GEORGIAN MEDICAL NEWS

ISSN 1512-0112

NO 1 (334) Январь 2023

ТБИЛИСИ - NEW YORK

ЕЖЕМЕСЯЧНЫЙ НАУЧНЫЙ ЖУРНАЛ

Медицинские новости Грузии

საქართველოს სამედიცინო სიახლენი

GEORGIAN MEDICAL NEWS

Monthly Georgia-US joint scientific journal published both in electronic and paper formats of the Agency of Medical Information of the Georgian Association of Business Press. Published since 1994. Distributed in NIS, EU and USA.

GMN: Georgian Medical News is peer-reviewed, published monthly journal committed to promoting the science and art of medicine and the betterment of public health, published by the GMN Editorial Board since 1994. GMN carries original scientific articles on medicine, biology and pharmacy, which are of experimental, theoretical and practical character; publishes original research, reviews, commentaries, editorials, essays, medical news, and correspondence in English and Russian.

GMN is indexed in MEDLINE, SCOPUS, PubMed and VINITI Russian Academy of Sciences. The full text content is available through EBSCO databases.

GMN: Медицинские новости Грузии - ежемесячный рецензируемый научный журнал, издаётся Редакционной коллегией с 1994 года на русском и английском языках в целях поддержки медицинской науки и улучшения здравоохранения. В журнале публикуются оригинальные научные статьи в области медицины, биологии и фармации, статьи обзорного характера, научные сообщения, новости медицины и здравоохранения. Журнал индексируется в MEDLINE, отражён в базе данных SCOPUS, PubMed и ВИНИТИ РАН. Полнотекстовые статьи журнала доступны через БД EBSCO.

GMN: Georgian Medical News – საქართველოს სამედიცინო სიახლენი – არის ყოველთვიური სამეცნიერო სამედიცინო რეცენზირებადი ჟურნალი, გამოიცემა 1994 წლიდან, წარმოადგენს სარედაქციო კოლეგიისა და აშშ-ის მეცნიერების, განათლების, ინდუსტრიის, ხელოვნებისა და ბუნებისმეტყველების საერთაშორისო აკადემიის ერთობლივ გამოცემას. GMN-ში რუსულ და ინგლისურ ენებზე ქვეყნდება ექსპერიმენტული, თეორიული და პრაქტიკული ხასიათის ორიგინალური სამეცნიერო სტატიები მედიცინის, ბიოლოგიისა და ფარმაციის სფეროში, მიმოხილვითი ხასიათის სტატიები.

ჟურნალი ინდექსირებულია MEDLINE-ის საერთაშორისო სისტემაში, ასახულია SCOPUS-ის, PubMed-ის და ВИНИТИ РАН-ის მონაცემთა ბაზებში. სტატიების სრული ტექსტი ხელმისაწვდომია EBSCO-ს მონაცემთა ბაზებიდან.

WEBSITE

www.geomednews.com

К СВЕДЕНИЮ АВТОРОВ!

При направлении статьи в редакцию необходимо соблюдать следующие правила:

1. Статья должна быть представлена в двух экземплярах, на русском или английском языках, напечатанная через **полтора интервала на одной стороне стандартного листа с шириной левого поля в три сантиметра**. Используемый компьютерный шрифт для текста на русском и английском языках - **Times New Roman** (**Кириллица**), для текста на грузинском языке следует использовать **AcadNusx.** Размер шрифта - **12**. К рукописи, напечатанной на компьютере, должен быть приложен CD со статьей.

2. Размер статьи должен быть не менее десяти и не более двадцати страниц машинописи, включая указатель литературы и резюме на английском, русском и грузинском языках.

3. В статье должны быть освещены актуальность данного материала, методы и результаты исследования и их обсуждение.

При представлении в печать научных экспериментальных работ авторы должны указывать вид и количество экспериментальных животных, применявшиеся методы обезболивания и усыпления (в ходе острых опытов).

4. К статье должны быть приложены краткое (на полстраницы) резюме на английском, русском и грузинском языках (включающее следующие разделы: цель исследования, материал и методы, результаты и заключение) и список ключевых слов (key words).

5. Таблицы необходимо представлять в печатной форме. Фотокопии не принимаются. **Все цифровые, итоговые и процентные данные в таблицах должны соответствовать таковым в тексте статьи**. Таблицы и графики должны быть озаглавлены.

6. Фотографии должны быть контрастными, фотокопии с рентгенограмм - в позитивном изображении. Рисунки, чертежи и диаграммы следует озаглавить, пронумеровать и вставить в соответствующее место текста **в tiff формате**.

В подписях к микрофотографиям следует указывать степень увеличения через окуляр или объектив и метод окраски или импрегнации срезов.

7. Фамилии отечественных авторов приводятся в оригинальной транскрипции.

8. При оформлении и направлении статей в журнал МНГ просим авторов соблюдать правила, изложенные в «Единых требованиях к рукописям, представляемым в биомедицинские журналы», принятых Международным комитетом редакторов медицинских журналов - http://www.spinesurgery.ru/files/publish.pdf и http://www.nlm.nih.gov/bsd/uniform_requirements.html
В конце каждой оригинальной статьи приводится библиографический список. В список литературы включаются все материалы, на которые имеются ссылки в тексте. Список составляется в алфавитном порядке и нумеруется. Литературный источник приводится на языке оригинала. В списке литературы сначала приводятся работы, написанные знаками грузинского алфавита, затем кириллицей и латиницей. Ссылки на цитируемые работы в тексте статьи даются в квадратных скобках в виде номера, соответствующего номеру данной работы в списке литературы. Большинство цитированных источников должны быть за последние 5-7 лет.

9. Для получения права на публикацию статья должна иметь от руководителя работы или учреждения визу и сопроводительное отношение, написанные или напечатанные на бланке и заверенные подписью и печатью.

10. В конце статьи должны быть подписи всех авторов, полностью приведены их фамилии, имена и отчества, указаны служебный и домашний номера телефонов и адреса или иные координаты. Количество авторов (соавторов) не должно превышать пяти человек.

11. Редакция оставляет за собой право сокращать и исправлять статьи. Корректура авторам не высылается, вся работа и сверка проводится по авторскому оригиналу.

12. Недопустимо направление в редакцию работ, представленных к печати в иных издательствах или опубликованных в других изданиях.

При нарушении указанных правил статьи не рассматриваются.

REQUIREMENTS

Please note, materials submitted to the Editorial Office Staff are supposed to meet the following requirements:

1. Articles must be provided with a double copy, in English or Russian languages and typed or compu-ter-printed on a single side of standard typing paper, with the left margin of **3** centimeters width, and **1.5** spacing between the lines, typeface - **Times New Roman** (**Cyrillic**), print size - **12** (referring to Georgian and Russian materials). With computer-printed texts please enclose a CD carrying the same file titled with Latin symbols.

2. Size of the article, including index and resume in English, Russian and Georgian languages must be at least 10 pages and not exceed the limit of 20 pages of typed or computer-printed text.

3. Submitted material must include a coverage of a topical subject, research methods, results, and review.

Authors of the scientific-research works must indicate the number of experimental biological species drawn in, list the employed methods of anesthetization and soporific means used during acute tests.

4. Articles must have a short (half page) abstract in English, Russian and Georgian (including the following sections: aim of study, material and methods, results and conclusions) and a list of key words.

5. Tables must be presented in an original typed or computer-printed form, instead of a photocopied version. **Numbers, totals, percentile data on the tables must coincide with those in the texts of the articles.** Tables and graphs must be headed.

6. Photographs are required to be contrasted and must be submitted with doubles. Please number each photograph with a pencil on its back, indicate author's name, title of the article (short version), and mark out its top and bottom parts. Drawings must be accurate, drafts and diagrams drawn in Indian ink (or black ink). Photocopies of the X-ray photographs must be presented in a positive image in **tiff format**.

Accurately numbered subtitles for each illustration must be listed on a separate sheet of paper. In the subtitles for the microphotographs please indicate the ocular and objective lens magnification power, method of coloring or impregnation of the microscopic sections (preparations).

7. Please indicate last names, first and middle initials of the native authors, present names and initials of the foreign authors in the transcription of the original language, enclose in parenthesis corresponding number under which the author is listed in the reference materials.

8. Please follow guidance offered to authors by The International Committee of Medical Journal Editors guidance in its Uniform Requirements for Manuscripts Submitted to Biomedical Journals publication available online at: http://www.nlm.nih.gov/bsd/uniform_requirements.html
http://www.icmje.org/urm_full.pdf
In GMN style for each work cited in the text, a bibliographic reference is given, and this is located at the end of the article under the title "References". All references cited in the text must be listed. The list of references should be arranged alphabetically and then numbered. References are numbered in the text [numbers in square brackets] and in the reference list and numbers are repeated throughout the text as needed. The bibliographic description is given in the language of publication (citations in Georgian script are followed by Cyrillic and Latin).

9. To obtain the rights of publication articles must be accompanied by a visa from the project instructor or the establishment, where the work has been performed, and a reference letter, both written or typed on a special signed form, certified by a stamp or a seal.

10. Articles must be signed by all of the authors at the end, and they must be provided with a list of full names, office and home phone numbers and addresses or other non-office locations where the authors could be reached. The number of the authors (co-authors) must not exceed the limit of 5 people.

11. Editorial Staff reserves the rights to cut down in size and correct the articles. Proof-sheets are not sent out to the authors. The entire editorial and collation work is performed according to the author's original text.

12. Sending in the works that have already been assigned to the press by other Editorial Staffs or have been printed by other publishers is not permissible.

Articles that Fail to Meet the Aforementioned Requirements are not Assigned to be Reviewed.

ავტორთა საყურადღებოდ!

რედაქციაში სტატიის წარმოდგენისას საჭიროა დავიცვათ შემდეგი წესები:

1. სტატია უნდა წარმოადგინოთ 2 ცალად, რუსულ ან ინგლისურ ენებზე,დაბეჭდილი სტანდარტული ფურცლის 1 გვერდზე, 3 სმ სიგანის მარცხენა ველისა და სტრიქონებს შორის 1,5 ინტერვალის დაცვით. გამოყენებული კომპიუტერული შრიფტი რუსულ და ინგლისურენოვან ტექსტებში - **Times New Roman (Кириллица)**, ხოლო ქართულენოვან ტექსტში საჭიროა გამოვიყენოთ **AcadNusx.** შრიფტის ზომა – 12. სტატიას თან უნდა ახლდეს CD სტატიით.

2. სტატიის მოცულობა არ უნდა შეადგენდეს 10 გვერდზე ნაკლებს და 20 გვერდზე მეტს ლიტერატურის სიის და რეზიუმეების (ინგლისურ, რუსულ და ქართულ ენებზე) ჩათვლით.

3. სტატიაში საჭიროა გაშუქდეს: საკითხის აქტუალობა; კვლევის მიზანი; საკვლევი მასალა და გამოყენებული მეთოდები; მიღებული შედეგები და მათი განსჯა. ექსპერიმენტული ხასიათის სტატიების წარმოდგენისას ავტორებმა უნდა მიუთითონ საექსპერიმენტო ცხოველების სახეობა და რაოდენობა; გაუტკივარებისა და დაძინების მეთოდები (მწვავე ცდების პირობებში).

4. სტატიას თან უნდა ახლდეს რეზიუმე ინგლისურ, რუსულ და ქართულ ენებზე არანაკლებ ნახევარი გვერდის მოცულობისა (სათაურის, ავტორების, დაწესებულების მითითებით და უნდა შეიცავდეს შემდეგ განყოფილებებს: მიზანი, მასალა და მეთოდები, შედეგები და დასკვნები; ტექსტუალური ნაწილი არ უნდა იყოს 15 სტრიქონზე ნაკლები) და საკვანძო სიტყვების ჩამონათვალი (key words).

5. ცხრილები საჭიროა წარმოადგინოთ ნაბეჭდი სახით. ყველა ციფრული, შემაჯამებელი და პროცენტული მონაცემები უნდა შეესაბამებოდეს ტექსტში მოყვანილს.

6. ფოტოსურათები უნდა იყოს კონტრასტული; სურათები, ნახაზები, დიაგრამები - დასათაურებული, დანომრილი და სათანადო ადგილას ჩასმული. რენტგენოგრამების ფოტოასლები წარმოადგინეთ პოზიტიური გამოსახულებით **tiff** ფორმატში. მიკროფოტოსურათების წარწერებში საჭიროა მიუთითოთ ოკულარის ან ობიექტივის საშუალებით გადიდების ხარისხი, ანათალების შეღებვის ან იმპრეგნაციის მეთოდი და აღნიშნოთ სურათის ზედა და ქვედა ნაწილები.

7. სამამულო ავტორების გვარები სტატიაში აღინიშნება ინიციალების თანდართვით, უცხოურისა – უცხოური ტრანსკრიპციით.

8. სტატიას თან უნდა ახლდეს ავტორის მიერ გამოყენებული სამამულო და უცხოური შრომების ბიბლიოგრაფიული სია (ბოლო 5-8 წლის სიღრმით). ანბანური წყობით წარმოდგენილ ბიბლიოგრაფიულ სიაში მიუთითეთ ჯერ სამამულო, შემდეგ უცხოელი ავტორები (გვარი, ინიციალები, სტატიის სათაური, ჟურნალის დასახელება, გამოცემის ადგილი, წელი, ჟურნალის №, პირველი და ბოლო გვერდები). მონოგრაფიის შემთხვევაში მიუთითეთ გამოცემის წელი, ადგილი და გვერდების საერთო რაოდენობა. ტექსტში კვადრატულ ფჩხილებში უნდა მიუთითოთ ავტორის შესაბამისი N ლიტერატურის სიის მიხედვით. მიზანშეწონილია, რომ ციტირებული წყაროების უმეტესი ნაწილი იყოს 5-6 წლის სიღრმის.

9. სტატიას თან უნდა ახლდეს: ა) დაწესებულების ან სამეცნიერო ხელმძღვანელის წარდგინება, დამოწმებული ხელმოწერითა და ბეჭდით; ბ) დარგის სპეციალისტის დამოწმებული რეცენზია, რომელშიც მითითებული იქნება საკითხის აქტუალობა, მასალის საკმაობა, მეთოდის სანდოობა, შედეგების სამეცნიერო-პრაქტიკული მნიშვნელობა.

10. სტატიის ბოლოს საჭიროა ყველა ავტორის ხელმოწერა, რომელთა რაოდენობა არ უნდა აღემატებოდეს 5-ს.

11. რედაქცია იტოვებს უფლებას შეასწოროს სტატია. ტექსტზე მუშაობა და შეჯერება ხდება საავტორო ორიგინალის მიხედვით.

12. დაუშვებელია რედაქციაში ისეთი სტატიის წარდგენა, რომელიც დასაბეჭდად წარდგენილი იყო სხვა რედაქციაში ან გამოქვეყნებული იყო სხვა გამოცემებში.

აღნიშნული წესების დარღვევის შემთხვევაში სტატიები არ განიხილება.

GEORGIAN MEDICAL NEWS
No 1 (334) 2023

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Abstract.

In this study, the liquid chromatographic (LC) parameters were optimized using response surface methodology (RSM) as a novel approach to achieve optimal separation of six vitamers of vitamin D and K during simultaneous estimation. Analytes were separated using an Accucore C18 column (50 x 4.6 mm, 2.6 µm), 0.1% aqueous formic acid (pH = 3.5), and methanol as mobile phase components. The Box-Behnken design (BBD) predicted the best combination of the selected critical quality attributes such as organic solvent composition in the mobile phase (90%), mobile phase flow rate (0.42 mL/min), and column oven temperature (40°C). Multiple regression analysis was used to fit the experimental data from 17 sample runs to a second-order polynomial equation. The adjusted coefficient of determination (R^2) for three desired responses were 0.983 (retention time of K_3 = R_1), 0.988 (resolution between D_2 and D_3 = R_2), and 0.992 (retention time of K_2-7 = R_3), all with significant probability values ($p<0.0001$), indicating a high significance for the regression model. Q-ToF/MS detection was interfaced with an electrospray combined ionization source. The optimized detection parameters delivered specific, sensitive, linear, accurate, precise, and robust quantification of all six analytes in the tablet dosage form.

Key words. Box-Behnken design, fat-soluble vitamins, optimization, response surface methodology, UPLC-Q-ToF/MS, validation.

Introduction.

Vitamins D and K (VDK) are essential micronutrients for calcium homeostasis. These vitamin deficiencies have been linked to chronic illnesses such as osteoporosis, cardiovascular disease, cancer, diabetes, autoimmune diseases, and depression [1]. VDK works synergistically in maintaining calcium homeostasis by absorbing calcium from the intestine and carboxylating matrix Gla protein (MGP) at the vascular wall and osteocalcin (OC, bone Gla protein) at the bone [2,3]. Inadequate VDK levels in the blood cause poor carboxylation of MGP and OC, which has been linked to negative outcomes such as cardiovascular disease, low bone mineral density, and osteoporosis [3]. Combining VDK in the form of supplements may be more effective for bone and cardiovascular health than taking either vitamin alone, according to genetic, molecular, cellular, and human research [4-7].

Analytical techniques such as HPLC-PDA [8,9], HPLC-FD [10], and LC-MS [11] for determining VDK in vitamin supplements have been reported. Accurate analysis of these vitamins in pharmaceutical supplements is critical for quality control [12]. Previously published analytical techniques detailed VDK quantification alone [9-11, 13] and also in combination with other vitamins [8,14]. A review of the literature using the WOS data base revealed that none of the LC-MS techniques were successful in achieving a clear baseline separation of ergocalciferol (D_2) and cholecalciferol (D_3) during simultaneous estimation due to their structural similarity [15]. Furthermore, due to the wide variation in the partition coefficient (*log P*) values of the selected vitamers, developing a simultaneous method, as well as ensuring resolution between D_2 and D_3, was difficult. The selection of an appropriate organic phase composition is critical in order to retain menadione (K_3), which is the least non-polar of the selected vitamers. Menaquinone-7 (K_2-7), on the other hand, is highly non-polar, and the method must minimize the retention time to avoid longer runs and higher solvent consumption. In our study, liquid chromatographic (LC) separation of the selected vitamers should be optimized to ensure analyte retention, resolved peaks, and the shortest possible runtime for simultaneous analysis.

Traditionally, optimization of a chromatographic technique looks at one factor at a time (OFAT) while leaving the others constant. As a result of OFAT, a large number of experiments are carried out without identifying the key parameters. Mathematically designed experiments are often used in studying interaction among factors and predicts outcomes within few experimental runs to improve the analytical quality (Analytical quality-by-design, AQbD) of a technique [16-19]. The most widely used technique for multivariate statistical methods is response surface methodology (RSM). RSM is a statistical and mathematical system that is based on data compatibility with polynomial models and should reveal the behaviour of all data with the goal of establishing a mathematical model for predictions [20,21]. RSM is applied and analysed using a variety of techniques, including experimental strategies, mathematical methods, and statistical inference. By examining the various variables and their effects, the RSM focuses on the optimal condition in food and pharmaceutical research [22-24]. Until now, no reported method has used RSM to optimize analytical parameters for fat-soluble vitamin analysis.

The current study was aimed to optimize LC conditions in order to achieve optimal separation of vitamins K_3, D_2, D_3, K_2-4, K_1, and K_2-7 while using RSM. To date, no studies were reported on the simultaneous estimation of the selected six fat-soluble

vitamins using RSM to determine optimum LC conditions with independent variables such as organic phase composition in mobile phase, flow rate, and column oven temperature have been conducted. Since LC-MS provides sensitive, selective, and selective determination, analytes were separated using UPLC and detected using Q-ToF/MS.

Experimental.

Chemicals and reagents: Analytical reference standards of ergocalciferol (D_2, 99.6%), cholecalciferol (D_3, 99.9%), menadione (K_3, 99.8%), phylloquinone (K_1, 99.6%), menaquinone-4 (K_2-4, 99.9%), and menaquinone-7 (K_2-7, 100%, USP Reference standard) were purchased from Sigma-Aldrich, India. The analyte structures are depicted in figure 1. Acetonitrile, methanol, and 2-propanol of LC-MS grade were purchased from Honeywell, USA. n-hexane, chloroform, and tetrahydrofuran of HPLC grade were purchased from SD Fine Chemicals, India. Formic acid, glacial acetic acid, and ammonium acetate of high purity were purchased from ThermoFischer Scientific, India. Mychiro (USV Private limited, Mumbai) tablets were used to establish the analyte recovery.

Stock solutions, calibration standards and quality control samples: Primary stock solutions (1 mg/mL) of D_2, D_3, K_2-4, K_2-7, K_3 and K_1 were prepared in methanol (diluent). Working standard stock solutions of 100 µg/mL, 10 µg/mL, 1 µg/mL, and 200 ng/mL were prepared by diluting appropriate volumes of primary stock solutions with methanol as a diluent. Calibration standards of 15.02, 22.5, 30.03, 75, 150, 300, 600, 900, 1200, and 1500 ng/mL were prepared. Quality control samples (QCs) with concentrations of 41.2 (Low-QC), 712.5 (Medium-QC),

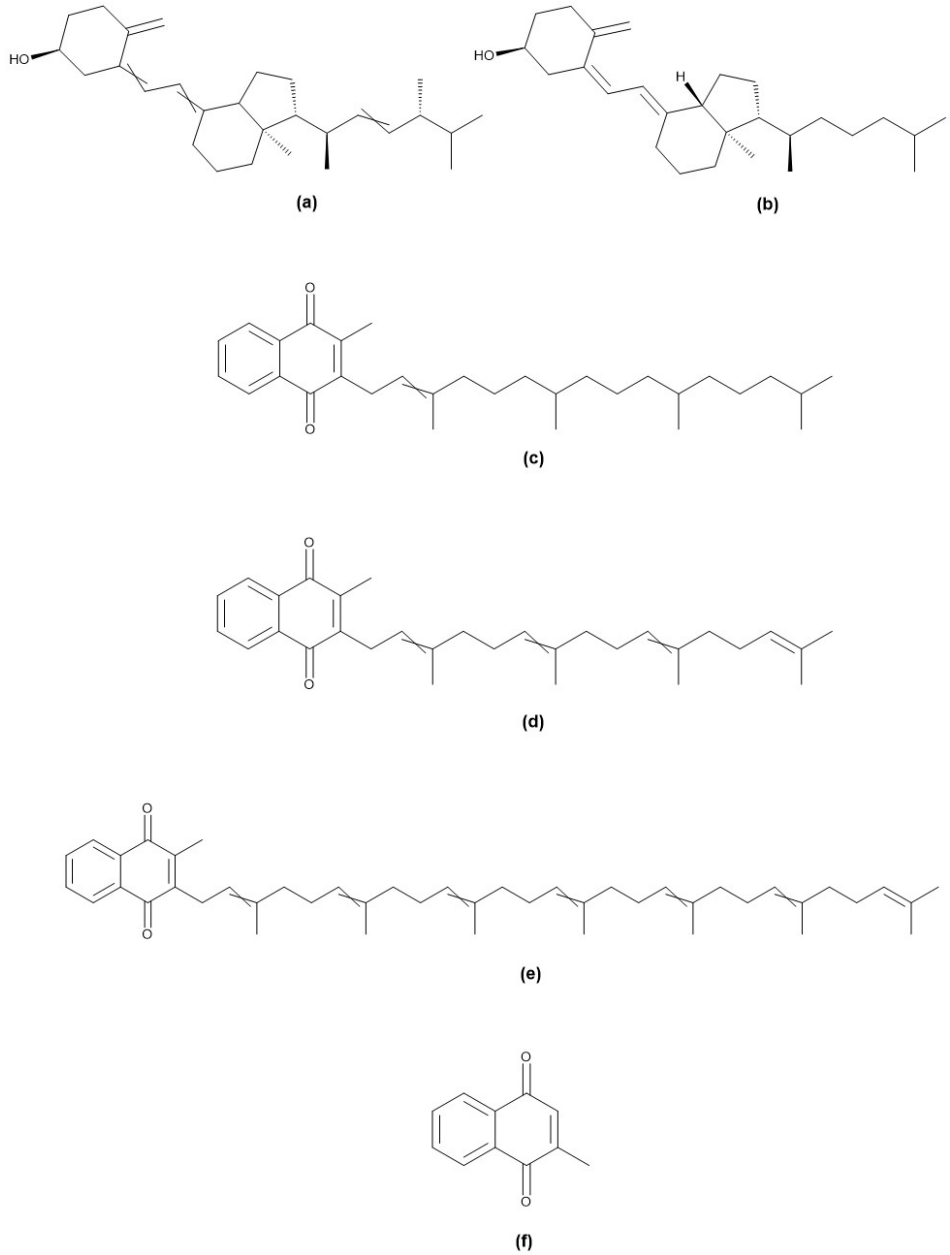

Figure 1. *Structures of fat-soluble vitamins (a) ergocalciferol (D_2), (b) cholecalciferol (D_3), (c) phylloquinone (K_1), (d) menaquinone-4 (K_2-4), (e) menaquinone-7 (K_2-7), (f) menadione (K_3).*

and 1095 (High-QC) ng/mL were prepared from appropriate standard stock solutions.

Primary stock solutions were prepared on weekly basis. Working standard stock solutions and QCs were freshly prepared prior to analysis. Stock solutions were stored under refrigeration at -20 °C. All samples were handled in amber borosilicate glassware of type A. To handle solvents and samples during dilutions, single channel micropipettes with capacities ranging from 0.01 to 10 mL were used (Transferpette S, Brand Scientific Equipment Pvt. Ltd, Wertheim, Germany).

Sample preparation: Placebo tablets were powdered (constituents: Myo-Inositol, D-Chiro-Inositol, and excipients) and 1 g of the powdered sample was spiked with six-fat soluble vitamins at two analyte concentration levels, 50 ng/g and 250 ng/g. Spiked sample was subjected to ultrasonication assisted solvent extraction. 30 mL of 2-propanol and n-hexane (65:35%, v/v) and 0.3 mL formic acid were added to the sample and sonicated at 60 KHz for 15 minutes while maintaining the temperature at 50 °C. The extract was brought to room temperature and centrifuged at 9000 rpm for 3 minutes. Supernatant was collected and centrifugation was repeated twice. The collected supernatant was subjected to solvent evaporation under gentle stream of nitrogen gas. The dried residue was reconstituted with 2 mL methanol. All samples were filtered using 0.45 µ polytetrafluoroethylene (PTFE) hydrophilic membrane filters prior to the analysis.

Ultraperformance liquid chromatography (UPLC): Acquity H-class UPLC system (Waters Corporation, Milford, MA, USA) equipped with an integrated vacuum degasser, binary solvent manager, thermos tatted column compartment, sample manager with autosampler injector was utilized to perform the LC separation. An Accucore C18 column (4.6 x 50 mm, 2.6 µm) was used as stationary phase. The mobile phase was composed of 0.1% formic acid in water (v/v, pH=3.5, A) and methanol (100%, B), and delivered in the following gradient time program: 0 to 2 minutes (90%, B), 2 to 3 minutes (90 to 100%, B), 3.01 to 17 minutes (100%, B), and 17.01 to 20 minutes (90%, B) at a flow rate of 0.5 mL/min. The sample injection volume was 5 µL. LC separation was monitored using diode array detection (DAD) at 269 nm.

Quadrupole time-of-flight mass spectrometry (Q-ToF/MS): Xevo G2-XS Q-ToF mass spectrometer (Waters Corporation, Wilmslow, UK) was used for mass spectrometric (MS) detection. The mass spectrometer was equipped with an electrospray combined ionization source (ESCi) capable of performing electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) simultaneously within a single run, which could be useful in determining analyte ionization efficiency within a single run when operated at a given polarity, i.e., positive, or negative. 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/hr cone gas (nitrogen) flow; 750 L/hr 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 µs 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.

Experimental design.

Preliminary trails: The trial-and-error method was used in preliminary trials to learn about the LC method's performance and to identify various significant independent parameters and their influence on dependent variables. The UPLC separation was focused on analyte peaks that were well retained and resolved. As a result, C18 column as a stationary phase, methanol as an organic modifier, and aqueous phase with 0.1%

Table 1. *Experimental runs of Box-Behnken design with their respective responses.*

Run	Methanol (%)	Flow rate (mL/min)	Column oven temperature (°C)	Retention time of K_3 (minutes)	Resolution between D_2 and D_3	Retention time of K_2-7 (minutes)
	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	90	0.5	32.5	0.96	1.88	22
2	90	0.5	32.5	0.98	1.83	21.7
3	100	0.5	25	0.83	1.1	14.6
4	100	0.7	32.5	0.83	0.92	13.8
5	100	0.5	40	0.83	1.05	14.7
6	90	0.7	40	0.86	1.46	18.8
7	80	0.5	40	1.2	2.1	30
8	90	0.5	32.5	0.95	1.84	22
9	80	0.5	25	1.18	2.21	29.2
10	90	0.3	25	1.15	2	26.3
11	90	0.5	32.5	0.94	1.82	21.8
12	80	0.3	32.5	1.5	2.6	33.6
13	90	0.7	25	0.83	1.52	19.5
14	80	0.7	32.5	1	2	25
15	90	0.3	40	1.1	1.94	25.2
16	100	0.3	32.5	0.83	1.2	16.8
17	90	0.5	32.5	0.93	1.83	22

formic acid (pH=3) as an additive were found to be significant for LC separation.

Box-Behnken design (BBD): RSM-BBD was used to further optimize the method's experimental conditions statistically due to its high efficiency and ability to reduce the number of trial-and-error experimental runs required. The independent factors chose for further optimization using BBD at three levels (-1, 0, +1) were: X_1 = initial (0 to 2 minutes) composition of methanol (%), X_2 = flow rate (mL/min), and X_3 = column oven temperature (°C). A sample set of 17 experimental runs (Table 1) were suggested by the Design Expert software with each run analyzed in triplicate to record the mean values of the desired responses: Y_1 = retention time of K_3 (minutes), Y_2 = resolution between D_2 and D_3, and Y_3 = retention time of K_2-7 (minutes). Experimental data was statistically analyzed using Design Expert software, version 12 (Stat-Ease, Minneapolis, USA). Response surface plots (Figures 2, 3 and 4) were used to examine how the independent variables interacted with one another and how those interactions affected the overall response. Two-way analysis of variance (ANOVA) was used to assess the model's suitability and the statistical significance of the regression coefficients (Tables 2 and 3). The quadratic regression equation for the independent and dependent variables is as follows: $Y_{1\text{-}3}=a+bX_1+cX_2+dX_3+eX_1^2+fX_2^2+gX_3^2+hX_1X_2+iX_1X_3+jX_2X_3$, where, $Y_{1\text{-}3}$ represents the responses, and X_1, X_2, and X_3 are the selected independent variables that effect the LC separation.

Method validation.

The developed UPLC-Q-ToF/MS method was validated to ensure its operational qualification by evaluating the parameters such as system suitability, linearity and range, limit of detection, limit of quantification, accuracy, precision, and robustness in accordance with USFDA's guidance [25,26].

System suitability: Six replicate injections of 1 µg/mL standard solution (all six analytes) were injected into the UPLC-DAD to assess system suitability. The outcomes of system suitability parameters on capacity factor (k'), injection repeatability, resolution (Rs), tailing factor (T), and theoretical plate number (N) were investigated by detecting the analyte retention at 269 nm (Table 4). The DAD at 269 nm was limited to system suitability evaluation.

To evaluate the remaining validation parameters, the detection was switched to Q-ToF/MS.

Sensitivity: The mean peak intensity units (n=3) obtained from the respective analytes' total ion chromatograms (TICs) were compared to the noise level in the preceding blank injection across the retention range of the analytes. The limit of detection (LOD) and limit of quantitation (LOQ) are determined using signal-to-noise (S/N) levels of 3:1 and 10:1 (Table 4).

Linearity: The method's linearity was tested at ten non-zero concentration points. Calibration standards of each analyte were prepared in the range of 15 to 1500 ng/mL. The calibration

Table 2. *Fitness summary of Box-Behnken design.*

Parameter	**Retention time of K_3 (minutes)**	**Resolution between D_2 and D_3**	**Retention time of K_2-7 (minutes)**
	Y_1	Y_2	Y_3
Model	Quadratic	Quadratic	Quadratic
F-value	104	159	222
p-value	<0.0001	<0.0001	<0.0001
R^2	0.992	0.995	0.996
Adjusted R^2	0.983	0.988	0.992
Predicted R^2	0.922	0.932	0.946
Lack of fit			
F-value	2.21	2.86	3.48
p-value	0.23	0.34	0.46

Table 3. *Regression coefficients (p-value = <0.5) in terms of coded factors.*

Regression coefficient	**Retention time of K_3 (minutes)**	**Resolution between D_2 and D_3**	**Retention time of K_2-7 (minutes)**
	Y_1	Y_2	Y_3
X_0	10.6	-2.75	129
X_1	-0.15	0.163	-1.06
X_2	-7.51	-4.19	-89.7
X_3	-0.002	0.078	-0.055
$X_1 * X_2$	0.062	0.04	0.7
$X_1 * X_3$	-0	0	-0.002
$X_2 * X_3$	0.013	0	0.066
X_1^2	0	-0.001	0
X_2^2	0.787	-0.562	9.06
X_3^2	0	-0.001	0.003

X_0 = Intercept; X_1 = Methanol (%); X_2 = Flow rate (mL/min); X_3 = Column oven temperature (oC); Y_1 = Retention time of K_3 (minutes); Y_2 = Resolution between D_2 and D_3; Y_3 = Retention time of K_2-7.

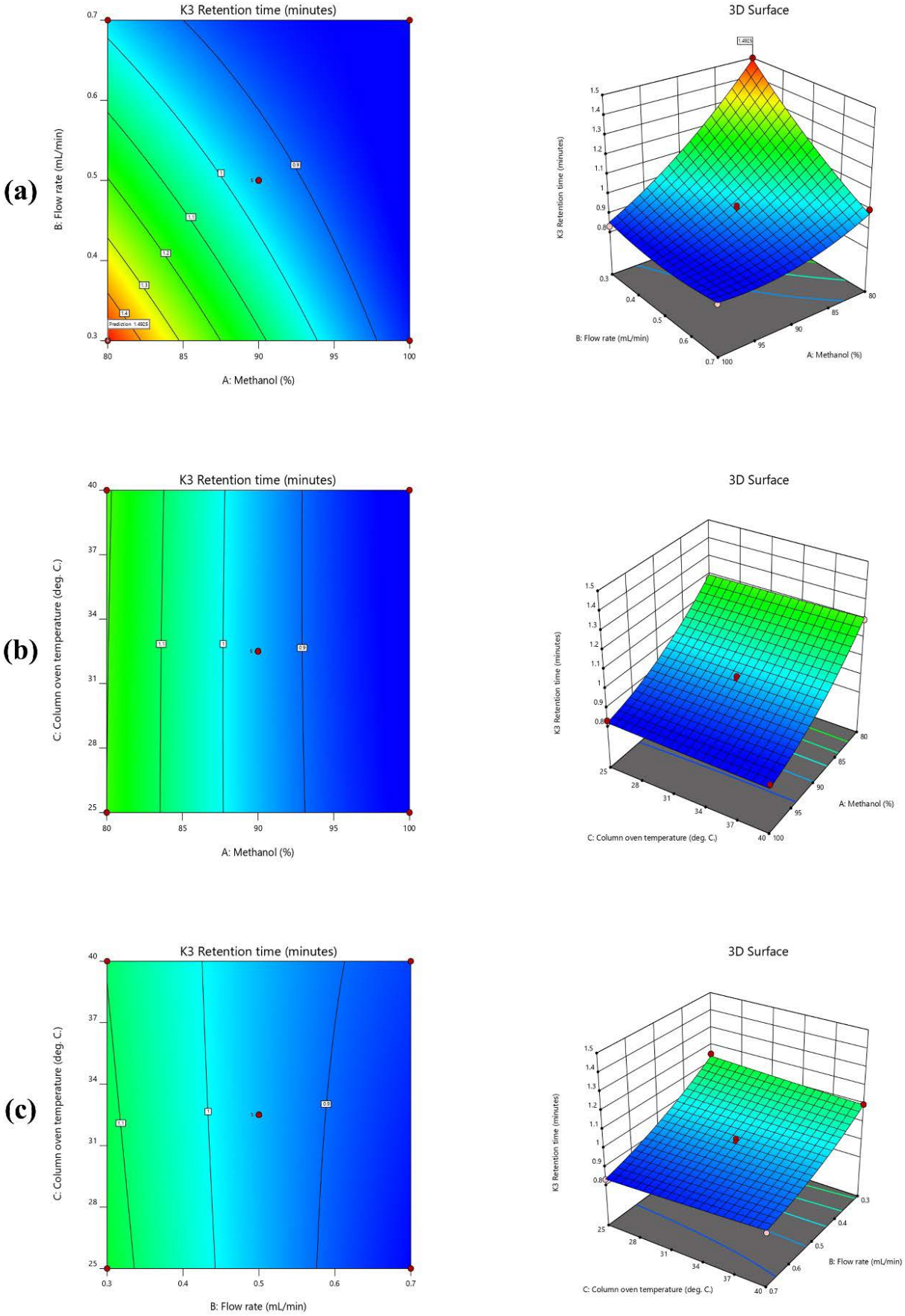

Figure 2. *Contour and response surface graphs of Y_1 versus (a) $X_1.X_2$, (b) $X_1.X_3$, (c) $X_2.X_3$*
Y_1 = retention time of K_3, X_1 = methanol (%), X_2 = flow rate (mL/min), X_3 = column oven temperature (^oC).

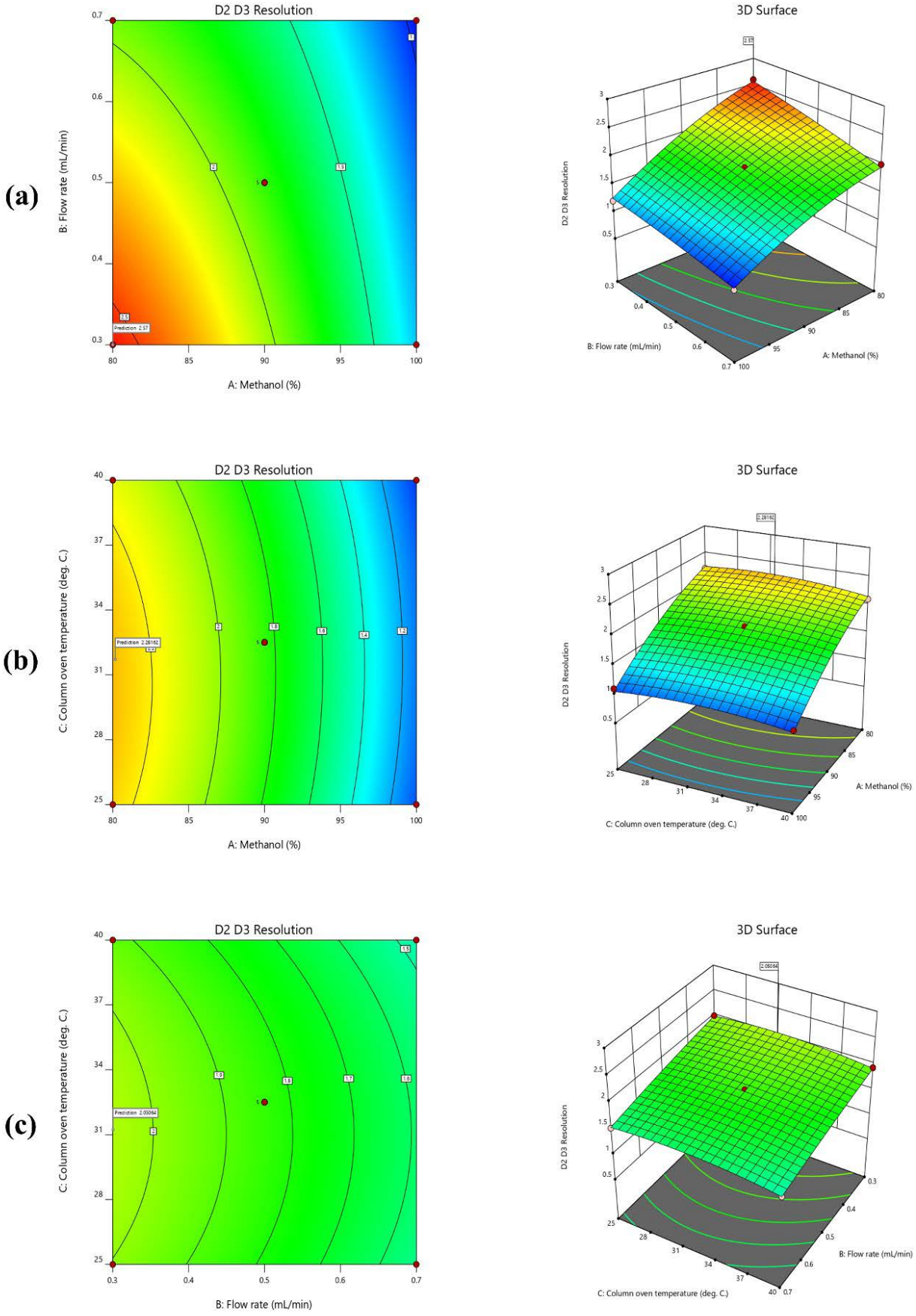

Figure 3. *Contour and response surface graphs of Y_2 versus (a) X_1.X_2, (b) X_1.X_3, (c) X_2.X_3*
Y_2 = resolution between D_2 and D_3, X_1 = methanol (%), X_2 = flow rate (mL/min), X_3 = column oven temperature (^oC).

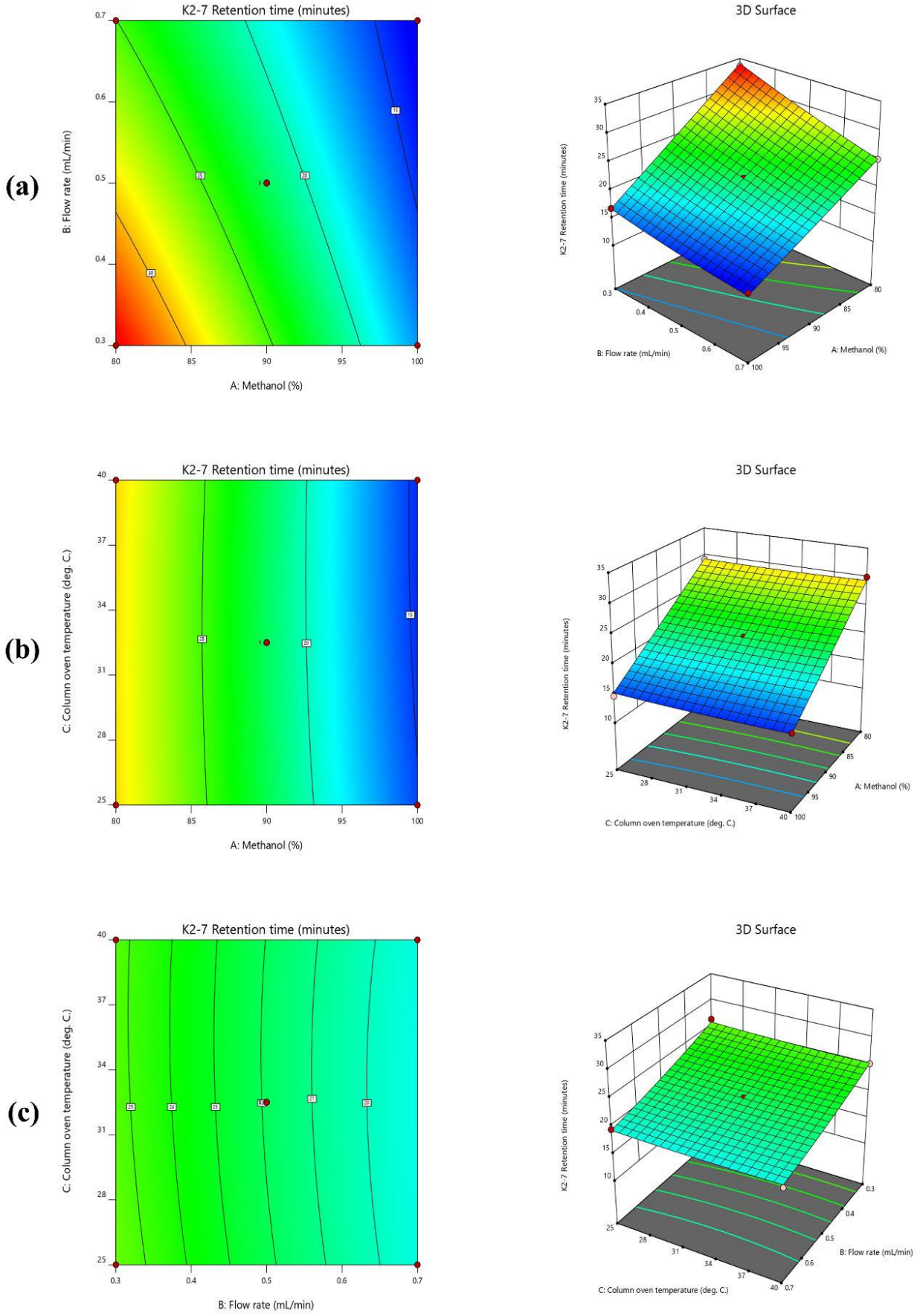

Figure 4. *Contour and response surface graphs of Y_3 versus (a) X_1.X_2, (b) X_1.X_3, (c) X_2.X_3*
Y_3 = retention time of K_2-7, X_1 = methanol (%), X_2 = flow rate (mL/min), X_3 = column oven temperature (°C).

Table 4. *Method validation summary.*

Parameter	K_3	D_2	D_3	K_2-4	K_1	K_2-7
Selectivity						
t_R	0.92	4.99	5.18	5.48	8.02	16.25
System suitability						
k'	0.11	5.01	5.24	5.60	8.66	18.58
Inj. Rep (%RSD)	0.273	0.102	0.152	0.213	0.382	0.657
T	0.95	0.98	0.97	1.02	1.06	1.13
N	4895	8709	8968	9250	16808	26684
R_s	-	3.51	1.68	3.39	6.63	7.77
Specificity						
MS1 (m/z)	173.04	397.36	385.36	445.32	451.36	649.53
MS2 (m/z)	105.02	379.34	367.34	445.32 187.08	451.36 187.07	607.41 187.08
Sensitivity						
LOD (ng/mL)	9	6.5	6	4.5	3.5	10
LOQ (ng/mL)	14.5	11.5	11	9.5	8.5	15
Linearity						
Regression equation	y=3.75x+5.05	y=7.64x+11.89	y=8.33x+39.72	y=8.93x+151	y=99.6x-312	y=6.68x-14.03
R^2	0.9999	0.9998	0.9999	0.9999	0.9999	0.9999
Spiked recovery						
50 ng/mL (%)	99.48	99.66	99.61	99.2	98.57	95.52
250 ng/mL (%)	99.61	99.85	99.84	99.29	99.17	96.05
Robustness (%RSD)						
Sample manager temperature (5, 15, and 25 °C)	0.16	0.11	0.24	0.18	0.33	0.46
Injection volume (3, 5, and 7.5 µL)	1.76	2.43	2.29	2.71	2.59	2.86
Ionization source condition (Before and after cleaning)	1.86	1.79	1.57	1.55	1.68	1.24

Table 5. *Confirmation of the optimized method parameters.*

Method parameters	Response	n	Predicted Mean responses	Observed responses (95% PI)	Std. Dev
X_1 = 90%; X_2 = 0.5 mL/min; X_3 = 40°C	Y_1	3	0.95	0.92	0.023
				0.91	
				0.92	
	Y_2	3	1.71	1.79	0.047
				1.80	
				1.81	
	Y_3	3	21.9	21.7	0.501
				21.0	
				21.5	

*X_1 = Methanol (%); X_2 = Flow rate (mL/min); X_3 = Column oven temperature (°C); Y_1 = Retention time of K_3 (minutes); Y_2 = Resolution between D_2 and D_3; Y_3 = Retention time of K_2-7; n = number of determinations; 95% PI = (1-0.05)*100%; Std. Dev = Square root of the residual mean square. Considered as an estimate of the standard deviation associated with the experiment.*

curves (CCs) were constructed by plotting response factor (RF=(Mean TIC value (n=5))/(Injected concentration)) of respective analyte against the concentration injected (Table 4). A simple linear regression equation (Y=mX+C) obtained from the calibration curves is used to study the relationship between analyte concentration (X) and response (Y).

Accuracy and precision: The precision of the developed method was assessed using intra-day and inter-day precision, and analyst-to-analyst repeatability. The intra-day precision and inter-day precision of the analytical method are determined in a single laboratory and analyst by evaluating three QC levels at respective concentrations of 41.25, 712.5, and 1095 ng/mL representative of the intended range of 15 to 1500 ng/mL over three weeks (Table 5). Analyst-to-analyst repeatability was assessed by each of the two analysts who prepared the stock solutions, QCs, operated the instrument, integrated the peaks,

and reported the data independently on the same day (Table 5).

Analyte spiked samples (50 ng/g of D_2, D_3, and K_3; 250 ng/g of K_1, K_2-4, and K_2-7) were prepared using placebo formulation and analyzed in triplicate and expressed as percentage mean absolute recovery (R) (Table 4).

Robustness: Robustness refers to the ability of an analytical method to remain unaffected by small variations in method parameters (mobile phase composition, column age, column temperature, and so on) and influential environmental factors (room temperature, air humidity, and so on) and characterize its reliability in routine analysis. This method's robustness is limited to factors viz., the sample manager temperature (5 to 25°C), injection volume (3 to 7.5 µL) and ionization source condition (before and after cleaning) (Table 4).

Method validation.

MassLynx (Version 4.1; Waters Corp., Milford, MA, USA) was used to acquire and process the UPLC-Q-ToF/MS data. Statistical design of experiment was performed using Design Expert (Version 12.0; Stat-Ease, Inc., MN, USA). Construction of calibration curves and interpolation of the standard calibration curves were performed using GraphPad (Version 9.0; GraphPad Software, Inc., CA, USA). Other calculations were carried out using Microsoft Excel 2019 (Microsoft Corp., USA).

Results and discussion.

Optimization of UPLC method parameters:

Preliminary trails:

In the preliminary trails, optimal analyte retention was observed with C-18 Accucore UPLC column (50 x 4.6 mm, 2.6µm). Water as a mobile phase component was introduced with caution due to the analytes' varying log P values (non-polar), as it has been observed to directly affect the retention of vitamins K_3 and K_2-7. Methanol and acetonitrile, as organic mobile phase modifiers, were tested for their effect on analyte retention. When compared to acetonitrile, using methanol (100%) as mobile phase was found to elute vitamin K_2-7 faster. Furthermore, higher methanolic compositions (100%) in mobile phase failed to retain K_3 and resolve D_2 and D_3 (Rs ≥1.5), indicating the effect of the initial (0 to 2 minutes) water/organic solvent ratio [27]. To avoid analyte degradation under alkaline conditions [28], formic acid (0.1% v/v in water) was used as a mobile phase additive. Among the selected fat-soluble vitamins, K_3, D_2, and D_3 are partially soluble in water, whereas vitamins K_2-4, K_1, and K_2-7 are insoluble. Thus, methanol (100%) was opted as choice of diluent for preparing primary stock solutions. We chose to investigate the effect of methanolic composition of mobile phase, flow rate, and column oven temperature on the retention of vitamins K_3 and K_2-7, as well as the resolution of vitamins D_2 and D_3 using response surface methodology. Other variables, such as sample injection volume (µL) and sample manager temperature (°C), were found to have no effect on analyte responses.

Response surface methodology – Box-Behnken design: As previously stated, 17 experimental runs were used to investigate the quadratic interactions between three independent actors (X_1,X_2,and X_3) and desired responses (Y_1,Y_2,and Y_3) through multiple regression analysis and ANOVA. The adjusted coefficients of determination (R_2) for the designed model were 0.983 (Y_1), 0.988 (Y_2) and 0.992 (Y_3), and the model's probability value of $p<0.001$ for all responses indicates that the regression model was highly significant. The statistically significant model terms ($p<0.001$) influencing the response Y_1 are X_1,X_2 and $X_1 \times X_2$. Similarly, significant terms influencing the response Y_2 and Y_3 are X_1 and X_2, respectively. Figures 2, 3 and 4 depicts the contour and response surface plots for the independent variables, which express their relationship with the dependent responses.

The response surface plot for the response Y_1 versus factors X_1 and X_2 predicted highest retention time for K_3 (Y_1) of 1.49 minutes at X_1=80% and X_2 of 0.3 mL/min (figures 2a, 2b and 2c). The column oven temperature (X_3) did not show any significant influence on the response (figure 2b and 2c). The response surface plots for the response Y_2 versus factors X_1,X_2 and X_3 predicted highest resolution between D_2 and D_3 (Y_2) at X_1 = 80%, X_2 = 0.3 mL/min, and X_3 = 31.5°C (figures 3a, 3b and 3c). The response surface plots for the response Y_3 predicted least retention time of 13.5 minutes at X_1 of 100% and X_2 of 0.7 mL/min (figure 3a). The column oven temperature (X_3) did not show any significant influence (figures 3b and 3c). X_2 and X_3 showed least interaction among the factors (figure 3c). Overall, the independent factors X_1 and X_2 had a significant influence on all responses. According to the response surface plots, methanol in the range of 88% to 91%, flow rate in the range of 0.45 to 0.52

Table 6. *Results of method precision.*

Parameter	QCs level (ng/mL)	K_3		D_2		D_3		K_2-4		K_1		K_2-7	
		R	***RSD***	***R***	***RSD***	***R***	***RSD***	***R***	***RSD***	***R***	***RSD***	***R***	***RSD***
Intra-day precision	*LQC (41.25)*	99.7	0.34	100	0.48	99.1	0.27	99.7	0.31	98.4	0.14	99.6	1.15
	MQC (712.5)	100	0.11	99.8	0.24	100	0.09	100	0.33	100	0.07	100.1	0.59
	HQC (1095)	100	0.04	100.2	0.14	100	0.01	100	0.16	100	0.07	99.7	0.33
Inter-day precision	*LQC (41.25)*	99.04	0.75	99.7	0.86	100.3	0.64	98.07	0.96	98.9	0.11	99.2	1.39
	MQC (712.5)	100.3	0.31	99.8	0.88	100.1	0.11	100.1	0.55	100.1	0.15	99.9	0.67
	HQC (1095)	100.1	0.06	100.2	0.25	100.2	0.02	100.1	0.28	100	0.08	99.9	0.18
Analyst-to- analyst repeatability	*LQC (41.25)*	99.9	1.13	99.9	0.2	100	0.35	99.2	0.43	98.7	0.13	98.5	1.41
	MQC (712.5)	100	0.66	100.3	0.16	100.1	0.23	100	0.14	100	0.09	99.8	0.83
	HQC (1095)	100.1	0.11	100	0.04	100.1	0.08	100	0.05	100	0.08	100	0.37

QCs = quality control samples; LQC = low quality control; MQC = median quality control; HQC = high quality control; R = absolute recovery (%); RSD = relative standard deviation.

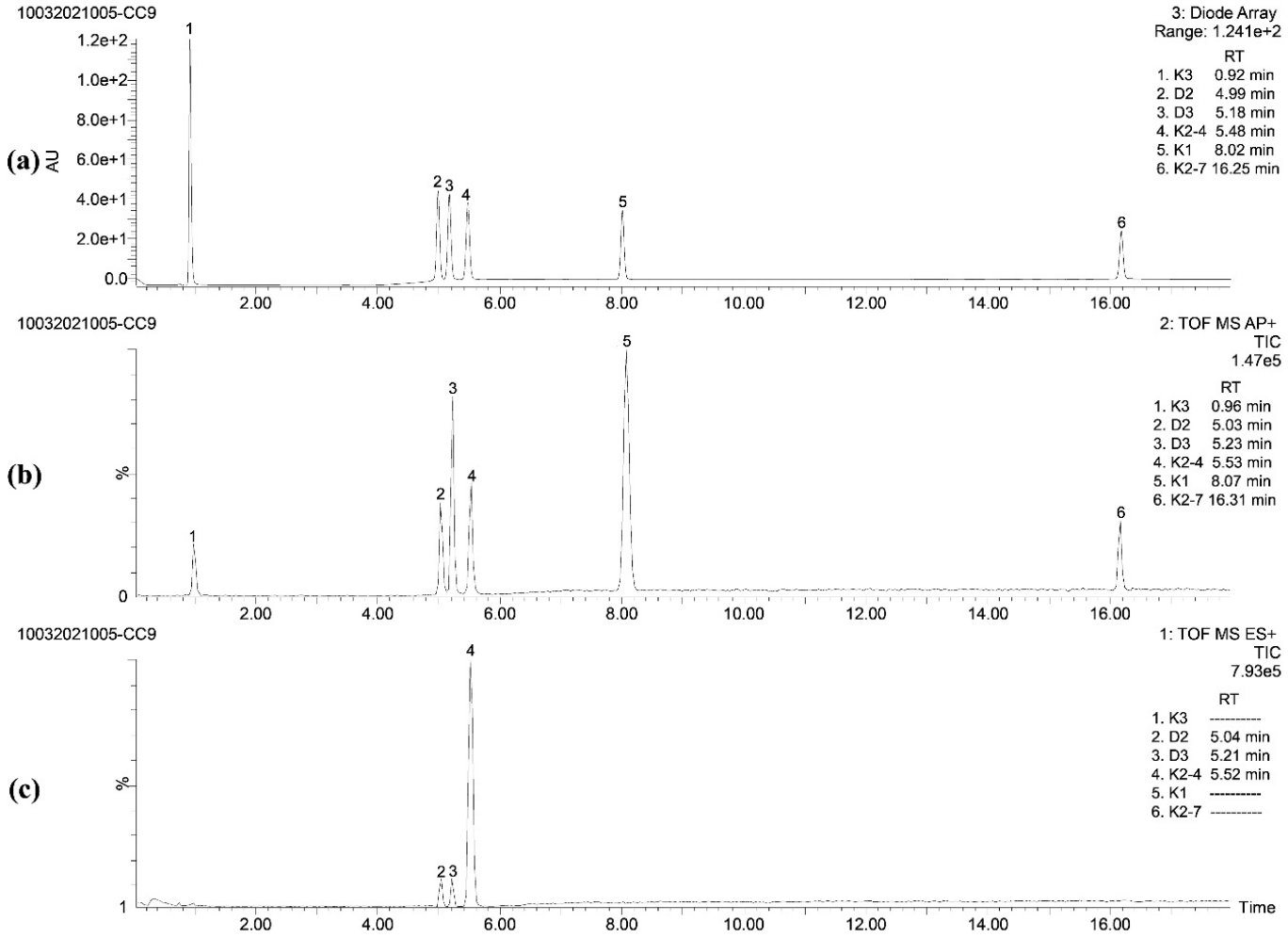

Figure 6. *Chromatograms (a) diode array detection (DAD), (b) total ion chromatogram (ESI+), (c) total ion chromatogram (APCI+).*

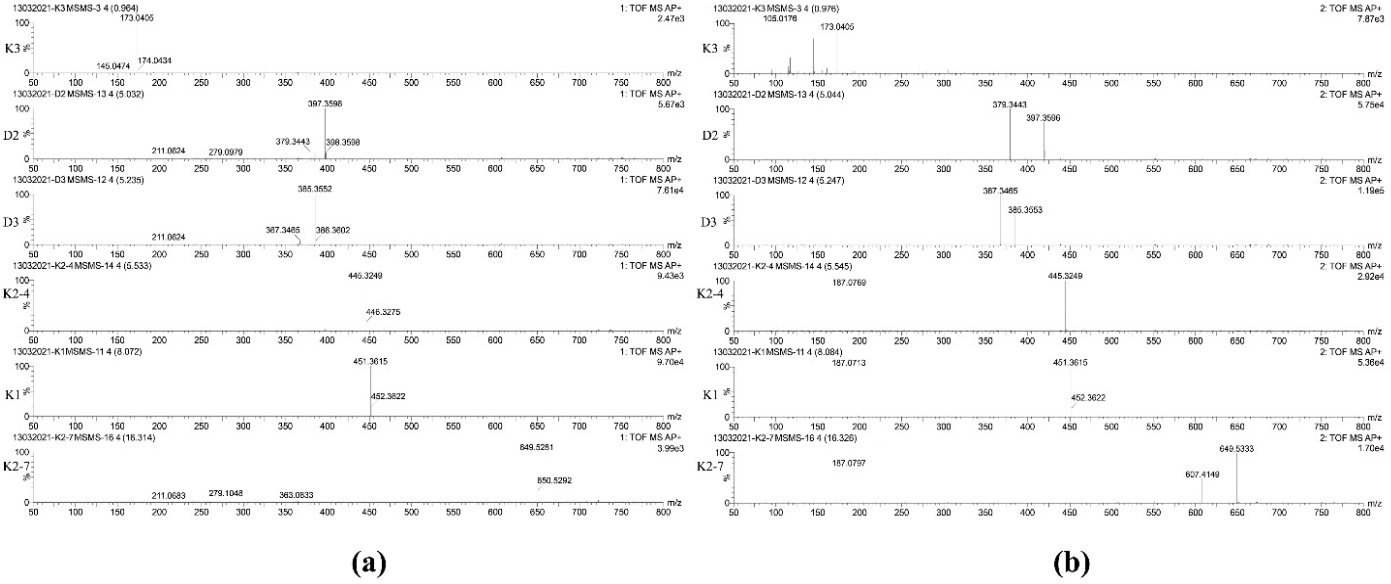

Figure 7. *Mass spectra (a) precursor ion spectra (b) product ion spectra of six-fat soluble vitamins K_3, D_2, D_3, K_2-4, K_1, K_2-7.*

mL/min., and column oven temperature in the range of 30 to 40 °C could achieve the desired results.

The experimental design's numerical optimization aided in achieving the desired results [29]. The design was numerically optimized for the factors: X_1 = 90%, X_2 = 0.5 mL/min., X_3 = 40°C to achieve desired responses: Y_1 = 1 minute, Y_2 = 1.7, Y_3 = 17 minutes. Based on these constraints, the predicted values for the responses were: Y_1 = 1 minute, Y_2 = 1.8, and Y_3 = 23.1 with a desirability value of 0.769 (Figure 5). UPLC separation using these constrains (mobile phase delivered isocratically) reported mean responses (n=3) with confidence (Confidence interval = 95%): Y_1 = 0.92 minutes, Y_2 = 1.81, and Y_3 = 21.4 minutes (Table 5). However, based on these observations, a gradient profile was created, as previously mentioned, to further reduce the chromatographic run time. As a result, the retention time of K_3 remained 0.92 minutes, the resolution between D_2 and D_3 was now 1.68, and the retention time of K_2-7 was reduced to 16.25 minutes (Figure 6).

Quadrupole time-of-flight mass spectrometry (Q-ToF/MS):

The efficient ionization of analyte molecules is critical in mass spectrometric analysis [30]. APCI operated in positive polarity resulted in better analyte ionization (Figure 6). As seen in the scan spectrum, all analytes produced molecular ion peaks with single protonation $[M+H]^+$ as well as double protonation $[M+2H]^+$ with the former being predominant (Figure 7). Collision induced dissociation of analytes resulted in product ion fragments of the analytes (Figure 7). When compared to the low energy mass spectrum, increasing the collision energy from 15 to 60 eV improved the ionization of vitamin K vitamers producing quantifiable daughter ions. Q-ToF-MRM-enhanced transmission mode and target enhancement improved ion counts of D_2, D_3, and K_1 but failed to show ionization improvements for K_3, K_2-4, and K_2-7 compared to generic full-scan mode. The loss of a water molecule $[M+H-18]^+$ from vitamins D_2 and D_3 molecules could be attributed to the presence of hydroxyl groups, resulting in an intense product ion at m/z 379 and 367, respectively. Carbon monoxide and acetylene losses from the vitamin K_3 molecule resulted in an intense product ion at m/z 105 $[M+H-28-26]^+$, whereas carbon monoxide loss alone resulted in a product ion at m/z 145 $[M+H-28]^+$. Vitamins K_1, K_2-4, and K_2-7 produced a common product ion at m/z 187, which could be attributed to the loss of side chain units attached to the naphthalene-1,4-dione moiety, namely $[M+H-265]^+$ for K_1, $[M+H-259]^+$ for K_2-4, and $[M+H-463]^+$ for K_2-7. A peak at m/z 607 in the product ion spectra of K_2-7 could be attributed to the loss of the C3H6· group $[M+H-42]^+$ at the terminal prenyl sidechain unit. The presence of molecular ion peaks $[M+H]^+$ in all analytes' product ion spectra corresponds to soft ionization of the APCI source, resulting in little fragmentation of the non-polar analytes [31]. The higher collision potentials used in this study may have contributed to the higher degree of fragmentation obtained. The intensities of the precursor and product ion peaks were used to calculate analyte responses for quantification of the respective analytes from the sample extracts.

The developed UPLC-Q-ToF/MS method was found to be selective (Figure 6) and specific (Figure 7) for the analytes of interest, with no interfering peaks at analyte retention times.

Method validation.

The minimum system suitability parameters evaluated (n=6) as a measure of a well-behaved chromatographic system were capacity factor (k'), precision/injector reproducibility (Relative standard deviation, RSD), resolution (Rs), tailing factor (T), and theoretical plate number (N) [25,26]. Except for the first peak i.e., K_3, k' of >2 indicates that the analyte peaks met the criteria for maintaining a significant distance from the void. Injection precision (n=5 per analyte) as an indication of UPLC performance, with RSD values not exceeding 0.657% for the analytes of interest, well within the desired limit of 1%. As a measure of peak separation for reliable quantitation, Rs values greater than 1.68 at the highest analyte concentration demonstrate the method's ability to resolve the D_2 and D_3 peaks, which has been a source of concern in many previously reported methods. The chromatographic resolution (Rs) between the analyte and the closest eluting peak is usually used to determine selectivity in LC–MS. The AOAC [27] requires Rs of at least 1.5, and the FDA [25,26] requires Rs of at least 2. With a peak tailing of ≤2, among the analytes of interest, the K_2-7 peak had the tailing of 1.13 which was highest among the analyte peaks, demonstrating that acceptable peak symmetry was maintained in the chromatographic separation, allowing for accurate quantitation. The analytical column efficiently retained the analytes of interest during a chromatographic run with N values ≥4895 using a fixed set of operating conditions such as peak position, particle size in column, flow rate of mobile phase, column temperature, and analyte molecular weight. The parameters evaluated met the method validation criteria, and the method's chromatographic suitability for the analytes of interest could be confidently claimed.

The method was found to be sensitive, as demonstrated by the LOD and LOQ values reported in the table 4. The calibration curves for analytes were created, and linearity was observed in the concentration range of 15-1500 ng/mL of each analyte, demonstrating an excellent correlation coefficient $^{r2} \geq 0.9998$. The method was found to be precise for intra-day and inter-day precision, and analyst-to-analyst repeatability evaluations with RSD values <1.41% (Table 5). The accuracy of the developed method was demonstrated by analyzing (n=3) analyte recovery by single extraction from a spiked placebo tablet. Analyte recovery ranging from 95.52% to 99.85% have been reported (Table 4).

The robustness of the UPLC-Q-ToF/MS method was evaluated by deliberately modifying chromatographic conditions. The robustness of the proposed method was assessed using factors such as ., the sample manager temperature (5 to 25°C), injection volume (3 to 7.5 µL) and ionization source condition (before and after cleaning). The influence of each of the factors was evaluated, and none of the factors exceeded the limits, demonstrating that the studied independent variables had no influence on the responses (Table 4).

Conclusion.

A selective, specific, sensitive, precise, accurate and robust UPLC-Q-ToF/MS method for the simultaneous estimation of D_2, D_3, K_1, K_2-4, K_2-7, and K_3 using response surface methodology to optimize the liquid chromatographic separation

was developed and validated. The Box–Behnken design allowed for simultaneous evaluation of the independent factors as well as the addition of interactions between the factors in order to optimize experimental conditions. According to the response surface plots, the retention of K_3, K_2-7, and the resolution between D_2 and D_3 were significantly influenced by methanol composition in mobile phase and flow rate. In terms of D_2 and D_3 resolution, column oven temperature had little interaction with other factors. It is recognized that using the design of experiment approach is an adaptable practice for reducing the total experimental runs required for the optimization and development of the UPLC method can generate profound data in a short period of time. The method appears to be robust and accurate, and it can resolve D_2 and D_3 during simultaneous D and K vitamer analysis. The proposed method was validated and could be a potential for use in routine laboratory analysis.

Author contributions.

Mr. Anoop Karthika designed and carried out the experiment, collected and interpreted the data, and wrote the manuscript. Ms. Kowmudi Gullapalli was involved in the experiment's execution, data interpretation, and proofreading the written manuscript. Dr. Krishnaveni Nagappan had overseen the execution of experiments as well as the accuracy of data collection and interpretation. Mr. Manohar Dronavajjula assisted with sample preparation and manuscript writing. Dr. Anilakumar Kandangath Raghavan provided insightful information on the performance of LC-MS experiments and proofread the manuscript. Dr. Ramalingam Peraman developed the response surface methodology (Box-Behnken design) and provided useful insights for carrying out the experimental design and proofread the manuscript.

Conflicts of interest.

All authors declare that they have no significant financial and other interests associated with this manuscript.

Acknowledgements.

We would like to express our gratitude to Dr. S. Chandra Nayaka, Scientist, Institution of Excellence, Vijnana Bhavan, University of Mysore, for allowing us to conduct LC-MS experiments at their facility.

We sincerely thank the Indian Council of Medical Research for awarding Mr. Anoop Karthika with a senior research fellowship [3/1/2/157/2019-(Nut)].

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