	1·88	1·12	1·50	1·77
98 : 2	1·88	2·28	1·22	1·50	1·24
96 : 4	1·72	2·23	1·32	1·60	1·56
94 : 6	1·78	2·75	1·03	1·75	1·65
MeOH/EtAc					
95 : 5	1·04	1·47	1·04	1·40	1·93
80 : 20	1·03	1·03	1·12	1·38	1·35
60 : 40	1·14	1·50	1·16	1·20	1·37
MeOH/AcN					
95 : 5	1·40	1·24	1·08	1·45	1·54
80 : 20	1·38	2·52	1·09	1·34	1·43
60 : 40	1·57	3·10	1·11	1·26	1·36
		Resolution (R_s)			
MeOH/H_2O					
100 : 0	2·73	3·62	1·02	4·30	6·25
98 : 2	3·37	6·92	2·46	6·17	6·60
96 : 4	5·37	8·38	3·21	7·37	6·95
94 : 6	7·07	16·13	4·40	10·52	7·32
MeOH/EtAc					
95 : 5	0·10	1·26	0·37	1·40	5·20
80 : 20	0·10	0·07	0·27	0·97	1·17
60 : 40	0·20	0·47	0·17	0·26	0·97
MeOH/AcN					
95 : 5	0·10	1·00	0·40	2·70	4·77
80 : 20	0·87	3·50	0·60	2·40	4·03
60 : 40	1·30	4·77	0·83	2·20	3·29

higher water concentration; stronger solvents led to α values very close to 1 and R_s values below 1, showing a worse separation.

Calibration graphs were obtained by plotting the peak area against the vitamin concentration and were linear in the ranges 0·05–30 μg ml^{-1} for vitamin A; 0·05–35 μg ml^{-1} for vitamin A acetate; 0·1–40 μg ml^{-1} for vitamin D_2; 1–60 μg ml^{-1} for vitamin E; 1–65 μg ml^{-1} for vitamin E acetate and 1–50 μg ml^{-1} for vitamin K_1. The relative standard deviations (RSD) were: for 0·58 μg ml^{-1} of vitamin A (10 determinations), 3·9%; for 0·80 μg ml^{-1} of vitamin A acetate, 3·8%; for 1·06 μg ml^{-1} of vitamin D_2, 4·7%; for 10·3 μg ml^{-1} of vitamin E, 3·5%; for 11·6 μg ml^{-1} of vitamin E acetate, 3·8%; and for 3·80 μg ml^{-1} of vitamin K_1, 4·7%.

HPLC determination of vitamins in paprika and paprika oleoresin

Addition of ethoxyquin (EQ), a non-natural antioxidant, to paprika is tolerated in several countries. EQ is a potentially interfering substance in the determination of the fat-soluble vitamins in paprika. However, with the methanol/water (98 : 2, v/v) mobile phase selected, EQ was eluted at the void volume and no interference was detected (Viñas *et al.*, 1991).

Vitamins were extracted from paprika after approximately 5 min stirring with ethyl acetate. To prove quantitative extraction, two successive extractions of the sample were performed. In the second solution, no peaks appeared at the retention times corresponding to the vitamins. Therefore, a single step with an extraction time of 10 min was selected.

Organic extracts of paprika contained many coloured substances which can overlap with the vitamins, thus preventing their selective determination. Consequently, the most suitable mobile phase composition for the separation of the different components present in paprika and paprika oleoresin was investigated. Non-aqueous reversed-phase chromatography (NARP) has been found useful for the analysis of lipophilic samples (Parris, 1978*a*, 1978*b*). Thus, the applicability of this method to the analysis of paprika under mild conditions, i.e. at room temperature without prior hydrolysis or isolation steps of the sample, was tried. Figure 3 illustrates the chromatograms obtained for the injection of a vitamin mixture (1) and a paprika sample (2) when different aqueous and non-aqueous mobile phases were compared under otherwise identical operating conditions. Chromatogram D for paprika samples shows two peaks at equal retention times to those of vitamin A acetate and vitamin E acetate and were therefore quantified in these forms. However, the possibility that these peaks correspond to a more complex ester mixture co-eluting at the same retention time cannot be excluded. Vitamin D exists in vegetables exclusively as D_2; thus, vitamin D found in paprika was quantified as vitamin D_2.

Figure 3 suggests that carotenoids and other pigments present in paprika samples require strong totally organic mobile phases to be eluted from the column. Thus, rapid and complete elution of the components of paprika was observed when methanol/ethyl acetate was used as the mobile phase (chromatograms A, B and C). Conversely, no elution of the sample components (only the vitamins can be detected) from the column was observed when a semi-aqueous methanol/water (98 : 2, v/v) mobile phase was used (chromatogram D). Systematic tests using 90%, 50% and 10% ethyl acetate in methanol clearly indicated the dependence of elution speed on the concentration of ethyl acetate; an increase in the concentration of this solvent in the mobile phase accelerated the elution of the sample components. Therefore, a gradient elution technique was applied. The optimized gradient elution programme is shown in Table 3. The initial methanol/water (98 : 2, v/v) solvent allows both the elution of the vitamins as a series of well resolved peaks in the early part of the chromatograms and the strong retention of the other sample components. This was followed by a rapid elution as a single peak, well resolved from the vitamins, when the ethyl acetate proportion was increased. Figure 4 shows the chromatogram for a paprika sample.

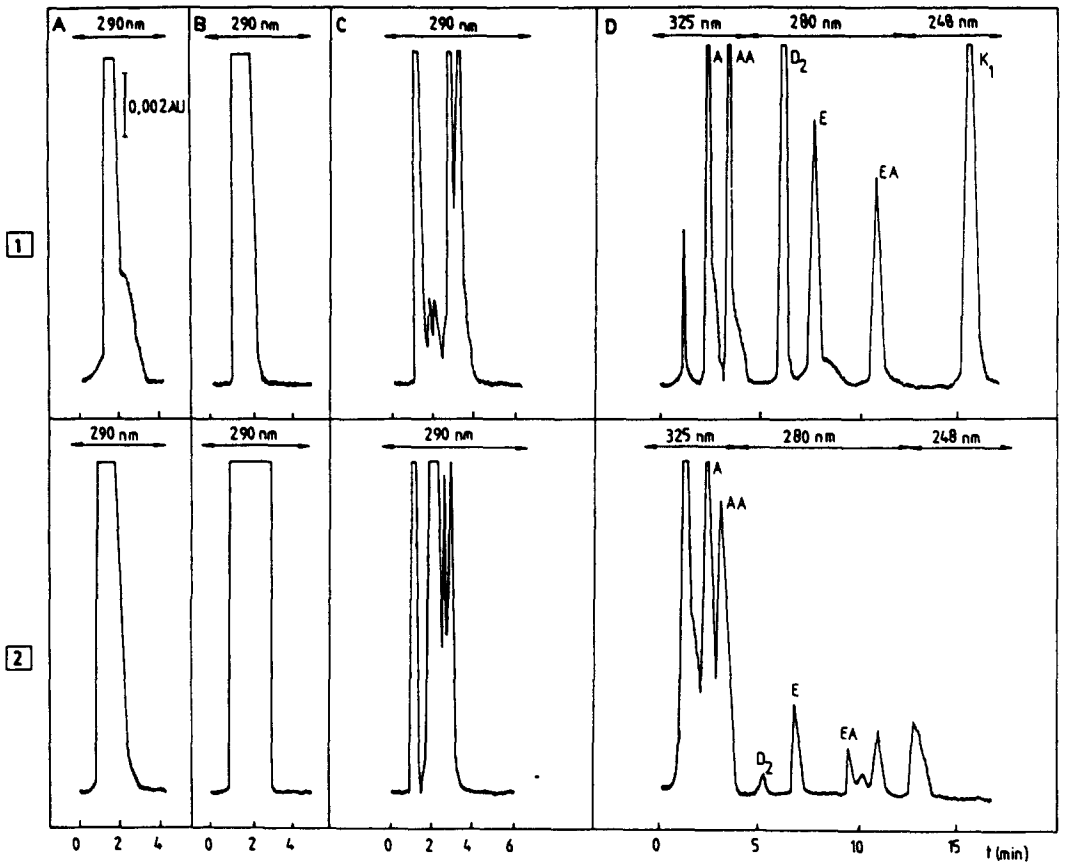

Fig. 3. Comparison of the elution profiles corresponding to a vitamins mixture (1) and a paprika sample (2) with different aqueous and non-aqueous mobile phases. Flow-rate, 1 ml min^{-1}; sample loop, 20 μl; UV detection, wavelength changes are reported at the top of the figure. Mobile phase (%): A, methanol/ethyl acetate (10 : 90, v/v); B, methanol/ethyl acetate (50 : 50, v/v); C, methanol/ethyl acetate (90 : 10, v/v); D, methanol/water (98 : 2, v/v).

The identity of the vitamin peaks was first confirmed by spiking samples with known amounts of standard vitamin solutions. Further peak characterization was achieved by varying the mobile phase composition and comparing the chromatograms of paprika with those of the corresponding vitamin standards. Attempts were made to confirm peak identities by fraction collection and mass spectrometry; however, sensitivity of the apparatus was not enough at these low concentrations.

Table 4 shows the results in sweet paprika and oleoresin samples. Four replicates were run for each sample and three injections of each extract were performed. Vitamin E is the more abundant. Vitamin D appears in a lower concentration and in some samples could not be detected (the minimum determinable content of vitamin D_2 in paprika is 2·5 μg g^{-1}). These findings are in close agreement with the common content in vegetables. Vitamin K_1 has not been found in any of the analyzed samples (minimum vitamin K_1 determinable content in paprika is 20 μg g^{-1}). The recovery of

Table 3. Gradient elution programme, flow-rate 1·0 ml min^{-1}

Stage	Time (min)	Mode	Elution solvent (%)		
			MeOH	Water	Ethyl acetate
Equilibrium	10	Isocratic	98	2	0
Start	17	Isocratic	98	2	0
	1	Linear to	5	0	95
End	3	Isocratic	5	0	95
	1	Linear to	98	2	0

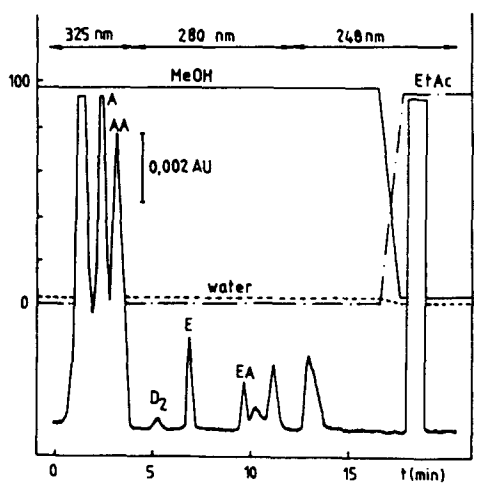

Fig. 4. Chromatograms of a paprika sample with the gradient elution programme. Flow-rate, 1 ml min^{-1}; sample loop, 20 μl; UV detection, wavelength changes are reported at the top of the figure.

Table 4. Fat-soluble vitamins content in paprika and paprika oleoresin ($\mu g\ g^{-1}$)

	Storage time	Vitamins ($\mu g\ g^{-1}$)				
		A	AA	D_2	E	EA
Paprika						
1	15 days	15	12	12	882	418
2		8	8	9	309	227
3		5	7	ND	322	122
4	6 months	4	6	ND	302	283
5		6	7	4	364	282
6		6	3	5	380	116
7	1 year	5	7	4	360	152
8		5	5	ND	228	90
9		5	8	6	161	105
Oleoresin						
1		112	271	78	13772	2547
2		80	165	178	2184	1265
3		277	349	174	9763	870

ND, Not detected.

vitamins was determined by analysis of paprika extracts spiked with 0·5 $\mu g\ ml^{-1}$ of vitamins A, A acetate and D, 5 $\mu g\ ml^{-1}$ of vitamins E and E acetate and 4 $\mu g\ ml^{-1}$ of vitamin K_1. Results showed recoveries for the vitamins ranging between 94 and 101%. Significant variations in all the vitamin levels were noted when the storage time of the paprika increased.

In the oleoresin samples, a wider variation in the vitamin E levels was detected. This would be expected due to variation in the content of the vegetable oils used to dilute the oleoresins; this procedure is commonly employed to obtain the final colour required.

Finally, the vitamin content of the dry pepper from which paprika is prepared was also determined. Previously, the different parts of the dry pepper—peel, seeds and peduncle—were separated and ground in a ball mill. Results showed that vitamins A, D and E were found in the peel. Vitamin E was found in the seeds while vitamins A and D were not detected. In the peduncle, only a minimal amount of vitamin E was found.

Several experiments were performed to establish the reproducibility of the method. Ten injections of the same paprika extract were used to determine the instrument stability. The relative standard deviations of the vitamins were in the range 1·8–5·9%. To calculate the precision of the whole procedure, 10 separate extractions of the same sample were performed and the solutions chromatographed (RSDs ranging from 3·2% to 6·5%).

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