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Research article

Simultaneous analysis of water-soluble and fat-soluble vitamins through RP-HPLC/DAD in food supplements and brewer's yeast

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ABSTRACT

The current study is focused on investigation and quantitation of seven commercially available on the Bulgarian market food supplements, containing multivitamin mixtures of water-soluble and fat-soluble vitamins. In addition, a second fermentation brewer's yeast is also analyzed. The analytical procedures are performed on a RP-HPLC/DAD using Purospher STAR C18 (Merck Millipore, Germany) 5 μm, 25 × 0.46 cm column, conditioned at 25 °C in a column oven. Dionex UltiMate 3000 high performance liquid chromatograph was carried out in diode array detector, set up at 270 nm for water-soluble vitamins, except for vitamin B_5, where 210 nm was applied as analytical wavelength. The fat-soluble vitamins were detected at 325 nm and 265 nm for vitamin A and vitamin E, respectively. Two general methods were developed where **Method 1** was based on gradient elution and **Method 2** was based on isocratic elution. Both methods identified stated by the manufacturer labeled amounts. The developed methods are applicable for routine analysis of vitamin contents both in multivitamin preparations and in brewer's yeast from secondary fermentation.

1. Introduction

Vitamins are a large group of low weight compounds that are involved in a numerous metabolic reaction and cellular processes in the human body. Depending on the physical property of solubility, these compounds are divided into two major groups: water-soluble and fat-soluble vitamins [1]. One of the biggest families of water-soluble vitamins is the B-group. This family consists of the substances thiamine (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_7), folic acid (B_9) and cyanocobalamin (B_{12}). Several B-group vitamins play an important role in human organisms mostly in the metabolic processes, but also as coenzymes to produce energy [2]. Additionally, vitamins are essential compounds to yeasts. Vitamins are involved in numerous yeast metabolic pathways, including those of amino acids, fatty acids, and alcohols, which suggests their notable implication in fermentation courses, as well as in the development of aromatic compounds [3].

Currently protein-rich yeast extracts have been often used as human dietary supplements, flavor enhancers or additives in fermentation processes and animal feed. Beyond the high protein content these extracts are also rich in amino acids, bioactive peptides and vitamins (B-vitamins, among others), which are responsible for some of the bioactive properties observed from these products [4].

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The highest challenge in estimation of group and self-amount of water-soluble B vitamin complexes is in the various types of matrices and specific factors related to the numerous food samples. In addition, the lack of significant interference risen from the matrix and the increased concentration variances of the analytes require the application of highly selective HPLC detectors [5].

The complicated nature of the sample matrix in brewer's yeast and the wide range in the concentrations of the different vitamins, contained in the evaluated mixtures along with the presence of both water-soluble and fat-soluble representatives is the main drawback in analysis of non-standardized mixtures. In addition, the presence of several biologically active forms of the vitamins, along with their chemical instability and heterogeneity is making their simultaneous analysis rather difficult [6]. Thus, the development and validation of applicable economically friendly, fast and reliable analytical procedure is essential and challenging task for researchers [7].

The dietary intake and/or nutritional care in each country may be generally supported in providing the best Food Composition Table requirements with accurate and reliable HPLC methods for estimation of vitamin B complex contents in foods and food supplements [8].

In addition, a number of biological functions in the body have been associated with the fat-soluble vitamins with an impact on the physiological functions of vitamins A and E [9-12]. The phytochemicals contained in the fat-soluble vitamins are known to express antioxidant, antibacterial, antifungal, antiviral, and anticarcinogenic properties [13], where an increased concentrations of α-tocopherol, are associated with a reduced risk of appearance of health disorders related to free radical accumulation, including cell damage, cancer and atherosclerosis, among others [14,15].

The quality control of multivitamin preparations in terms of vitamin content is very important due to the various pharmacological effects of vitamins. A great necessity for strict follow up on the substance content in the marketed preparations is the instability of vitamins during manufacture and storage [16,17]. A cornerstone in multivitamin analysis is the fact that each vitamin has its own unique structure affecting its physicochemical properties, which often causes serious drawbacks in simultaneous multivitamin mixture analysis [18]. The decreased amounts of the analytes along with heterogeneity of the matrices is another common constraint in multivitamin analysis, thus requiring precise and reliable analytical methods, able to identify small amounts and even traces of analytes in multicomponent samples. This is a challenging task in selecting an appropriate analytical technique often requiring the application of the more complex and demanding high resolution mass spectral (MS) methodologies and gas chromatography coupled with mass spectrometry (GC-MS) methods [19].

The performed literary survey indicated insufficient information on existence of appropriate HPLC-DAD methods for analysis of mixtures of more than 4 water-soluble vitamins in multivitamin preparations, foods, food supplements and other related samples, with the only alternative being the more sophisticated and unfeasible for routine analysis LC-MS methods [20-22].

Some literary data indicate the application of C18-based solid phases for simultaneous differentiation of water-soluble vitamins from complex matrices, requiring the usage of FLD, ECD and MS/MS as detection techniques [23-25]. These methods obtained good separation and optimal quantitation, but unfortunately are economically unprofitable; require highly qualified personnel and special working conditions, which increase the cost and timeframe of the analysis.

In addition, attention should be also pointed to the contents of the used mobile phase since the reagents used may cause problems with co-elution, base-line and time drifts, complicated backgrounds, etc. [7,26].

This determined the objective of this study to develop and apply a fast, precise and sensitive RP-HPLC/DAD method for routine qualitative and quantitative analysis simultaneously of water-soluble and fat-soluble vitamins in multivitamin preparations, food supplements and brewer's yeast.

2. Materials and methods

2.1. Chemicals, reagents, and preparations

Thiamine hydrochloride (98.5–101.5%), Riboflavin (98%), Nicotinamide (98%), D-biotin (99%), Folic acid (99%) and cyanocobalamin (99%) were purchased from Fisher (Germany). Vitamins B_5 (Pantothenic acid) and B_6 (Pyridoxine hydrochloride) were purchased from Chem-Lab (Zedelgem, Belgium) and Fluorochem (United Kingdom) respectively. HPLC grade methanol, acetonitrile, chloroform (≥99.9%, containing amylenes as stabilizer) and salts for preparing the buffer solution were supplied by Sigma-Aldrich (Steinheim, Germany). Water used throughout the analyses was from a Millipore system.

2.2. Buffer preparation

For buffer preparation, 5.04 g of Disodium hydrogen phosphate and 3.01 g of Potassium dihydrogen phosphate were dissolved in distilled water, and the volume was made up to 1000 mL with distilled water. Orthophosphoric acid was used to adjust the pH to 4. The buffer was filtered through a membrane filter (0.20 μm) using a Millipore glass filter holder.

2.3. Sample preparation

2.3.1. Preparation of standard solutions

Stock solutions containing vitamins B_1 (100 μg/mL), B_2 (2 mg/mL), B_3 (2 mg/mL), B_6 (2 mg/mL) and B_{12} (50 μg/mL) were prepared by dissolving accurately weighed amounts of reference standards in distilled water. Stock solutions of vitamins B_5 (2 mg/mL), B_7 (100 μg/mL) and B_9 (100 μg/mL) were diluted in a buffer with pH 9. Thereafter, accurate amounts of the eight individual stock

solutions were diluted with distilled water to obtain 6 μg/mL (vitamin B_{12}), 20 μg/mL (vitamins B_1; B_7; B_9), 200 μg/mL (vitamin B_3) and 400 μg/mL (vitamin B_2; B_5; B_6). Individual standard solutions of fat-soluble vitamins A (100 μg/mL) and E (1 mg/mL) were prepared with chloroform. The solvent for further dilution to work concentrations was mobile phase.

2.3.2. Preparation of samples containing water-soluble vitamin mixtures

The analyzed multivitamin preparations were purchased from the local market and are summarized in Table 1. All pharmaceutical formulations were capsules, except the Phyto pharma product, which was soft gelatin capsules. For sample preparation, the capsule contents were ground into fine powder by porcelain mortar and an appropriate amount was dissolved in 10 mL distilled water. Prior to injection, all samples were filtered using PVDF syringe filter, 25-mm diameter, 0.22-μm porosity.

2.3.3. Preparation of samples containing fat-soluble vitamin mixtures

Three food supplements containing retinol and tocopherol were purchased (Table 1). The soft gelatin capsules were cut off and their contents were dissolved in chloroform. The following dilution to the work concentration was prepared with the mobile phase and filtered through the above-mentioned filter.

2.3.4. Yeast sample preparation

In this study, brewer's yeast from secondary fermentation was used. An appropriate amount of 1 g of the yeast was weighted on an analytical balance. For pretreatment of the sample, 26 mL 1 N NaOH was added and the obtained solution was incubated for 1 h at 50 °C [27]. Further on, the solution was acidified with HCl to pH 5.6 then was centrifuged at 4000 rpm for 5 min and the supernatant was processed by liquid-liquid extraction (LLE). The water fraction was double filtered consequently firstly through 0.47 μm PVDF syringe filters followed by filtration through 0.22 μm PVDF syringe filters. 10 μL of the obtained solution was injected into the HPLC system to analyze the water-soluble vitamins. The chloroform fraction was treated using the same steps to analyze the fat-soluble vitamins contents.

2.4. Chromatographic system and analytical procedures

The HPLC system consists of UltiMateDionex 3000 SD pump connected to UltiMateDionex DAD 3000 detector, all from Termo Fischer Scientific® San Jose, CA, USA. The HPLC separations were performed on a reversed-phase Purospher STAR C18 (Merck Millipore, Germany) 5 μm, 25 × 0.46 cm column, conditioned at 25 °C in a column oven. Data were recorded and evaluated by Chromeleon 7.2 software (Thermo Fisher Scientific, San Jose, CA, USA). The applied mobile phase for separation of water-soluble vitamins consists of solvent A: phosphate buffer (pH = 4): acetonitrile (98:2, v/v) and solvent B: methanol:dist. water (50:50, v/v). A gradient elution program was used as follows: 0–3 min eluent B increased to 20% from 15%; 3–30 min eluent B increased to 30% while eluent A decreased linearly; 30–31 min eluent B decreased to 15% and finished with 85% solvent A and 15% solvent B an isocratic elution for 4 min. The total run time was 35 min with 0.8 mL/min flow rate and 20 μL injection volume. For separation of fat-soluble vitamins was used isocratic mode for 20 min with a mobile phase consisting of acetonitrile:methanol (98:2, v/v). For this method, the flow rate was set to 1 mL/min with injection volume of 20 μL.

2.5. Validation procedures

Both methods were validated following the ICH guidelines Q2 (R1) with respect to accuracy, repeatability, intermediate precision, range, linearity and specificity [28].

2.5.1. Accuracy

Accuracy was determined by analysis of three different levels of the target concentration (50%, 100% and 150%). Each level was prepared in triplicate and their peak areas were used to calculate the average recovery using the following formula: Recovery % = (amount found/amount added) × 100. To evaluate the accuracy of the proposed method, percentage recovery and %RSD values should be ranging from 98.0% to 102.0%, and not more than 2.0%, respectively.

Table 1
Analyzed multivitamin products.

Multivitamin product	Label content
Probien b-complex, Fortex, Bulgaria	B_1, B_2, B_3, B_6·B_{12}
Magnematrix, Vitagold, Bulgaria	B_1, B_2, B_5, B_6 and Magnesium
Vitamins B-complex + Folic acid, Danhson, Bulgaria	B_1, B_2, B_3, B_5, B_6, B_7, folic acid, B_{12}
B-complex, Phyto pharma, Bulgaria	B_1, B_2, B_3, B_5, B_6, B_7, B_9, B_{12}
Multivitamin product	**Label content**
Geritamin, Teva, Bulgaria	Vit A, Vit E
Gericaps, Adipharm, Bulgaria	Vit A, Vit E
Vitamin E, Phyto pharma, Bulgaria	Alpha-tocopheryl acetate

2.5.2. Linearity

Linearity of the methods was determined by injecting five concentrations, covering about 50%, 75%, 100% 125% and 150% of certain vitamins. In order to prove the linearity of a method after using the method of the calibration curve, the correlation coefficient must be above 0.99.

2.5.3. Precision

For investigation of within-day repeatability and between-day precision, six samples of individual standard solutions at 100% level were analyzed in 1 day (repeatability) and in three consecutive days (between-day precision) to calculate the relative standard deviation, which was expected to be $\leq$2. The specificity is defined as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

2.5.4. Specificity and sensitivity

Specificity is the ability to distinguish the analyte in the presence of other components in the sample matrix. Specificity of the methods was detected by injecting solutions of standard, sample and blank separately. To evaluate the sensitivity, limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration graph, according to the equations: LOD = 3.3 × (sa/S); LOQ = 10 × (sa/S), where sa is the standard deviation of the intercept a and S is the slope.

3. Results and discussion

Thus far, various extraction techniques have been developed to optimize the isolation of a certain analyte from the matrix. Nowadays, several liquid and gas chromatography methods coupled with mass spectrometry (MS) have been widely used for the determination of fat- and water-soluble vitamins because of their higher accuracy, selectivity, and sensitivity compared with classic methods permitting the detection of vitamins in traces [29,30]. High-resolution MS methodologies have little been used for routine analysis of vitamins but constitute a promising technique that will replace the conventional MS and tandem mass spectrometry as the determination of all the vitamins in a single run will be possible [19]. However, the application of MS detection and/or MS/MS detection in a routine analysis has its drawbacks and limitations, requiring costly apparatuses, skilled technicians and the limitations in recognizing hydrocarbons that generate parallel ions. In addition, this type of detectors are unable to separate optical and geometric isomers [31].

Based on the close chemical and physicochemical properties of water-soluble group B vitamins and the wide range of their concentrations in multivitamin preparations, along with high price of the sensitive but costly MS detector, the development and validation of fast, sensitive, and applicable for routine analysis RP-HPLC/DAD method would be useful tool for pharmaceutical practice and in manufacture of food supplements, beverages and other types of commercial multivitamin products.

3.1. Methods development

Various marketed multivitamin products are currently available, containing a mixture of water-soluble and fat-soluble vitamins and needing of a simplified method for quality control [32]. In response to this we developed an RP-HPLC/DAD procedure based on a single stationary phase, with changes only in the elution type and mobile phase parameters, allowing application of common solvents as mobile phase constituents.

A number of mobile phases and various elution modes were applied, attempting to achieve good separation for both water-soluble and fat-soluble vitamin mixtures [33,34]. The results from the performed analytical procedures determined that two different methods for the two groups of vitamin mixtures are necessary. This perplexity was due to the limitations in the selectivity arousing from the high number of water-soluble vitamins content in the mixture (a total of 7 per item). Thus the method development procedure included an initial determination of a suitable stationary phase applicable to the analysis of both types of vitamins and subsequent establishment of a proper elution mode and mobile phase composition.

Firstly, our development procedure was aimed at identification of a suitable reversed phase analytical column allowing for the separation to be done on one and the same stationary phase. A number of stationary phases were tested: Phenomenex Luna C18 [35], aminopropyl-loaded silica gel column in the reverse-phase mode [36], Phenomenex Gemini C18 110 A column (Nova 2013), whereas as most appropriate for the retention and separation of both water-soluble and fat-soluble vitamin mixtures was found to be Purospher STAR C18 (Merck Millipore) 5 μm, 25 × 0.46 cm column assuring optimal retention and separation of the evaluated mixtures.

The next step of our development was focused on identification of proper elution mode and mobile phase.

3.1.1. Development of analytical procedure for analysis of mixture of water-soluble vitamins - Method 1

The basic differences in the physicochemical properties of the studied water-soluble vitamin mixtures determined the method development as the most challenging task in the current study. Additional point break was found to be the wide range of concentration levels of the vitamins in the evaluated preparations. The method was developed and optimized by evaluating the changes in chromatographic conditions including stationary and mobile phase contents, flow rate, temperature and wavelength of the detector. The selection of an optimal mobile phase was based on evaluation of the behavior of the analyzed vitamins. The close polarity of the studied molecules defined the necessity of buffering the mobile phase, which was achieved by introduction of the corresponding phosphate buffer into its composition. A series of trials of various pH values (from 2 to 7) of the applied phosphate buffer solutions were performed. The results were found to be consistent with recently published data [22] that the retention times of vitamins B_1 and B_6

strongly depend on the pH of the mobile phase, while the retention of vitamin B_3 is not affected. As most appropriate for separation of these 3 components was determined to be the phosphate buffer with pH = 4.0.

As organic modifiers methanol and acetonitrile were tested, where the best selectivity was obtained by their mixture. A gradient elution mode consisting of a mobile phase mixture of solvents A and B in different ratios was applied for the optimal retention of the components in the total mixture. The solvent A is a mixture of phosphate buffer (pH = 4.0):acetonitrile (98:2, v/v) and solvent B is methanol:distilled water (30:70, v/v), following the gradient program enlisted in materials and methods section. The applied gradient approach assured good and suitable separation of all water-soluble vitamin components in the evaluated mixtures.

3.1.2. Development of analytical procedure for analysis of mixture of fat-soluble vitamins - Method 2

The simultaneous liquid chromatographic analysis of water- and fat-soluble vitamins is challenging because of their wide polarity range. Most of the techniques focus on separating either fat- or water-soluble vitamins and only few methods are intended to separate lipophilic and hydrophilic vitamins simultaneously.

Our experiments determined that the developed gradient approach for separation and evaluation of mixtures of water-soluble vitamins is inapplicable for separation of mixtures of fat-soluble vitamins. In this means we developed an isocratic analytical procedure for analytical determination of vitamin A and vitamin E as single preparations and in a mixture. During the development procedure various mobile phases, flow rates, temperatures and wavelengths were rated and the most appropriate were determined to be the following conditions: column temperature 25 °C, mobile phase acetonitrile:methanol (98:2, v/v), flow 1 mL/min, run time - 20 min, injection volume 20 μL and wavelengths 265 nm (vitamin E) and 325 nm (vitamin A).

3.2. Methods validation

The developed RP-HPLC methods were validated according to the following parameters: specificity, linearity, precision, accuracy and sensitivity.

Analysis of the placebo solution demonstrated that it does not give interfering peaks at the retention time of the vitamins in standard solution, which proved the methods' specificity. To evaluate the linearity, linear regression to process the calibration plot was applied, with a subsequent calculation of the linear equation and the correlation coefficient. The results of R^2 presented in Table 2 indicated good correlation between the peak areas and the range of the concentrations analyzed in the concentration ranges. Calibration curves and linear equations were also used for calculation the values of LOD and LOQ. The accuracy of the developed methods was evaluated by calculating the mean percentage recovery of individual standard solutions from three different concentration levels (50, 100, 150%), injected three times. The percentage recovery was in the range 97.6–101.0 (Table 2) which revealed that the methods are accurate. To access the within-day repeatability and between-day precision, relative standard deviations were calculated and the results show that the developed methods were precise within the acceptable limits as presented in Table 2.

3.3. Method application

The developed and validated methods were further applied to vitamin analysis in commercially available food supplements in various dosage forms. The extraction procedures were developed and adjusted for the particular pharmaceutical formulation, i.e. tablets, capsules and food supplements, including brewer's yeast.

3.3.1. Method application: vitamins assay in commercial multivitamin preparations of water-soluble vitamins

Based on the available information, the capsules were processed by saturation and homogenization. An accurate amount of each powder formulation was dissolved in distilled water. To improve the stability and absorption of pantothenic acid, d-biotin and folic acid, their calibration and work standard solutions were diluted with buffer pH = 9.0. Prior to injection, all samples were filtered using a PVDF syringe filter.

For the objective of the sample analysis, the wavelength was also evaluated. Chromatograms were recorded in the 200–400 nm range using DAD. Seven of the water-soluble vitamins absorb UV at 270 nm, except for pantothenic acid. On the other hand, absorption at 210 nm provides much cleaner chromatograms of vitamin B_5 as it is presented in Fig. 1. Therefore, we selected 270 nm and 210 nm

Table 2
Validation data for Method 1 and Method 2.

Vitamin	Range (mg/mL)	Recovery (%)	Repeatability	Intermediate precision	R^2	LOD (μg/mL)	LOQ (μg/mL)
B_1	0.025–0.125	99.4–100.1	0.745	0.935	0.999	4.00	12.00
B_2	0.200–1.000	98.9–100.1	0.953	1.153	0.999	4.47	13.45
B_3	0.100–0.500	99.7–101.0	0.536	0.894	1.000	0.03	0.08
B_5	0.200–1.000	99.1–100.3	0.614	1.288	0.999	9.23	27.30
B_6	0.200–1.000	98.7–100.0	0.946	1.371	0.996	6.38	19.14
B_7	0.003–0.015	98.5–100.2	1.037	1.946	0,999	0.12	0.37
B_9	0.010–0.050	98.8–100.0	1.164	2.191	0.997	1.40	4.20
B_{12}	0.003–0.015	97.6–99.9	1.269	2.345	0.996	0.36	1.10
A	0.010–0.050	99.6–100.9	0.543	1.424	0.997	1.22	3.70
E	0.200–1.000	99.7–100.1	0.379	0.986	0.999	0.86	2.60

as the most adequate wavelengths.

The proposed method identified for some of the analyzed food supplements only traces of vitamin B_{12}. This may be due to the low concentration of the substance along with the multistage sample preparation. Thus the sample preparation procedure in this case should be optimized additionally.

3.3.2. Method application: vitamins assay in commercial multivitamin preparations of fat-soluble vitamins

Sample treatment procedure for fat-soluble vitamins started with a release of the tested vitamins from the investigated dosage forms, which were in a form of soft gelatin capsules. After cutting the latter, the gelatin shell was washed carefully with chloroform and dissolved to the work concentration with the same solvent [37]. The further processing of the working solution showed that chloroform is not an appropriate solvent for this chromatographic system, due to worsening in the peak appearance. We consider this to be due to the poor miscibility of the solvent and the higher polar mobile phase of acetonitrile and methanol. Consequently, the working solution was further processed with a fresh filtered mobile phase and 20 μL was injected into the HPLC system.

Based on literary data [38] confirmed additionally with our investigations, the maximum UV absorbance of the individual vitamin were selected as detection wavelengths. Vitamin A was examined at 325 nm, while the Vitamin E absorption was negligible. Therefore, tocopherol was recorded at 265 nm (Fig. 2).

3.3.3. Method application: vitamins assay in brewer's yeast

Brewer's yeast has been proposed mainly as a nutritional supplement due to the fact it is inexpensive source of vitamins, nucleic acids and β-glucans [39]. A great number of pretreatment methods of vitamins are developed [40-43] where the general pretreatment techniques, including liquid–liquid extraction (LLE), solid-phase extraction (SPE), ultrasonic assisted extraction (UAE), supercritical fluid extraction (SFE), SPE, LLE and dispersive liquid–liquid microextraction (DLLME) methodology [44-49]. Nevertheless, it is obvious that the sample extraction and purification approach is of vital importance for the proper pretreatment process, since it can significantly improve the analytical performance in means of selectivity, sensitivity, accuracy etc. [50]. In addition, the utilization of more advanced eco-friendly and effective sample preparation techniques can significantly decrease the personnel costs and time consumed, but still the applied pretreatment methods heavily depend on the type of matrix used [50].

There is a limited number of publications related to simultaneous identification and quantitation of water-soluble and fat-soluble vitamins contained in secondary fermentation brewer's yeast extracts. As already mentioned, some literary survey is related mainly to the extraction and purification techniques [40–49]. Other contributes to identification of other biologically active substances contained in these matrices as polyphenols [51], palmitoleic acids and triacylglycerols [52]. This pointed our attention towards development of a methodology which would allow identifying and simultaneously analyzing water-soluble and fat-soluble vitamins content in secondary fermentation brewer's yeast extracts.

The applied purification procedure of analyzed brewer's yeast consists of alkaline hydrolysis with subsequent neutralization to pH 6.8 with 1 N HCl. For the removal of the interfering fraction the prepared neutralized solution was centrifuged until the clear supernatant was obtained. In an attempt to improve the quality of the solution the method was optimized by including liquid-liquid extraction. Different combinations of solvents were applied and water: chloroform was selected as most appropriate for the analyzed vitamins. Both fractions were double filtered through PVDF syringe filters (firstly through 0.47 μm, and through 0.22 μm).

The purified by the above developed procedure samples were chromatographed under the conditions following the developed by us **Method 1** for evaluation of water-soluble vitamin contents. The results of the multicomponent analysis in the brewer's yeast are

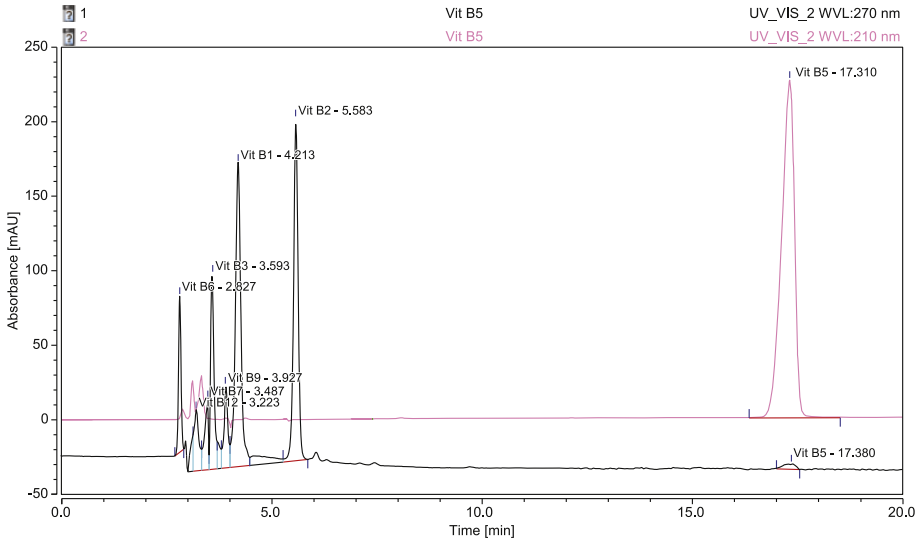

Fig. 1. Representative chromatogram of the RP-HPLC/DAD analysis of mixture of all evaluated water-soluble vitamins in two wavelengths.

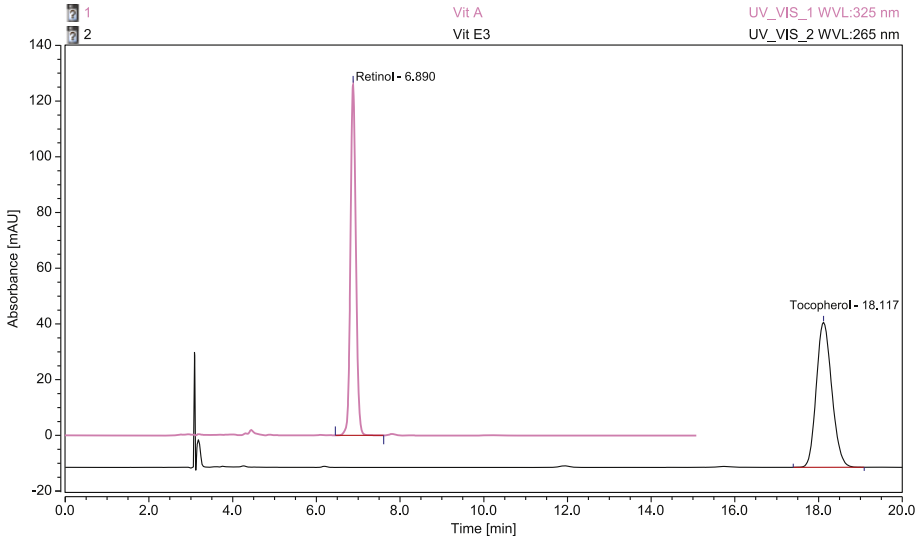

Fig. 2. Representative chromatogram of the RP-HPLC/DAD analysis of mixture of all evaluated fat-soluble vitamins in two wavelengths.

presented on Figs. 3 and 4.

The chromatogram recorded at 270 nm identifies a total of seven peaks corresponding to the retention times of the expected pyridoxine (B_6), cyanocobalamin (B_{12}), d-biotin (B_7), niacin (B_3), folic acid (B_9), thiamine (B_1) and riboflavin (B_2), respectively. An additional chromatogram was recorded at 210 nm to detect the expected pantothenic acid (B_5) (Fig. 4).

Thus, the obtained results demonstrate that the analyzed 1 g of brewer's yeast contains all of the evaluated B-group vitamins in the amounts given in Table 3.

In addition, the chloroform fraction was also evaluated by the developed **Method 2** for identification and quantitation of lipid-soluble vitamins in the brewer's yeast. The results are presented on Fig. 5.

The results identified the presence of vitamins A and E in contents given in Table 3.

A certain drawback and limitation of our study is related to the limited number of water soluble vitamins, successfully differentiated in the brewer's yeast extract. Unfortunately, after a high number of experiments we managed to optimally divide only 7 water-soluble vitamins. The authors believe that the main reason being co-elution of the non-dividable substances with close lipophilicity, along with possible interactions between them.

Another limitation is considered to be the very low amount of some of the analytes (as B_{12}) which requires additional processing of the mixture.

The obtained results determined the developed RP-HPLC/DAD methods for evaluation of water-soluble and fat-soluble vitamin mixtures in food supplement preparations and brewer's yeast from secondary fermentation as applicable for routine control.

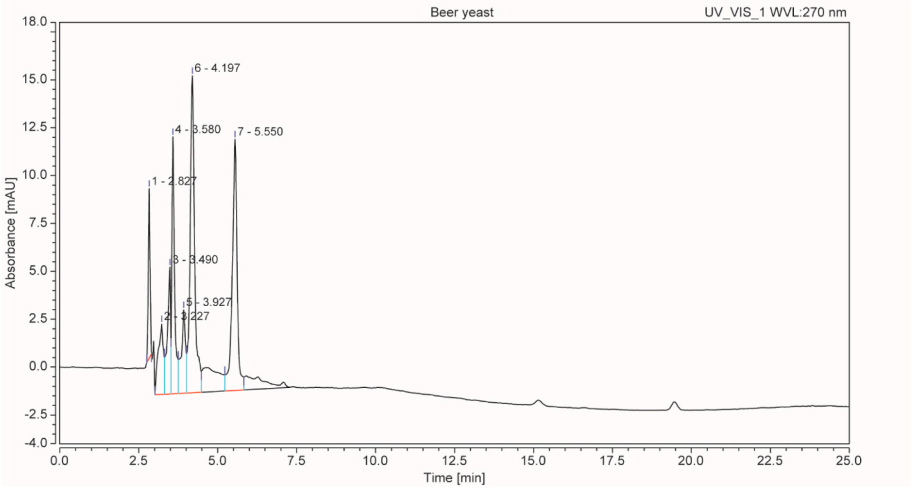

Fig. 3. Representative HPLC-DAD chromatogram of the analysis of the content of water-soluble vitamins in the evaluated brewer's yeast at 270 nm.

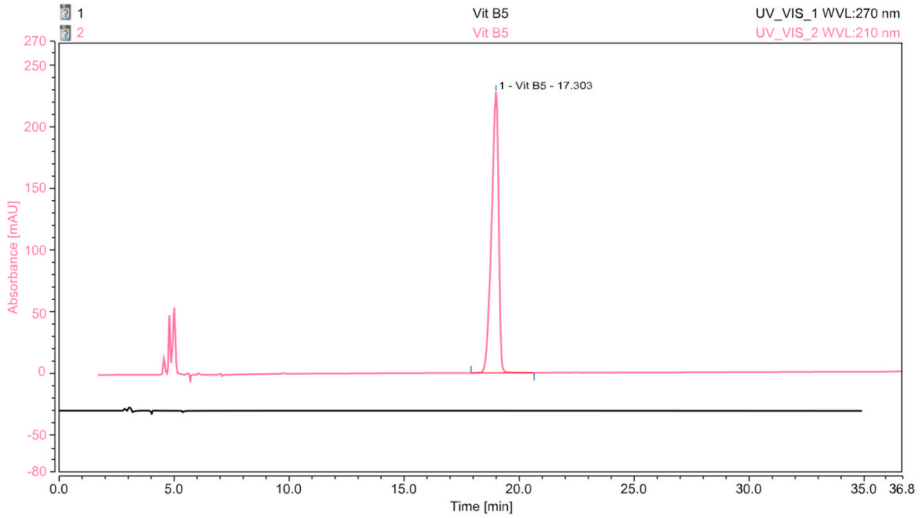

Fig. 4. Representative HPLC-DAD chromatogram of the analysis of the content of water-soluble vitamin B_5 in the evaluated brewer's yeast at 210 nm.

Table 3
Determined vitamin content in commercial preparations and brewer's yeast, presented along with method recovery (n = 3).

		B_1	B_2	B_3	B_5	B_6	B_7	B_9	B_{12}	A	E
Probien b-complex	Label mg	1.5	1.5	5	–	1.5	–	–	0.0016	–	–
	Found (mean value ± SD)	1.46	1.42	4.87	–	1.46	–	–	[a]	–	–
		0.027	0.015	0.076		0.042					
	Recovery (%)	96.0	94.9	97.4	–	96.0	–	–	–	–	–
Magnematrix	Label mg	2.1	2.1	–	6	4.5	–	–	–	–	–
	Found (mean value ± SD)	2.00	1.98	–	6	4.46	–	–	–	–	–
		0.031	0.074			0.096					
	Recovery (%)	94.3	94.3	–	100.0	99.11	–	–	–	–	–
B-complex, Danhson	Label mg	4.2	4.8	18	18	6	0.6	0.45	0.003	–	–
	Found (mean value ± SD)	4.17	4.78	17.99	17.83	5.95	0.584	0.442	0.0028	–	–
		0.078	0.067	0.149	0.222	0.093	0.024	0.009	0.001		
	Recovery (%)	99.3	99.6	99.9	99.1	99.2	97.3	98.2	94.6	–	–
B-complex, Phyto parma	Label mg	0.66	0.84	9.6	3.6	0.84	0.03	0.120	0.0015	–	–
	Found (mean value ± SD)	0.653	0.841	9.63	3.57	0.837	0.032	0.119	0.0014	–	–
		0.042	0.027	0.165	0.207	0.031	0.004	0.018	0.001		
	Recovery (%)	98.9	100.1	100.3	99.2	99.6	105.9	99.2	95.3	–	–
Geritamin	Label mg	–	–	–	–	–	–	–	–	1.5	67
	Found (mean value ± SD)	–	–	–	–	–	–	–	–	1.51	66.9
										0.047	0.064
	Recovery (%)	–	–	–	–	–	–	–	–	100.6	99.8
Gericaps	Label mg	–	–	–	–	–	–	–	–	1.5	67
	Found (mean value ± SD)	–	–	–	–	–	–	–	–	1.47	66.5
										0.014	0.075
	Recovery (%)	–	–	–	–	–	–	–	–	98.1	99.3
Vitamin A	Label mg	–	–	–	–	–	–	–	–	1.5	–
	Found (mean value ± SD)	–	–	–	–	–	–	–	–	1.52	–
										0.053	
	Recovery (%)	–	–	–	–	–	–	–	–	101.3	–
Vitamin E	Label mg	–	–	–	–	–	–	–	–	–	100
	Found (mean value ± SD)	–	–	–	–	–	–	–	–	–	101
											0.146
	Recovery (%)	–	–	–	–	–	–	–	–	–	101.0
Brewer's yeast 1 g	Label mg	0.12	0.2	1.2	0.1	0.04	0.05	0.002	[a]	0.92	1.6
	Found (mean value ± SD)	0.114	0.183	1.18	0.087	0.033	0.039	0.001	–	0.86	1.52
		0.007	0.022	0.051	0.019	0.017	0.008	0.001		0.068	0.013
	Recovery (%)	95.0	91.5	98.3	87	82.5	78	85.1	–	93.5	95.2

[a] Traces of the corresponding substance.

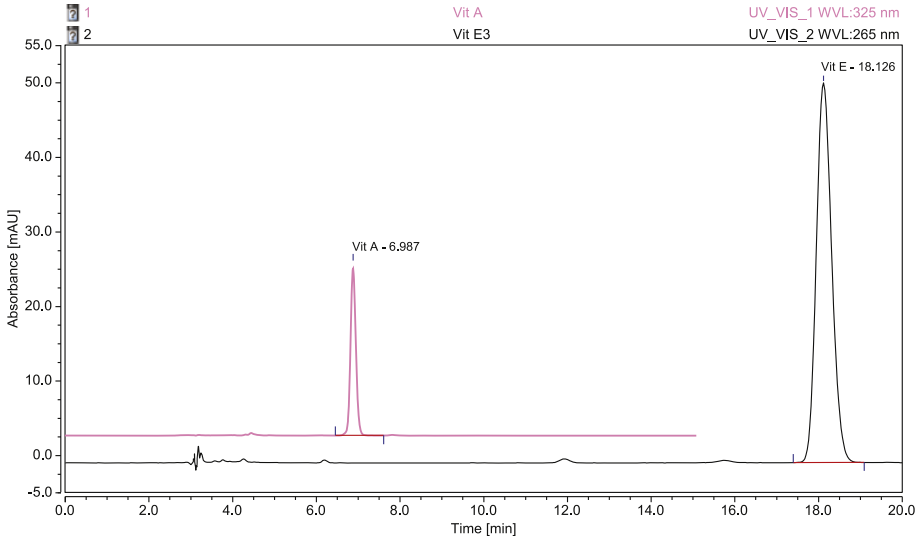

Fig. 5. Representative HPLC-DAD chromatogram of the analysis of the content of fat-soluble vitamins in the evaluated brewer's yeast at 325 nm for vitamin A and 265 nm for vitamin E.

4. Conclusion

Rapid, precise and selective single column RP-HPLC/DAD methods for routine analysis of water-soluble and fat-soluble multivitamin mixtures in food supplements and brewer's yeast are developed and validated as per ICH guidelines. The methods are based on a Purospher STAR C18 (Merck Millipore) 5 μm, 25 × 0.46 cm column assuring optimal retention and separation. A common and available gradient elution with solvent A of phosphate buffer (pH = 4.0):acetonitrile (98:2, v/v) and solvent B of methanol:distilled water (30:70, v/v) (Method 1), along with isocratic methanol:acetonitrile (98:2, v/v) mobile phase (Method 2) was applied to achieve suitable separation. Seven commercially available food supplements and a second fermentation brewer's yeast were quantified by the developed and validated procedures. The obtained results identified that the amount corresponds to the labeled content, determining the methods as applicable for routine analysis.

CRediT author statement

Alexandrina Mateeva: Performed the experiments, Analyzed and interpreted the data, Wrote the original draft. **Magdalena Kondeva-Burdina**: Contributed reagents, materials, analysis tools or data, Performed the experiments. **Lily Peikova**: Contributed reagents, materials, analysis tools or data, Performed the experiments, Analyzed and interpreted data. **Silvia Guncheva**: Performed the experiments, Analyzed and interpreted data. **Alexander Zlatkov**: Conceived and designed the experiments, Analyzed and interpreted data. **Maya Georgieva**: Conceived and designed the experiments, Analyzed and interpreted data, Wrote the paper.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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