separations

MDPI

Article

Comparison of Validation Parameters for the Determination of Vitamin D3 in Commercial Pharmaceutical Products Using Traditional and Greener HPTLC Methods

Mohammed H. Alqarni [1], Faiyaz Shakeel [2], Ahmed I. Foudah [1], Tariq M. Aljarba [1], Aftab Alam [1], Sultan Alshehri [2] and Prawez Alam [1,*]

[1] Department of Pharmacognosy, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia
[2] Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
* Correspondence: p.alam@psau.edu.sa or prawez_pharma@yahoo.com

Abstract: Several analytical methods are documented for the estimation of vitamin D3 (VD3) in pharmaceuticals, food supplements, nutritional supplements, and biological samples. However, greener analytical methods for VD3 analysis are scarce in the literature. As a consequence, attempts were made to design and validate a greener "high-performance thin-layer chromatography (HPTLC)" method for VD3 estimation in commercial pharmaceutical products, as compared to the traditional HPTLC method. The greenness indices of both approaches were predicted by utilizing the "Analytical GREENness (AGREE)" method. Both traditional and greener analytical methods were linear for VD3 estimation in the 50–600 ng band^{-1} and 25–1200 ng band^{-1} ranges, respectively. The greener HPTLC strategy outperformed the traditional HPTLC strategy for VD3 estimation in terms of sensitivity, accuracy, precision, and robustness. For VD3 estimation in commercial tablets A–D, the greener analytical strategy was better in terms of VD3 assay over the traditional analytical strategy. The AGREE index of the traditional and greener analytical strategies was estimated to be 0.47 and 0.87, respectively. The AGREE analytical outcomes suggested that the greener analytical strategy had a superior greener profile to the traditional analytical strategy. The greener HPTLC strategy was regarded as superior to the traditional HPTLC methodology based on a variety of validation factors and pharmaceutical assays.

Keywords: AGREE; greener HPTLC; traditional HPTLC; validation; vitamin D3

Citation: Alqarni, M.H.; Shakeel, F.; Foudah, A.I.; Aljarba, T.M.; Alam, A.; Alshehri, S.; Alam, P. Comparison of Validation Parameters for the Determination of Vitamin D3 in Commercial Pharmaceutical Products Using Traditional and Greener HPTLC Methods. *Separations* **2022**, *9*, 301. https://doi.org/10.3390/separations9100301

Academic Editor: Attilio Naccarato

Received: 9 September 2022
Accepted: 4 October 2022
Published: 9 October 2022

1. Introduction

Vitamin D3 (VD3), also known as "cholecalciferol", is a fat-soluble vitamin used in the treatment of rickets [1–3]. It metabolizes to an active metabolite "25-hydroxyvitamin D3 (calcifediol)" which plays an important role in several biochemical processes [4,5]. Most of the population of Saudi Arabia suffers from VD3 deficiency [6,7]. VD3 is present in several pharmaceutical products, food supplements, and plant products. As a result, the determination of VD3 in a variety of products, including pharmaceutical products, is necessary both qualitatively and quantitatively.

An exhaustive literature survey demonstrated several analytical approaches for VD3 analysis in commercial pharmaceutical products, food supplements, and biological fluids. For the determination of VD3 in various food, feed, pharmaceutical, and environmental samples, a spectrophotometry method was reported [8]. Several "high-performance liquid chromatography (HPLC)" methods were reported for VD3 analysis in various food products, nutritional supplements, pharmaceutical products, and edible fungus [9–17]. A number of HPLC approaches were also reported to determine VD3 and its metabolites in human plasma and serum samples [18–20]. Additionally, various "liquid-chromatography mass-spectrometry (LC-MS)" assays were reported for the determination of VD3 and its metabolites in foodstuffs, plasma, and serum samples [12,21,22]. An ultra-high-performance liquid

chromatography method was used for the detection of VD3 in dietary supplements [23]. A fast supercritical fluid chromatography (SFC) method was also reported for the quantitative determination of VD3 and its related impurities [24]. A few SFC-mass spectrometry (SFC-MS) methods were proposed for the determination of VD3 and its metabolites in human milk and plasma samples [25,26]. An electrochemical strategy was also utilized for VD3 estimation in dosage forms [27]. A single "high-performance thin-layer chromatography (HPTLC)" method was reported for VD3 analysis in fish oil [28]. A single greener HPLC approach was reported for the determination of VD3 in thermodynamic solubility samples [29]. The range of analytical approaches for VD3 analysis was found in published literature. Some green analytical methods, such as SFC, SFC-MS, and HPLC methods, were utilized for the quantification of VD3 in a variety of sample matrices [24–26,29]. However, the greenness indices of the literature pharmaceutical assays were not determined. Furthermore, no VD3 detection has been carried out using the greener HPTLC approach. The literature has employed a variety of qualitative and quantitative methods to evaluate the analytical assays' greenness profiles [30–34]. Although, only the "Analytical GREENness (AGREE)" methodology utilizes all twelve green analytical chemistry (GAC) principles for the determination of the greenness profile [32]. Accordingly, the "AGREE approach" was utilized for the evaluation of the greenness profile of the present analytical assays [32].

Based on these assumptions, the objective of the current research was to create and verify a greener reverse-phase HPTLC strategy for VD3 detection in pharmaceutical products in comparison to the traditional normal-phase HPTLC strategy. The traditional solvent combinations were utilized as the mobile phase in the traditional analytical strategy. However, the greener analytical strategy used green solvent combinations as the mobile phase. Traditional and greener analytical strategies for VD3 detection have proven effective using "The International Council for Harmonization (ICH)" Q2-R1 recommendations [35].

2. Materials and Methods

2.1. Materials

VD3 sample (purity > 98%) was procured from "Sigma Aldrich (St. Louis, MO, USA)". The HPLC-grade solvents such as chloroform (CHL), diethyl ether (Et2O), ethanol (E2OH), and methanol (MeOH) were procured from "E-Merck (Darmstadt, Germany)". The HPLC-grade water was obtained from the Milli-Q unit. The commercial tablets of VD3 (A–D) (each tablet containing 5000 IU or 125 µg VD3) were procured from the local pharmacy shop in Riyadh, Saudi Arabia. All other materials and reagents used were of analytical grades.

2.2. Instrumentation and Analytical Conditions

The "HPTLC CAMAG TLC system (CAMAG, Muttenz, Switzerland)" was utilized for the VD3 analysis in commercial tablets A–D. The samples were prepared and spotted as 6 mm bands utilizing a "CAMAG Automatic TLC Sampler 4 (ATS4) Sample Applicator (CAMAG, Geneva, Switzerland)". The "CAMAG microliter Syringe (Hamilton, Bonaduz, Switzerland)" was attached with the sample applicator. The application rate for VD3 detection was set at 150 nL s^{-1} and remained constant. The TLC plates were developed in a "CAMAG automated developing chamber 2 (ADC2) (CAMAG, Muttenz, Switzerland)" in linear ascending mode at an 80 mm distance. The preparation chamber was saturated with the appropriate mobile phase vapors for 30 min at 22 °C. VD3 was identified at a wavelength of 272 nm. Scan speed was set at 20 mm s^{-1}, and the slit size was adjusted to 4×0.45 mm^2. For each experiment, three or six replicates were used. The software used was "WinCAT's (version 1.4.3.6336, CAMAG, Muttenz, Switzerland)".

Both the traditional normal-phase HPTLC strategy and the greener reverse-phase HPTLC strategy utilized the same analytical conditions and instruments. The main distinctions between traditional and greener analytical strategies were the TLC plates and mobile phase mixtures. The TLC plates used in the traditional HPTLC strategy were "glass plates (plate size: 10×20 cm^2) pre-coated with normal-phase silica gel (particle size: 5 µm) 60F254S plates (E-Merck, Darmstadt, Germany)" while the TLC plates used in the

greener HPTLC strategy were "RP-60F254S plates (E-Merck, Darmstadt, Germany)". The traditional mobile phase in the traditional analytical strategy was CHL-Et2O (90-10, v v^{-1}), whereas the greener mobile phase in the greener analytical strategy was E2OH-water (70-30, v v^{-1}). Due to the use of RP-TLC plates and green solvent mixtures in the greener analytical method, it is considered a reverse-phase HPTLC method.

2.3. Calibration Curves and Quality Control (QC) Sample for VD3

The necessary quantity of VD3 was dispensed into the specified volume of the mobile phase to create the VD3 stock solution, which had a final concentration of 100 µg mL^{-1}. The traditional HPTLC strategy was used to obtain VD3 concentrations in the 50–600 ng $band^{-1}$ range, whilst the greener analytical strategy—which entailed adjusting the amount of VD3 stock solution—was used to obtain concentrations in the 25–1200 ng $band^{-1}$ range. For the traditional and greener analytical strategies, 200 µL of each concentration of VD3 were spotted onto normal-phase and reverse-phase TLC plates, respectively. Both methods were used to record the VD3 concentration spot area. Plotting VD3 concentrations versus observed spot area over six replicates (n = 6) resulted in the creation of VD3 calibration curves. For the evaluation of many validation parameters, three separate QC samples were produced fresh.

2.4. Sample Processing for the Estimation of VD3 in Marketed Tablets A–D

The average weight of ten marketed tablets of each brand (A–D) (each containing 125 µg of VD3) was noted. The VD3-containing tablets were crushed and finely pulverized using a glass pestle and mortar. MeOH was utilized to extract the weight of powder containing 250 µg of VD3. Each brand (A–D) separately had 50 mL of MeOH redispersed into it after the MeOH had been evaporated at 40 °C [36]. The collected sample served as a test sample for both methods to figure out the quantity of VD3 in the marketed tablets.

2.5. Validation Parameters

Traditional and greener analytical methods for VD3 estimation were validated for different parameters following the ICH-Q2-R1 guidelines [35]. By graphing VD3 concentrations versus measured spot area, VD3 linearity was discovered. In the 50–600 ng $band^{-1}$ range (n = 6), the linearity of the traditional analytical strategy for VD3 was determined. For the greener analytical strategy, VD3 linearity was determined in the 25–1200 ng $band^{-1}$ range (n = 6).

The determination of the retardation factor (R_f), asymmetry factor (As), and theoretical plate number per meter (N m^{-1}) were utilized to assess the system suitability parameters for traditional and greener analytical methods for VD3 analysis. The "R_f, As, and N m^{-1}" values for both processes were determined using their published equations [34].

Utilizing the percent recovery method, the accuracy of traditional and greener analytical strategies for the analysis of VD3 was assessed. To assess the accuracy of the traditional analytical strategy, VD3 was measured at three QC concentrations of standard VD3 solution: low QC (LQC; 100 ng $band^{-1}$), middle QC (MQC; 300 ng $band^{-1}$), and high QC (HQC; 600 ng $band^{-1}$). To assess the accuracy of the greener analytical strategy, VD3 was also measured at three QC concentrations of standard VD3 solution: LQC (50 ng $band^{-1}$), MQC (400 ng $band^{-1}$), and HQC (1200 ng $band^{-1}$). For both analytical strategies at each QC level, the percent recovery for VD3 was computed (n = 6).

The intra/inter-assay precision of traditional and greener analytical strategies was compared for VD3. The estimation of freshly produced VD3 samples at LQC, MQC, and HQC on the same day for both analytical strategies (n = 6) was used to determine the intra-assay precision for VD3. The assessment of freshly produced VD3 samples at LQC, MQC, and HQC for three consecutive days for both strategies (n = 6) allowed for the determination of the VD3 inter-assay precision (n = 6).

By purposefully changing the mobile phase compositions, the VD3 robustness was assessed for both analytical strategies. For the traditional analytical strategy, the traditional

mobile phase CHL-Et2O (90-10, v v^{-1}) for VD3 was changed to CHL-Et2O (92-8, v v^{-1}), and CHL-Et2O (88-12, v v^{-1}), and the variations in measured response and R_f values were recorded (n = 6). Additionally, the changes in measured response and R_f values were recorded (n = 6) when the greener mobile phase E2OH-water (70-30, v v^{-1}) for VD3 was changed to E2OH-water (72:28, v v^{-1}) and E2OH-water (68-32, v v^{-1}) for the greener analytical strategy.

Using a "standard deviation" methodology, the sensitivity of the traditional and greener analytical methods for VD3 was evaluated in terms of "limit of detection (LOD) and limit of quantification (LOQ)". The VD3 "LOD and LOQ" values were obtained using their reported formulae for both analytical procedures (n = 6) [35].

To assess the specificity of the traditional and greener analytical strategies for VD3 estimation, the R_f values and UV absorption spectra of VD3 in the marketed formulations A–D were compared to a VD3 standard.

2.6. Application of Traditional and Greener Analytical Strategies in the Estimation of VD3 in Marketed Tablets A–D

For the traditional analytical procedure, the processed samples of commercial tablets A–D were applied to normal-phase TLC plates and reversed-phase TLC plates for the greener analytical procedure. For both analytical procedures, the chromatographic responses were recorded using the same experimental procedures utilized for the determination of standard VD3 (n = 3). For analytical procedures, the percent assay of VD3 in commercial tablets A–D was obtained using a VD3 calibration curve.

2.7. Greenness Evaluation

The greenness profile for the traditional and greener analytical strategies for VD3 estimation was assessed using the AGREE methodology [32]. The AGREE index (0.0–1.0) for the traditional and greener analytical strategies was determined using "AGREE: The Analytical Greenness Calculator (version 0.5, Gdansk University of Technology, Gdansk, Poland, 2020)".

2.8. Statistical Analysis

Several validation parameters of the traditional and greener analytical methods were determined and compared utilizing the Student's *t*-test, which was determined using MS Excel 2013 program. A value of $p < 0.05$ was taken as a significant value.

3. Results and Discussion

3.1. Method Development

In order to develop a suitable band for VD3 estimation by the traditional analytical procedure, different concentrations of CHL and Et2O, including CHL-Et2O (40-60, v v^{-1}), CHL-Et2O (50-50, v v^{-1}), CHL-Et2O (60-40, v v^{-1}), CHL-Et2O (70-30, v v^{-1}), CHL-Et2O (80-20, v v^{-1}), and CHL-Et2O (90-10, v v^{-1}) were evaluated as the traditional mobile phase mixtures. The chamber saturation conditions were applied to develop all mobile phase compositions. A typical TLC plate for the standard and commercial formulations is presented in Figure 1.

It was discovered that the traditional mobile phases, including CHL-Et2O (40-60, v v^{-1}), CHL-Et2O (50-50, v v^{-1}), CHL-Et2O (60-40, v v^{-1}), CHL-Et2O (70-30, v v^{-1}), and CHL-Et2O (80-20, v v^{-1}), provided unfavorable VD3 chromatographic peaks with higher As values (As >1.15). It was discovered that the traditional mobile phase CHL-Et2O (90-10, v v^{-1}) provided a well-resolved and intact VD3 chromatographic peak at $R_f = 0.34 \pm 0.01$ (Figure 2A) when tested. VD3 was also found to have an As values of 0.97, which is acceptable. As a consequence, the CHL-Et2O (90-10, v v^{-1}) was optimized as the final traditional mobile phase for the traditional analytical method of VD3 measurement.

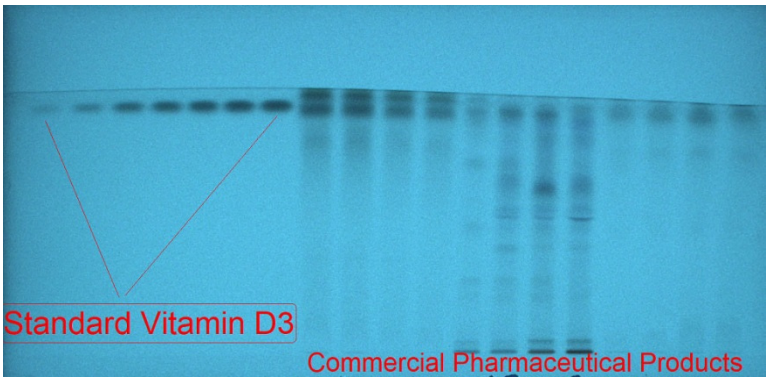

Figure 1. A typical thin-layer chromatography (TLC) plate of standard vitamin D3 (VD3) and commercial formulations developed using ethanol-water (70:30 v v^{-1}) as the greener mobile phase for the greener high-performance TLC (HPTLC) method.

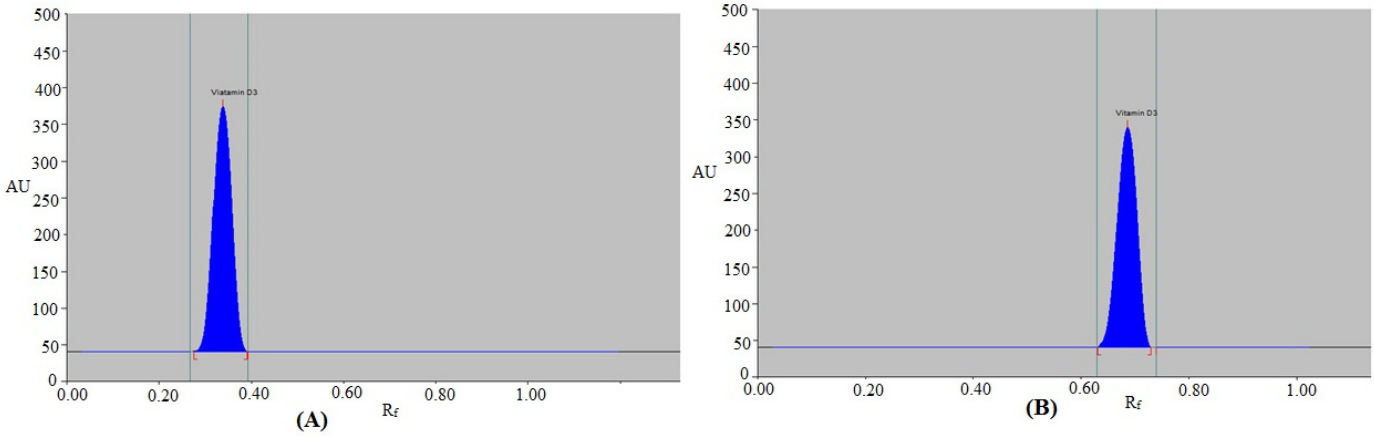

Figure 2. Representative chromatograms of standard VD3 recorded using (**A**) traditional normal-phase HPTLC and (**B**) greener reversed-phase HPTLC methods.

In order to develop a suitable band for VD3 estimation using the greener analytical method, different concentrations of E2OH and water, such as E2OH-water (40-60, v v^{-1}), E2OH-water (50-50, v v^{-1}), E2OH-water (60-40, v v^{-1}), E2OH-water (70-30, v v^{-1}), E2OH-water (80-20, v v^{-1}), and E2OH-water (90-10, v v^{-1}), were evaluated as the greener mobile phase mixtures. It was discovered that the greener mobile phase mixtures, including E2OH-water (40-60, v v^{-1}), E2OH-water (50-50, v v^{-1}), E2OH-water (60-40, v v^{-1}), E2OH-water (80-20, v v^{-1}), and E2OH-water (90-10, v v^{-1}), provided unfavorable VD3 chromatographic peaks with higher As values (As >1.20). It was discovered that the greener mobile phase E2OH-water (70-30, v v^{-1}) provided a well-resolved and intact VD3 chromatographic peak at $R_f = 0.69 \pm 0.02$ (Figure 2B) when tested. VD3 was also found to have an As values of 1.04, which is acceptable. As a consequence, the E2OH-water (70-30, v v^{-1}) was optimized as the final greener mobile phase for the greener analytical method of VD3 measurement. When the spectral bands for VD3 were recorded in densitometry mode, the greatest TLC response for VD3 was discovered at a wavelength of 272 nm. Thus, the complete VD3 study was performed at 272 nm.

3.2. Validation Parameters

The ICH-Q2-R1 recommendations were used to obtain a number of parameters for VD3 measurement [35]. The outcomes of the linear regression analysis of the VD3 calibration curves for both analytical methods are shown in Table 1. The VD3 calibration curve for the traditional analytical strategy was linear in the 50–600 ng $band^{-1}$ range. The VD3 calibration curve was linear in the 25–1200 ng $band^{-1}$ range for the greener analytical

strategy. The determination coefficient (R^2) and regression coefficient (R) for VD3 were estimated to be 0.9919 and 0.9959, respectively, for the traditional analytical assay. The R^2 and R values for VD3 were predicted to be 0.9955 and 0.9977, respectively, for the greener analytical assay. The findings showed a significant correlation between the measured area and VD3 levels. All these outcomes demonstrated the reliability of both analytical approaches for VD3 estimation. On the other hand, the greener analytical assay was linear over a wider range than the traditional analytical assay.

Table 1. Results for the linearity of vitamin D3 (VD3) for the traditional normal-phase high-performance thin-layer chromatography (HPTLC) and greener reversed-phase HPTLC methods (mean ± SD; n = 6).

Parameters	Traditional HPTLC	Greener HPTLC
Linearity range (ng band^{-1})	50–600	25–1200
Regression equation	y = 16.975x + 922.55	y = 18.446x + 1213.2
R^2	0.9919	0.9955
R	0.9959	0.9977
Traditional error of slope	0.40	0.41
Traditional error of intercept	11.81	4.63
95% confidence interval of slope	15.25–18.69	16.65–20.23
95% confidence interval of intercept	871.70–973.39	1193.25–1233.14
LOD ± SD (ng band^{-1})	17.54 ± 0.24	8.47 ± 0.12
LOQ ± SD (ng band^{-1})	52.62 ± 0.72	25.41 ± 0.36

R^2: determination coefficient; R: regression coefficient; LOD: limit of detection; LOQ: limit of quantification.

Table 2 presents the system suitability parameters for both the traditional and greener analytical assays. For VD3 estimation, the R_f, As, and N m^{-1} values for the traditional analytical assay were obtained as 0.34, 0.97, and 4875, respectively, which were acceptable. For the greener analytical assay, the R_f, As, and N m^{-1} results for VD3 estimation were 0.69, 1.04, and 4798, respectively, which were also acceptable values.

Table 2. System suitability parameters of traditional and greener HPTLC methods for VD3 estimation (mean ± SD; n = 3).

Parameters	Traditional HPTLC	Greener HPTLC
R_f	0.34 ± 0.01	0.69 ± 0.02
As	0.97 ± 0.01	1.04 ± 0.02
N m^{-1}	4875 ± 4.19	4798 ± 4.12

R_f: retardation factor; As: asymmetry factor; N m^{-1}: number of theoretical plates per meter.

The accuracy of both analytical methods for VD3 estimation was measured in terms of percent recovery. Table 3 illustrates the accuracy outcomes for both analytical methods. The % recoveries of VD3 at three QC concentrations were uncovered as 94.83–103.52% using the traditional analytical assay. The VD3 % recoveries at three QC concentrations were uncovered as 98.74–100.85% for the greener analytical assay. Both assays were expected to be accurate for VD3 estimation based on these outcomes. However, the % recoveries of VD3 using the greener analytical assay were significant compared to the traditional analytical assay ($p < 0.05$). As a result, for VD3 estimation, the greener analytical assay was demonstrated to be more accurate than the traditional analytical assay.

The intra/inter-assay precision of both analytical assays was studied, and the data for VD3 estimation were expressed as the percent of the relative standard deviation (% RSD). For both analytical assays of VD3 estimation, Table 4 illustrates the outcomes of the intra/inter-day precisions. The % RSD of VD3 for intra-day precision was uncovered as 2.69–3.13% for the traditional analytical assay. The % RSD of VD3 for inter-day precision was uncovered as 2.99–3.14% for the traditional analytical assay. For the greener analytical assay, the % RSD of VD3 for intra-day precision was uncovered as 0.61–0.77%. For the greener analytical assay, the % RSD of VD3 for inter-day precision was uncovered as 0.61–0.86%. These outcomes revealed that both assays for VD3 estimation were precise. The precisions of VD3 using the greener analytical assay were significant compared to the

traditional analytical assay ($p < 0.05$). Therefore, the greener analytical assay showed to be more precise than the traditional analytical assay for VD3 estimation.

Table 3. Accuracy analysis of VD3 for traditional and greener HPTLC methods (mean ± SD; n = 6).

Conc. (ng band^{-1})	Conc. Found (ng band^{-1}) ± SD	Recovery (%)	RSD (%)
Traditional HPTLC			
100	103.24 ± 3.23	103.24	3.12
300	284.51 ± 8.65	94.83	3.04
600	621.14 ± 16.97	103.52	2.73
Greener HPTLC			
50	49.91 ± 0.38	99.82	0.76
400	394.98 ± 2.97	98.74	0.75
1200	1210.23 ± 7.61	100.85	0.62

Table 4. Evaluation of VD3 intra/inter-day precision for traditional and greener HPTLC methods (mean ± SD; n = 6).

Conc. (ng band^{-1})	Intra-Day Precision			Inter-Day Precision		
	Conc. Found (ng band^{-1}) ± SD	Standard Error	RSD (%)	Conc. Found (ng band^{-1}) ± SD	Standard Error	RSD (%)
Traditional HPTLC						
100	94.87 ± 2.97	1.21	3.13	93.61 ± 2.94	1.20	3.14
300	316.54 ± 9.12	3.72	2.88	318.21 ± 9.68	3.95	3.04
600	581.45 ± 15.67	6.39	2.69	618.31 ± 18.54	7.57	2.99
Greener HPTLC						
50	50.23 ± 0.39	0.15	0.77	50.64 ± 0.44	0.17	0.86
400	405.61 ± 3.01	1.22	0.74	393.65 ± 3.10	1.26	0.78
1200	1194.51 ± 7.35	3.00	0.61	1206.32 ± 7.41	3.02	0.61

The robustness of both analytical methodologies for VD3 estimation was determined by intentionally altering mobile phase components. Table 5 illustrates the outcomes of the robustness analysis for both analytical strategies. The VD3 % RSD for the traditional analytical strategy was uncovered as 3.63–3.71%. The VD3 R_f values were predicted to be 0.33–0.36 for the traditional analytical strategy. For the greener analytical strategy, the % RSD for VD3 was uncovered as 0.67–0.71%. The VD3 R_f values were uncovered as 0.68–0.70 for the greener analytical strategy. These outcomes demonstrated that both analytical strategies for VD3 estimation were robust. When compared to the traditional analytical strategy, the greener analytical strategy significantly reduced the %RSD of VD3 ($p < 0.05$). Accordingly, the greener analytical strategy fared better than the traditional analytical strategy when it came to VD3 estimation.

Table 5. Measurement of VD3 robustness for traditional and greener HPTLC methods (mean ± SD; n = 6).

Conc. (ng band^{-1})	Mobile Phase Mixture (Chloroform-Diethyl Ether)			Results		
	Original	Used		Conc. (ng band^{-1}) ± SD	RSD (%)	R_f
Traditional HPTLC						
300	90:10	92:8	+2.0	288.71 ± 10.12	3.50	0.33
		90:10	0.0	294.61 ± 10.95	3.71	0.34
		88:12	−2.0	308.41 ± 11.21	3.63	0.36
Greener HPTLC Mobile phase mixture (ethanol-water)						
400	70:30	72:28	+2.0	389.51 ± 2.64	0.67	0.68
		70:30	0.0	394.25 ± 2.75	0.69	0.69
		68:32	−2.0	403.67 ± 2.89	0.71	0.70

To assess the sensitivity of both VD3 estimation assays, the "LOD and LOQ" were utilized. Table 1 illustrates the outcomes of the "LOD and LOQ" calculations for VD3 utilizing both analytical strategies. According to the traditional HPTLC strategy, VD3s "LOD and LOQ" were found to be 17.54 ± 0.24 and 52.62 ± 0.72 ng band^{-1}, respectively. The "LOD and LOQ" of VD3 using the greener HPTLC strategy were determined to be 8.47 ± 0.12 and 25.41 ± 0.36 ng band^{-1}, respectively. These outcomes demonstrated that both analytical strategies were sensitive to VD3 estimation. When compared to the traditional analytical strategy, the "LOD and LOQ" values of VD3 when employing the greener analytical strategy were significant ($p < 0.05$). Accordingly, the greener analytical strategy was demonstrated to be more sensitive than the traditional analytical strategy for VD3 estimation.

The specificity of the proposed analytical strategies of VD3 estimation was evaluated by comparing the R_f values and UV absorption spectra of VD3 in commercial tablets A–D with that of standard VD3. Figure 3 shows the overlaid UV absorption spectra of standard VD3 and VD3 in commercial tablets A–D. The peak response of standard VD3 and commercial tablets A–D was measured at 272 nm. By recoding the similar UV absorption spectra, R_f values, and wavelengths of VD3 in standard and commercial formulations A–D, we demonstrated the specificity of both analytical strategies for VD3 determination.

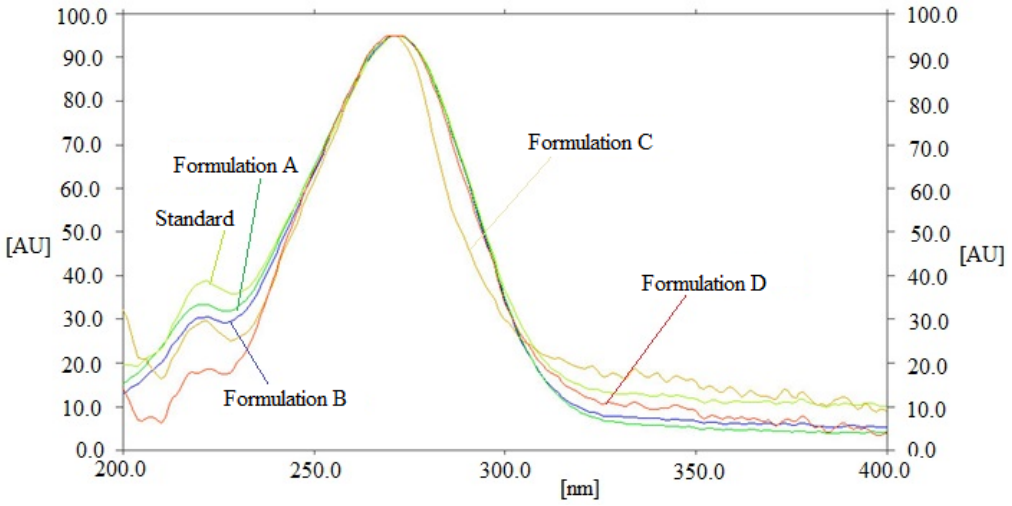

Figure 3. Superimposed UV absorption spectra of standard VD3 and various commercial formulations.

3.3. Application of Traditional and Greener HPTLC Strategies in the Estimation of VD3 in Marketed Tablets A–D

For the estimation of VD3 in commercial tablets A–D, both analytical strategies were applied as alternative approaches to routine liquid chromatography methods. The chromatograms of VD3 from commercial formulations A–D were identified by comparing the TLC spot at $R_f = 0.34 \pm 0.01$ for VD3 with the standard VD3 utilizing the traditional analytical strategy. The chromatographic peaks of VD3 in commercial tablets A–D were similar to that of standard VD3 when using the traditional analytical assay. The chromatograms of VD3 from commercial formulations A–D were also identified by comparing the TLC spot at $R_f = 0.69 \pm 0.02$ for VD3 with the standard VD3 utilizing the greener analytical strategy. The chromatographic peaks of VD3 in the commercial tablets A–D were also similar to that of standard VD3 when using the greener analytical strategy. Furthermore, no additional peaks of excipients were found in the commercial tablets using both analytical strategies, indicating no interaction between VD3 and tablet excipients. The calibration curve of VD3 was used to determine its content using traditional and greener analytical strategies, and the results are illustrated in Table 6. Using the traditional analytical assay, the % assay of VD3 in the commercial tablets A–D ranged from 87.64–95.36%. Using the greener analytical strategy, the % assay of VD3 in commercial tablets A–D ranged from 98.19–101.12%.

Table 6. Application of traditional and greener HPTLC strategies in the estimation of VD3 in commercial tablets A–D.

Samples	Label Claim (µg)	Content Found (µg) ± SD	Assay (%)
Traditional HPTLC			
Formulation A	125	119.21 ± 2.12	95.36
Formulation B	125	116.41 ± 2.06	93.12
Formulation C	125	111.51 ± 1.97	89.20
Formulation D	125	109.56 ± 1.88	87.64
Greener HPTLC			
Formulation A	125	126.41 ± 2.18	101.12
Formulation D	125	125.98 ± 2.14	100.78
Formulation C	125	124.14 ± 2.13	99.31
Formulation D	125	122.74 ± 2.15	98.19

Using the greener analytical strategy rather than the traditional analytical strategy, it was discovered that all commercial tablets had more VD3. This observation may have been made feasible by the employment of different solvent mixtures in the traditional and greener analytical methods. Overall, the greener analytical strategy was considered superior to the traditional analytical strategy for VD3 pharmaceutical assay.

3.4. Greenness Assessment

For the evaluation of analytical techniques' greenness, various qualitative and quantitative methodologies are presented [30–34]. However, only AGREE makes use of all twelve GAC components for evaluating greenness [32]. Accordingly, the greener profiles of both analytical strategies were evaluated using the AGREE method. Figure 4 illustrates a representative pictogram of the AGREE index of the traditional and greener analytical strategies. For traditional and greener analytical strategies, the AGREE index was predicted to be 0.47 and 0.87, respectively. These results showed that the greener analytical strategy for VD3 analysis had a superior greenness profile to the traditional analytical strategy.

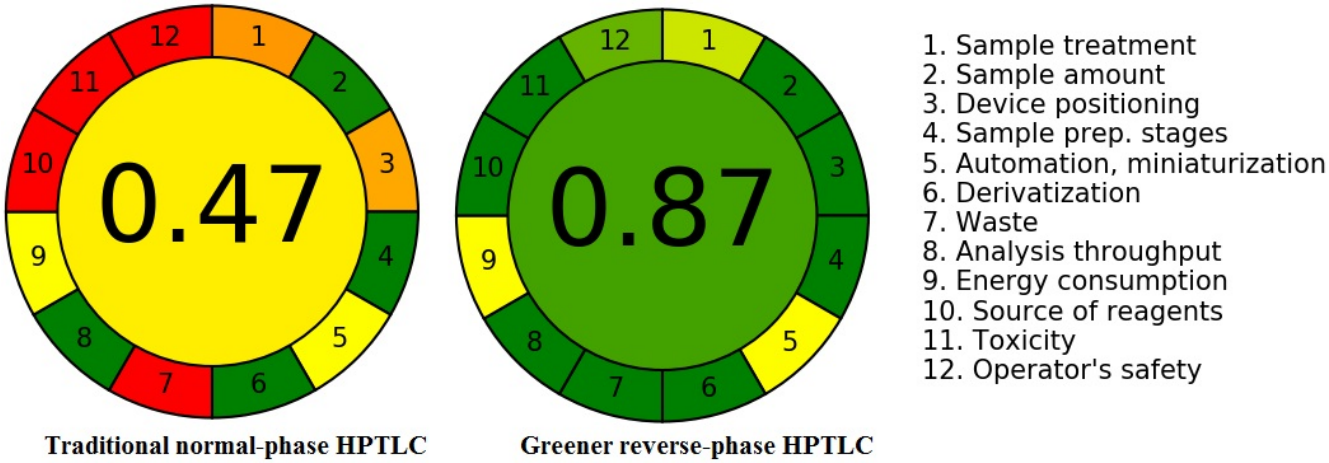

Figure 4. The pictograms of the AGREE indices for the traditional and the greener HPTLC strategies recorded utilizing the "AGREE: The Analytical Greenness Calculator".

4. Conclusions

There is a scarcity of greener analytical assays for VD3 estimation in the literature. In contrast to the traditional HPTLC methodology, this research aimed to design and validate a sensitive and greener HPTLC assay for VD3 estimation in marketed tablets. The amount of VD3 in all commercial tablets was discovered to be much higher in terms of % assay when comparing the greener analytical method to the traditional analytical strategy. According to the AGREE outcomes, the greener analytical strategy had a higher level of greenness than

the traditional analytical strategy. Based on a number of validation criteria and the results of pharmaceutical assays, the greener HPTLC approach was declared to be superior to the traditional HPTLC approach for VD3 estimation in commercial tablets. In conclusion, these outcomes suggest that the greener HPTLC assay can be used for the estimation of VD3 in commercially available products. Overall, the greener HPTLC strategy is more accurate, precise, robust, and sensitive than the traditional HPTLC strategy for the determination of VD3 in commercial formulations.

Author Contributions: Conceptualization, M.H.A. and P.A.; methodology, A.A., P.A. and T.M.A.; software, A.I.F. and F.S.; validation, S.A. and M.H.A.; formal analysis, A.I.F. and T.M.A.; investigation, P.A. and A.A.; resources, S.A.; data curation, A.I.F. and F.S.; writing—original draft preparation, F.S.; writing—review and editing, M.H.A., P.A. and S.A.; visualization, S.A.; supervision, P.A.; project administration, P.A.; funding acquisition, S.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Researchers Supporting Project (number RSP-2021/146) at King Saud University, Riyadh, Saudi Arabia and The APC was funded by RSP.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors are thankful to the Researchers Supporting Project (number RSP-2021/146) at King Saud University, Riyadh, Saudi Arabia for supporting this research.

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

References

1. Christakos, S.; Dhawan, P.; Benn, B.; Porta, A.; Hediger, M.; Oh, G.T.; Jeung, E.B.; Zhong, Y.; Ajibade, D.; Dhawan, K.; et al. Vitamin D-molecular mechanism of action. *Ann. N. Y. Acad. Sci.* **2007**, *1116*, 340–348. [CrossRef]
2. Pike, J.W. Vitamin-D3 receptors-structure and function in transcription. *Annu. Rev. Nutr.* **1991**, *11*, 189–216. [CrossRef]
3. Holick, M.F. Resurrection of vitamin D deficiency and rickets. *J. Clin. Investig.* **2006**, *116*, 2062–2072. [CrossRef] [PubMed]
4. Deluca, H.D. Metabolism and molecular mechanism of action of vitamin-D. *Biochem. Soc. Trans.* **1982**, *10*, 147–158. [CrossRef]
5. Jurutka, P.W.; Bartik, L.; Whitfield, G.K.; Mathern, D.R.; Barthel, T.K.; Gurevich, M.; Hsieh, J.C.; Kaczmarska, M.; Haussler, C.A.; Haussler, M.R. Vitamin D receptor: Key roles in bone mineral pathophysiology, molecular mechanism of action, and novel nutritional ligands. *J. Bone Miner. Res.* **2007**, *22*, V2–V10. [CrossRef] [PubMed]
6. Alsuwdia, A.O.; Frag, Y.M.; Sayyari, A.A.; Mousa, D.H.; Alhijaili, F.F.; Al-Harbi, A.S.; Housawi, A.A.; Mittal, B.V.; Singh, A.K. Prevalence of vitamin D deficiency in Saudi adults. *Saudi Med. J.* **2013**, *34*, 814–818.
7. AlBuhairan, F.S.; Tamim, H.; Al-Dubayee, M.; AlDhukair, S.; Al-Shehri, S.; Tamimi, W.; El-Bcheraoui, C.; Magzoub, M.E.; de Vries, N.; Al-Alwan, I. Time for an adolescent health surveillance system in Saudi Arabia: Findings from "Jeeluna". *J. Adolesc. Health* **2015**, *57*, 263–269. [CrossRef] [PubMed]
8. Rahman, A.; Rahman, M.M.; Hossain, M.S.; Jahan, M.S.; Akter, N.J.; Bari, M.L. A simple and alternative UV spectrometric method for the estimation of vitamin D3. *Microb. Bioact.* **2019**, *2*, 98–105.
9. Johnsson, H.; Halen, B.; Hessel, H.; Nyman, A.; Thorzell, K. Determination of vitamin D3 in margarines, oils and other supplemented food products using HPLC. *Int. J. Vitam. Nutr. Res.* **1989**, *59*, 262–268.
10. Sarioglu, K.; Celebi, S.S.; Mutlu, M. A rapid method for determination of vitamins D2 and D3 in pharmaceutical preparations by HPLC. *J. Liq. Chromatogr. Relat. Technol.* **2001**, *24*, 973–982. [CrossRef]
11. Jakobsen, J.; Clausen, I.; Leth, T.; Ovesen, L. A new method for the determination of vitamin D3 and 25-hydroxyvitamin D3 in meat. *J. Food Compos. Anal.* **2004**, *17*, 777–787. [CrossRef]
12. Bilodeau, L.; Dufresne, G.; Deeks, J.; Clement, G.; Bertrand, J.; Turcotte, S.; Rbichaud, A.; Beraldin, F.; Fouquet, A. Determination of vitamin D3 and 25-hydroxyvitamin D3 in foodstuffs by HPLC UV-DAD and LC–MS/MS. *J. Food Compos. Anal.* **2011**, *24*, 441–448. [CrossRef]
13. Kumar, S.; Chawla, D.; Tripathi, K. An improved and sensitive method for vitamin D3 estimation by RP-HPLC. *Pharm. Anal. Acta* **2015**, *6*, E1000410.
14. Temova, Z.; Roskar, R. Stability-indicating HPLC–UV method for vitamin D3 determination in solutions, nutritional supplements and pharmaceuticals. *J. Chromatogr. Sci.* **2016**, *54*, 1180–1186. [CrossRef]
15. Farag, A.M.; Rizk, M.S.; El-Bassel, H.A.; Youssif, M.H. Determination of vitamin D3 content in high, low and zero fat food using high performance liquid chromatography. *Med. J. Cairo Univ.* **2018**, *86*, 3911–3918.

16. Huang, B.-F.; Pan, X.-D.; Zhang, J.-S.; Xu, J.-J.; Cai, Z.-X. Determination of vitamins D2 and D3 in edible fungus by reversed-phase two-dimensional liquid chromatography. *J. Food Qual.* **2020**, *2020*, E8869279. [CrossRef]
17. Rashidi, L.; Nodeh, H.R.; Shahabuddin, S. Determination of vitamin D in the fortified sunflower oil: Comparison of two developed methods. *Food Anal. Methods* **2022**, *15*, 330–337. [CrossRef]
18. Brunetto, M.R.; Obando, M.A.; Gallignani, M.; Alarcon, O.M.; Nieto, E.; Salinas, R.; Burguera, J.L.; Burguera, M. HPLC determination of vitamin D3 and its metabolite in human plasma with on-line sample cleanup. *Talanta* **2004**, *64*, 1364–1370. [CrossRef]
19. Keyfi, F.; Nahid, S.; Mokhtariye, A.; Nayerabadi, S.; Alaei, A.; Varasteh, A.-R. Evaluation of 25-OH vitamin D by high performance liquid chromatography: Validation and comparison with electrochemiluminescence. *J. Anal. Sci. Technol.* **2018**, *9*, E25. [CrossRef]
20. Babat, N.; Turkmen, Y. Determination of serum vitamin D3 level by high performance liquid chromatography (HPLC) in patients with coronary artery ectasia. *Cardiol. Cardiovasc. Med.* **2020**, *4*, 97–104. [CrossRef]
21. Mirza, T.; Qadeer, K.; Ahmad, I. Clinical analysis of vitamin D and metabolites. *J. Baqai Med. Univ.* **2009**, *12*, 25–28.
22. Shah, I.; James, R.; Barker, J.; Petroczi, A.; Naughton, D.P. Misleading measures in vitamin D analysis: A novel LC-MS/MS assay to account for epimers and isobars. *Nutr. J.* **2011**, *10*, E46. [CrossRef]
23. Becze, A.; Fuss, V.L.B.; Scurtu, D.A.; Tomoaia-Cotisel, M.; Mocanu, A.; Cadar, O. Simultaneous determination of vitamins D3 (calcitriol, cholecalciferol) and K2 (menaquinone-4 and menaquinone-7) in dietary supplements by UHPLC. *Molecules* **2021**, *26*, 6982. [CrossRef]
24. Andri, B.; Lebrun, P.; Dispas, A.; Klinkenberg, R.; Streel, B.; Ziemons, E.; Marini, R.D.; Hubert, P. Optimization and validation of a fast supercritical fluid chromatography method for the quantitative determination of vitamin D3 and its related impurities. *J. Chromatogr. A* **2017**, *1491*, 171–181. [CrossRef]
25. Oberson, J.M.; Benet, S.; Redeuil, K.; Campos-Gimenez, E. Quantitative analysis of vitamin D and its main metabolites in human milk by supercritical fluid chromatography coupled to tandem mass spectrometry. *Anal. Bioanal. Chem.* **2020**, *412*, 365–375. [CrossRef]
26. Socas-Rodriguez, B.; Pilarova, V.; Sandahl, M.; Holm, C.; Turner, C. Simultaneous determination of vitamin D and its hydroxylated and esterified metabolites by ultrahigh-performance supercritical fluid chromatography−tandem mass spectrometry. *Anal. Chem.* **2022**, *94*, 3065–3073. [CrossRef]
27. Durovic, A.; Stojanovic, Z.; Kravic, S.; Kos, J.; Richtera, L. Electrochemical determination of vitamin D3 in pharmaceutical products by using boron doped diamond electrode. *Electroanalysis* **2020**, *32*, 741–748. [CrossRef]
28. Demchenko, D.V.; Pozharitskaya, O.N.; Shikov, A.N.; Makarov, V.G. Validated HPTLC method for quantification of vitamin D in fish oil. *J. Planar Chromatogr.* **2011**, *24*, 487–490. [CrossRef]
29. Almarri, F.; Haq, N.; Alanazi, F.K.; Mohsin, K.; Alsarra, I.A.; Aleanizy, F.S.; Shakeel, F. An environmentally benign HPLC-UV method for thermodynamic solubility measurement of vitamin D3 in various (Transcutol + water) mixtures. *J. Mol. Liq.* **2017**, *242*, 798–806. [CrossRef]
30. Abdelrahman, M.M.; Abdelwahab, N.S.; Hegazy, M.A.; Fares, M.Y.; El-Sayed, G.M. Determination of the abused intravenously administered madness drops (tropicamide) by liquid chromatography in rat plasma; an application to pharmacokinetic study and greenness profile assessment. *Microchem. J.* **2020**, *159*, E105582. [CrossRef]
31. Duan, X.; Liu, X.; Dong, Y.; Yang, J.; Zhang, J.; He, S.; Yang, F.; Wang, Z.; Dong, Y. A green HPLC method for determination of nine sulfonamides in milk and beef, and its greenness assessment with analytical eco-scale and greenness profile. *J. AOAC Int.* **2020**, *103*, 1181–1189. [CrossRef]
32. Pena-Pereira, F.; Wojnowski, W.; Tobiszewski, M. AGREE-Analytical GREEnness metric approach and software. *Anal. Chem.* **2020**, *92*, 10076–10082. [CrossRef]
33. Alam, P.; Salem-Bekhit, M.M.; Al-Joufi, F.A.; Alqarni, M.H.; Shakeel, F. Quantitative analysis of cabozantinib in pharmaceutical dosage forms using green RP-HPTLC and green NP-HPTLC methods: A comparative evaluation. *Sustain. Chem. Pharm.* **2021**, *21*, E100413. [CrossRef]
34. Foudah, A.I.; Shakeel, F.; Alqarni, M.H.; Alam, P. A rapid and sensitive stability-indicating green RP-HPTLC method for the quantitation of flibanserin compared to green NP-HPTLC method: Validation studies and greenness assessment. *Microchem. J.* **2021**, *164*, E105960. [CrossRef]
35. International Conference on Harmonization (ICH). *Q2 (R1): Validation of Analytical Procedures–Text and Methodology*; ICH: Geneva, Switzerland, 2005.
36. Alam, P.; Shakeel, F.; Ali, A.; Alqarni, M.H.; Foudah, A.I.; Aljarba, T.M.; Alkholifi, F.K.; Alshehri, S.; Ghoneim, M.M.; Ali, A. Simultaneous determination of caffeine and paracetamol in commercial formulations using greener normal-phase and reversed-phase HPTLC methods: A contrast of validation parameters. *Molecules* **2022**, *27*, 405. [CrossRef]