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Development and Validation of a RP-HPLC/UV Method for Analysis of Vitamin A, C and E in Pharmaceuticals, Cosmetics, and Beverages

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Abstract

Vitamins A, E, and C are used in many pharmaceuticals, cosmetic products, and beverages to compensate for their daily intake and better skin health. In the presented study, an isocratic reversed-phase HPLC method was developed for the simultaneous analysis of these three vitamins. Different experimental conditions were optimized and the developed method was validated as per ICH guidelines. Furthermore, the developed method was applied for the analysis of vitamins A, C, and E in samples of pharmaceuticals, cosmetics, and beverages. The mobile phase acetonitrile and water containing 1% phosphoric acid (65:35, v/v) were pumped at 1 ml/minute and the analytes were analyzed at 280 nm. These vitamins were extracted from a USP syrup base (containing 10% ethanol), emulsion (w/o), and tablet matrix. The linearity of the analytes in different matrices was excellent with a correlation coefficient (r2) of 0.99. The % recovery was above 90 % from all the matrices except for vitamin E from the syrup matrix. The precision was well within range by calculating the % RSD. The vitamins were stable when stored at -4 ˚C in amber-colored tubes. Due to the variable concentration of the analytes in commercial products, dilution integrity was performed and the results were according to the ICH guidelines. The method was successfully applied for the quantification of vitamins A, E, and C in pharmaceutical products (Syrups and tablets), cosmetics both medicated and non-medicated, and food products including energy drinks and ketchup.

KEYWORDS

Vitamins A, E, & C; HPLC/UV; Cosmetics; Pharmaceuticals

1.0 INTRODUCTION

Vitamins are amongst the most extensively used supplements, consumed in almost every part of the world to cover their probable deficiency and for the maintenance of a balanced diet [1-3]. The list of reasons for their extensive consumption includes the poor availability of organic food items like fruits, vegetables, and dairy products, excessive use of packaged food items, hybridization tendencies in food commodities, unhealthy lifestyle attitudes, excessive workload, and much more [4]. Due to the importance of vitamins in almost all physiological and metabolic activities, it has been considered as an obligation by health care practitioners and nutritionists to include and consume this important entity in our daily food to avoid metabolic disorders and maintain a healthy well-being [5].

Vitamins are mainly classified into two groups based on their solubility in their respective solvents, that are, fat-soluble and water-soluble vitamins [6-8]. The fat-soluble vitamins include Vitamin A (All-trans retinol), Vitamin D (Cholecalciferol), Vitamin E (Tocopherols) and Vitamin K (alpha-phylloquinone), while the aqueous soluble vitamins include Vitamin C (Ascorbic acid) and Vitamin B complex; a huge class of vitamins like Thiamine, Riboflavin, Niacin, Pyridoxine, Cyanocobalamin, and Folic acid [9, 10].

Almost all vitamins present in nature have their importance and carry substantial metabolic functions [11-14], however, currently, the vitamins having antioxidant activity are not only under the limelight of pharmaceutical and nutraceutical industries, but researchers and nutritionists have also developed a keen interest due to their potential benefits. Among these antioxidant vitamins, Vitamin A, having a molecular formula of $C_{20}H_{30}O$, also known as all-trans-retinol, is present in animals' animal-origin foods as Retinyl esters and plant-origin food as provitamin A carotenoids (α-carotene, β-carotene, and β-cryptoxanthin. Vitamin A carries a proven physiological and metabolic role in plants, animals, and humans. In humans, it plays a vital role in the vision cycle, cell differentiation of epithelium, osteogenesis, reproductive pathways, and immunity [15]. As compared to α-tocopherol, the beta carotene activity as an antioxidant is relatively weak. In humans' low-density lipoproteins (LDL), β-carotene are about 1/20th of the concentration of Vitamin E [16], and Vitamin A is utilized only when tocopherol is exhausted [17]. Similarly, vitamin C (L-Ascorbic acid) with a molecular formula $C_6H_8O_6$, a hydrophilic compound with a strong role as a coenzyme, cofactor, collagen synthesis & protection, and a geroprotector that is an anti-aging agent. Vitamin C is considered as one of the most widely studied and consumed antioxidant agents, because of its ability to react with free radicals produced in the body due to different ongoing chemical reactions and stop them from further reactions causing any potential damage to cells and tissues [18]. The L-ascorbate radical has the potential to act as an electron donor, effectively speeding up redox reactions when transition metals like iron or copper are present. Consequently, in the context of atherosclerotic plaques containing ferric iron, vitamin C could function as a pro-oxidant rather than its typical role as an antioxidant [19]. Similarly, another lipophilic vitamin that is Vitamin E (α-tocopherol), with a molecular formula $C_{29}H_{50}O_2$, a protein kinase C inhibitor, is abundantly found in sunflower and olive oils with proven ability as an antioxidant, anticoagulant, immunomodulator, and antiatherogenic agent. Currently, vitamin E along with Vitamin C has been exclusively researched for their antioxidant roles and their probable involvement in alleviating different life-threatening conditions & ailments like Diabetes mellitus, cardiovascular conditions, ophthalmic diseases, neurological disorders [19], cancer of different nature [20,21] and multiple dermatological problems [22]. It is important to mention that despite the claimed benefits of these naturally occurring vitamins as antioxidants, some clinical studies also showed no substantial evidence for alleviating the discussed ailments [23].

Keeping in mind the importance of these natural antioxidant agents and their extensive use in different commonly used packaged food items (energy drinks & sauces), pharmaceuticals, and cosmetics (medicated & non-medicated), different researchers across the globe have so far developed various chromatographic techniques for the quick, reliable and authentic detection and quantification of these potential antioxidant agents/vitamins for regulatory purposes. Some of the notable studies conducted was the simultaneous detection of Vitamin A and Vitamin E in human serum by using HPLC/UV with different columns like Supelco (C18) LC-18, Kromasil 100 C18, and Brownlee analytical (Perkin Elmer) C18 column [24]. Simultaneous detection studies of fat-soluble and water-soluble vitamins in a single run are not easy to conduct, and they are very rarely reported [25, 26]. Furthermore, no such study, which includes the detection and quantification of these antioxidant vitamins (Vitamin A, E, and C) in marketed pharmaceutical products, cosmetics (medicated & non-medicated), and packaged food items (juices and sauces) simultaneously, has been reported. This study aimed to detect and quantify the two fat and water-soluble antioxidant vitamins in a variety of marketed products.

2.0 MATERIALS AND METHODS

2.1 Chemicals

All-trans-retinol (Vitamin A), DL-α-tocopherol (Vitamin E), and Ascorbic acid (Vitamin C) were purchased from Sigma-Aldrich. Diclofenac sodium lauryl sulfate was provided by Stanley Pharmaceutical (Pvt), Pakistan. HPLC grade Methanol, ethanol, acetonitrile, and phosphoric acid (H_3P_O4) were obtained from Merck (Merck, Darmstadt, Germany). The analytical grade distilled water was obtained by using a water purification system, Milliford, USA.

2.2 Instrumentation

This study of detecting antioxidant vitamins simultaneously was conducted on HPLC (Alliance Waters e-2695 Separations Module, Milford, USA), controlled by Empower software, having an optional column heater having series e2695 XE, optional sample heater with series e2695 XC, and an autosampler. The column used for the analysis was Thermo Fisher Scientific HPLC Acclaim Reversed phase C18 (250 mm × 4.6 mm, 5µm). The detector linked with the system was the Waters 2489 UV/VIS detector.

2.3 Optimization of Experimental Conditions

2.3.1 Selection of Solvent and Preparation of Standard solution

Stock solution of all-trans-retinol (Vitamin A), Ascorbic acid (Vitamin C), DL-α-tocopherol (Vitamin E), and diclofenac sodium (internal standard) each 1 mg/mL was prepared in a mixture of acetonitrile and 1% phosphoric acid (65: 35). The solutions were stored at -20ºC in amber-colored glass vials. For calibration, working solutions were prepared from the already prepared stock solution and the volume was adjusted with mobile phase. For Vitamin A and E, the concentrations were 0.05, 0.1, 0.2, 0.4, 1.0 and 2.0 µg/ml. For Vitamin C the concentrations were 0.1, 0.2,0.5, 1.0, 1.5, and 3.0 µg/ml. For accuracy, precision, and stability, spiked samples were prepared from different stock solutions.

2.3.2 Selection of mobile phase and optimization of its pH

Different mobile compositions were evaluated to get best peak shapes and resolution. Combination of methanol and water, acetonitrile and water, and acetonitrile and water containing H_3PO_4 were evaluated. The mobile phase selected was the combination of acetonitrile and water containing 1 % phosphoric acid (65:35, v/v). The analytes were run through the system in an isocratic mode, keeping the temperature ambient.

2.3.3 Selection of Detector wavelength

Different wavelengths in the range of 250 to 310 nm were checked for optimum results i.e. 250, 260, 270, 280, 290, 300, and 310 nm. 280 nm was selected as the optimum wavelength.

2.3.4 Optimization of mobile phase flow rate

Flow rates of 0.8, 1.0, 1.2, 1.4, and 1.6 ml/ minute were evaluated. During this evaluation not only, the peaks shapes and resolution were observed but the HPLC pump back pressure was also under observation. 1 ml/min was selected as the optimum flow rate.

2.3.5 Selection of internal standard

Diclofenac sodium, naproxen, and levosulpiride were evaluated as internal standard. The diclofenac sodium showed good response and no interference with the analytes at 280 nm with flow rate of 1 ml/min.

2.4 Extraction Procedure

The method was proposed for analysis of vitamin A, C and E in pharmaceutical products, cosmetics and beverages. Sample were extracted from three different matrices. The matrices were prepared in laboratory for spiking the sample. One was simple syrup USP containing 10 % ethanol which served as a matrix for vitamin A, C, and E in pharmaceutical liquid formulations and juices. Another matrix was a base of a solution that contained water and oil with sodium lauryl sulfate as an emulsifier. The third matrix was a starch and magnesium stearate mixture which mimics a tablet matrix. Simple dilution method was used for extraction. From simple syrup (also used a matrix for beverages) and water and oil emulsion, 500 µl was taken in a test tube, to this known concentration of the analytes was added. 1.5 ml of acetonitrile (containing 1 % phosphoric acid) was used as an extracting solvent. After adding the extracting solvent, the sample was vortexed for 3 minutes. The samples were then centrifuged at 4000 rpm for 15 minutes. The supernatants were separated and 10 µl of it was injected into the HPLC system. The same procedure was used for the solid matrix by taking 100 mg starch and magnesium stearate powder, a known concentration of analytes was added, and 1.5 ml of extracting solvent was added to it. Vigorous vertexing was followed by centrifugation.

2.5 Validation of the developed method

2.5.1 Linearity

Mixtures of the analytes were made from the stock solution of the analytes. The mixtures contained in vitamin C in the concentrations of 0.1, 0.2, 0.5, 1.0, 1.5 and 3.0 µg/ml. The concentrations of vitamins A and E were 0.05, 0.1, 0.2, 0.4, 1.0 and 2 µg/ml. To each mixture, 0.5 µg/ml of diclofenac sodium as internal standard was added. The same concentration ranges were extracted from the three different matrices.

Calibration curves were constructed for the analytes by plotting the peak response ratio (peak area of analyte divided by peak area of internal standard) vs concentration of the analytes. The curve was constructed for both the standard and extracted (spiked) samples from the three matrices. The regression line and linearity (r2) were determined by using MS Excel 2016.

2.5.2 Specificity

The specificity of the method was determined by analyzing the analytes prepared in the mobile phase, blank matrices, and analytes added to the matrices. The peaks separation was observed and the resolution between two consecutive peaks was calculated. Interferences from the matrices were also evaluated.

2.5.3 Accuracy

The accuracy was calculated from the concentration of analytes recovered from the analyte and percent recovery. The formula shown in equation 1, was used for percent recovery where the peak response ratio is the peak area of the analyte divided by the peak area of the internal standard. Four concentration levels were used for accuracy of the method

$$\% \, recovery = \frac{Peak\ response\ ratio\ in\ matrix}{Peak\ response\ ratio\ in\ Standard} \times 100 \text{----------------1}$$

2.5.6 Precision

The precision was calculated by percent relative standard deviation (%RSD). This was done by performing intra-day and inter-day studies, by analyzing the analytes at three different concentration levels in different matrices. The concentrations used for Vitamin C were 2.5, 1.0, and 0.5 µg/ml while for Vitamin A and E the concentrations were 1.5,1.0, and 0.2 µg/ml. The intraday study was performed by analyzing the samples in triplicate (n = 3) at three different times of the day i.e. 08: 00, 16:00 and 23 hours. The inter-day precision was performed by analyzing the samples for three consecutive days.

2.5.7 Sensitivity

The limits of detection and limits of quantification for the three analytes were determined by the serial dilution method. The signal-to-noise ratio was 3:1 for LOD and 10:1 for LOQ

2.5.8 Stability of spiked samples

The stability studies were performed by calculating the percent loss in concentration by comparing the concentration of the freshly prepared spiked analytes with the sample stored for 24 hours at room temperature, 4 ˚C and -20 ˚C. Long term stability was performed for one month when the samples were stored at -20 ˚C and underwent three freeze-thawed cycles. Each freeze thaw cycle was separated by 12 hours from another cycle. Three concentration levels were used for stability of each analyte.

2.5.9 Dilution integrity

The commercial products analyzed were having variable concentrations ranges. Dilution integrity were performed to validate the dilution for concentrations beyond the concentrations of analytes in the calibration curve. Ten times higher concentration were used then upper limit of quantification of all the analytes i.e. 30 µg/ml of vitamin C and 10 µg/ml of Vitamin A and E. 10- and 20-fold dilutions were used for these concentrations.

2.6 Application of the method

2.6.1 Sample collection

The samples were collected from the local market of Distt. Swabi, Distt. Bannu, Distt. Lakki Marwat, Distt. Mardan and Distt. Peshawar (Khyber Pakhtunkhwa), Pakistan. The developed method was used for quantification of the vitamins in different pharmaceuticals, cosmetics and beverages collected from the market.

3. RESULTS

The two fat-soluble vitamins (A & E) and water-soluble vitamin C were analyzed using RP-HPLC/UV. After evaluating the different compositions of mobile phases, acetonitrile and water containing 1% phosphoric acid were selected as the optimum mobile for analysis of the three vitamins. These three vitamins are usually present either alone or in combination in different cosmetic products (both medicated and non-medicated), food products, and in pharmaceuticals or nutraceutical formulations. Diclofenac sodium was used as an internal standard. The elution order was vitamin C, A, E, and internal standard (diclofenac sodium) with retention times of 3.02, 4.67, 5.55 and 8.52 minutes, respectively.

3.1 Extraction

Vitamins A and E are insoluble in simple syrups. To facilitate the solubility of vitamin A and E, a base of simple syrup USP with a sufficient quantity of ethanol was added to make ethanol's final concentration 10 % in syrup. To this syrup base vitamin, A and E six different concentrations in the range of 0.05 to 2 µg/ml were added. Similarly, Vitamin C in the concentration levels 0.1 to 3.0 µg/ml was added to the syrup base. All three analytes were extracted from the syrup base with a simple liquid-liquid extraction technique. 0.1 % Phosphoric in acetonitrile was used as an extraction solvent. The three analytes were extracted in the same concentration ranges from the emulsion base and the mixture of powder containing starch and magnesium stearate. Diclofenac sodium with the final constant concentration of 0.5 µg/ml was added to all the standard solutions as well as the different matrices. The extraction scheme is given in supplementary Figure 1.

3.2 Linearity

The linearity of the three analytes in standard solutions (prepared in mobile phase) and extracted from the matrices were determined. The simple linear regression equation (y = a + bx) was used and r2 (correlation coefficient) was determined for all the analytes using MS Excel, 2016. All the analytes showed good linearity in standard solutions and the three different matrices. The details are present in Table 1 and Figure 1(showing the calibration curve).

Table-1. Calibration range, linearity, and sensitivity of the HPLC method for Vitamin C, A and E

	Vitamin C	Vitamin A	Vitamin E
Concentration	0.1-3.0 µg/ml	0.05-2.0 µg/ml	0.05-2.0 µg/ml
Linearity			
Standard mixtures			
Regression equation	y =1.517x + 0.091	y=2.817x + 0.088	y = 0.616x + 0.049
Correlation coefficient	0.999	0.999	0.998
Spiked samples in emulsion			
Regression equation	y =1.450x + 0.082	y=2.773x + 0.064	y = 0.557x + 0.048
Correlation coefficient	0.999	0.998	0.997
Spiked samples in powder			
Regression equation	y =1.484x + 0.061	y=2.748x + 0.091	y = 0.582x + 0.046
Correlation coefficient	0.997	0.999	0.999
Spiked samples in Syrup			
Regression equation	y = 1.456x + 0.095	y=2.385x+0.105	y = 0.519x + 0.034
Correlation coefficient	0.998	0.998	0.998
Sensitivity			
LOQ (µg/ml)	0.100	0.05	0.05
LOD (µg/ml)	0.036	0.015	0.023

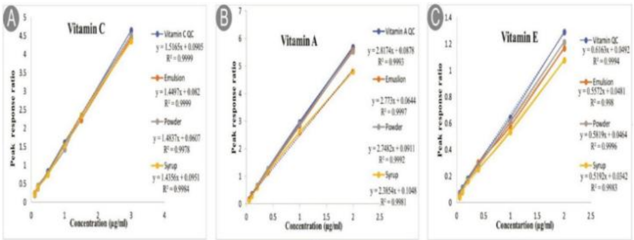

Fig. 1: Calibration curves of standard (prepared in mobile phase), spiked emulsion, powder and syrup A) vitamin C, B) vitamin A, and C) vitamin E.

3.3 Specificity

The chromatograms of the standard and spiked analytes in three matrices showed no interferences from the matrix. All the four peaks i.e. Vitamin C, A, E, and internal standard were well separated from one another. The resolution between the peaks was as;

Peak of Vitamin C and A, 8.19
Peak of Vitamin A and E, 2.93
Peak of Vitamin E and IS, 8.688

The chromatogram in Figure 2 shows all three analytes and internal standard in a standard sample prepared in mobile phase.

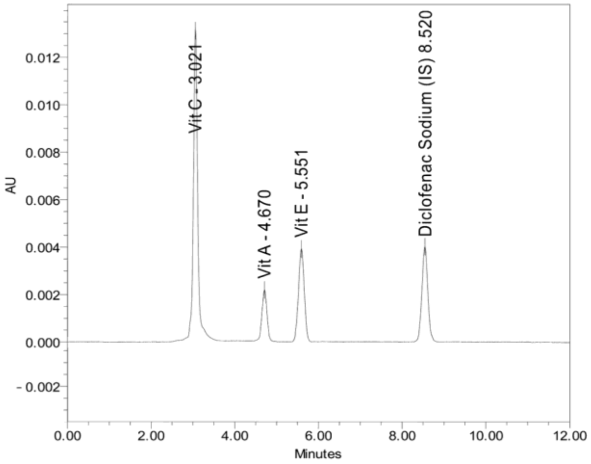

Fig. 2: Chromatogram showing peaks of Vitamins C (1.0 µg/ml), A (0.5 µg/ml), and E (0.5 µg/ml) in QC sample.

3.4 Accuracy

Accuracy was determined from the concentration recovered from the different matrices. The % recovery for the three analytes from the three matrices was calculated at four different concentration levels, including a high concentration level, two medium concentrations and a low concentration level (presented in Table 2). The four different concentration levels were used for spiking in the three different matrices and the extraction technique described in Figure 2 was used. The vitamin C was best recovered from the powder matrix with a recovery above 95%. The recovery of vitamin C from both syrup and emulsion was above 93 %. The Vitamin A recovery from emulsion, powder, and syrup was approximately above 95%, 97%, and 91%, respectively. The recovery of Vitamin E from emulsion, powder and syrup was approximately 93 %, 96 %, and 84 %, respectively. The % relative standard deviation (%RSD) values were within the range (not greater than 15 %) according to the ICH guidelines [27].

Table-2. Accuracy (recovery) of Vitamin C, A, and E from different matrices, each sample was analyzed as triplicate (n = 3)

	Concentration added (μg/ml)	Concentration recovered mean ± SD	% recovery mean ± SD	%RSD
Vitamin C				
Emulsion	3.0	2.870 ± 0.051	95.67 ± 1.71	1.778
	1.5	1.435 ± 0.013	95.69 ± 0.84	0.882
	0.5	0.476 ± 0.004	95.27 ± 0.82	0.863
	0.1	0.093 ± 0.002	93.33 ± 1.70	1.821
Powder	3	2.911±0.027	96.95±0.88	0.913
	1.5	1.435 ± 0.031	95.71 ± 1.99	2.087
	0.5	0.484 ± 0.005	96.86 ± 0.98	1.016
	0.1	0.095 ± 0.003	95.00 ± 2.94	3.099
Syrup (USP)	3	2.809±0.010	93.64±0.329	0.351
	1.5	1.423 ± 0.005	94.86 ± 0.34	0.358
	0.5	0.477 ± 0.004	95.43 ± 0.864	0.906
	0.1	0.0950 ± 0.001	94.87 ± 0974	1.027
Vitamin A				
Emulsion	2	1.910 ±0.008	95.48± 0.411	0.430
	1	0.955 ± 0.005	95.53 ± 0.531	0.556
	0.4	0.385 ± 0.005	96.33 ± 1.328	1.379
	0.1	0.097 ± 0.001	96.70 ± 0.374	0.387
Powder	2	1.938 ± 0.024	96.90± 1.21	1.244
	1	0.976 ± 0.005	97.60 ± 0.455	0.466
	0.4	0.392 ± 0.005	97.91 ± 1.247	1.274
	0.1	0.098 ± 0.001	98.40 ± 0.4	0.462
Syrup	2	1.825±0.019	91.23±0.93	1.021
	1	0.913±0.005	91.30±0.50	0.537
	0.4	0.368±0.008	91.91±1.94	2.111
	0.1	0.091±0.004	91.33±4.19	4.588
Vitamin E				
Emulsion	2	1.868±0.034	93.40±1.716	1.837
	1	0.937 ± 0.006	93.70 ± 0.589	0.628
	0.4	0.379 ± 0.003	94.66 ± 0.849	0.898
	0.1	0.094 ± 0.002	94.33 ± 2.05	2.178
Powder	2	1.935± 0.006	96.73 ± 0.306	0.317
	1	0.966 ± 0.005	96.60 ± 0.454	0.471
	0.4	0.387±0.005	96.75 ± 1.43	1.477
	0.1	0.097 ± 0.001	96.66 ± 1.247	1.290
Syrup	2	1.715±0.007	85.73 ± 0.37	0.432
	1	0.857±0.005	85.73±0.531	0.620
	0.4	0.340±0.004	85.00±0.891	1.047
	0.1	0.084±0.002	84.333±2.054	2.437

3.5 Precision

The precision of the method (intraday and interday) was calculated by measuring the quantity recovered as mean ± SD and the % relative standard deviation (% RSD). The results indicated an acceptable precision for all the analytes at the concentration given in Table 3. The % RSD for intraday was in range of 0.261-2.044 % for all the samples. The % RSD for inter-day precision varied from 0.762 to 3.819. The variation retention time was also evaluated. The inter-day mean ± SD (RSD) retention time (minutes) for Vitamin C, A, E and internal standard were 3.016 ± 0.012 (0.390), 4.673 ± 0.012 (0.266), 5.541 ±0.008 (0.148), and 8.530 ±0.002 (0.019), respectively. These values were well within limits according to international guidelines [28].

Table-3. HPLC method intraday and interday precision

Matrix	Vitamin	Concentration added to matrix	Intraday, (Concentration recovered) Mean ± SD (%RSD)	Interday (Concentration recovered) Mean ± SD (%RSD)
Emulsion	Vitamin C	2.5 μg/ml	2.38 ± 0.031 (1.281)	2.33 ± 0.058 (2.48)
		1 μg/ml	0.961 ± 0.007 (0.771)	0.950 ± 0.013 (1.33)
		0.5 μg/ml	0.478 ± 0.001 (0.261)	0.471 ± 0.006 (1.300)
	Vitamin A	1.5 μ/ml	1.435 ± 0.013 (0.881)	1.416 ± 0.021 (1.451)
		1 μg/ml	0.956 ± 0.004 (0.429)	0.952 ± 0.010 (1.029)
		0.2 μg/ml	0.190 ± 0.001 (0.497)	0.184 ± 0.007 (3.546)
	Vitamin E	1.5 μg/ml	1.44 ± 0.029 (2.044)	1.416 ± 0.034 (2.399)
		1 μg/ml	0.926 ± 0.004 (0.397)	0.921 ± 0.008 (0.842)
		0.2 μg/ml	0.189 ± 0.002 (1.136)	0.180 ± 0.004 (2.319)
Powder	Vitamin C	2.5 μg/ml	2.41 ± 0.029 (1.21)	2.37 ± 0.029 (1.212)
		1 μg/ml	0.964 ± 0.006 (0.595)	0.956 ± 0.010 (0.997)
		0.5 μg/ml	0.481 ± 0.003 (0.686)	0.474 ± 0.006 (1.291)
	Vitamin A	1.5 μg/ml	1.421 ± 0.009 (0.666)	1.404 ± 0.020 (1.435)
		1 μg/ml	0.974 ± 0.006 (0.633)	0.955 ± 0.018 (1.861)
		0.2 μg/ml	0.191 ± 0.002 (0.891)	0.184 ± 0.007 (3.819)
	Vitamin E	1.5 μg/ml	1.437 ± 0.021 (1.430)	1.410 ± 0.037 (2.654)
		1 μg/ml	0.955 ± 0.005 (0.520)	0.923 ± 0.007 (0.762)
		0.2 μg/ml	0.190 ± 0.001 (0.429)	0.185 ± 0.005 (2.894)
Syrup	Vitamin C	2.5 μg/ml	2.37 ±0.021 (0.868)	2.337 ± 0.029 (1.227)
		1 μg/ml	0.964 ± 0.006 (0.595)	0.956 ± 0.010 (0.997)
		0.5 μg/ml	0.480 ± 0.003 (0.687)	0.474 ± 0.006 (1.291)
	Vitamin A	1.5 μg/ml	1.353 ± 0.021 (1.518)	1.328 ± 0.033 (2.491)
		1 μg/ml	0.915 ± 0.013(1.443)	0.898 ± 0.009(1.013)
		0.2 μg/ml	0.182 ± 0.001(0.684)	0.177 ± 0.002(1.163)
	Vitamin E	1.5 μg/ml	1.282 ± 0.01 0(0.780)	1.258 ± 0.023 (1.832)
		1 μg/ml	0.852 ± 0.006(0.705)	0.829 ± 0.898(1.083)
		0.2 μg/ml	0.170 ± 0.002(0.960)	0.167 ± 0.003(1.76)

3.6 Sensitivity

The sensitivity was determined in terms of LOD and LOQ. A series of dilutions were prepared and analyzed by using the signal-to-noise ratio as described in the material and method section. The LOD and LOQ (lower limit of quantification) values are given in Table 1. The LOQ is low enough to quantify the concentration of the vitamins in different products.

3.7 Stability

Short-term stability of the extracted samples was evaluated at room temperature, 4 ˚C, and -20 ˚C. The percent loss concentration after 24 hours at room temperature for Vitamin C, A, and E are given in Supplementary Table 1. The percent loss at 4 ˚C was comparatively less than at room temperature. The % loss of all the samples stored at -20 ˚C was less than that of samples stored at 4 ˚C. Similarly, the samples were stable for one month when stored at -20 ˚C which also included three freeze-thawed cycles before analysis. There was a difference of 12 hours between each freeze-thawed cycle. The concentration recovered were compared with freshly prepared (extracted) spiked samples.

3.8 Dilution integrity

30 µg/ml of vitamin C and 10 µg/ml of vitamin A and E were diluted 10- and 20 folds in the spiked samples of all the three matrices. The accuracy (% recovery) was within ± 15 and precision (% RSD) did not exceed 15 %. The data is shown in Table 4.

Table-4. Dilution integrity of 10 times increased concentration of vitamin C, A, and E in the three matrices.

Matrix	Analyte	Dilution	Conc. recovered Mean ±SD	Accuracy (% recovery)	%RSD
Emulsion	Vitamin C (30 µg/ml)	10-fold	2.946 ± 0.136	98.22 ± 4.532	4.615
		20-fold	1.466 ± 0.051	97.70 ± 3.407	1.466
	Vitamin A (10 µg/ml)	10-fold	0.994 ± 0.104	99.43 ± 10.38	10.445
		20-fold	0.498 ± 0.026	99.63 ± 5.117	5.135
	Vitamin E (10 µg/ml)	10-fold	0.950 ± 0.098	95.00 ± 9.801	6.77
		20-fold	0.487 ± 0.015	97.43 ± 3.034	7.826
Powder	Vitamin C (30 µg/ml)	10-fold	2.861 ± 0.137	95.38 ± 4.551	4.772
		20-fold	1.450 ± 0.045	96.70 ± 2.986	3.087
	Vitamin A (10 µg/ml)	10-fold	0.923 ± 0.061	92.83 ± 6.086	6.594
		20-fold	0.481 ± 0.032	96.13 ± 6.381	6.637
	Vitamin E (10 µg/ml)	10-fold	0.944 ± 0.059	94.383 ± 5.996	6.352
		20-fold	0.487 ± 0.032	97.53 ± 6.311	6.471
Syrup	Vitamin C (30 µg/ml)	5-fold	2.838 ± 0.132	94.60 ± 4.400	4.651
		10-fold	1.457 ±0.072	97.11 ± 4.800	4.94
	Vitamin A (10 µg/ml)	5-fold	0.902 ± 0.066	90.20 ± 6.636	7.357
		10-fold	0.489 ± 0.036	97.83 ± 7.108	7.266
	Vitamin E (10 µg/ml)	5-fold	0.887 ± 0.054	88.65 ± 5.366	6.053
		10-fold	0.474 ± 0.036	94.70 ± 7.059	7.453

3.9 Application of the method

The developed method was applied for the analysis of the three vitamins in different commercial products including pharmaceuticals, cosmetic products, and food items. 500 µl was taken from liquid and semisolid/emulsion products. The tablets were triturated and 100 mg powder was weighed and further extraction procedure was performed. To each sample, the internal standard was added in sufficient quantity so that its final concentration remains 0.5 µg/ml. Adjustment to high concentrations of vitamins were made through dilution integrity.

The products analyzed are presented in Table 5 along with the quantities of vitamins present in these products. In the food products, the mango nectar juice and ketchup contained Vitamin C, A, and E. The mango nectar juice and ketchup (tomato ketchup) claimed to have only vitamin C. The vitamins A and E in these products may have been present due to the use of mango and tomato from the natural source. Vitamins A, E and C are naturally present in mangoes and tomatoes. Representative chromatograms of the samples analyzed are shown in Figure 3.

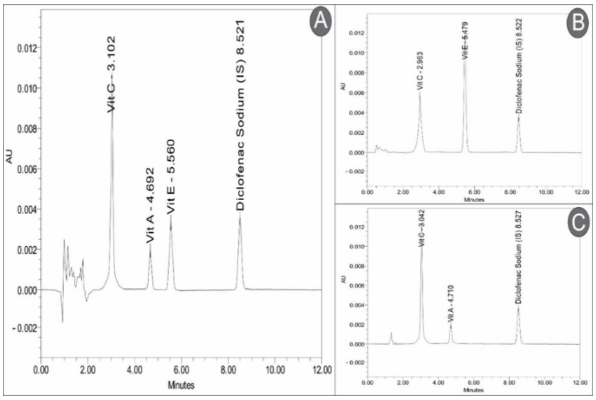

Fig. 3: Chromatograms of, (A) tablet sample containing all the three vitamins, (B) emulsion sample containing vitamin C and E, (C) Syrup sample containing vitamin C and A only. Internal standard (Diclofenac) is eluted in the last.

4. DISCUSSION

Vitamin A, E, and C are used in topical formulations, oral formulations (liquid and solid pharmaceutical formulation), and food products especially juices to compensate for their dietary deficiencies. Vitamins C and E have antioxidant properties and Vitamin A is considered an indirect antioxidant. The daily intake of these vitamins is necessary for people of all age groups. The vitamin supplements manufactured by the pharmaceutical and nutraceutical industry claim their quantities on the labels. Due to their antioxidant properties, these vitamins are used in topical preparations to improve the stability of the product as well as nourishment to the skin[29]. Cosmetics and juices most of the time only claim that certain vitamins are present without mentioning their quantities. The claim sometimes comes as "rich in vitamins". This is a problem in developing countries and in some countries misbranded and substandard products are also available in the market which do not follow the instructions of regulatory authorities.

A plethora of literature is available on the quantification of Vitamin A, E, and C. But mostly HPLC methods are developed for either fat-soluble vitamins or water-soluble vitamins separately. Review has been published regarding the extraction of vitamins from different matrices but most of the studies are either on single vitamins or same class of vitamins[30-32]. Similarly, non-chromatographic analysis of vitamins are also available but they are individual methods and do not involve simultaneous determination[33]. In literature one HPLC method was found for determination of eight water and fat soluble vitamins using multiple wavelengths and with variable range of percent recover from 78 to 116 %.[34] The previously reported method for the three vitamins by Paulo et al., has a longer analysis time and the switching of wavelength was another dry back as compared to the current method[25]. Klejdus et al., developed a gradient RP-HPLC method for water- and fat-soluble vitamins[26]. Moreno et al, also analyzed fat- and water-soluble vitamins using an isocratic method involving switching of wavelengths and solid phase extraction technique[34]. In this study, we developed a versatile reverse-phase HPLC method for a water-soluble vitamin (Vitamin C) and two fat-soluble vitamins (A and E). The vitamins were spiked in three different and complex matrices. The percent recovery from all the matrices was satisfactory except of Vitamin E from syrup which was below 90 %. Vitamin A recovery from syrup was also less but it was above 90 %. The comparatively low recovery of vitamin E from syrup may be attributed to its stability in syrup. Vitamin A and E have stability issues while vitamin C is more stable as compared to the two fat-soluble vitamins[35]. The recovery of vitamins A and E was very good from emulsion along with vitamin C. Vitamin A and E are more stable in topical formulations (emulsion type formulation) and they also enhance the stability of the product[29]. The ICH guidelines recommend the stability of samples at different temperatures for bioanalytical techniques. However, for the non-clinical dose formulations such stability studies are usually not performed. Henry et al., suggests that processed stability and freeze thaw stability can be performed to evaluate the sample and compare its concentration to freshly prepared samples[36]. The processed samples/extracted samples (processed stability) were stored at different temperatures as shown in table-4. Both short-term and long-term stability showed that the percent losses for all the three analytes were below 8 % when stored at -80 ˚C for 24 hours and one month. There is a recent study in which dilution integrity was performed for a non-bioanalytical technique[37]. The dilution integrity is usually performed for bioanalytical method validation but due to variable high and low concentrations of vitamins in different products in the current study the dilution integrity was successfully applied for quantification.

Table-5. Quantification of vitamin A, E and C in different products

	Formulation type	Vitamin C Claimed (quantity)	Vitamin C quantified	Vitamin A Claimed (quantity)	Vitamin A quantified	Vitamin E Claimed (quantity)	Vitamin E quantified
Pharmaceuticals	Tablet	C (60 mg/tablet)	59.4 mg/tablet	C (0.75 mg/tablet)	0.762 mg/tablet	C (100 mg/tablet)	99.56 mg/tablet
	Tablet	C (90 mg/tablet)	90.504 mg/tablet	C (1.5 mg/tablet)	1.513 mg/tablet	C (20 mg/tablet)	20.107 mg/tablet
	Tablet	C (72 mg/tablet)	72.347 mg/tablet	NC	ND	C (13.400 mg/tablet)	13.425 mg/tablet
	Syrup	C (125 mg/5 ml)	128 mg/ 5ml	NC	ND	NC	ND
	Syrup	C (20 mg/5 ml)	19.342 mg/5 ml	C (0.36 mg/5ml)	0.33mg/5 ml	6mg/5ml	5.92mg/ ml
	Syrup	C (50 mg/5ml)	48.765 mg/5ml	C (0.9 mg/5 ml)	0.887 mg/5 ml	NC	ND
Medicated cosmetics	Sunblock cream	C**	88 µg/g	NC	ND	C**	20 µg/g
	Cream	C**	51µg/g	C**	297 µg/g	C**	1021 µg/g
	Lotion	NC	ND	NC	ND	C**	53 µg/ml
	Moisturizer cream	NC	ND	NC	ND	C**	195 µg/g
	Face Wash	NC	ND	NC	ND	C**	24 µg/ml
Non-medicated cosmetics	Body scrub	C**	10 µg/g				
	Moisturizing lotion	C**	124 µg/ml	NC	ND	C**	13 µg/ml
	Face mask	NC	ND	NC	ND	C**	75 µg/ml
	Skin Care cream	NC	ND	C**	78 µg/ml	C**	95 µg/ml
	Beauty cream	NC	ND	NC	ND	C**	320 µg/ml
	Mango nectar juice	C	805 µg/ ml	NC	0.5 µg/ ml	NC	0.17 µg/ ml

*C=claimed, NC=not claimed, ND= not detected, C**=claimed but quantity not mentioned on label

5. CONCLUSION

In the present method, all the analytes were eluted within 9 minutes. The method showed a linear response for all three vitamins in standard solutions and the three different matrices. The method showed good sensitivity (LOQ and LOD), however, the concentration ranges used for the calibration curve were higher because usually, the concentration of these vitamins is higher in pharmaceuticals, topical products, and juices. Still, some concentrations in some commercial products were beyond the calibration range and the dilution integrity was successfully applied. The analysis time was shorter using a simple isocratic mobile phase and a single wavelength. All the peaks were well resolved. Even when the commercial products (cosmeceuticals, Pharmaceuticals, nutraceuticals, and food items) were analyzed there were no interferences from the matrices or the other claimed co-ingredients. The products were stable when stored at -20 ˚C in amber colored Eppendorf tubes for shorter period as well as for longer period.

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