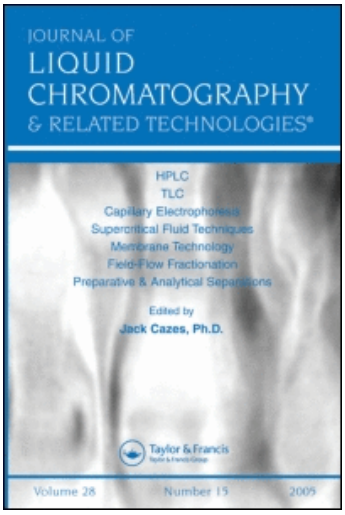

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Sławomir Wieliński[a]; Andrzej Olszanowski[a]
[a] Institute of Chemical Technology and Engineering, Poznań University of Technology, Poznan, Poland

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DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF FAT-SOLUBLE VITAMINS IN CAPSULES

Sławomir Wieliński* and Andrzej Olszanowski

Institute of Chemical Technology and Engineering, Poznań University of Technology, Pl. M. Skłodowskiej-Curie 2, 60-965 Poznan, Poland

ABSTRACT

A very simple nonaqueous reverse phase HPLC was developed for determining retinol acetate, retinol palmitate, cholecalciferol, alpha-tocopherol acetate, alphacalcidol, and phylloquinone in capsules without the need for saponification. A reverse phase (Lichrospher C8, 4.6 mm, id.) column was used with a mobile phase of acetonitrile–methanol (95:5, v/v) and flow rate of 1 mL/min. Sample treatment consisted only of the dilution of the capsule contents with *n*-hexane and methanol. The range of the assay method has been set at 2.5–150% of finished product label claim. The limit of detection of each vitamin was between 0.259 μg/mL (K_1) and 2.379 μg/mL (alphacalcidol). The minimum average amount recovered was 98.1% (D_3). The precision (relative standard deviation of label claim among six sample preparation as repeatability) was

*Address correspondence to S. Wieliński. E-mail: SW84260@glaxowellcome.co.uk

not more than 0.82% (A palmitate) and, as intermediate ($N = 24$), was not more than 1.76% (E acetate). The Standard Preparation (at -15°C) and Sample Preparation (at 4°C) were stable for up to 48 and 24 h, respectively. This method is suitable for routine quantification in industrial quality assurance laboratories. The principal aspects of method validation are examined and discussed, and the validation studies indicate that the method is specific, accurate, precise, and robust over the concentration range investigated.

INTRODUCTION

The World Health Organisation (WHO) emphasizes the importance of adequate dietary intake of the vitamins A, D, and E. Vitamins are vital substances for normal growth, development, and health. Their determination is of interest to biochemistry, pharmaceuticals, and the food sciences. High Performance Liquid Chromatography (HPLC) has been applied successfully to the separation of fat-soluble vitamin in pure standard solution (1), pharmaceutical formulation (2), human serum (3,4), and food samples (5) by either gradient elution or isocratic separation techniques.

The traditional assay methods for vitamins A, D, E, and alphacalcidol in multivitamin preparation involve the separate analysis of each vitamin, using different biological or physicochemical techniques (6–9). Many of these methods include very sophisticated and time-consuming procedures for sample preparation and purification. In most cases, the methods also lack selectivity, high precision, and accuracy because of interference caused by other ingredients in the formulation.

In the first part of this work (10–12), we show that an initial scan of a mixture with a gradient elution program offers a very rapid and easy procedure for predicting the isocratic conditions. Practical optimization, using a gradient elution for rapid selection and mixture-design statistical technique for the separation of fat-soluble vitamins in capsules from a single sample extract, using HPLC reverse phase and column backflushing technique, was described. Finally, the separation of fat-soluble vitamins under various chromatographic conditions has been investigated.

The best separation was achieved using octyl-bonded phase and methanol–acetonitrile mobile phase coupled with column backflushing technique.

In this work, we have developed our method and it was validated (13) in accordance with Wang et al.'s procedure. Thus, was developed a sensitive and precise analytical method for the simultaneous determination of vitamin A (retinol acetate, palmitate), vitamin D_3 (cholecalciferol), vitamin E (alpha- tocopherol acetate) and alphacalcidol, and vitamin K_1 (phylloquinone) in capsules from a single sample extract, using HPLC reverse phase and column backflushing

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technique. The procedure eliminates saponification, lengthy extractions, sample cleanup, is specific to the compounds of interest in the presence of interference, and has very good internal precision as compared to current compendial methods.

This technique provides the high degree of resolution, reproducibility, and quantitative determination required in the industrial assurance laboratory. Increased use of such a new method will greatly reduce the imprecision and inaccuracies associated with vitamin analysis.

EXPERIMENTAL

Chemicals and Reagents

Vitamin K_1 (phylloquinone) and A (all-*trans*-retinol acetate, all-*trans*-retinol palmitate) were obtained from Promochem (USP, BP-standard). Vitamin D_3 (cholecalciferol) and vitamin E (*dl*-α-tocoferol acetate) were purchased from Sigma. Alphacalcidol was purchased from Infarm Poland. All other chemicals, such as *n*-hexane, acetonitrile, methanol, and tetrahydrofuran, were commercially available (Merck, Baker). Water was obtained from a Maxima purification system (conductivity below 0.45 μS/cm).

Instrumentation and Equipment

The liquid chromatograph used was obtained from Hewlett Packard. The apparatus consisted of a pump module (HP1100), a photodiode-array detector (HP1100), an autosampler (HP1100), a degasser, and chromatography data station Chemstation rev. 6.03. The chromatographic system used for validation was made of an HP1050 pump module, an HP1050 autosampler, an HP1050 VWD detector, and chromatography data station Chemstation rev. 5.01

Standard Preparation

An accurately weighed substance, estimated to contain 20-mg retinol palmitate (A palmitate) and 10-mg retinol acetate (A acetate), 7.5-mg cholecalciferol (D_3) and alphacalcidol, 60-mg phylloquinone (K_1), and 30-mg tocopherol acetate (E acetate), was dissolved in 10-mL *n*-hexane and diluted to 100-mL with methanol. The next standard was sonicated for 15 min at 30°C. Air should be avoided and excluded by working in an atmosphere of pure nitrogen so as to guard against oxidative degradation.

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Sample Solution Preparation

A multivitamin capsule (containing all capsule components) was formulated to quantitative recovery. The contents of one capsule was transferred quantitatively into a 100-mL standard flask, dissolved in 10-mL *n*-hexane, and diluted to 100-mL with methanol. The sample was then placed in an ultrasonic bath for 10 min at 30°C.

Chromatographic Condition

HPLC separation was performed on a 25 cm × 4.6 mm, id, stainless steel column packed with octyl stationary phase (5 μm Lichrospher 100). The column was thermostatted at 33°C during all experiments. Isocratic elution was performed with acetonitrile–methanol (95:5 (v/v)) with flow rate of the mobile phase at 2.0 mL/min. Solvent was vacuum degassed individually from reservoirs with an in-line degasser. Sample injections (10 μL) were made using a loop injection valve and autosampler.

All data were obtained as chromascan from which spectra and chromatograms could be taken using a Chemstation Data Station. Detection was performed at 285 nm with a photodiode array detector. The hold-up time (t_0) was estimated to be 1.35 min by using replicate injections of 10^{-5} *M* KI. Peak recognition was performed by injection of each individual solute. Column backflushing was used to remove strongly held components from the top of the column after the fat-soluble vitamins have been analyzed. The sample was injected at the top of the column.

After elution of the solute under analysis, the switching valves were used to reverse the elution flow enabling the eluent to flow into the bottom of the column and out of the top to the waste. By doing this, strongly held components are quickly removed from the top of column, thus, extending the life of the column. Peak homogeneity was assessed by using DAD.

RESULTS AND DISCUSSION

Validation of the Test Procedure

The objective of validation of an analytical procedure is to demonstrate that it is adequate for its intended purpose. To meet current pharmaceutical regulatory guidelines (i.e., ICH, USP, BP) a number of parameters must be investigated in order to validate analytical methods. According to the USP24 ⟨1225⟩

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Table 1. System Suitability

	t_R (min)	Area (mAUs)	High (mAU)	N (m^{-1})	T (–)	k' (–)	R_s (–)	α (–)	%RSD ($n=5$)
Limit	–	–	–	NLT 9000	NMT 2.0	1–10	NLT 1.6	–	
A acetate	1.853	632.1	177.4	25968	0.834	1.1	–	–	0.76
D_3	2.214	614.7	151.0	28475	0.799	1.67	3.74	1.68	0.43
Alfa-D_3	2.93	671.1	138.4	36632	0.831	1.91	6.48	1.81	0.39
E acetate	3.298	1004.5	191.0	36154	0.74	2.44	2.44	1.19	0.39
K_1	3.574	705.8	127.3	29431	0.803	2.70	2.45	1.18	0.57
A palmitate	4.641	1245.3	181.5	42481	0.895	2.50	6.76	1.47	0.21

guidelines, analytical methods for quantification of major components of bulk drug substance or preservatives in finished pharmaceutical products fall under Assay Category I.

Data elements required for Assay Category I include precision, accuracy, specificity, range, linearity, and ruggedness. The method for fat-soluble vitamins satisfies all these requirements.

System Suitability Criteria

The system suitability results were calculated according to the USP24 ⟨621⟩ from typical chromatograms. In all cases, the column efficiency was greater than 25,500 theoretical plates per meter. The tailing factors were not more than 1.2. The resolution was between 2.44 (E acetate) and 6.76 (A palmitate). Instrument precision determined by five successive injections of the standard preparation exhibited a maximum residual standard deviation (RSD) of 0.76%. Table 1 illustrates the system suitability results obtained over 24 independent runs spanning 2 months. Typical chromatograms obtained from a 10-μL injection of Standard Preparation are illustrated in Figure 1. The overall chromatographic run time was 10 min.

Specificity and Selectivity

The specificity in terms of interferences from the reagents was assessed by performing a blank. All the components except the matrix were subjected to the analytical procedure. Freedom from interference by all fat-soluble vitamins was assessed by injecting a standard solution containing only one vitamin. Freedom from interference by degradation products was assessed exposing standard solutions of each vitamin in glass to a lamp emitting at 350 nm (at a distance of approximately 8–10 cm), or by storing the vials in an oven at 45 ± 1°C (E acetate). The solutions

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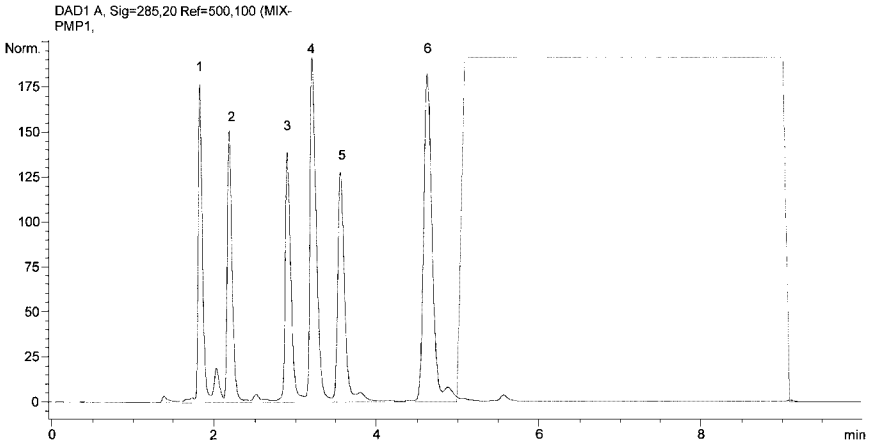

Figure 1. Standard Preparation of the fat-soluble vitamins (1 – A acetate, 2 – D_3, 3 – Alphacalcidol, 4 – E acetate, 5 – K_1, 6 – A palmitate) with detection at 285 nm.

were analyzed after various time intervals, using the DAD. Analyte spectra were compared with those stored in the library after each run.

The selectivity of assay was determined by analysis of a placebo. Placebo multivitamin capsule formulations containing all the normal ingredients except vitamin were prepared for this study. These were treated in the same manner as the normal samples, and chromatograms were inspected for interferences from the fat-soluble vitamins or from any of the other ingredients in capsule formulation.

Peak homogeneity is also a way of determining selectivity. The overlay of all vitamin spectra captured automatically at the apex, up slope, and down slope from a test solution was good. Absorbance ratios recorded across the chromatographic peak with two pairs of wavelengths did not give any significant deviation in ratio under the conditions used. Applying the same difference functions to a test solution of placebo did not give any significant deviation in the baseline at the retention time of all components, which means that there was no detectable interference from the matrix under the analyzed peak.

Linearity of Assay

A linear relationship was evaluated across the range of analytical procedure. The linearity of the response ratios of vitamin was determined with the standard preparation of these vitamins having a concentration ranging from 0 to 150% working level. Calibration curves were determined by linear regression. The assays exhibited linearity between the response (y) (peak area) and the corresponding

Table 2. Linearity of Fat-Soluble Vitamins[a]

Vitamin	Coefficient of Correlation	Bias (%)
A Palmitate	0.9982	0.3
A Acetate	0.9999	0.2
D_3	0.9994	0.3
Alphacalcidol	0.997	0.4
K_1	0.998	0.2
E Acetate	0.9995	0.3

[a]Coefficient of correlation acceptance criteria: NLT 0.995. Bias acceptance criteria: ±3%.

concentration of vitamin. The results of linear regression analysis show that the correlation coefficients of all standard curves are ≥0.997 and the bias was 0.2% (Tab. 2). The slope value is close to one, which confirms the accuracy of the method over the range investigated.

Range

The range of the assay method has been set at 2.5–150% of the finished product label claim, since the method has been shown to be precise, accurate, and linear within this region.

Sensitivity of Assay

Limit of detection (LOD) was evaluated as the lowest concentration of a mixed standard solution of each vitamin, which gave a signal-to-noise ratio of three. The LOD of each vitamin determined from a mixed standard solution was 1.627 μg/mL for retinol palmitate, 1.689 μg/mL for retinol acetate, 2.141 μg/mL for cholecalciferol, 2.379 μg/mL for alphacalcidol, 0.587 μg/mL for phylloquinone, and 0.259 μg/mL for tocopherol acetate.

Limit of quantitation (LOQ) was evaluated as the lowest concentration of mixed standard solution of each vitamin, which gave a signal-to-noise ratio of 10 (Fig. 2).

The repeatability of measurement of a mixed standard solution at the LOQ concentration was assessed by performing six successive injections of the same solution. LOQ is 5.423 μg/mL for retinol palmitate, 5.63 μg/mL for retinol acetate, 7.137 μg/mL for cholecalciferol, 7.93 μg/mL for alphacalcidol, 1.957 μg/mL for phylloquinone, and 0.863 μg/mL for tocopherol acetate.

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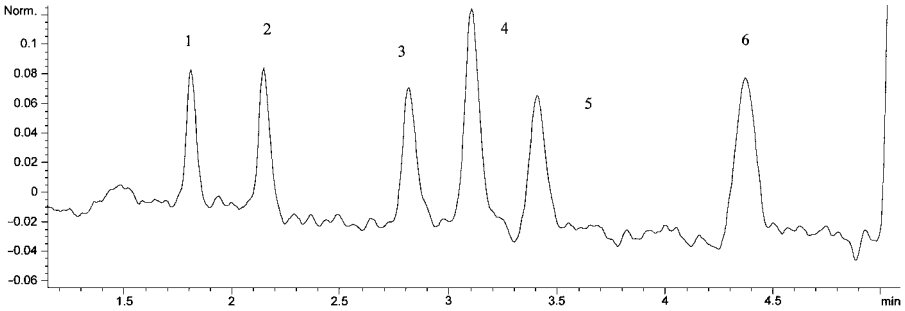

Figure 2. Chromatogram of quantitation limit standard solution (number as in Fig. 1).

The RSD values were 0.65, 0.45, 0.87, 0.71, 0.91, and 0.88% ($n = 6$ injections), respectively.

Accuracy of Assay

Accuracy was established across the specified range of an analytical procedure. The accuracy of the method was shown by analyzing spiked finished product samples of known concentration, in placebo, and by comparing the analytical result to the known added value.

The average percentage recovery was calculated at each concentration level. The average amounts recovered are shown in Table 3. Since the results obtained are within the acceptable range of 97.0–103.0%, the method is deemed to be accurate.

Table 3. Assay Accuracy[a]

Recoveries	A Palmitate	A Acetate	D_3	Alphacalcidol	K_1	E Acetate
2.5	97.6	97.6	97.3	97.0	98.1	98.4
25	98.2	97.8	97.1	97.2	98.5	99.2
50	98.9	98.5	98.5	98.2	98.9	97.4
75	98.9	99.0	98.2	97.9	97.2	98.2
100	99.6	99.5	98.1	102.2	98.9	99.1
125	99.1	99.3	97.9	101.9	99.2	98.2
150	102.1	103.0	99.7	102.1	98.7	99.8
RSD (%)	1.4	1.8	0.9	2.4	0.7	0.8
Overall recovery ($n = 7$)	99.2	99.2	98.1	99.5	98.5	98.6

[a] Accuracy acceptance criteria: 97.0–103.0%. Precision acceptance criteria: 3% within each level.

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Table 4. Assay Precision: Repeatability and Intermediate[a]

	A Palmitate	A Acetate	D_3	Alphacalcidol	K_1	E Acetate
Repeatability of six assay ($n = 6$)	0.82	0.6	0.42	0.42	0.45	0.17
Day 1, Chemist I (HPLC System I, Reagents I)	0.82	0.6	0.42	0.42	1.17	1.47
Day 2, Chemist II (HPLC System I, Reagents I)	0.85	0.55	0.51	0.52	0.98	1.35
Day 3, Chemist I (HPLC System II, Reagents I)	0.95	0.89	0.98	0.96	1.15	1.41
Day 4, Chemist II (HPLC System II, Reagents II)	1.1	1.04	0.96	0.92	0.88	1.39
Intermediate precision ($n = 24$)	1.34	1.12	1.21	1.18	1.37	1.76

[a]Repeatability acceptance criteria: 1.5%. Intermediate precision acceptance criteria: NMT 2%.

Precision of Assay

The precision (repeatability and intermediate precision) of the method was determined from one batch of synthetic mixtures.

Repeatability

Six assay preparations were analyzed in a single session by Chemist I with a HPLC System I (equipment + column). The RSD of the six results was between 0.17% (E acetate) and 0.82% (A palmitate), which was within the limit 1.5% (Tab. 4).

Intermediate Precision

Intermediate precision was evaluated using typical variations such as days, analysts, HPLC System, and reagents. It was evaluated by making six replicate injections from the same batch of synthetic mixtures. The RSD of each individual

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Table 5. Stability of Analytical Solution[a]

Time (h)	A Palmitate	A Acetate	D_3	Alphacalcidol	K_1	E Acetate
16	0.7/0.8	0.4/1.0	0.9/0.9	1.1/1.3	0.4/1.1	0.2/0.1
24	1.2/1.9	0.5/1.7	1.1/1.8	1.3/1.8	0.7/1.3	0.4/0.5
48	1.6/2.8	1.8/2.3	1.9/3.2	1.7/3.2	1.1/2.0	0.8/0.7
96	2.4/3.4	2.6/3.1	3.2/4.7	4.1/4.9	1.3/2.9	1.0/1.1

[a]Values are potency % change, which are given as Standard Preparation (store at −15°C)/Sample Solution Preparation (store at 4°C).

precision run was not more than 2% (Tab. 4). Analysis of the data yielded RSD between 1.12% (A acetate) and 1.76% (E acetate).

The low scatter in the data supports the high degree of robustness of the analytical method.

Stability of Analytical Solutions

The stability of the analytical solution was determined from the standard preparation (prepared from an in-house reference standard) at −15°C and from a sample solution preparation (prepared from a finished product) at 4°C. These solutions were analyzed at 0, 24, 48, and 96 h. Solutions were analyzed against a freshly prepared standard at each time interval. Vitamin concentrations were examined as a function of time (Tab. 5). These data were evaluated for percentage change of potency from time zero. The standard preparation and sample solution preparation were found to be stable for 48 and 24 h, respectively. Since the percentage change in potency is within ±2%, the solutions are considered stable at −15°C (standard preparation) and 5°C (sample solution preparation).

Robustness

To demonstrate the robustness of the chromatographic method, a designed experiment was performed that focused on seven factors considered to be potentially important to method's robustness. Each factor was varied around the nominal value as presented in the defined method. These nominal values and range of each factor tested are presented in Table 6.

The effect of altering the above factors was determined in an eight-run, statistically designed experiment (Plackett–Burman factorial experiment design), which looked at concentration calculated by peak areas and peak heights, minimum resolution, and tailing.

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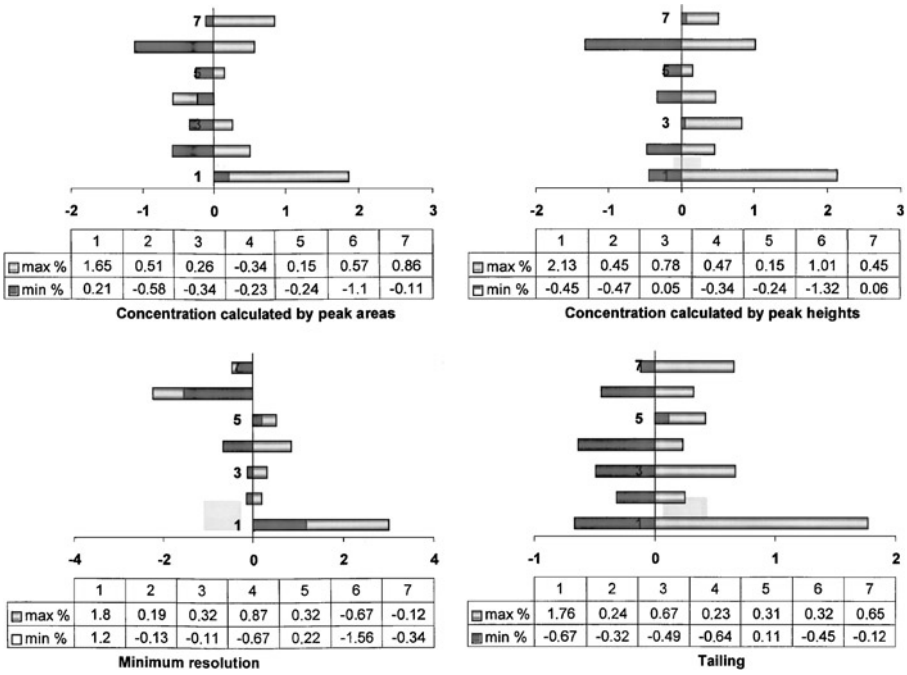
Table 6. Method Robustness

Factor	Nominal Value	Range Examined
Column (% carbon loading)	9	7% (Hypersil); 9% (Spherisorb); 10% (Techsphere)
Response time of detector (s)	0.500	0.120–2
Flow (mL/min)	2	1.5–2.5
Column temperature (°C)	33	23–43
Detection wavelength (nm)	285	280–290
Methanol concentration (%)	5	2–7
Injection volume (μL)	10	5–15

The main effects of changing each chromatographic component are shown in Figure 3.

One of the most important effects observed is due to column changes. The Hypersil column demonstrated about a 2% increase in minimum resolution and concentration calculated by peak areas and peak heights as compared to Spherisorb.



	1	2	3	4	5	6	7
max %	1.65	0.51	0.26	-0.34	0.15	0.57	0.86
min %	0.21	-0.58	-0.34	-0.23	-0.24	-1.1	-0.11

	1	2	3	4	5	6	7
max %	2.13	0.45	0.78	0.47	0.15	1.01	0.45
min %	-0.45	-0.47	0.05	-0.34	-0.24	-1.32	0.06

	1	2	3	4	5	6	7
max %	1.8	0.19	0.32	0.87	0.32	-0.67	-0.12
min %	1.2	-0.13	-0.11	-0.67	0.22	-1.56	-0.34

	1	2	3	4	5	6	7
max %	1.76	0.24	0.67	0.23	0.31	0.32	0.65
min %	-0.67	-0.32	-0.49	-0.64	0.11	-0.45	-0.12

Figure 3. The main effects of concentration calculated by peak areas and peak heights, minimum resolution, and tailing factor.

These effects are unexpectedly high considering the similarity of specification for these columns.

Fortunately, none of the factors studied had any significant effect on the concentration calculated by peak areas. The results obtained are excellent and indicate that the method is robust.

CONCLUSIONS

The described nonaqueous HPLC method for the analysis of fat-soluble vitamins has been evaluated for system suitability, specificity, linearity, precision, accuracy, stability of solution, and robustness. The chromatographic method developed and optimized allows resolution of vitamin A (retinol acetate, palmitate), vitamin D (cholecalciferol), vitamin E (alpha-tocopherol acetate), and alphacalcidol in capsules from a single sample extract, using a HPLC reverse phase and column backflushing technique.

The present method can be considered a relatively simple, convenient, and cost-effective procedure for routine determinations of fat-soluble vitamins in capsules. The sample preparation is very simple and analytical time short and, thus, can be used in industrial quality assurance laboratories.

The conclusion from the robustness test of the chromatographic method for the analysis of fat-soluble vitamins in capsules is that the method is robust concerning the analytical results of the vitamins.

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