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Short communication

# Separation and determination of Vitamins E and A in multivitamin syrup using micellar liquid chromatography and simplex optimization

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**Abstract**

A rapid method has been described for separation and determination of Vitamins A and E using micellar liquid chromatography (MLC). Influence of temperature of column and addition of organic modifiers on separation efficiency was investigated. A temperature of 30 °C and 1-butanol modifier was selected. Optimization of the parameters affecting the separation including percent of organic modifier, pH of the mobile phase, concentration of surfactant, and flow rate of the mobile phase was performed simultaneously using the super-modified simplex (SMS) procedure. Results showed that 11.7% 1-butanol, 76.9 mM sodium dodecyl sulphate (SDS), pH of 6.73 and a flow rate of 1.35 ml $min^{-1}$ are the best conditions for separation of these compounds. The analytical parameters including linearity, $r > 0.9990$; limit of detection 1.71 and 4.52 μg $ml^{-1}$ for A and E, respectively; precision of the method, R.S.D. < 2.85%; and recovery, more than 90%, show that the method is useful for measuring these compounds in pharmaceutical preparations.


*Keywords:* Micellar liquid chromatography; Fat-soluble vitamins; Sodium dodecyl sulfate; Super-modified simplex; Multivitamin syrup

## 1. Introduction

Vitamins are a diverse group of compounds, both chemically and analytically, because they comprise a range of biomolecules whose common properties reside solely in the fact that they are essential dietary components. These compounds are needed in relatively small amounts to sustain life and good health. Vitamins have been broadly divided into fat-soluble and water-soluble groups. Each of these groups has been further subdivided, mainly based on their functionalities [1]. Due to nutritional importance of fat-soluble vitamins, several high-performance liquid chromatographic (HPLC) methodologies have been reported for their determinations in foods, pharmaceuticals and body fluids [2–13]. In most of these methods, large amounts of organic solvents are used as the mobile phase, which result in an environmental impact.

In spite of its low efficiency, low sensitivity and relatively high detection limit, micellar liquid chromatography (MLC) has been recently developed as a useful chromatographic method. This is due to the ability of MLC for simultaneous separation of ionic and nonionic compounds, possibility of direct injection of biological fluids, such as serum and plasma, the enhancing effect of micelle formation on luminescence intensity, its low cost, low volatility of mobile-phase constituents and much less amount of toxic organic solvents (environmentally benign) [14].

The primary step in the development of MLC separations is to optimize the chromatographic performance through adjustment of the experimental parameters, such as temperature, and type and amount of the organic modifier [14]. HPLC separations are mainly affected by varying the composition of the mobile phase. The principal parameters in MLC are concentration of organic modifier, pH, and concentration of the surfactant [14]. Ordinarily, only one factor is varied at fixed values of the other parameters. The one factor at a time method requires a very large number of experiments. When there are many parameters and especially if the factors in-

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teract, the true optimum may not be achieved. A number of different experimental designs have been developed to reduce the number of experiments and amounts of chemicals. One of the most efficient optimization designs is the simplex method. Simplex optimization was first introduced in HPLC separations by Morgan and Deming [15,16]. The modified simplex procedure was developed to overcome the disadvantages of the original simplex [17]. The super-modified simplex (SMS) procedure, which was introduced by Routh et al. [18], is a modified version of the modified simplex method. Recently, we examined the performance of SMS method for optimization of separation of water-soluble vitamins using MLC [19].

In the present work, we report a method for separation and determination of fat-soluble Vitamins A and E using MLC. The method is rapid, facile and more environmentally benign because of using much lower amounts of toxic organic solvents. The effects of the dependent parameters including sodium dodecyl sulphate (SDS) concentration, pH, 1-butanol percent and flow rate of the mobile phase were optimized by the SMS method. The optimized method was applied to the analysis of these vitamins in a multivitamin syrup sample.

## 2. Experimental

### 2.1. Reagents

Analytical-grade retinol palmitate (Vitamin A) and tocopherol acetate (Vitamin E) were supplied by Roche (Basel, Switzerland). HPLC-grade methanol and acetonitrile, analytical-grade α-tocopherol and reagent-grade ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-2-butanol, 1-pentanol, 2-pentanol, SDS, sodium dihydrogen phosphate and sodium monohydrogen phosphate, all were purchased from Merck (Darmstadt, Germany). Doubly distilled, deionized water was used in all experiments.

Stock standard solution of each vitamin and α-tocopherol were prepared in 1-butanol containing 1% of ascorbic acid to provide a concentration of 30 mg ml$^{-1}$ for all the compounds. These solutions were freshly prepared each time and stored below 4 °C and protected from light. The solutions were diluted with ethanol to the desired concentration levels just before performing the analysis. A 500 mM stock solution of SDS was prepared in filtered water. Organic solvents and phosphate buffer were added to the mobile phase where necessary. All mobile phases and solutions were filtered using 0.45 μm Nylon membranes (Millipore, Bedford, MA, USA).

### 2.2. Apparatus

The chromatographic measurements were carried out with a crystal 200 series HPLC pump (ATI Unicam, Cambridge, UK) equipped with a Rheodyne (Cotati, CA, USA) Model 7125 manual injector with a 50 μl loop, a PU4225 UV detector (Philips, Cambridge, UK), a Spherisorb ODS analytical column (5 μm, 100 mm × 3.9 mm) from Unicam (Cambridge, UK) and a RP-18 guard column (7 μm, 15 mm × 3.2 mm) from Applied Biosystems (San Jose, CL, USA). The analytical column was water-jacketed and thermostated with a NB-33722 Ultra-Thermostat (Colora, Lorch, Germany). The chromatographic calculations were performed using a Unicam 4880 data handling system.

### 2.3. Sample preparation

Five millilitres of multivitamin syrup (Amin Pharmaceutical, Isfahan, Iran) was quantitatively transferred to a 25 ml volumetric flask. Two and a half millilitres of a 10 mg ml$^{-1}$ α-tocopherol was added to this solution as an internal standard and the resulting mixture was diluted with water. This solution was filtered through the 0.45 μm membrane and stored below 4 °C, protected from light.

## 3. Results and discussion

### 3.1. Temperature effect on efficiency

Although it is not generally possible to set valid rules for the influence of temperature on HPLC separations, but the column temperature affects pressure, analysis time and separation [20]. The lower efficiency of MLC as compared to conventional reversed-phase liquid chromatography (RPLC) is due to higher viscosity of micellar mobile phase. The efficiency of column could be improved at higher temperatures due to faster mass transfer of solute between mobile and stationary phases. However, the efficiency may decrease with temperature due to several reasons, such as decomposition of sample or mobile phase, risk of bubble formation, effect on temperature-dependent chromatographic equilibria and increasing of silica solubility [20]. The influence of column temperature on two basic parameters [21] including the number of theoretical plates, *N*; and asymmetry factor, *B*/*A*; was investigated in the range of 25–40 °C. The results are presented in Table 1. It can be seen that the most appropriate temperature is 30 °C for separation of these compounds. It is worth to mention that due to high lipophilisity of these compounds no peak was observed for micellar solution in absence of modifier, even at high surfactant concentration (ca. 200 mM).

Table 1
Effect of temperature on chromatographic performance of vitamins

| Temperature (°C) | $t_R$ (min) | | *B*/*A* | | *N* | |
|---|---|---|---|---|---|---|
| | A | E | A | E | A | E |
| 25 | 40.0 | 12.3 | 1.20 | 2.60 | 235 | 63 |
| 30 | 36.0 | 12.0 | 1.21 | 1.70 | 348 | 370 |
| 35 | 32.0 | 11.2 | 2.20 | 2.10 | 127 | 160 |
| 40 | 29.5 | 11.2 | 2.27 | 1.90 | 126 | 177 |

Conditions: mobile phase was 50 mM SDS and 10% (v/v) 1-butanol; 20 μl sample; flow rate, 2 ml min$^{-1}$; λ, 285 nm; AUFS, 0.05; column, 100 mm × 3.9 mm spherisorb ODS (5 μm).

Table 2
Effect of organic modifiers on chromatographic performance of vitamins

| Modifier | $t_R$ (min) | | *B/A* | | *N* | |
|---|---|---|---|---|---|---|
| | A | E | A | E | A | E |
| 1-Butanol | 36.0 | 12.0 | 1.21 | 1.70 | 348 | 370 |
| 2-Butanol | 40.3 | 11.3 | 2.20 | 2.10 | 79 | 79 |
| 2-Methyl-2-butanol | 53.2 | 15.6 | 1.90 | 16.0 | 33 | 43 |

Conditions: 50 mM SDS and 10% (v/v) of organic modifier as mobile phase and column temperature was 30 °C. Other conditions same as Table 1.

### 3.2. Effect of organic modifier on efficiency

Addition of alcohols to the micellar mobile phase reduces the film thickness of the surfactant molecules covering the stationary phase and thus, produces an enhancement in efficiency [22]. The presence of an alcohol in the micellar mobile phase also alters the retention mechanism by shifting equilibria of the solutes from the stationary phase, and from the micelle toward the bulk aqueous phase. This leads to a reduction in the capacity factors [23,24].

Different compounds including methanol, acetonitrile, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-2-butanol, 1-pentanol and 2-pentanol were examined as modifier. Among these solvents, only 1-butanol, 2-butanol and 2-methyl-2-butanol could elute vitamins. Other modifiers either did not show enough elution strength including methanol, acetonitrile, ethanol, 1-propanol and 2-propanol, or enough solubility in the mobile phase including1-pentanol and 2-pentanol.

Here, again the two basic parameters, *N* and *B/A*, were used for selection of the best organic modifier (Table 2). The results show that a considerable improvement in chromatographic efficiency was obtained by addition of the organic solvent to micellar mobile phase. The efficiency was increased in the following order for different modifiers:

1-butanol > 2-butanol > 2-methyl-2-butanol.

### 3.3. Simultaneous optimization of 1-butanol percent, SDS concentration, pH and flow rate of the mobile phase

The SMS program, which was written using GW-Basic, was used for optimization of the variables considered to be most effective for separation of the mixture of vitamins. These variables were: modifier percent (1-butanol), SDS concentration, pH and flow rate of the mobile phase. The chromatographic response function (CRF), given by Eq. (1), was chosen as the criterion for the optimization process [25]:

$$\mathrm{CRF} = \sum_{i=1}^{n-1} R_i + n^a - b|T_A - T_L| + c\,(T_1 - T_0) \qquad (1)$$

where $R_i$ is the resolution between adjacent peak pairs; $n$ the number of peaks detected; $T_A$ a specified analysis time (60 min in this work); $T_L$ and $T_1$ the retention times of the last and the first peaks, respectively; $T_0$ a specified minimum retention time (2 min in this work); and $a$, $b$ and $c$ operator-selectable weights that were selected to be 2, 0.2 and 0.1, respectively.

Table 3
The progress of SMS towards optimum in the analysis of vitamins

| Experiment number | SDS concentration (mM) | 1-Butanol (%, v/v) | pH | Flow rate (ml min$^{-1}$) | CRF |
|---|---|---|---|---|---|
| 1 | 50.0 | 7.0 | 3.00 | 2.00 | 11.20 |
| 2 | 70.0 | 8.0 | 4.00 | 2.00 | 8.96 |
| 3 | 85.0 | 9.5 | 5.50 | 1.50 | 17.79 |
| 4 | 100.0 | 12.0 | 7.00 | 1.00 | 18.91 |
| 5 | 60.0 | 11.0 | 6.00 | 2.25 | 18.10 |
| 6 | 77.5 | 11.8 | 6.75 | 1.38 | 27.53 |
| 7 | 77.4 | 11.7 | 6.71 | 1.38 | 27.10 |
| 8 | 71.4 | 9.83 | 5.31 | 1.67 | 17.58 |
| 9 | 77.8 | 10.7 | 6.00 | 1.58 | 17.38 |
| 10 | 83.3 | 11.4 | 6.60 | 1.49 | 18.00 |
| 11 | 81.4 | 11.2 | 6.39 | 1.52 | 18.32 |
| 12 | 100.0 | 12.0 | 7.00 | 1.00 | 18.91 |
| 13 | 81.3 | 10.9 | 6.22 | 1.53 | 17.45 |
| 14 | 78.1 | 12.1 | 6.83 | 1.55 | 26.70 |
| 15 | 77.4 | 12.3 | 6.96 | 1.56 | 26.30 |
| 16 | 82.0 | 11.5 | 6.47 | 1.78 | 16.70 |
| 17 | 79.0 | 11.6 | 6.62 | 1.52 | 17.53 |
| 18 | 80.4 | 11.6 | 6.55 | 1.64 | 27.10 |
| 19 | 81.1 | 11.6 | 6.52 | 1.70 | 17.00 |
| 20 | 78.3 | 11.7 | 6.70 | 1.46 | 27.40 |
| 21 | 76.9 | 11.7 | 6.73 | 1.35 | 27.97 |
| 22 | 76.8 | 11.6 | 6.54 | 1.62 | 17.10 |
| 23 | 81.8 | 11.7 | 6.70 | 1.44 | 18.10 |
| 24 | 83.2 | 11.7 | 6.71 | 1.29 | 17.46 |
| 25 | 84.4 | 12.2 | 6.99 | 1.28 | 18.10 |

The super-modified simplex program was started by introducing lower and upper boundary conditions for the above four variables; 1-butanol, 7–15%; SDS concentration, 50–100 mM; pH, 3–7; flow rate, 1–2.5 ml min$^{-1}$. The initial simplex consisted of the first five vertices (one more than the number of variables) that were chosen randomly. Experiments 1–5 in Table 3 show the values of the four parameters designated as the initial simplex conditions. The experimental and calculated CRF values are also presented in Table 3. Experiment number 21 indicates the optimum conditions with the highest CRF value. All calculations were performed using worksheets. The simplex was halted at experiment 25, as there was no further significant improvement towards maximization of the CRF value. The optimum conditions were found to be 76.9 mM SDS, 0.02 M phosphate buffer, pH 6.73, 11.7% (v/v) 1-butanol and a flow rate of 1.35 ml min$^{-1}$.Using the optimized mobile phase, a good separation of the vitamins was achieved within 20 min. Fig. 1 shows the chromatogram obtained under the optimized conditions.

### 3.4. Analytical parameters

The proposed method at the optimized conditions, i.e., column temperature 30 °C; a mobile phase containing 76.9 mM SDS, 11.7% (v/v) 1-butanol and 0.02 M phosphate buffer at

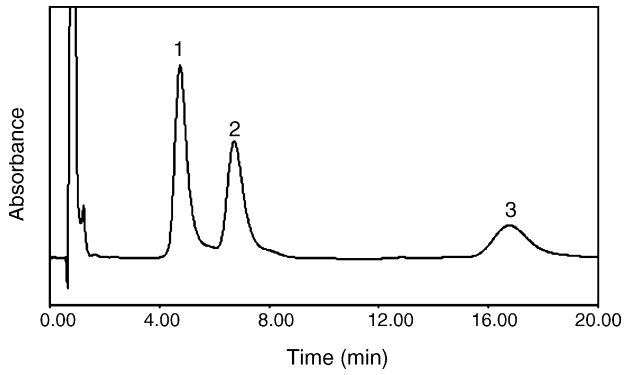


Fig. 1. Chromatogram obtained under the optimized conditions (vertex number 21). Conditions: mobile phase, 76.9 mM SDS, 11.7% 1-butanol (v/v), 0.02 M phosphate buffer pH 6.73; 20 μl sample; flow rate l.35 ml $min^{-1}$; 30 °C; λ, 285 nm; AUFS, 0.1. α-Tocopherol (1), Vitamin E (2), Vitamin A (3).

pH 6.73 and flow rate of 1.35 ml $min^{-1}$; UV detection at 285 nm and absorbance unit full scale (AUFS) of 0.1 has been validated with respect to repeatability, linearity, limit of detection, precision and accuracy by using standards and a reference sample supplied by Amin Pharmaceutical, and the results are presented in the following sections.

### 3.4.1. Repeatability

Complete analysis was performed in triplicate on two vitamins to calculate the average deviations as a measure of chromatographic reproducibility. The relative standard deviations obtained in the analysis of the vitamins are presented in Table 4. The results show relatively high precision of the proposed method at the optimized condition, as the standard deviations were <5% R.S.D.

### 3.4.2. Linear range

A series of five standard solutions at low- and high-concentration levels were prepared. Each solution was injected three times and regression analysis was performed by the method of least-squares. Table 5 summarizes the parameters of the calibration curves obtained by measuring peak areas for each vitamin eluted with micellar mobile phase. Linear regression coefficients ($r$) were always more than 0.9990 for the two vitamins.

Table 4
Quantification of Vitamins A and E

| Vitamin | Vitamin content (IU[a] 5 $ml^{-1}$) | Amount found[b] (IU 5 $ml^{-1}$) | S.D.[c] (IU) | R.S.D. (%) | Recovery (%) |
|---|---|---|---|---|---|
| A | 3500 | 3521 | 146 | 4.34 | 94 |
| E | 15 | 16.00 | 0.16 | 1.00 | 90 |

[a] International unit.
[b] Values are the average of triplicate analysis.
[c] Standard deviation of triplicate analysis.

Table 5
The result of linear least-squares analysis of the calibration data in the measurement of Vitamins A and E

| Vitamin | Linear range (mg $ml^{-1}$) | $r$ | Slope | Intercept | Detection limit (μg $ml^{-1}$) |
|---|---|---|---|---|---|
| A | 0.15–1.5 | 0.9990 | 2.3363 | 0.0317 | 1.71 |
| E | 2–8 | 0.9992 | 0.0183 | −0.0394 | 4.52 |

### 3.4.3. Limits of detection

The detection limits were assessed using internal standards. According to the calibration graphs, detection limits for each vitamin ($3\sigma$ criterion), were calculated using $S_{y/x}$ instead of $\sigma$ and intercept instead of blank signal [26]. The obtained values are presented in Table 5 and were well below those required for the analysis of the vitamins in pharmaceuticals. The method LODs are comparable with other similar hydro-organic HPLC methods that have been reported for the analysis of fat-soluble vitamins [7,10].

### 3.4.4. Precision

Method precision was determined by measuring repeatability (within-day precision) for each vitamin at the same concentration levels using the same reagents and apparatus. The precision of the method for determination of vitamins in multivitamin syrup regarding the coefficients of variation was excellent. The R.S.D. values obtained were 1.71 and 2.85% for Vitamins A and E, respectively.

### 3.4.5. Accuracy

Method validation regarding recovery was achieved using a standard vitamin sample and the results are illustrated in Table 4. The recoveries are more than 90% with the average R.S.D values not more than 2.90%.

### 3.5. Real sample analysis

In order to evaluate the applicability of the proposed method, multivitamin syrup was analyzed and results were compared with those reported by manufacturer. As can be seen in Table 4, there is a good agreement between the results obtained by the proposed method and reported values. Fig. 2 shows a typical chromatogram obtained for the multivitamin syrup.

In conclusion, application of the SMS optimization procedure together with advantages of MLC resulted in successful separation and determination of Vitamins A and E with good sensitivity, and less toxicity in a reasonable time. The results of the work confirmed the usefulness of the proposed method for analysis of these vitamins in pharmaceutical preparations. The method is green and uses minimum amount of toxic organic solvent.

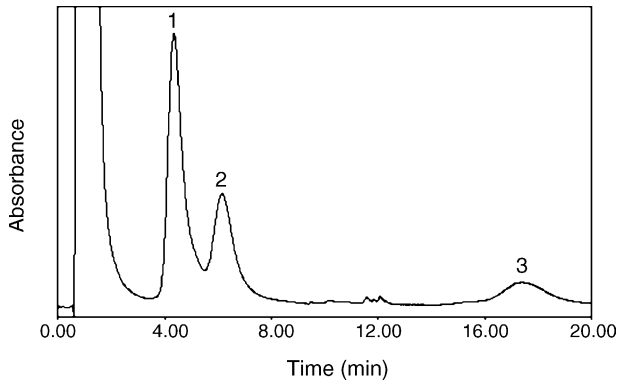


Fig. 2. The chromatogram of Vitamins A and E in multivitamin syrup. Conditions are the same as in Fig. 1.

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