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ORIGINAL PAPER

Optimization of ultrasonication-assisted extraction conditions using RSM-I-Optimal experimental design to recover vitamin D_2 and K_1 from selected green leafy vegetable samples

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Abstract
This study employed the response surface methodology to optimize the extraction conditions for recovering vitamins D_2 and K_1 from green leafy vegetables using ultrasonication-assisted extraction. The vitamin content was determined using an Accucore C18 column and a UPLC-Q-ToF/MS method. An RSM-I-Optimal design was used for designing the experiment to find the best combination of solvent level (mL), sonication time (min), sonication frequency (kHz), and temperature (°C). The experimental data from a 25-sample set were fitted to a second-order polynomial equation using multiple regression analysis. The extraction models had R^2 values of 0.895 and 0.896, respectively, and the probability values ($p < 0.0001$) indicated that the regression model was highly significant. The optimal extraction conditions were: solvent level of 65 mL, sonication time of 45 min, sonication frequency of 70 kHz, and temperature of 45 °C. Under these conditions, the predicted recovery (%) values for vitamins D_2 and K_1 were 90.7% and 90.4%, respectively. This study has the potential to use the reported extraction method for routine quantification of vitamins D_2 and K_1 in the laboratory using UPLC-Q-ToF/MS.

Keywords Response surface methodology (RSM) · I-optimal design · Ultrasonication-assisted extraction (UAE) · Ultraperformance-liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-ToF/MS) · Vitamin D_2 · Vitamin K_1

Introduction

Vitamin D (VD) and vitamin K (VK) are micro nutrient that is required in the diet. The deficiency of these vitamins is common in people of all ages, genders, races, and geographical locations [1]. When compared to adequate VD and VK status, low VD and VK status is associated with an increased risk of all-cause mortality and possibly cardiovascular mortality and events [2]. Both VD and VK have been proposed as SARS-CoV-2 disease modifiers [3]. As a result, it is critical to develop an efficient extraction method for utilizing vitamins D and K (VDK) from widely available sources in order to maximize its utilization by the population.

A significant amount of bioavailable VD is required to be metabolized efficiently to 1,25-hydroxyvitamin D, which is functional in promoting bone mineralization as well as various biological activities in the innate and adaptive immune system, heart functioning and blood pressure regulation, insulin secretion by pancreatic cells, brain and fetal development, and so on [4–6]. VK is required for the formation of liver zymogen and has the ability to regulate and control the synthesis of coagulation factors II, VII, IX, and X. Bleeding disorders are treated with vitamin K_1 (VK_1) [7]. Furthermore, it can inhibit cancer [8, 9], prevent vascular calcification [10, 11], participate in bone metabolism [10, 11], prevent chronic kidney disease [12], and aid in the treatment of pertussis syndrome [12]. The richest dietary sources of vitamin D_2 (VD_2) and VK_1 are green leafy vegetables and condiments [13]. VK_1 accounts for more than 80% of total

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VK in the human diet, and most of our current knowledge on VK is focused on VK_1 [14].

VD_2 is a fat-soluble vitamin obtained from plants, particularly mushrooms and yeast. Traditionally, liquid–liquid extraction was used to extract VD_2 and its metabolites from various sources. During these procedures, organic solvents such as acetonitrile, ethanol, chloroform, methanol, diethyl ether, tetrahydrofuran (THF), hexane, and others are commonly used [15–18]. Different types of pure and mixed solvents with a wide range of solubility and selectivity are used in liquid–liquid extraction. Furthermore, the separation steps involved in this type of extraction are straightforward. However, because of the large volume of organic solvents used during these extraction processes, waste with low biodegradability and high volatility is generated, polluting the environment.

VK_1 is a fat-soluble, antihemorrhagic vitamin produced by plants, green algae, and some cyanobacteria species. It belongs to a class of naphthoquinone derivatives with chlorophyll biological activity [19, 20]. Traditional VK_1 pretreatment methods include saponification [21], enzymatic hydrolysis [22], and liquid–liquid extraction [14]. However, because VK_1 is easily decomposed by alkali, VK_1 determination by saponification is inaccurate [23]. Due to the complexity of the procedures, the recovery rate is typically low, and the precision of the detection result is poor. Meanwhile, liquid–liquid extraction takes a long time and a lot of solvent, so the extraction efficiency is low.

As a result, there is a growing demand for newer extraction techniques that demonstrate lower solvent consumption and shorter extraction times. As a result, various advanced extraction techniques have been investigated, including supercritical fluid extraction (SFE), microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), and solid phase extraction (SPE). These techniques can be used instead of traditional methods to reduce operation time and increase extractable yields [17]. Consequently, a new method for extracting VD_2 and VK_1 from green leafy vegetables is required. Solid-phase extraction (SPE) is a sample pretreatment technology with high recovery rates and simple operation [24, 25]. Furthermore, ultrasound-assisted extraction (UAE) is widely used in sample preparation for analytical chemistry [26], as it is inexpensive, efficient, adjustable, applicable, and simple to use. According to previous research, UAE can improve the extraction efficiency of many target compounds [27]. As a result, UAE followed by SPE may be a viable option for extracting and purifying VD_2 and VK_1 from green leafy foods.

Response surface methodology (RSM) I-optimal design was considered to determine the optimal conditions for the UAE procedure through a series of experimental runs. I-optimal design is random and exact, and it aids in minimizing the integral of prediction variance across factor space [28]. It could be used to make the method more cost-effective since the information is obtained through fewer experiments [29, 30]. Statistical experimental designs can save time by accurately predicting experiment results, provide information about the impact of a specific factor on response, and be used to optimize processes [31]. Various design methods have been suggested based on the design requirements. These designs have the advantage of being able to consider a larger number of variables to determine their commutative effect on process output. A limited number of experiments can provide better optimal conditions for a desired range of process output. Such an approach can save time and resources while also reducing material waste. To find optimal factors, the optimal design must meet three primary criteria: D-optimal, G-optimal, and I-optimal. The D-optimal provides better estimation, while the G- and I-optimal provide better predictions; this allows you to compare the performance of the best design to others. Our goal of obtaining prediction variance of G- and I-optimal efficiency is good, but G-optimal takes longer than I-optimal [29]. As a result, I-optimal was chosen for the RSM technique.

The proposed method was developed using the RSM-I-optimal design to determine the optimal conditions for the UAE procedure, ensuring reduced time and cost of operation and achieving better analyte recoveries.

Materials and methods

Chemicals, reagents and samples

Analytical reference standards of ergocalciferol (VD_2, 99.6%) and phylloquinone (VK_1, 99.6%) were purchased from Sigma-Aldrich, India. Methanol, and 2-propanol of LC–MS grade were purchased from Honeywell, USA. n-heptane (HPLC grade) was purchased from SD Fine Chemicals, India. Formic acid, 99% (LC–MS grade, Fischer Scientific) was purchased.

To optimize UAE, vitamin-free kelp powder (*Thallus laminariae*, SRM 3232, NIST) was used as a matrix. Drumstick leaves (*Moringa oleifera*), fenugreek leaves (*Trigonella foenum graecum*), ponnaganni (*Alternanthera sessilis*), and curry leaves (*Murraya koenigii*, Condiment) were procured from local farms in Kallikoppalu village (12.4277° N, 76.7207° E), Karnataka, India. The agronomic practises used to cultivate green leafy vegetables are summarized in Table 1. The matured leaf samples were collected in the month of August, 2021 between 6 and 8 a.m. from the test plants with mean recorded temperatures of 26.4 ± 0.9 °C.

Collected leaves were cleaned and freeze dried at − 48 °C for 24 h (ilShinBioBase Co. Ltd., Korea). Dried leaves were powdered prior to the extraction.

Table 1 Summary of the agronomic practices used prior to the harvest of test plants

Test plant	Soil	Frequency of irrigation	Harvesting age
Moringa oleifera	Well drained loamy soil	Once in every 10 days	12 months
Trigonella foenum graecum L	Well drained loamy soil	Once in every 4 days	24 days
Alternanthera sessilis	Well drained loamy soil	Once in every 2 days	90 days
Murraya koenigii	Well drained loamy soil	Once in a week	4 months

Table 2 Independent variables and their levels used for RSM-I-Optimal modeling

Variable	Units	Coded factors; X_i	I-Optimal design coded levels	
			−1	+1
Solvent level	mL/g sample	X_1	30	100
Sonication time	min	X_2	20	80
Sonication frequency	KHz	X_3	20	70
Sonication temperature	°C	X_4	20	70

Table 3 Experimental runs of RSM-I-optimal design and their respective responses

Run	X_1	X_2	X_3	X_4	Vitamin D_2 (Recovery, %)	Vitamin K_1 (Recovery, %)
1	67	20	41	41	71	65
2	68	56	70	40	94	94
3	100	80	57	57	80	80
4	100	35	20	57	70	66
5	30	42	52	52	49	52
6	30	80	70	70	60	58
7	30	42	52	52	52	50
8	30	42	52	52	51	49
9	67	20	41	41	73	69
10	68	56	70	40	96	95
11	100	20	70	70	70	66
12	86	80	20	20	51	52
13	67	56	40	70	66	66
14	100	35	57	20	88	88
15	67	56	40	70	82	81
16	100	80	20	63	65	67
17	34	80	20	55	27	23
18	100	80	61	20	82	82
19	64	78	42	41	70	72
20	34	80	55	20	36	33
21	30	80	20	20	35	34
22	93	35	20	20	65	68
23	30	20	20	70	25	17
24	35	37	20	20	27	20
25	30	20	70	20	40	38

Experimental design

The RSM-I-optimal design is used in the experimental design analysis (RSM) to optimize response variables related to quantitative impartial variables [32, 33]. A four factor (X_1, X_2, X_3, and X_4), two-level (− 1 and + 1) RSM-I-Optimal design was applied to obtain optimal conditions and identify correlation between extraction variables. Table 2 shows the experimental ranges of the selected variables. Twenty-five experimental runs from I-Optimal design were detailed in

Table 4 Analysis of variance (ANOVA) for determination of RSM-I-optimal design fitness

Parameter	Vitamin D_2 recovery (%)	Vitamin K_1 recovery (%)
Model	Quadratic	Quadratic
F-value	32.5	32.8
p-value	< 0.0001	< 0.0001
R^2	0.895	0.896
Adjusted R^2	0.867	0.868
Predicted R^2	0.813	0.817
Lack of fit		
F-value	2.55	3.19
p-value	0.154	0.103

Table 5 Regression coefficients (p-value ≤ 0.5) in terms of coded factors

Regression coefficient	Vitamin D_2 recovery (%)	Vitamin K_1 recovery (%)
X_0 (Intercept)	78.7	78.3
X_1	17.3	18.5
X_2	2.08	4.08
X_3	12.6	13.2
X_1^2	− 14.5	− 14.3
X_2^2	− 10.7	− 13.1

Table 3. The recovery of vitamins D_2 and K_1 was determined using a UPLC-Q-ToF/MS method. Each experiment was performed in triplicate, and the mean data were used to calculate the response, *Y*. Experimental data was statistically analyzed using Design Expert software, version 12 (Stat-Ease, Minneapolis, USA). Response surface plots examined how the independent variables interacted with one another and how those interactions affected the overall response. Two-way ANOVA was used to assess the model's suitability and the statistical significance of the regression coefficients (Tables 4 and 5). The quadratic regression equation for the independent and dependent variables (Table 6) is as follows:

$$Y_{AB} = a + bX_1 + cX_2 + dX_3 + eX_4 + fX_1^2 + gX_2^2 + hX_3^2 + iX_4^2 + jX_1X_2 + kX_1X_3 + lX_1X_4 + mX_2X_3 + nX_2X_4 + oX_3X_4 \quad (1)$$

where, Y_{AB} is the recovered (%) VD_2 and VK_1 content, and X_1, X_2, X_3, and X_4 are the selected independent variables that effect the extraction.

Ultrasonication-assisted extraction conditions

The ultrasonic extraction of VD_2 and VK_1 from kelp powder (SRM 3232) was carried out using an ultrasonic bath (Enertech Pvt. Ltd., India). Kelp powder (1 g) was spiked with D_2 (2.5 µg/g) and K_1 (2.5 µg/g) vitamins and gently mixed (Labomixer, Hosokawa, USA) for 5 min. The analyte spiked kelp powder was sonicated at 70 kHz frequency with 65 mL of 2-propanol and n-heptane (80:20%) for 45 min at 45 °C. The kelp extract was centrifuged at 5000 rpm for 10 min after extraction (Avanti, Beckman Coulter Inc., USA). Solvent was evaporated by concentrating the extract under vacuum at 40 °C to obtain dried residue (Rotavapor, BÜCHI Labortechnik AG, Switzerland). The dried residue was reconstituted with methanol and purified further using the previously described solid phase extraction (SPE) technique (Xu et al. 2020). SPE silica was activated using 6 mL of n-heptane and the extract was passed through the column at a flow rate of 1.0 mL/min. At a flow rate of 1.0 mL/min, the cartridge (Oasis HLB, Waters Co., USA) was rinsed with 6 mL of n-hexane. Finally, 8 mL of a 97:3 v/v n-heptane/diethyl ether solution was added, and the eluate was collected. Following nitrogen drying of the eluent, 1 mL of methanol was added, followed by vortexing. A 5 µL sample was injected into the LC–MS instrument for analysis after being filtered through a 0.22 µm hydrophilic polyvinylidene fluoride (PVDF) membrane filter (Merck KGaA, Darmstadt, Germany).

Table 6 Polynomial equation in terms of actual factors (p-value ≤ 0.5) calculated by RSM-I-optimal design and optimized UAE conditions for vitamins D_2 and K_1 extraction

Response	Second order polynomial	R^2	Optimized conditions				Estimated response (Mean recovery, %)	Observed response (Mean recovery, %)[a]
			X_1 (mL)	X_2 (min)	X_3 (KHz)	X_4 (°C)		
Vitamin D_2 recovery (%); Y_A	$-59.6 + 2.03X_1 + 1.26X_2 + 0.5X_3 - 0.01X_1^2 - 0.01X_2^2$	0.895	65	45	70	45	90.6	95.8 ± 5.3
Vitamin K_1 recovery (%); Y_B	$-72.1 + 2.04X_1 + 1.59X_2 + 0.52X_3 - 0.01X_1^2 - 0.01X_2^2$	0.896	65	45	70	45	90.4	95.2 ± 6.9

[a]The mean recovery was calculated using data from single injections of multiple extractions (n = 5) of a single sample

UPLC-Q-ToF/MS analysis and method validation

A UPLC-Q-ToF/MS system (Waters Co., Milford, MA, USA) equipped with an Accucore C18 column (50 × 4.6 mm, 2.6 µm; ThermoFisher Scientific, USA), auto-sampler, binary solvent manager, diode-array detector (DAD), thermostatted column compartment, degasser, electrospray combined ionization (ESCi) source as interface is capable of ionizing analytes in both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), quadrupole time of flight mass spectrometer (Q-ToF/MS) was used to simultaneously analyze VD_2 and VK_1. For the liquid chromatographic (LC) separation, the mobile phase was composed of 0.1% formic acid solution (A) and methanol (B), was delivered in gradient time program: 0 to 2 min (85 to 95%B), 2 to 3 min (95 to 100%B), 3.01 to 8 min (100%B), and 8.01 to 10 min (85%B) with a flow rate of 0.5 mL/min, injection volume of 5 µL, column temperature of 40 °C, and detection wavelength of 269 nm. Q-ToF/MS detection of analytes was carried out using the following instrument and acquisition parameters: 450 °C probe temperature; 25 to 60 V sampling cone voltage ramp; 120 °C source temperature; 100 V source offset voltage; 50 L/h cone gas (nitrogen) flow; 750 L/h desolvation gas (nitrogen) flow; the collision energy ramp ranges from 15 to 60 eV (Argon, collision gas); the sample infusion flow rate is 5 µL/min; 20 s dwell time (MS1 and MS2); 20 µs and 40 µs ramp time for MS1 and MS2, and a mass range of 50 to 1500 m/z. Mass Lynx software version 4.1 (Waters Co., Milford, MA, USA) was used to acquire and process the data.

To evaluate the linearity of the analytical method, calibration curves were plotted using 10 different concentrations (15.02, 22.5, 30, 75, 150, 300, 600, 900, 1200, and 1500 ng/mL) prepared from a stock solution (1 mg/mL) in methanol for each analyte. The LOD and LOQ values were calculated using the following equations to assess the method's sensitivity: LOD = 3.3 (calibration curve standard deviation/slope) and LOQ = 10 (calibration curve standard deviation/slope). VD_2 and VK_1 at three different concentrations (41.2, 712.5, and 1095 ng/mL) were analyzed with five replicates on the same day and twice a week for 3 weeks to determine intra-day precision and inter-day precision. Both precision values are given in terms of relative standard deviations (RSD, %).

The UPLC-Q-ToF/MS method for VD_2 and VK_1 analysis in spiked kelp powder SRM was validated in terms of detection and quantification limits (LOD and LOQ), linearity, inter- and intra-day precision, and accuracy.

Statistical analysis

The mean ± standard deviation (SD) of three measurements is used to express quantitative data (Microsoft Excel 2019, USA). The data was analyzed using Pearson correlation analysis ($p < 0.01$) and the Design-Expert software (Stat-Ease Inc., MN, USA) to determine the effect of the variables and how they interacted. The significant terms for the response model were determined using ANOVA. Non-significant terms ($p > 0.05$) were removed from the initial models using stepwise selection because they were not addressed by the ANOVA. The experimental data were then fixed to finalize the model, and response surface plots were used to determine the interactions between factors.

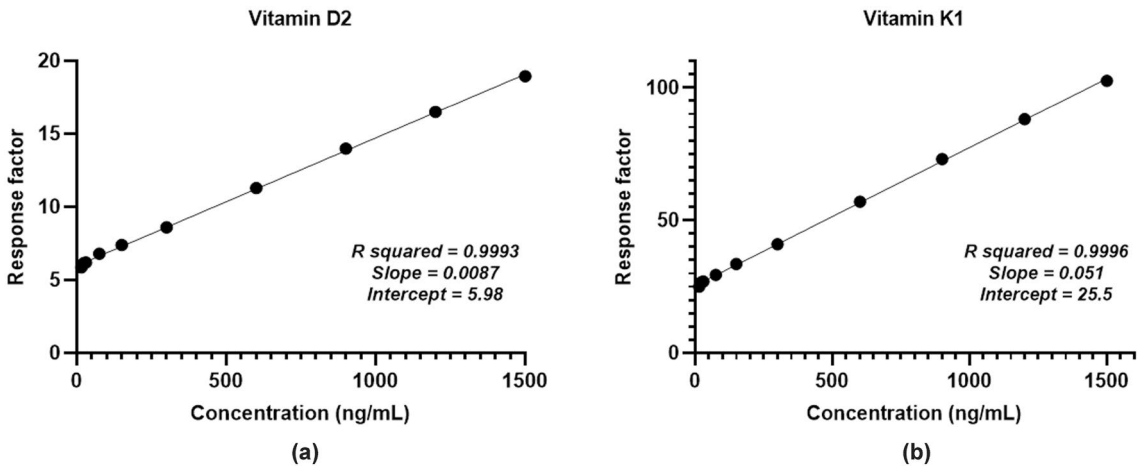

Fig. 1 Representative calibration curves of the analytes in the concentration range of 15–1500 ng/mL (**a**) Vitamin D_2 and (**b**) Vitamin K_1

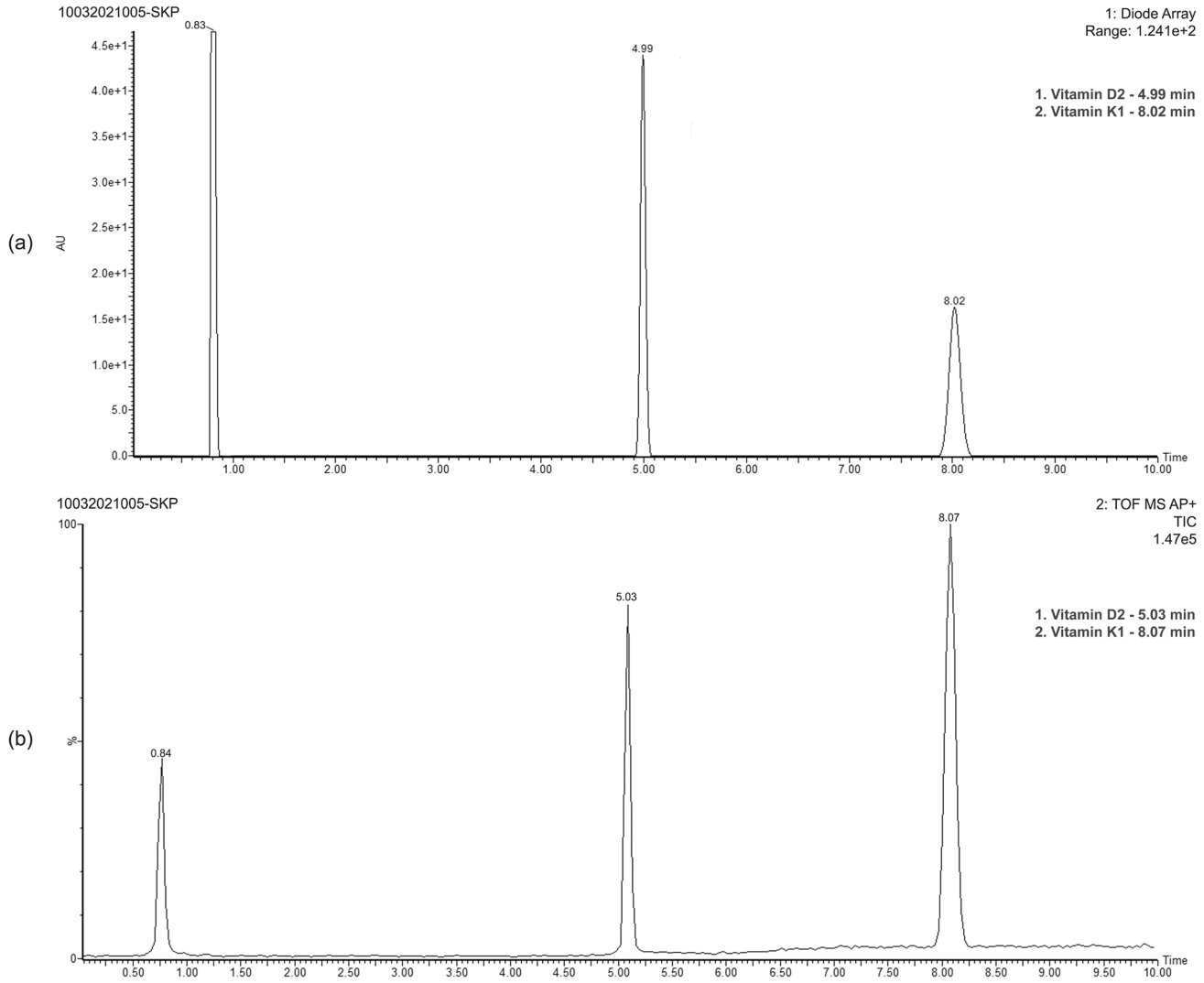

Fig. 2 Representative chromatograms of analytes (**a**) diode-array detection (DAD) and (**b**) total ion chromatogram (APCI+)

Results and discussion

Quantification of vitamins D_2 and K_1

The UPLC-Q-ToF/MS method for quantification of VD_2 and VK_1 was validated. Chromatograms of VD_2 and VK_1 in kelp powder SRM are shown in Fig. 1. Linearity was determined by performing triplicate analyses on ten samples with concentrations ranging from 15 to 1500 ng/mL, which corresponds to the range that could be detected in green leafy vegetable samples. The calibration curves (Fig. 1) revealed linear regression equations with good correlation coefficients ($r^2 = 0.9993$ and 0.9996, for VD_2 and VK_1). The LOD and LOQ were calculated to be 7 and 11 ng/mL for VD_2, 3 and 8 ng/mL for VK_1, respectively. The intra-day and inter-day variation was used to assess the precision and accuracy of the UPLC-Q-ToF/MS method. The relative standard deviation (RSD, %) was used to express the repeatability, which was determined from five consecutive samples spiked at three different concentration levels (41.3, 712 and 1095 ng/mL). The RSD values for inter-day variation ranged from 0.02 to 1.39%, while the RSD values for intra-day variation ranged from 0.01 to 1.15%. The inter-day recoveries ranged from 98.9 to 100.3%, while the intra-day recoveries ranged from 98.4 to 100.1%. The low RSD values indicate that the method is repeatable, implying that it has good precision and accuracy for VD_2 and VK_1 analysis. Analytes was clearly and effectively separated using a UPLC with a Accucore C18 column set at 40 °C and detected at 269 nm at RT = 4.99 min and 8.02 min for VD_2 and VK_1, respectively (Fig. 2a and b). Mass detection (APCI, + ve) confirmed analyte specificity, with m/z values of 397 and 451 for VD_2 and VK_1, respectively (Fig. 3a and b).

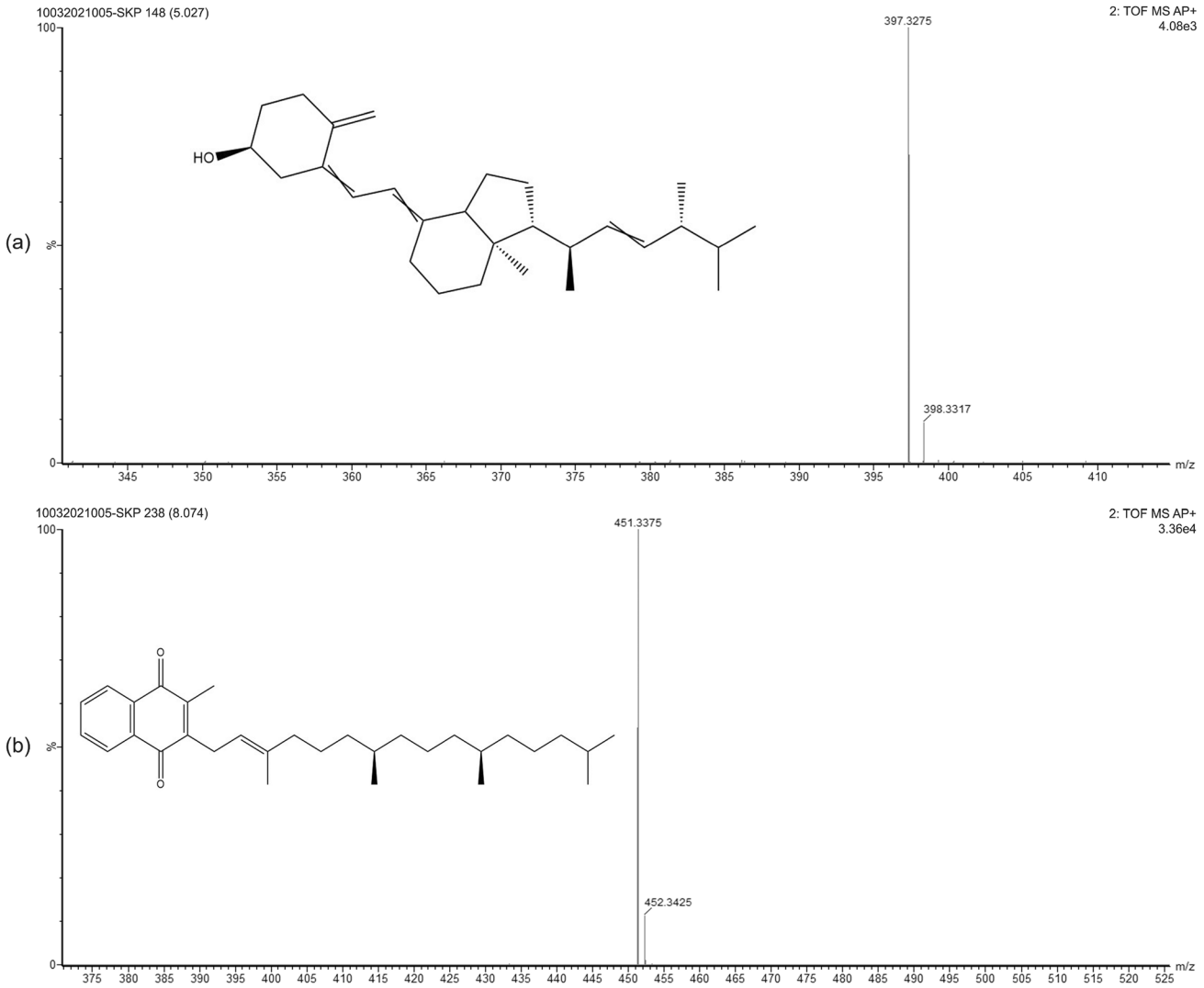

Fig. 3 Representative mass spectra of analytes (**a**) vitamin D_2 and (**b**) vitamin K_1

Vitamins D_2 and K_1 recovery using UAE and quantification

Organic solvent extraction using solvents such as hexane or acetone has generally been used for the extraction of fat-soluble vitamins due to its low cost and high yields, despite concerns about chemical contamination and hazardous working environments. With recent advancements in extraction technology, however, several aspects concerning labour safety and environmental contamination have been carefully considered. UAE is beneficial in increasing sample fluidity and allowing the target to be rapidly and completely transferred to the extractant [26, 27].

We attempted to find the most effective conditions for extracting VD_2 and VK_1 using UAE in this study by taking into account extracting solvents, temperature, ultrasonication frequency, and extraction time. The most commonly used extraction solvent for various food matrices has been a mixture of 2-propanol and n-hexane. [34–36]. In this study, n-hexane was substituted for the more environmentally friendly n-heptane. Because VK_1 is tightly bound to chloroplast membranes in plant cells, vortexing with a mechanical apparatus, sonification, or boiling are frequently used to achieve a more efficient extraction [34, 36]. UAE has recently been shown to efficiently recover fat-soluble vitamins in food matrix [26, 37]. According to Yueqing et al. (2020), the material-liquid ratio and ultrasonication—time, power, and temperature—had a significant influence on VK_1 recovery from fat-containing foods [37]. Liangxiao et al. (2019) discovered that ultrasonic temperature and power had a direct relationship with fat-soluble vitamin recovery, while extraction efficiency increased initially and then decreased as ultrasonic time increased [26]. Increased ultrasonication temperature and power improves matrix diffusion rates and

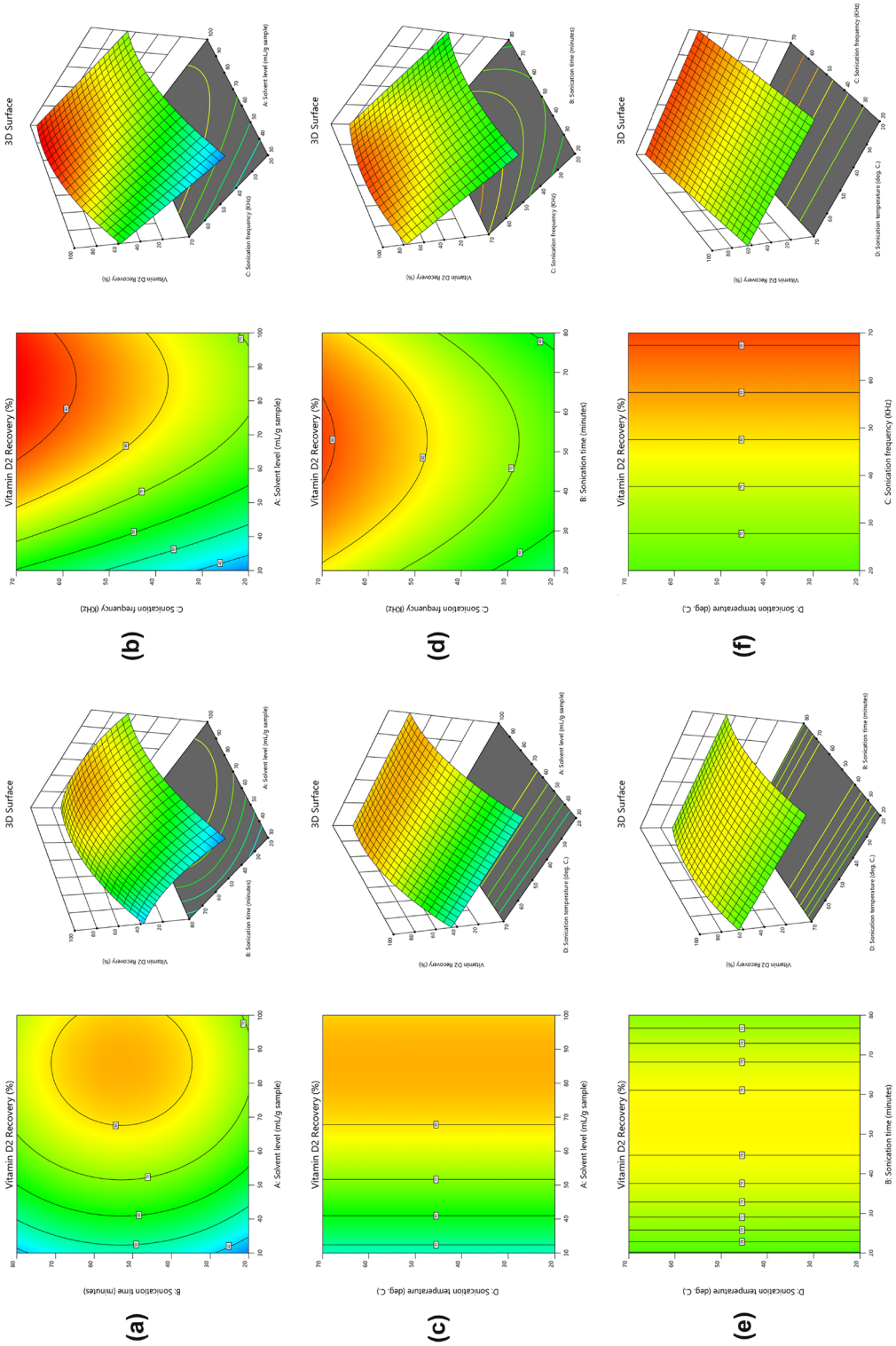

Fig. 4 Contour and response surface graphs of recovery (%) of vitamin D_2 (Y_1) versus **a** solvent level (mL) (X_1) and sonication time (min) (X_2), **b** solvent level (mL) (X_1) and sonication frequency (kHz) (X_3) (**c**) solvent level (mL) (X_1) and temperature (°C) (X_4), **d** sonication time (min) (X_2) and sonication frequency (kHz) (X_3), **e** sonication time (min) (X_2) and temperature (°C) (X_4) and **f** sonication frequency (kHz) (X_3) and temperature (°C) (X_4)

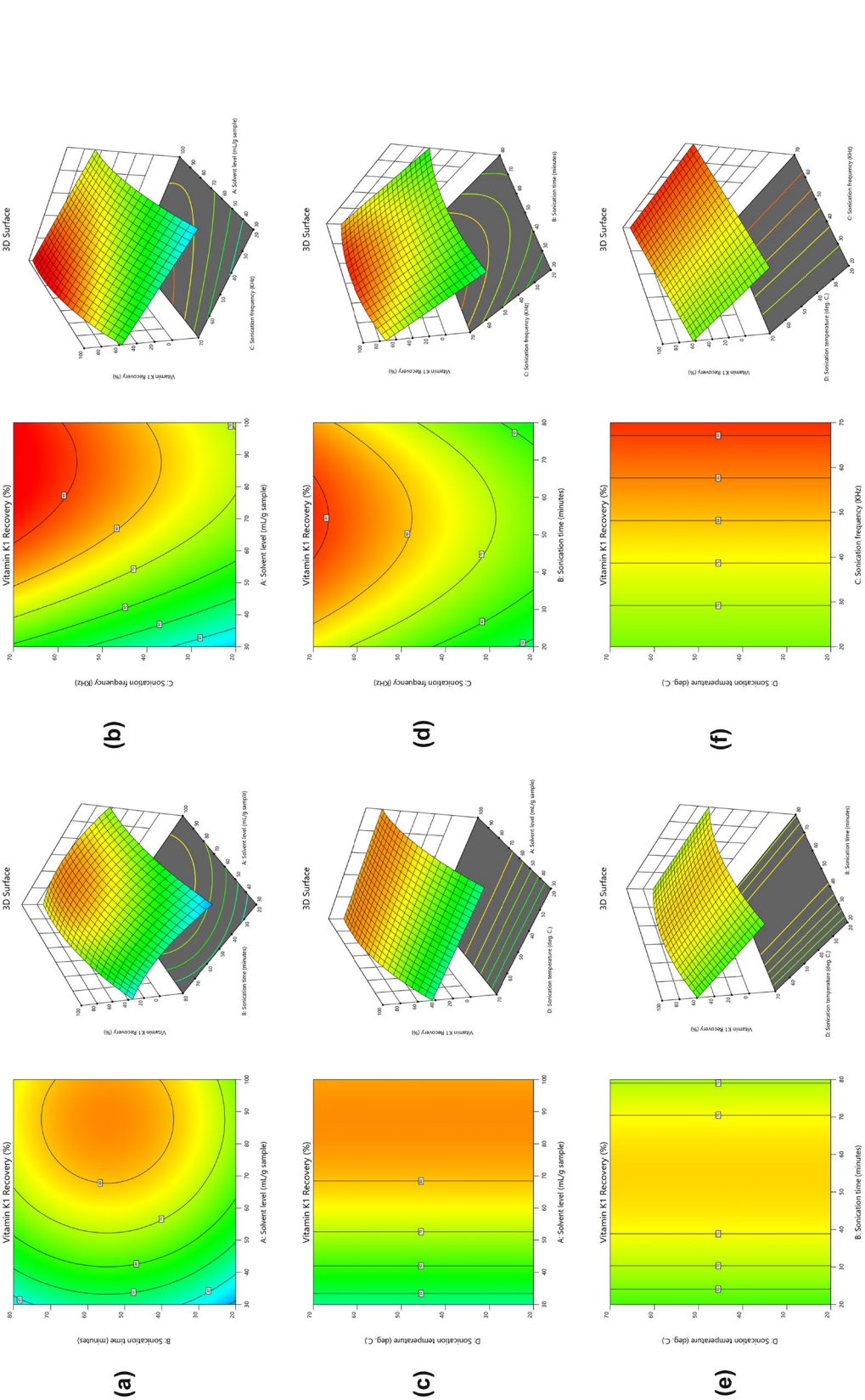

Fig. 5 Contour and response surface graphs of recovery (%) of vitamin K_1 (Y_2) versus **a** solvent level (mL) (X_1) and sonication time (min) (X_2) **b** solvent level (mL) (X_1) and sonication frequency (kHz) (X_3), **c** solvent level (mL) (X_1) and temperature (°C) (X_4), **d** sonication time (min) (X_2) and sonication frequency (kHz) (X_3), **e** sonication time (min) (X_2) and temperature (°C) (X_4), **f** sonication frequency (kHz) (X_3) and temperature (°C) (X_4)

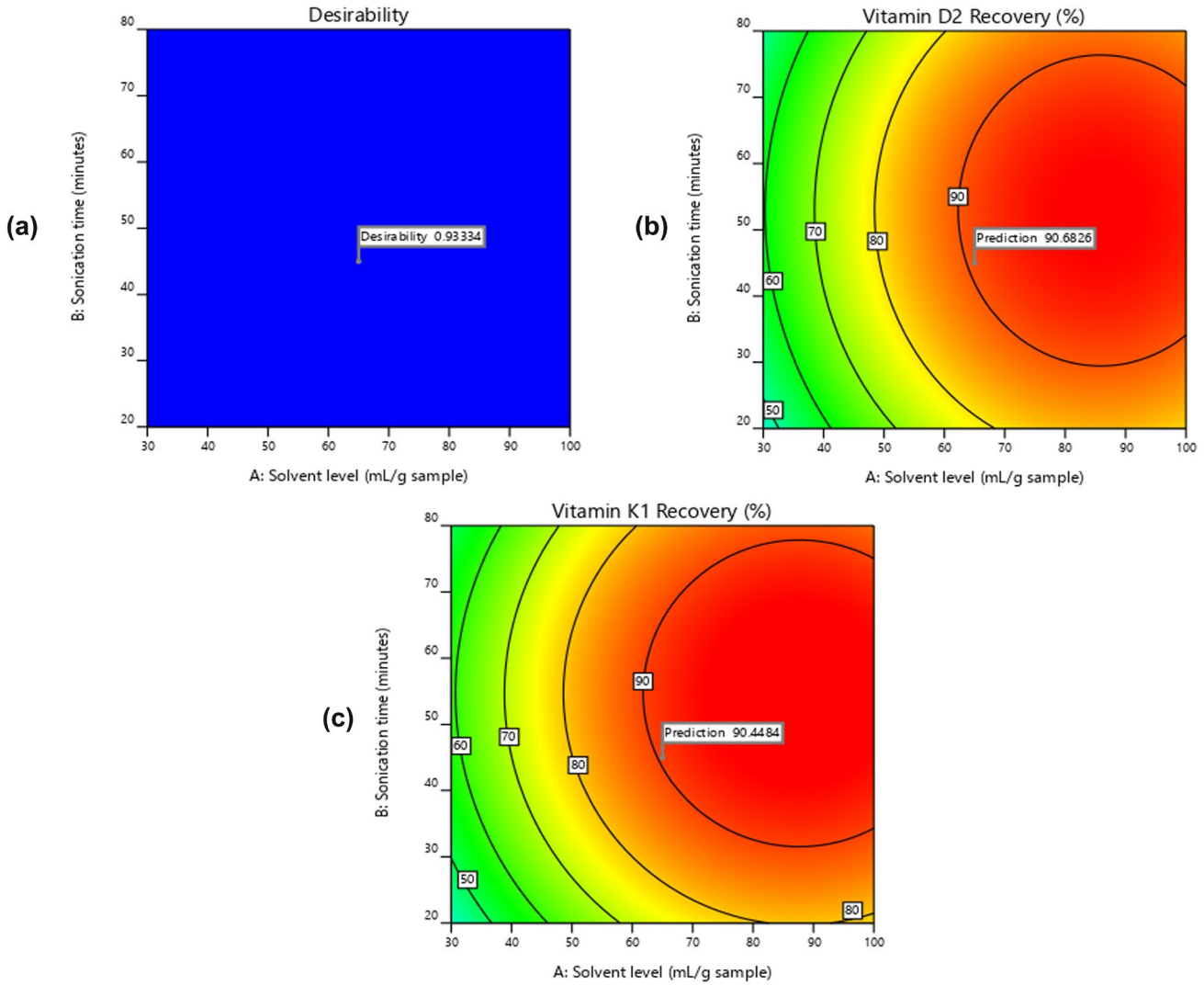

Fig. 6 Desirability graphs **a** desirability function, **b** recovery (%) of vitamin D_2 (Y_1), **c** recovery (%) of vitamin K_1 (Y_2)

extraction solvent solubility. The goal of this study is to optimize UAE parameters by using a mixture of 2-propanol and n-heptane (80:20, %) as a solvent to improve the recovery of VD_2 and VK_1 from green leafy matrix. SRM 3232 Kelp Powder (*Thallus laminariae*) was used to optimize the UAE because it was already certified for VK_1 content [38].

To extract VD_2 and VK_1 from kelp powder using UAE, the main factors were solvent level (mL), sonication time (min), sonication frequency (kHz) and temperature (°C), and the extractions were performed using an RSM-I-optimal design with a four-factor, two-level design (Table 2). Table 6 shows the best extraction conditions and analyte recoveries. Figures 4 and 5 depicts the variable response surface plots (surface and contour plots). The convex response surface suggested optimal variables. The extraction with the variable combination of solvent level: 68 (mL), sonication time: 56 min, sonication frequency: 70 kHz, and temperature: 40 °C resulted in high recovery of VD_2 (95%) and VK_1 (94%), whereas the extraction with the variable combination of solvent level: 30 (mL), sonication time: 20 min, sonication frequency: 20 kHz, and temperature: 70 °C resulted in low recovery of VD_2 (25%) and VK_1 (17%). The recovery (%) of VD_2 and VK_1 in the highest set was roughly four times that of the lowest set.

Optimization of vitamins D_2 and K_1 extraction using RSM

Multiple regression analysis and ANOVA were used to analyze twenty-five experimental data sets, as shown in Table 4. The extraction conditions for vitamins D_2 and K_1 recovery from kelp powder were optimized using RSM-I-Optimal

Table 7 Vitamins D_2 and K_1 content (µg/g) in the spiked SRM 3232 kelp powder and leaf samples (S)

Analyte	SRM 3232		S1	S2	S3	S4
	2.5 µg/g[a]	5 µg/g[b]	µg/g sample			
Vitamin D_2	2.39 ± 0.05	4.79 ± 0.11	0.16	0.27	< 0.01*	1.4 ± 0.04
Vitamin K_1	2.38 ± 0.07	4.74 ± 0.13	5.94 ± 0.14	5.25 ± 0.11	7.31 ± 0.1	3.24 ± 0.07

[a]Analyte spiked in SRM 3232 at a concentration of 2.5 µg/g

[b]Analyte spiked in SRM 3232 at a concentration of 5 µg/g

*Vitamin D_2 content was found to be less than the limit of quantification (11 ng/mL)

S1 = Drumstick leaves; S2 = Fenugreek leaves; S3 = Ponnaganni leaves; S4 = Curry leaves

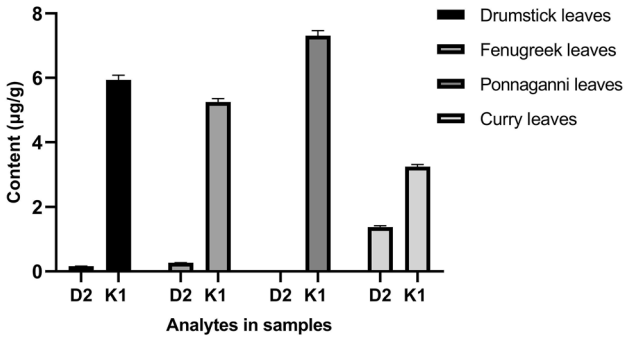

Fig. 7 Content of vitamin D_2 and K_1 in samples extracted using UAE and analyzed using UPLC-Q-ToF/MS

design, and quadratic regression equations were established (Table 6). To fit the second-order polynomial equation shown in Table 6, an ANOVA of the quadratic model's response surface was performed. The adjusted coefficient of determination (R^2) values for the VD_2 and VK_1 extraction model were 0.867 and 0.868, and the probability value ($p \leq 0.0001$) indicated that the regression model was highly significant.

Solvent level (X_1), sonication frequency (X_2) and the interactions: X_1^2 and X_2^2 are statistically significant variables ($p \leq 0.05$). Figure 3 depicts the contour and surface plots for the independent variables, which express the relationships between the VD_2 and the variables. The response surface plot for VD_2 recovery versus solvent level and sonication frequency, as shown in Fig. 4b, predicted a higher VD_2 recovery of 96.5% at a solvent level of 87 mL and a sonication frequency of 70 kHz. Furthermore, as shown in the response surface plots for VD_2 recovery versus sonication time and frequency, and VD_2 recovery versus sonication frequency and temperature (Figs. 4d and f), higher sonication frequency was a key contributor to achieve better recovery (Figs. 4b, d and f). Similar trends were seen for VK_1 recovery, with the best recovery of 97.5% obtained at a solvent level of 88 mL and a sonication frequency of 70 kHz, as shown in the response surface plots for VK_1 recovery versus solvent level, sonication time, sonication frequency, and temperature (Fig. 5a–f).

As a result, the response surface optimization for VD_2 and VK_1 recovery yielded desirable conditions (Fig. 6a–c) of solvent level = 65 mL, sonication time = 45 min, sonication frequency = 70 kHz, and temperature = 45 °C with a predicted recovery of 90.7% and 90.4% for VD_2 and VK_1.

Sample analysis

Selected samples of green leafy vegetables such as drumstick leaves, fenugreek leaves, ponnaganni leaves and condiments such as curry leaves were extracted using the optimized UAE conditions and purified using SPE. Purified samples were analyzed for the content (Table 7) of VD_2 and VK_1 using UPLC-Q-ToF/MS method (Fig. 7). According to the Indian Food Composition Tables (2017), the reported national wide (India) average content (µg/g) of VD_2 and VK_1 in the analyzed samples were: 0.14 and 4.79 in drumstick leaves, 0.24 and 4.28 in fenugreek leaves, 0.006 and 5.74 in ponnaganni leaves, 1.17 and 2.75 in curry leaves [13]. The difference in the contents of VD_2 and VK_1 in the current study and previously published values could be attributed to a variety of factors, including the geographical location of the farming site, the agronomical practices used in cultivation, harvesting conditions, the age of the test plants, the extraction and analytical procedures used, which could be confirmed by additional research in this area.

Conclusion

We demonstrated in this study that response surface methodology could be used to improve the quality of VD_2 and VK_1 extraction from green leafy matrix. Given that most conventional methods rely on liquid–liquid extraction and saponification principles, which require more solvent and pose a risk of analyte degradation, these findings are extremely significant. In the selected samples, we also discovered the best UAE conditions for VD_2 and VK_1 extraction. Traditionally, VD_2 and VK_1 were extracted from green leafy vegetables using organic non-polar solvents such as chloroform, n-hexane, petroleum ether, and others. This method was made

more environmentally friendly by substituting n-heptane for n-hexane. The use of optimal conditions obtained using the RSM-I-Optimal experimental design significantly reduced extraction time and improved VD_2 and VK_1 recovery from the matrix analyzed using UPLC-Q-ToF/MS.

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Declarations

Competing interests The authors state that they have no financial or non-financial interests.

Ethical approval Not applicable.

References

1. S. Omeed, K. Swapnil, G. Amandeep, B. Pankaj, G. Amy, *Vitamin D Deficiency* (StatPearls Publishing, Treasure Island, 2021)
2. A.J. van Ballegooijen, J.W.J. Beulens, L.M. Kieneker, M.H. de Borst, R.T. Gansevoort, I.P. Kema, L.J. Schurgers, M.G. Vervloet, S.J.L. Bakker, Eur. J. Nutr. **60**, 1645 (2021)
3. M.P.J. Visser, A.S.M. Dofferhoff, J.M.W. van den Ouweland, H. van Daal, C. Kramers, L.J. Schurgers, R. Janssen, J. Walk, Front. Nutr. **8**, 761191 (2022)
4. S. Acar and B. Özkan, in *Vitamin D*, edited by Ö. Özdemir (IntechOpen, 2021).
5. V.J. Jasinghe, C.O. Perera, Food Chem. **92**, 541 (2005)
6. V.J. Jasinghe, C.O. Perera, Food Chem. **95**, 638 (2006)
7. M. Kamao, Y. Suhara, N. Tsugawa, T. Okano, J. Chromatogr. B **816**, 41 (2005)
8. V.M. Brandenburg, L.J. Schurgers, N. Kaesler, K. Püsche, R.H. van Gorp, G. Leftheriotis, S. Reinartz, R. Koos, T. Krüger, Atherosclerosis **240**, 10 (2015)
9. X. Jinghe, WJCC **3**, 757 (2015)
10. J.K.D. Villa, M.A.N. Diaz, V.R. Pizziolo, H.S.D. Martino, Crit. Rev. Food Sci. Nutr. **57**, 3959 (2017)
11. I.J. Riphagen, J.C. van der Molen, M. van Faassen, G. Navis, M.H. de Borst, F.A.J. Muskiet, W.H.A. de Jong, S.J.L. Bakker, I.P. Kema, Clin. Chem. Lab. Med. (CCLM) (2016). https://doi.org/10.1515/cclm-2015-0864
12. W. Chen, Y. Guo, Exp Ther Med (2019).
13. T. Longvah, R. Ananthan, K. Bhaskarachary, and K. Venkaiah, *Indian Food Composition Tables* (National Institute of Nutrition, Hyderabad, 2017).
14. L.J. Schurgers, C. Vermeer, Pathophysiol. Haemos. Thromb. **30**, 298 (2000)
15. T. Aburjai, S. Al-Khalil, M. Abuirjeie, Phytochemistry **49**, 2497 (1998)
16. V. Justová, Z. Wildtová, V. Pacovský, J. Chromatogr. A **290**, 107 (1984)
17. J.L. Luquegarcia, M.D. Luquedecastro, J. Chromatogr. A **935**, 3 (2001)
18. S. Masuda, T. Okano, T. Kobayashi, Food Chem. **45**, 215 (1992)
19. J.P. Karl, X. Fu, G.G. Dolnikowski, E. Saltzman, S.L. Booth, J. Chromatogr. B **963**, 128 (2014)
20. J.G. Basset, S. Latimer, A. Fatihi, E. Soubeyrand, A. Block, Mini-Rev. Med. Chem. **17**, 1028 (2017)
21. A. Gentili, A. Miccheli, P. Tomai, M.E. Baldassarre, R. Curini, V. Pérez-Fernández, J. Food Compos. Anal. **47**, 21 (2016)
22. Y. Shao, L.F. Zhai, H.X. Wang, P.H. Chai, China Dairy Ind **44**, 57 (2016)
23. R.B. Jäpelt, J. Jakobsen, Food Chem. **192**, 402 (2016)
24. I. Boegh Andersen, C. Lohman Brasen, J. Skov Madsen, A. Schmedes, J. Chromatogr. B **1117**, 41 (2019)
25. M.-C. Hennion, J. Chromatogr. A **856**, 3 (1999)
26. L. Zhang, S. Wang, R. Yang, J. Mao, J. Jiang, X. Wang, W. Zhang, Q. Zhang, P. Li, Food Chem. **289**, 313 (2019)
27. D. Wang, L. Zhang, Y. Xu, X. Qi, X. Wang, X. Wang, Q. Zhang, P. Li, Antioxidants **8**, 321 (2019)
28. A.A. MahaboobAli, B. Momin, P. Ghogare, Prep. Biochem. Biotechnol. **50**, 445 (2020)
29. R. Prasad, R. Venugopal, L.A. Kumaraswamidhas, C. Pandey, S.K. Pan, Min. Metall. Explor. **37**, 1703 (2020)
30. A. Pattanaik, V. Rayasam, Adv. Powder Technol. **29**, 3404 (2018)
31. S. Balicki, I. Pawlaczyk-Graja, R. Gancarz, P. Capek, K.A. Wilk, ACS Omega **5**, 20854 (2020)
32. S. Palani, P. Lakshmanan, G. Kumanan, Mater. Today **46**, 1033 (2021)
33. M. Rajesh, K. Rajkumar, V.E. Annamalai, Mater. Manuf. Process. **36**, 329 (2021)
34. S.L. Booth, J.W. Suttie, J. Nutr. **128**, 785 (1998)
35. M.L. Dismore, D.B. Haytowitz, S.E. Gebhardt, J.W. Peterson, S.L. Booth, J. Am. Diet. Assoc. **103**, 1650 (2003)
36. T.J. Koivu-Tikkanen, V. Ollilainen, V.I. Piironen, J. Agric. Food Chem. **48**, 6325 (2000)
37. Y. Xu, L. Zhang, R. Yang, X. Yu, L. Yu, F. Ma, H. Li, X. Wang, P. Li, Molecules **25**, 839 (2020)
38. L.L. Yu, J.F. Browning, C.Q. Burdette, G.C. Caceres, K.D. Chieh, W.C. Davis, B.L. Kassim, S.E. Long, K.E. Murphy, R. Oflaz, R.L. Paul, K.E. Sharpless, L.J. Wood, J.H. Yen, R. Zeisler, Anal. Bioanal. Chem. **410**, 1265 (2018)