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Determination of Vitamins K_1, K_2 MK-4, MK-7, MK-9 and D_3 in Pharmaceutical Products and Dietary Supplements by TLC-Densitometry.

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Received: 30 June 2020; Accepted: 16 July 2020; Published: 18 July 2020

Abstract: Vitamin K is a group of lipophilic molecules. Forms of vitamin K play an essential role in the activation of specific proteins involved in blood clotting cascade or bone metabolism. Another molecule belonging to the fat-soluble vitamins group that also plays an important role in calcium metabolism is vitamin D_3. The dietary supplements containing vitamins K and D_3 are one of the most frequently consumed by patients. The objective of this work was to develop a simple, fast and sensitive thin-layer chromatography (TLC)-densitometric procedure for the simultaneous quantitative analysis of vitamins K and D_3 in pharmaceutical products and dietary supplements. The analysis of vitamins was performed on the silica gel RP-18 $F_{254}S$ plates with methanol-ethanol-isopropanol in a volume ratio of 15:1:4 as a mobile phase. The densitometric measurements were made at 254 nm. The method was validated by checking the specificity, linearity, precision, recovery, limit of detection, limit of quantification and robustness in accordance with International Conference on Harmonization (ICH) guidelines. The method was shown to be specific, accurate (recoveries were from 95.78 to 104.96%), linear over the tested range (correlation coefficient, exceeding 0.99), and precise (precision and intermediate precision RSD below 2.70% for all analytes). The satisfactory results of the validation of the method indicate that it can be used in the quality control of dietary supplements and pharmaceutical products containing vitamins K and D_3.

Keywords: vitamin K_1; vitamin K_2; vitamin K_3; vitamin D_3; TLC-densitometry; quantification; dietary supplements and pharmaceutical products

1. Introduction

Vitamin K is a group of lipophilic molecules, of which vitamin K_1 (phylloquinone) constitutes the major part of our diet [1]. The chemical structure of vitamin K consists of a 2-methyl-1,4-naphthoquinone structure with a side-chain in the third position [2]. According to the structure of the side-chain at the third position of the naphthoquinone ring, vitamin K can be divided into different subtypes, such as K_1, K_2, K_3 and K_4. Vitamin K_1 has a singular form but menaquinones (vitamin K_2) include fourteen isoforms (MK-1 to MK-14) with a variable side-chain of n isoprene units (Figure 1), generating a series of isoforms referred to MK-n [3,4].

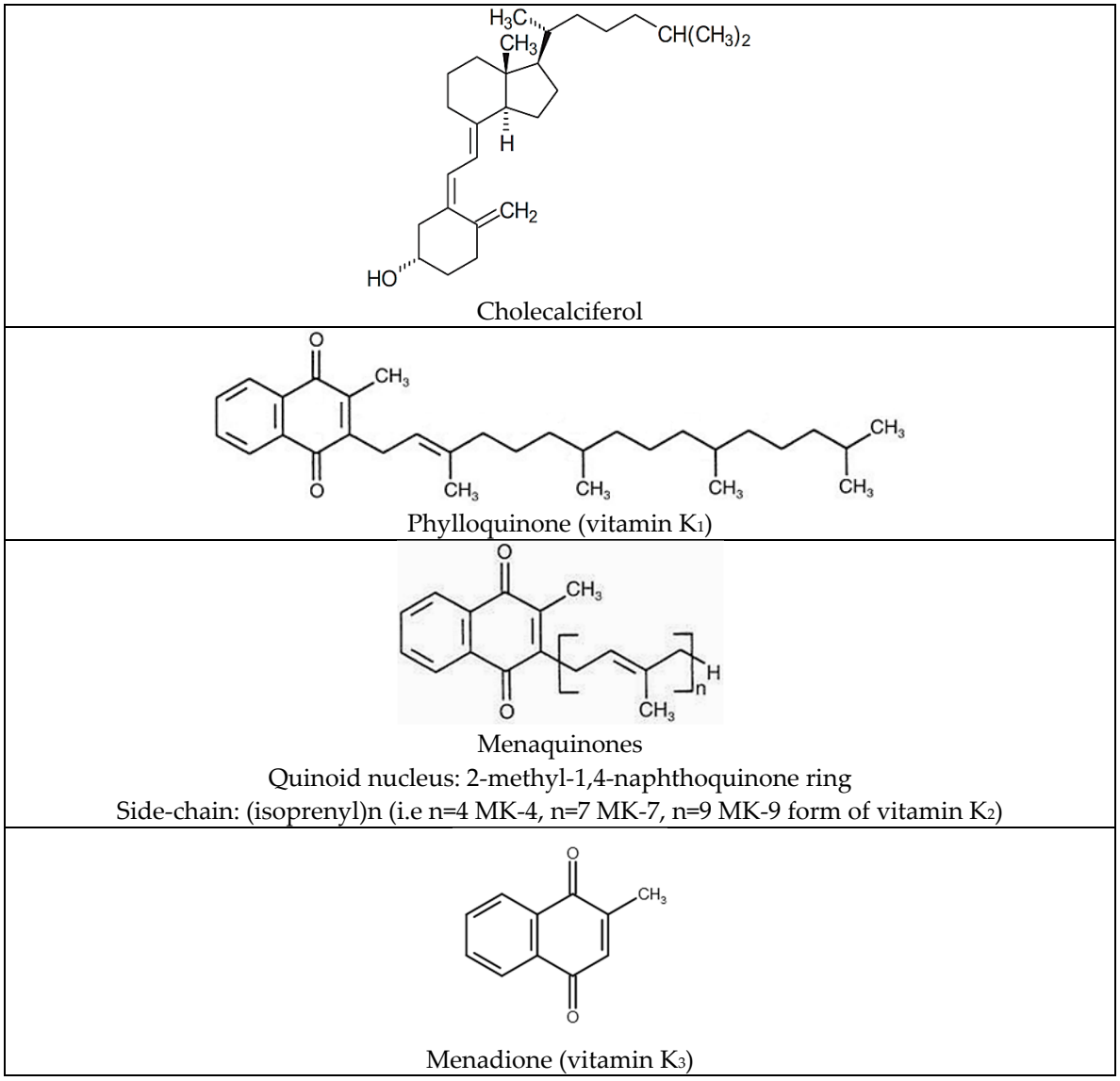

Figure 1. Chemical structure of analyzed compounds.

Vitamin K_3 (menadione) is a used synthetic member of the naphthoquinone family and becomes biologically active by its conversion in the human body to MK-4, but may be toxic [3,5,6] and is also considered as an anticancer agent [7]. All forms of vitamin K have one well-known function: it plays an essential role in the activation of specific proteins involved in blood clotting cascade or bone metabolism, such as osteocalcin and prothrombin, as a cofactor for the post-translational enzyme γ-glutamyl carboxylase [8,9] Moreover, vitamin K_2 (menaquinone) is considered as an agent in the prevention of osteoporosis [3,10].

Another molecule belonging to the fat-soluble vitamins group, also playing an important role in calcium metabolism is vitamin D [11]. It is mainly composed of two chemical forms including vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) [12]. It has been defined that vitamin D_2 has a more minor role than vitamin D_3 [13]. Vitamin D and its metabolites regulate calcium and phosphate homeostasis [14,15]. Besides, vitamin D_3 by its active form—calcitriol (1,25-dihydroxyvitamin D_3)—displays different actions, controlling blood pressure, insulin secretion, regulation of immune system, and controlling cell differentiation [15,16]. The human organism is capable of synthesizing the active form of vitamin D from 7-dehydrocholesterol (7DHC) upon UV irradiation [17].

Rich sources of vitamin K_1 are green leafy vegetables, vegetable oils like soybean and olive [18]. Menaquinones (MKs) are primarily synthesized by bacteria and are found in much lower amounts in the food supply in dairy, meat, and fermented food products also converted from dietary phylloquinone [18–20]. Vitamin D and its analogues are found in animal food products, such as meat,

offal, eggs, fish, milk and dairy products [21]; similarly, vitamin D_2 is found in fortified milk and other dairy products but also in yeast and mushrooms [22]. However, sun exposure is a major contributor to vitamin D in humans [23].

It is estimated that, on an annual basis, 40% of the European population is either vitamin D insufficient or deficient [24]. Based on a scientific opinion of the European Food Safety Authority (EFSA) panel, on the dietary reference values for vitamin K, it was concluded that the available evidence on occurrence, absorption, function, and content in the body or organs of menaquinones is insufficient [25]. In contrast, the menaquinones are present in the food supply, although there are limited food composition data available [18]. The optimal concentrations of both vitamin K and D are beneficial for bone and cardiovascular health, as supported by some human and other studies. Although, based on the current studies, there is not enough evidence to recommend combined vitamins K and D supplementation in the prevention of certain diseases [11,26]. Nevertheless, the use of dietary supplements is increasing in many European countries. The fat-soluble vitamins were one of the most frequently consumed ingredients, which accumulate in the body, raising the risks of overdosing and potential negative effects of these supplements [27]. Moreover, in many countries, no dietary supplement databases were available, and labelled values have limitations [27,28].

In the available literature, there are various methods for the analysis of vitamin K, D and its analogues, such as immunoassay [29], spectrophotometry [30], fluorimetry [31], electrochemistry [32], capillary electrophoresis [33], and chromatography. Chromatographic methods are suitable and widely used for the determination of fat-soluble vitamins [29]. Thin-layer chromatography (TLC) has been applied for the determination of vitamin K_1 [34], K_2 MK-4, K_2 MK-7, and K_1 [35] and vitamin D_3 [36]. In the majority of the studies on vitamins K and D, high-performance liquid chromatography (HPLC) using different types of detection methods has been applied, including electrochemical [37], spectrophotometric, fluorimetric [38,39] or mass spectrometry/tandem mass spectrometry (LC-MS) [40–42]. Among the chromatographic methods, gas chromatography (GC) is lesser used in vitamin K and D analysis [43,44]. However, TLC is an important investigative technique for the analysis of lipophilic vitamins and together with densitometry gives sensitive and precise quantification of vitamins K and D compounds on TLC plates [45].

The objective of this work was to develop a simple, fast and sensitive TLC-densitometric procedure for the simultaneous quantitative analysis of vitamins K and D_3 in pharmaceutical products and dietary supplements.

2. Materials and Methods

2.1. Chemicals

Ethanol, methanol, isopropanol, and acetonitrile were purchased either from Merck (Darmstadt, Germany) or POCH (Gliwice, Poland), and all were of analytical grade

2.2. Standard Solutions and Substance

All standard substances were HPLC grade: cholecalciferol (vitamin D_3) (≥ 99.8% purity, cat. No. PHR1237); vitamin K_1 (≥ 99.0% purity, sum of isomers, cat. No. 95271); menaquinone 4 (vitamin K_2), (99.9% purity, cat. No. 47774); menadione (vitamin K_3), (99.9% purity, cat. No. 47775) were from Sigma-Aldrich (Steinheim, Germany). Menaquinone 7 (vitamin K_2) (99.8% purity, cat. No. ASB-00022822-005) was from ChromaDex (Irvine, CA, USA) whereas menaquinone 9 (vitamin K_2) (97% purity, cat. No. M218610) was from LGC Standards GmbH (Wesel, Germany).

Standard solutions in methanol were prepared in concentration ranges: D_3 from 8.92 to 124.83 $\mu g \cdot mL^{-1}$; K_1 and K_2 MK-4 from 8.33 to 116.67 $\mu g \cdot mL^{-1}$, K_3 from 8.50 to 119.00 $\mu g \cdot mL^{-1}$, K_2 MK-7 from 13.17 to 131.67 $\mu g \cdot mL^{-1}$, K_2 MK-9 from 15.17 to 151.67 $\mu g \cdot mL^{-1}$.

2.3. Pharmaceutical Products, Dietary Supplements and Solutions

The following pharmaceutical products were used:

- Vigantoletten 1000 (Merck, Serono, Switzerland)—Tablets containing 25 μg of cholecalciferol. Excipients: D,L–α-tocopherol, hydrogenated soybean oil, gelatin, sucrose, corn starch, microcrystalline cellulose, colloidal anhydrous silica, sodium starch glycolate (type C), talc, glycerol tristearate.
- Vitacon (Polfa Warszawa S.A., Poland)—Tablets containing 10 mg of phytomenadione. Excipients: colloidal hydrated silica, microcrystalline cellulose, croscarmellose sodium, lactose monohydrate, magnesium stearate, povidone K-30, carmellose sodium, sucrose, colloidal anhydrous silica, talc, polysorbate 80, titanium dioxide (E171), quinoline yellow (E104), Capol wax.

The following dietary supplements were used:

- Vitamin K_2 MK-4, (Carlson Laboratories Inc. Arlington Heights, IL, USA)—Beef gelatin capsules containing 5 mg vitamin K_2 as MK-4 (menatetrenone). Other ingredients: microcrystalline cellulose, magnesium stearate (veg.), silicon dioxide.
- Vitamin D_3 5,000 IU Vitamin K_2 100mcg, (Oxford Vitality, Oxfordshire, UK)—Tablets containing 125 μg of vitamin D_3 (as cholecalciferol equivalent to 5,000 IU) and 100 μg of vitamin K_2 (as menaquinone MK-9). Other ingredients: microcrystalline cellulose, magnesium stearate, dicalcium phosphate, silicon dioxide.
- Kinon (Valentis AG, Agno – Lugano, Switzerland)—Tablets containing 75 μg of vitamin K_2 (as menaquinone-7). Other ingredients: microcrystalline cellulose, calcium phosphate, cross-linked sodium carboxymethylcellulose, magnesium stearate (veg.), talc, silicon dioxide.
- Kinon D_3 (Valentis AG, Agno – Lugano, Switzerland)—Tablets containing 50 μg of cholecalciferol and 100 μg of vitamin K_2 (as menaquinone-7). Other ingredients: microcrystalline cellulose, calcium phosphate, cross-linked sodium carboxymethylcellulose, magnesium stearate, talc, silicon dioxide.

All preparations and supplements were purchased at local pharmacies or online stores, and, during the study, the expiry date was not less than 1 year.

Solutions of the tested supplements—Four tablets were powdered and all powdered tablet mass was transferred quantitatively into a 25 mL volumetric flask, dissolved in 10 mL of ethanol and sonicated for 20 min and filled up to the mark with the same solvent. The obtained solutions were filtered using 0.45 μm nylon filter disks. For supplement Vitamin K_2 MK-4 the content of four capsules was transferred into a 25 mL volumetric flask, dissolved using 10 mL of ethanol, sonicated for 20 min and filled up to the mark with the same solvent.

Solution of the Vigantoletten—Ten tablets were powdered and all powdered mass was transferred quantitatively into a 10 mL volumetric flask. The powder was dissolved in 5 mL of ethanol, sonicated for 20 min and filled up to the mark with the same solvent. Next, the solution was filtered using 0.45 μm nylon filter disks.

Solution of the Vitacon—Ten tablets were weighed on an analytical balance, and the average tablet weight was calculated. The tablets were powdered and 26.76 mg of powdered mass was accurately weighed (accuracy of 0.1 mg) and transferred quantitatively into a 50 mL flask. The powder was dissolved in 20 mL of ethanol, sonicated for 20 min and filled up to the mark with the same solvent. The obtained solution was filtered using 0.45 μm nylon filter disks. Then, 2.5 mL of the solution was diluted with ethanol to 10 mL.

2.4. Instrumentation and Chromatographic Conditions

TLC separation was performed on 12.0 × 10 cm aluminum foil-backed TLC silica gel 60 RP-18 F_{254S} plates (Merck, Darmstadt, Germany; #1.05559.0001), without pretreatment. Standards (5-8 μL) and sample solutions (5–16 μL) were applied onto the plates as 5-mm bands by Linomat V (CAMAG, Muttenz, Switzerland) semiautomatic sample applicator (150 nL/s dosage speed) equipped with a 100 μL syringe (Hamilton, Bonaduz, Switzerland). The distances on plates were all 10 mm: between tracks; from the edges and from the bottom edge. The plates were developed using mobile phases composed of methanol-ethanol-isopropanol (15:1:4, v/v/v) in a glass chromatographic chamber (17.5 × 16 × 6.2 cm Sigma-Aldrich, Laramie, WY, USA #Z204161). The chamber was previously saturated

with mobile phase vapours for only 2 min at room temperature. The development distance was 115 mm. The plates were dried at 25 °C for 15 min. Next, the plates were viewed under a UV cabinet 3 (CAMAG Muttenz, Switzerland) and scanned in absorption–reflection mode at 254 nm with a TLC Scanner 3 (CAMAG Muttenz, Switzerland). The densitometric scanning conditions were as follows: slit dimensions 4.00 × 0.45 mm, scanning speed 20 mm/s and date resolution 100 μm/step. A winCats 1.4.8 version software was used for imaging and saving the chromatograms. The peak areas and absorption spectra of the examined compounds were recorded for quantitative and qualitative analysis.

2.5. Method Validation

The newly developed method was validated by checking the specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and robustness according to the procedure specified in the International Conference on Harmonization (ICH) guidelines. [46].

2.5.1. Specificity

The specificity of the method was assessed by comparing densitograms obtained for standard solutions and for sample solutions, a densitogram of blank solution and a blank densitogram. In the obtained densitograms, the retardation factor (R_f) values of analyzed compounds, the capacity factor (k), separation factor (α), resolution factor (R_s), and peak areas were taken into account. The capacity factor was calculated according to the equation $k = (1 - R_f)/R_f$. The separation factor was calculated with the equation $\alpha = k_1/k_2$ with $k_1 \geq k_2$; its value should be higher than 1.0. The resolution factor was calculated according to the formula $R_s = 2d/(w_1 + w_2)$, where d is the distance between two adjacent peaks centers, while w_1 and w_2 are peak widths at base. The R_s determines the resolution obtained in the developed chromatographic conditions for two neighboring peaks and its value should be not less than 1.

2.5.2. Linearity

The linearity of the method was assigned by analysis of eight (vitamins K_1, K_3, K_2 MK-4, D_3) or six (vitamin K_2 MK-7 and MK-9) standard solutions at different concentrations. The concentration range of the standard solutions is given in Section 2.2. The solutions were applied to the plates in an amount of 6 μL as bands, then the plates were developed under the conditions given in Section 2.4 and evaluated densitometrically. The test was performed twice for each substance. The calibration curves were constructed based on the relationship between the registered peak areas and the amount of vitamin in micrograms per band. The linear model was fitted to the calibration data. The slope of regression line, y-intercept, standard deviation of slope and intercept, correlation coefficient (R), determination coefficient (R^2), and the standard error of the estimate of the calibration plot were calculated using the program Statistica 13.3 (TIBCO Software, USA). The Shapiro–Wilk test was used, in order to determine whether the residuals have normal distribution.

2.5.3. The limit of detection (LOD) and limit of quantification (LOQ)

For the determination of LOQ, standard solutions at the concentration ranges: 4.17–16.68 μg mL^{-1} for K_1 and K_2 MK-4, 6.58–26.33 μg mL^{-1} for K_2 MK-7, 7.58–30.33 μg mL^{-1} for K_2 MK-9, 4.25–17.0 μg mL^{-1} for K_3, 4.46–17.84 μg mL^{-1} for D_3 were applied (6 μL) on the TLC plates. The limit of detection and determination of the method was determined by visual assessment based on the signal-to-noise ratio. The signal to noise ratio of 3 to 1 was adopted for the LOD and 10 to 1 for the LOQ.

2.5.4. Precision and intermediate precision

The analysis of six replicates of standard solutions of tested vitamins were used to determine the repeatability of the method. The study was done for 100% concentration level (0.40 μg per band of MK-7, MK-4 and D_3; 0.46 μg per band of MK-9; 0.35 μg per band of K_1; 0.35 μg per band of K_3). The intermediate precision (reproducibility) was obtained for the same concentration of freshly prepared

solutions by different analysts who performed the analysis over a period of one week. Peak areas were compared, and data were statistically analyzed. The results were expressed as the percentage of relative standard deviation (%RSD).

2.5.5. Accuracy

Accuracy was assessed by determination of the recovery (%) of the drugs or dietary supplements. Accurately weighed amounts of the vitamin standards (at level 80, 100 and 120% of the declared content) were added to solutions of tested pharmaceutical products or supplements, and percentage recovery was calculated based on the determined amounts of added standards under the conditions of the developed method in relation to the quantity weighed. As ICH recommends, three determinations were performed for each level.

2.5.6. Robustness

The robustness of the TLC-densitometric procedure was evaluated by making small, but deliberate, changes to the method parameters. The conditions changed were: the chamber type (17.5 × 16 × 6.2 cm, 17.5 × 16 × 8.2 cm), saturation time of the chamber (± 2 min) and the distance of development (± 0.5 cm). The influence of small changes of isopropanol in the composition of the mobile phase (± 0.2 mL) for chromatographic separation was also studied.

2.5.7. Analysis of pharmaceutical products and dietary supplements

The solutions of tested preparations and dietary supplements were prepared as specified in Section 2.3. The determination of vitamins in tested pharmaceutical products and dietary supplements was carried out according to the procedure outlined in Section 2.4. Five measurements were performed for each determination and the mean value was taken for further calculations.

3. Results and Discussion

As described in the introduction, vitamin K_2 plays an important role in regulating blood calcium levels and bone metabolism, while vitamin K_1 mainly affects the blood coagulation process. The beneficial effect of vitamin K on bone mineralization can be enhanced by simultaneous supplementation with vitamin D_3, which is largely responsible for calcium metabolism. Patients are overwhelmed with information about various dietary supplements containing vitamins K and D_3 that will quickly cure them or help prevent disease. Besides, using ready-made preparations available at the pharmacy is much easier than applying a healthy and well-balanced diet. No wonder then that dietary supplements containing vitamins K and D_3 are among the most consumed by patients. In addition, it should be remembered that many dietary supplements are not subjected to sufficient quality control, much less scientific research confirming their effectiveness. The most common form of vitamin K found in dietary supplements is K_2MK-7, much less often MK-4 and MK-9. Moreover, MK-7 and MK-9 are almost always combined with vitamin D_3. In turn, vitamin K_1 is either the only active ingredient in pharmaceutical preparations or one of two together with vitamin D_3 in dietary supplements. Vitamin K_3 is not used in preparations intended for humans, but it can be a component of products administered to animals.

Therefore, the objective of this work was to develop a new fast and universal TLC-densitometric procedure for the simultaneous determination of various forms of vitamin K and vitamin D_3.

Firstly, optimal conditions for the separation of vitamin K_1, K_2 MK-4, MK-7, MK-9, K_3 and D_3 were established. We examined the suitability of TLC silica gel 60 F_{254} plates and silica gel plates with octadecylsilyl groups (RP-18 F_{254s}) as the stationary phase. The usefulness of a number of mobile phases of various compositions was also checked.

The most optimal conditions allowing the separation of all tested vitamins were obtained using a mobile phase with a methanol-ethanol-isopropanol (15:1:4, v/v/v) mobile phase and TLC RP-18 F_{254s} plates as the stationary phase (Figure 2).

The wavelength for densitometric scanning was selected based on absorption spectra for the analyzed compounds, recorded directly from the chromatograms. The absorption spectra for vitamin K_1 and K_2 were similar in shape with two maxima at wavelengths of approximately 240 and 260 nm (Figure 3). The spectrum of vitamin K_3 showed maxima at 240 and 254 nm, whereas vitamin D_3 had only one maximum at a wavelength of about 257 nm. Therefore, the wavelength of 254 nm was considered the most optimal and was used for recording the peak areas of all compounds tested.

The application of densitometric detection showed that peaks in the chromatograms were symmetrical, well resolved, easy to identify and determine. The retardation factors obtained under the developed conditions for the analyzed vitamins are listed in Table 1.

Table 1. Separation data obtained for analyzed vitamins.

Substance	R_f	k	α	R_s
MK-9	0.08	11.5	-	-
MK-7	0.15	5.7	2.0	1.2
K1	0.24	3.2	1.8	1.6
MK-4	0.33	2.0	1.6	1.5
D_3	0.36	1.8	1.1	0.9
K_3	0.68	0.5	3.6	4.7

In the latest available literature, only a few publications were found describing the use of the TLC method to determine vitamin K or D_3. Pyka et al. applied reversed-phase thin-layer chromatography for the analysis of vitamin K_1 purity on RP-$8F_{254s}$ plates using methanol as the mobile phase [34].

Atia et al. separated vitamin K_1 and menaquinones MK-4 and MK-7 using HPTLC silica gel $G60F_{254}$ plates and a mixture of methanol-ethanol-isopropanol-water (75:5:5:15, v/v/v/v) in the absorbance mode at 254 nm [35]. In this work, in contrast to Atia et al., we propose a different stationary phase and a modified composition of the mobile phase; this enables us to achieve the separation of four forms of vitamin K and vitamin D_3.

According to the ICH recommendations for the assessment of the reliability of the developed method, the following parameters were determined: linearity, specificity, recovery, robustness, limits of quantitation and detection [46].

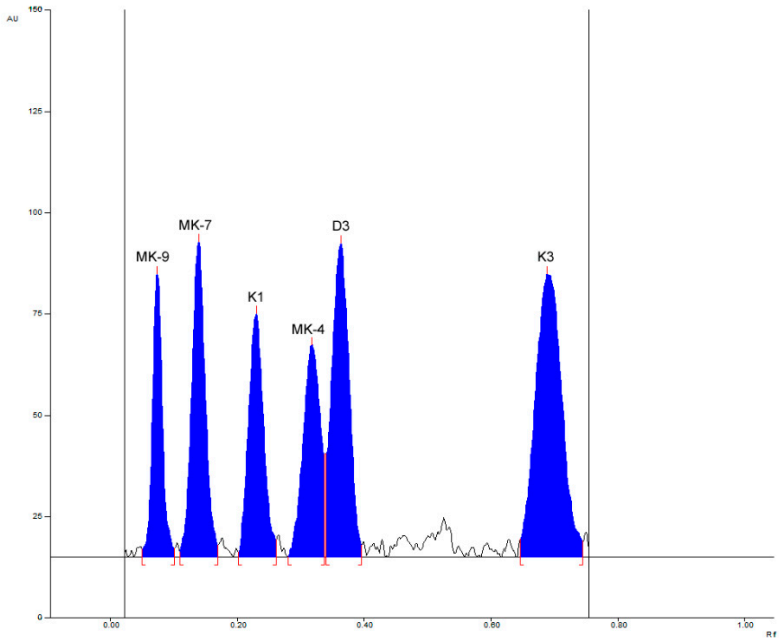

Figure 2. Example densitogram registered for a solution of a mixture of standards at 254 nm.

The developed method was specific against studied compounds. There are no peaks on densitograms recorded for standard solutions and for sample solutions and a blank densitogram, where studied components occur. Peaks of tested substances were also well resolved. The resolution factors obtained for K_1, K_2 MK-9, MK-7, and K_3 were greater than 1.1 and meet the acceptance criteria. The value of the resolution factor for two adjacent peaks corresponding to K_2 MK-4 and D_3 was 0.9 but no dietary supplements were found in which these vitamins would be placed together. The regression analysis results obtained for examined compounds are presented in Table 2.

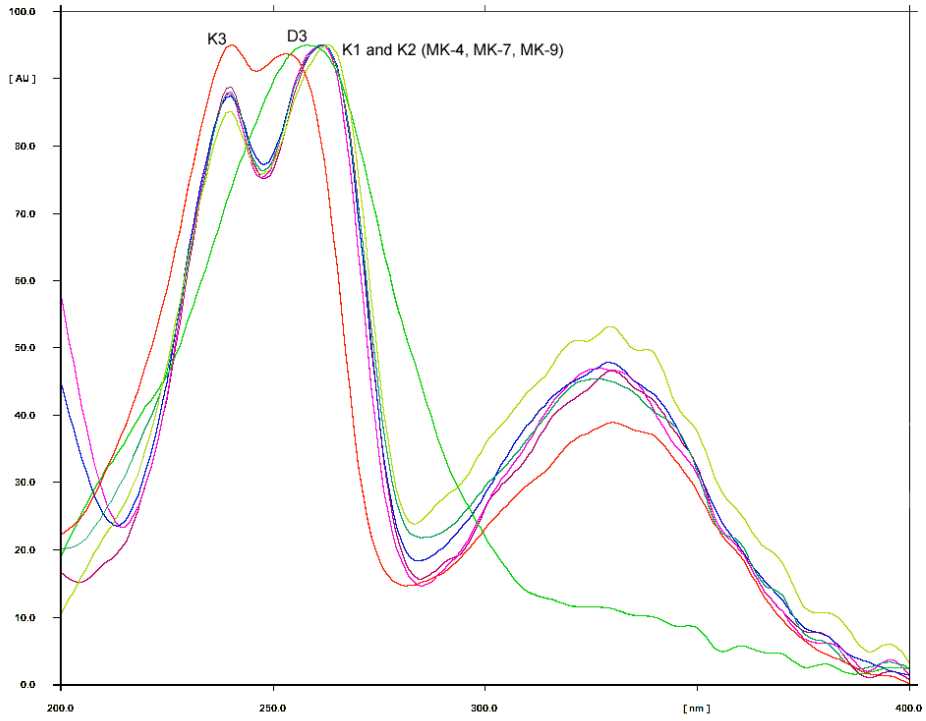

Figure 3. Overlay UV absorption spectra of standards registered directly from the densitogram.

The correlation coefficients (R) and determination coefficients (R^2) obtained for the linear model for all examined vitamins were greater than 0.99 and 0.98, respectively. The y-intercept of the linear equations was statistically insignificant.

The distribution of the residuals can be approximated with a normal distribution as it is shown by the p-value ($p > 0.05$) of the Shapiro–Wilk normality test. Based on regression analysis, it was assumed that the calibration data fitted well to the linear model.

The sensitivity of the method was good. The LOD values obtained were found to range from 25.0 ng per band for K_1 to 53.5 ng per band for D_3. In contrast, LOQ values were found to range from 50.0 ng per band for K_1 to 107.0 ng per band for D_3. The results received for intermediate precision and precision expressed as RSD% do not exceed 2.70%. The %RSD values indicate a small scatter between the series of measurements obtained and confirm the precision of the developed TLC-densitometric method.

The accuracy of the method expressed as percent of recovery at three concentration levels was from 95.78% to 104.96%. Detailed results are presented in Table 2. The values of the recovery for all vitamins, located within the range from 95% to 105%, confirm the accuracy of the proposed method.

The robustness of the proposed procedure was evaluated by introducing small changes in chromatographic conditions, such as chamber size, saturation time of the chamber, the distance of development, and volumes of the mobile phase components. In all the deliberately varied conditions, the R_f, R_s values, and peak areas for analysed vitamins showed no significant changes.

Table 2. Method validation results.

Parameter	MK-9	MK-7	K_1	MK-4	D_3	K_3
LOD (ng/band)	45.5	35.0	25.0	50.0	53.5	25.5
LOQ (ng/band)	91.0	79.0	50.0	100.0	107.0	51.0
Linearity range, (µg/band)	0.091–0.910	0.079–0.790	0.050–0.700	0.100–0.700	0.107–0.749	0.051–0.714
Slope ($a \pm S_a$)	5,334.0 ± 105.7	6,892.9 ± 238.8	9,414.8 ± 319.2	8,500.3 ± 268.1	7,895.7 ± 50.0	12,918.0 ± 466.4
Intercept ($b \pm S_b$)	117.1 ± 56.4	241.5 ± 114.0	181.2 ± 133.6	139.5 ± 112.3	-36.578 ± 111.8	279.3 ± 199.2
$t = b/S_b$	2.07	2.11	1.35	1.24	-0.33	1.40
Normality of residuals (Shapiro-Wilk test)	0.9842 (p = 0.9952)	0.8999 (p = 0.1580)	0.9264 (p = 0.2139)	0.8949 (p = 0.0665)	0.9748 (p = 0.9091)	0.9761 (p = 0.9254)
Correlation coefficient, R	0.9982	0.9941	0.9921	0.9931	0.9931	0.9910
R^2 value	0.9960	0.9871	0.9830	0.9853	0.9852	0.9808
Precision (% RSD)	1.77	2.20	1.14	2.70	2.33	2.30
Intermediate precision (% RSD)	2.63	1.64	1.38	2.21	1.53	2.47
Recovery levels (%) n=3						
80%	100.45% RSD = 2.18%	95.78% RSD = 1.65%	99.89% RSD = 1.87%	99.70% RSD = 2.10%	98.64% RSD = 1.32%	99.13% RSD = 0.68%
100%	102.00% RSD = 1.60%	101.40% RSD = 1.80%	100.63% RSD = 2.33%	102.20% RSD = 2.26%	104.96% RSD = 1.96%	100.02% RSD = 0.86%
120%	101.92% RSD = 1.43%	100.29% RSD = 1.75%	103.30% RSD = 1.59%	101.00% RSD = 1.81%	101.56% RSD = 1.50%	101.97% RSD = 0.64%

S_a, S_b—standard deviations of the slope and the intercept; t—calculated Student's t-test statistics; normal distribution of residuals ($p > 0.05$).

The proposed reversed-phase TLC-densitometric method was successfully applied for the determination of K_1, K_2 MK-4, MK-7 MK-9 and D_3 in the commercially available pharmaceutical products and dietary supplements. The obtained results of the quantitative analysis together with the statistical assessment are presented in the Table 3. The results are in agreement with the labelled value of the analysed vitamins in tablet or capsule dosage forms. Besides, the results indicate that the proposed TLC method was found to be simple, specific, sensitive, precise, and accurate for the estimation of four forms of vitamin K and vitamin D_3 in pharmaceutical formulations or dietary supplements.

Table 3. Results of vitamins' determination in pharmaceutical products and dietary supplements with statistical evaluation.

Preparation	Declared concentration	Determined concentration
Vigantoletten 1000	25 μg D_3/tablet	x_m = 26.24 SD = 0.54 RSD = 2.06% μ = x_m ± 0.67
Vitacon	10 mg K_1/tablet	x_m = 9.63 SD = 0.25 RSD = 2.55% μ = x_m ± 0.30
Vitamin K_2 MK-4	5 mg MK-4/capsule	x_m = 5.11 SD = 0.14 RSD = 2.80% μ = x_m ± 0.18
Vitamin D_3 5,000 IU Vitamin K_2 100 mcg	125 μg D_3/tablet	x_m = 118.75 SD = 3.32 RSD = 2.80% μ = x_m ± 4.13
	100 μg MK-9/tablet	x_m = 102.06 SD = 1.61 RSD = 1.57% μ = x_m ± 2.00
Kinon	75 μg MK-7/tablet	x_m = 76.02 SD = 1.34 RSD = 1.76% μ = x_m ± 1.66
Kinon D_3	50 μg D_3/tablet	x_m = 47.17 SD = 0.79 RSD = 1.68% μ=x_m ± 0.99
	100 μg MK-7/tablet	x_m = 103.23 SD = 1.09 RSD = 1.06% μ = x_m ± 1.36

x_m—arithmetic mean; SD—standard deviation; RSD—relative standard deviation (%); μ—confidence interval of the mean value at significance level 95%.

4. Conclusions

The newly developed simple, reversed-phase TLC-densitometric method for the simultaneous determination of six vitamins (K_1, K_2 MK-4, MK-7, MK-9, K_3 and D_3) was presented. The obtained validation results of the method, both in terms of the quality of chromatographic separation and statistical evaluation, are satisfactory and meet ICH guidelines for methods for quantitative analysis. Therefore, it can be concluded that the developed analytical procedure may be an alternative to other separation techniques, i.e., HPLC or LC-MS, which are more time consuming, labor intensive, and expensive.

Author Contributions: conceptualization, U.H.; validation, A.P., B.Ż.W.; investigation, U.H., A.P., B.Ż.W., M.S.; writing—original draft preparation, M.S. and B.Ż.W.; writing—review and editing, U.H.; supervision, U.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding

Conflicts of Interest: The authors declare no conflict of interest.

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