

SEPARATION AND DETERMINATION OF A MIXTURE OF FAT-SOLUBLE VITAMINS A, D₂, AND E AND β-CAROTENE BY GRADIENT THIN-LAYER CHROMATOGRAPHY

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A procedure for determining and separating a mixture of fat-soluble vitamins by gradient thin-layer chromatography was developed. It was shown that a theoretical approach could be used to select the optimum thin-layer chromatographic conditions for determining tocopherol acetate, retinol acetate, ergocalciferol, and β-carotene. The developed procedure was validated in terms of detection limit, specificity, efficiency, and precision. The procedure could be used for quality control of complex multivitamin preparations, medicinal plant raw material, and vegetable oils, oil extracts, and dietary supplements based on them.

Keywords: tocopherol acetate, retinol acetate, ergocalciferol, β-carotene, thin-layer chromatography, gradient elution.

Vitamins represent a very important class of essential dietary components and are biologically active compounds (BAC) of various chemical natures. A deficiency or excess of vitamins in the body disrupts drastically various metabolic processes, which leads to serious diseases [1, 2].

The variety of vitamin medicines is currently rather broad. Their compositions are complicated and multi-faceted. Fat-soluble vitamins (FSV) are used as independent preparations and are also very important minor constituents of vegetable oils (VO).

HPLC methods for identifying, separating, and quantifying FSV in drug substances, single and multi-component dosage forms, premixes, dietary supplements, and microorganism cultures are known [3 – 8]. Spectral analytical methods such as photoelectrocolorimetry, which is based on measurements of the optical density of vitamin solutions after adding specific reagents that form colored reaction products [9, 10], are also widely used. Direct and differential spectrophotometry is widely used to analyze drug substances in order to determine the identity, purity, and quantitative content [11, 12]. Drawbacks of these spectral methods are the awkwardness and length of the determinations, the instability of the colored reaction products, insufficient sensitivity and selectivity, the inability to determine vitamins A, E, and

D₂ and β-carotene in mixtures, and the large experimental uncertainty. A drawback of HPLC is the expensive equipment, reagents, materials, and standards. TLC has all the advantages of chromatographic methods and is widely used because of its celerity, availability, sufficient sensitivity, selectivity, low cost, and analytical simplicity.

The goal of the work was to study various eluting systems and the possibility of a theoretical approach to selecting the optimum chromatographic conditions for separating and determining mixtures of FSV by TLC.

EXPERIMENTAL PART

Standard samples of the studied FSV were used to develop the method [11 – 14]. The detector was chosen considering requirements such as specificity, high sensitivity, availability, and high-quality chromatogram. Table 1 lists the reagents (PMA = phosphomolybdic acid) for detecting the chromatographic bands.

Table 2 lists the eluents used for chromatography of standard solutions of ergocalciferol (vitamin D₂, FSP 42-0008018000), retinol acetate (vitamin A, FS 42-7811-97), tocopherol acetate (vitamin E, Reg. 42-7843-97), and β-carotene (VFS 42-3128-98) [11 – 14]. Samples were placed on plates using micropipettes (1 and 10 μL, MSh-1 and -10,

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TABLE 1. Detectors for TLC Determination of FSV

Sample No.	Reagent	Vitamin A	Vitamin E	Vitamin D ₂	β-Carotene
1	conc. HNO ₃	–	Reddish-orange bands on white background	–	–
2	SbCl ₃	Rapidly disappearing blue color	–	Pink bands on white background	Rapidly disappearing blue color
3	conc. HClO ₄	–	–	Brownish-orange bands on white background	–
4	conc. H ₂ SO ₄	Dark-violet bands on white background	–	–	Rapidly disappearing blue color
5	5% PMA in alcohol	Dark-blue bands on yellowish-green background			–
6	Visible light	–	–	–	Yellowish-orange bands on white background

Russia). Eluents and detectors were prepared using chemically pure and analytically pure solvents and reagents (ZAO Vekton, St. Petersburg, Russia).

The influence of eluent polarity on the chromatographic mobility of the vitamins in a thin layer had to be studied in order to select the optimum chromatographic conditions for separating ergocalciferol, β-carotene, and retinol and tocopherol acetates. Greater than 10 eluting systems with various polarities were studied experimentally (Table 2). Eluents proposed in the literature [1, 2, 15, 16] were investigated. New chromatographic systems were certified. The polarity (P') and chromatographic parameters of the vitamins such as relative migration rate in the absorbent layer (R_f value), distribution coefficient (K), and absorption selectivity index (L) were calculated for each eluting system.

The optimum chromatographic conditions for separating a model mixture of standard samples using gradient elution were Sorbfil PTSKh-P-A silica gel plates (5 × 10 cm, Krasnodar, Russia); chamber saturated with eluent (1 and 2) vapor for 20 min; optimum sample volume, 10 μL of β-carotene (1 mg/mL) in EtOH; solutions (0.5 μL) in EtOH of vitamins A, E, and D₂ containing 1, 10, and 0.035 mg/mL, respectively; eluent 1, hexane:CHCl₃ (19:1); run height 8 cm with drying of the plate in air at room temperature to evaporate solvent; eluent 2, hexane:CHCl₃ (3:1), run height 6 cm; total elution time, 55 min; β-carotene identified by the characteristic color in visible light; vitamin detector, PMA solution (5%) in EtOH; plate storage time at $t \geq 80^\circ\text{C}$ after detection, 3 – 5 min.

The developed method was tested on complex multivitamin preparations Aevit (OOO Lyumi, Ekaterinburg, Russia;

TABLE 2. R_f Values of Standard FSV A, E, and D₂ and β-Carotene

System	Eluent composition	P'	R_f value			
			retinol acetate	tocopherol acetate	ergocalciferol	β-carotene
1	Hexane	0	0.01 ± 0.01	0.01 ± 0.01	0.025 ± 0.01	0.19 ± 0.01
2	Hexane:benzene (29:1)	0.15	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.38 ± 0.01
3	Hexane:benzene (19:1)	0.22	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.57 ± 0.01
4	Hexane:benzene (14:1)	0.29	0.01 ± 0.01	0.08 ± 0.01	0.03 ± 0.01	0.69 ± 0.01
5	Hexane:CHCl ₃ (19:1)	0.22	0.02 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.78 ± 0.02
6	Hexane:CHCl ₃ (10:1)	0.40	0.04 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.91 ± 0.01
7	Hexane:CHCl ₃ (5:1)	0.73	0.13 ± 0.02	0.46 ± 0.02	0.27 ± 0.02	0.98 ± 0.01
8	Hexane:CHCl ₃ (4:1)	0.88	0.24 ± 0.02	0.52 ± 0.02	0.35 ± 0.02	0.98 ± 0.01
9	Hexane:CHCl ₃ (3:1)	1.10	0.36 ± 0.02	0.76 ± 0.02	0.67 ± 0.02	0.98 ± 0.01
10	Hexane:CHCl ₃ (2:1)	1.47	0.85 ± 0.02	0.92 ± 0.02	0.85 ± 0.02	0.98 ± 0.01
11	Hexane:CHCl ₃ (1:1)	2.20	0.92 ± 0.02	0.95 ± 0.02	0.93 ± 0.02	0.98 ± 0.01
12	CHCl ₃	4.40	0.95 ± 0.02	0.98 ± 0.02	0.99 ± 0.01	0.99 ± 0.01

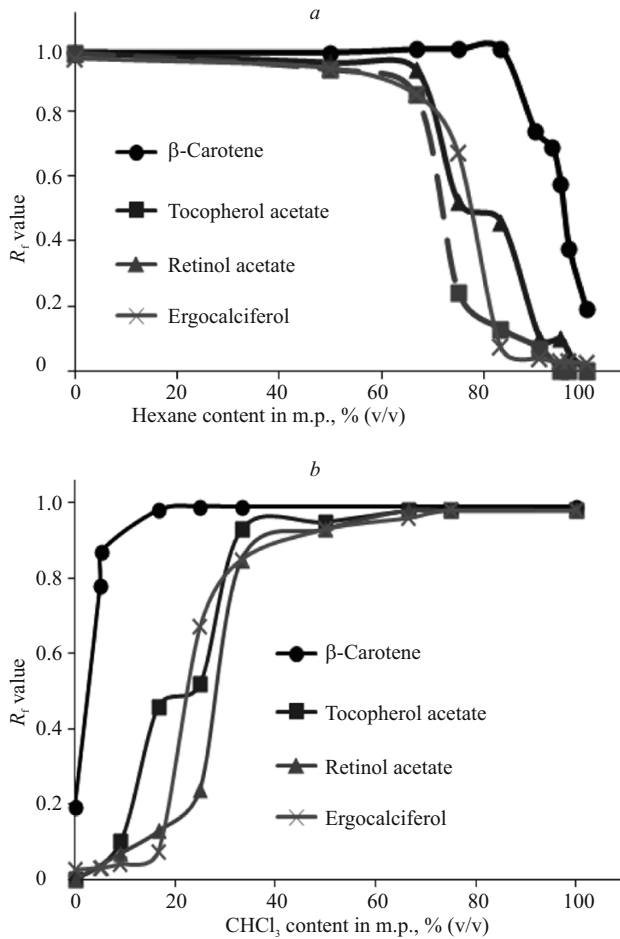


Fig. 1. Relative FSV migration rate as a function of content: hexane in mobile phase (silica gel stationary phase) (a); CHCl_3 in mobile phase (silica gel stationary phase) (b).

capsules of 0.2 g in No. 10 blisters); Aekol (ZAO Altaivitaminy, Biisk, Russia; 100-mL vials); and VO from sea buckthorn fruit (ZAO Altaivitaminy, Biisk, Russia; 50-mL vials). FSV were isolated from the preparations by repercolation. The extractant was EtOH (96%), which did not mix with the oil phase and was a good solvent for the FSV.

Determination in Aekol preparation. A weighed portion (900 mg) [17] was divided into three equal parts (300 mg each). The first part was treated with pure extractant

TABLE 3. Detection Limits for Studied FSV

Sample No.	FSV	DL, g
1	Vitamin A	10^{-8}
2	Vitamin E	10^{-6}
3	Vitamin D ₂	7×10^{-9}
4	β -Carotene	10^{-5}

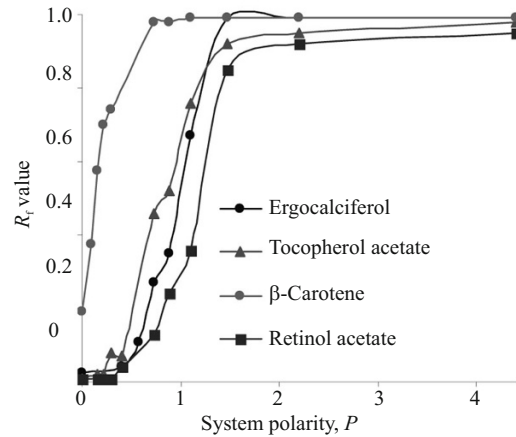


Fig. 2. R_f values of tocopherol acetate, retinol acetate, ergocalciferol, and β -carotene as functions of eluent polarity.

(96% EtOH, 10 mL). The mixture was shaken vigorously for 5 min to produce a fine emulsion of the oil phase in the EtOH. The container was tightly sealed and left for 1 d in a cool dark place. The EtOH extract was separated from the oil phase using a separatory funnel. Each sequential portion of the test preparation was extracted with the extract obtained from the preceding step.

Determination in Aevit preparation. The contents of one capsule of Aevit preparation [18] were treated with pure extractant (96% EtOH, 10 mL). The mixture was shaken vigorously for 5 min to produce a fine emulsion of the oil phase in EtOH. The container was tightly sealed and left for 1 d in a cool dark place. The EtOH extract was separated from the oil phase using a separatory funnel. The contents of a second and third capsule were extracted with the extract obtained from the preceding step.

Determination in sea buckthorn oil. A weighed portion of sea buckthorn oil (1.2 g) [19] was divided into three equal parts (400 mg each). The first part was treated with pure extractant (96% EtOH, 10 mL). The mixture was shaken vigorously for 5 min to produce a thin emulsion of the oil phase in EtOH. The container was tightly sealed and left for 1 d in a cool dark place. The EtOH extract was separated from the oil

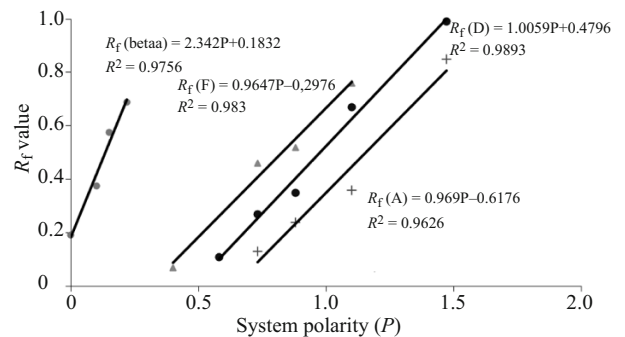


Fig. 3. Linear dependences of FSV R_f value on eluent polarity.

TABLE 4. Determination of Method Specificity

R_f values of FSV standards				$R_{f(\text{oil-placebo})}$ value
A	E	D ₂	β -carotene	
0.36 ± 0.001	0.76 ± 0.001	0.69 ± 0.020	0.98 ± 0.010	0.01 ± 0.001
				0.04 ± 0.010
				0.1 ± 0.010
				0.15 ± 0.020
				0.64 ± 0.030

**Fig. 4.** Chromatogram of Aekol preparation in EtOH (3 μ L) with gradient chromatography [system 1, hexane:CHCl₃ (19:1), 8-cm run; system 2, hexane:CHCl₃ (3:1), 6-cm run].**Fig. 5.** Chromatogram of sea buckthorn oil EtOH extract (5 μ L) with gradient chromatography.

phase using a separatory funnel. Each sequential portion of the test preparation was extracted with the extract obtained from the preceding step.

RESULTS AND DISCUSSION

Table 1 shows that none of the detectors could be used for simultaneous detection of the studied FSV. The plates after chromatography should be viewed in visible light and then treated with PMA solution (5%) in EtOH.

Plots of the R_f values as a function of the percent content of each component in the eluent were obtained by varying the hexane:CHCl₃ ratio in the two-component mobile phase (Fig. 1). The results established that the following hexane:CHCl₃ volume ratios gave the optimum R_f values [20] (70 – 80/20 – 30, for vitamin A; 75 – 85/15 – 25, for vitamin E; 75 – 77/23 – 25, for vitamin D₂; and 94 – 96/3.3 – 6, for β -carotene).

A plot of the relative FSV mobility (Fig. 2) vs. the system polarity (P') in the range from 0 to 4.4 units was constructed using the results. Table 2 and Fig. 2 show that eluents with small polarities (from 0.15 to 0.30 units) must be used in order to attain the optimum R_f values for β -carotene [21]. However, vitamins A, E, and D₂ remain at the origin at these polarities and are strongly retained by the absorbent. The optimum R_f values for them were obtained using sys-

tems 6 – 8 (Table 2), where the polarity of the mobile phase was 0.73 – 1.10. This meant that these vitamins could not be separated and a highly efficient separation could not be achieved in one chromatography step. However, this problem could be solved by using gradient elution. The influence of the system polarity on the R_f value for each FSV was studied in more detail in order to select the range of eluent P' values in which these functions were linear (from 0 to 0.2 system polarity units for β -carotene; from 0.4 to 1.1, vitamin E; from 0.58 to 1.47, for ergocalciferol; and from 0.7 to 1.5, for vitamin A) (Fig. 3).

Various systems for separating a mixture of the studied FSV by TLC could be selected using the proposed functions

TABLE 5. Calculated Method Plate Efficiency

Sample No.	FSV	H , μ m	N
1	Vitamin A	50	1200
2	Vitamin E	93	645.16
3	Vitamin D ₂	55	1090.91
4	β -Carotene	138	601.45

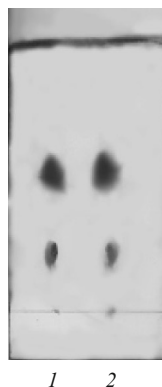


Fig. 6. Chromatogram of Aevit preparation in EtOH (track 1, 0.5 μ L) and a mixture of retinol acetate (0.5 μ L) and tocopherol acetate (0.5 μ L) standards (track 2) using hexane:CHCl₃ (3:1).

so that the R_f values would fall in the optimum ranges. Therefore, the equations of the lines (Fig. 3) indicated that the eluent polarity should be varied over rather narrow ranges from 0.05 to 0.168 units (for β -carotene); from 0.62 to 0.93 (for vitamin E); from 0.76 to 1.07 (for ergocalciferol), and from 0.95 to 1.26 (for vitamin A).

The developed method was validated in terms of detection limit (DL), specificity, efficiency, and precision.

The sensitivity of the method was established from the minimum observed amount of compound in a spot that appeared visually after detection. Table 3 lists the detection limits of the studied FSV using the selected reagents. The selected techniques for detecting bands in the chromatograms were rather sensitive, i.e., comparable with HPLC, and also economically feasible.

The specificity was determined from the R_f value of a spot in a control track that should correspond to the R_f value of the spots for the standards. The specificity was also determined using a placebo experiment (refined soy oil, which is often used as a solvent for producing FSV oil preparations). EtOH solutions of this oil were chromatographed using hexane:CHCl₃ (3:1) in parallel with FSV standards. Five chromatographic bands with R_f values (0.01 ± 0.001), (0.04 ± 0.01), (0.1 ± 0.02), (0.15 ± 0.02), and (0.64 ± 0.03)

TABLE 7. Chromatographic Separation Parameters for FSV in Sea Buckthorn Oil

Sample No.	Chromatographic separation parameter	D ₂	E	β -Carotene
1	R_f value	0.61 ± 0.02	0.76 ± 0.02	0.78 ± 0.02
2	Distribution coefficient (K)	2.57	0.39	0.28
3	Absorption selectivity (L)	5.59		1.40

TABLE 6. Chromatographic Separation Parameters for FSV in Aekol Preparation

Sample No.	Chromatographic separation parameter	A	E	β -Carotene
1	R_f value	0.33 ± 0.02	0.72 ± 0.02	0.78 ± 0.02
2	Distribution coefficient (K)	2.03	0.39	0.28
3	Absorption selectivity (L)	5.21		1.40

were observed. Table 4 presents the results and indicates that the developed method satisfied the acceptance criteria. The chromatogram of the placebo lacked spots at the band levels of the standards.

The plate efficiency was determined from the number of theoretical plates for the standard bands (Table 5). This parameter satisfied the allowed acceptance criterion (>500).

The obtained total extract from Aekol preparation was chromatographed (3 μ L) using system 1 (8-cm run) and then system 2 (6-cm run). It is noteworthy that bands of pure compounds on chromatograms of FSV mixtures isolated from oil phases of the preparations (Aekol and sea buckthorn oil) were situated slightly lower (Tables 6 and 7 and Figs. 4 and 5) than those for elution of the pure standards (Table 2). This may have been due to interaction of the FSV and β -carotene in the complicated mixture. Apparently, this caused a slight lag of the compounds during migration in the thin absorbent layer. Figure 4 shows the chromatograms. Table 6 presents the chromatographic parameters for separating FSV and β -carotene in Aekol preparation.

Total extract from Aevit preparation (0.5 μ L) was placed at the origin of a chromatography plate and chromatographed using system 2 (8-cm run) because the preparation did not contain β -carotene. Figure 6 shows the chromatogram of the Aevit solution and a mixture of standard retinol acetate and tocopherol acetate. Table 8 presents the chromatographic separation parameters for FSV in Aevit preparation.

The obtained total extract from VO (5 μ L) was placed at the origin of a chromatography plate and chromatographed using system 1 (8-cm run) and then system 2 (6-cm run). Figure 5 shows the chromatogram of the EtOH extract of sea buckthorn fruit oil with gradient chromatography. Table 7

TABLE 8. Chromatographic Separation Parameters for Vitamins A and E in Aevit Preparation

Sample No.	Chromatographic separation parameter	A	E
1	R_f value	0.36 ± 0.02	0.76 ± 0.02
2	Distribution coefficient (K)	1.79	0.29
3	Absorption selectivity (L)		6.17

TABLE 9. Determination of Method Precision

Sample	R_f value						
	FSV standards				FSV in dosage forms		
	A	E	D ₂	β -carotene	Aevit	Aekol	sea buckthorn oil
1	0.36	0.76	0.64	0.78	0.36	0.33	0.61
2	0.35	0.77	0.67	0.79	0.76	0.72	0.76
3	0.37	0.75	0.68	0.77		0.78	0.78
RSD, %	4.29	3.03	4.86	0.906	–	–	–
ε_{av} , %	5.33	3.44	7.73	1.12			

presents the chromatographic separation parameters for FSV and β -carotene in sea buckthorn oil.

The results indicated that the FSV chromatographic bands were separated satisfactorily (because the separation efficiency criterion was $L > 1$) and that the use of this method was justified.

The precision was determined by performing the full separation and FSV identification three times for samples of finished products. Table 9 presents the results.

Spots with approximately identical intensities and R_f values were obtained in the tests. The calculated RSDs were less than the acceptance criteria of 5%. This indicated that the method was reproducibly precise.

Thus, a method for determining and separating mixtures of FSV using gradient TLC was developed and could be used for quality control of complex multivitamin preparations, medicinal plant raw material, and VO, oil extracts, and dietary supplements based on them.

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