Identification and quantitation of Choline in food supplements using high performance liquid chromatography

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Abstract. A reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the separation and quantitative determination of choline in dietary supplements. The method optimization involved the selection of a Purospher C18 column and an acetonitrile:water (75:25, v/v) mobile phase, with detection at 205–208 nm. Validation followed ICH Q2 guidelines, assessing specificity, linearity, accuracy, precision, and sensitivity. The method demonstrated excellent linearity (R^2 > 0.9900) over the range of 50–150 µg/mL, with recovery rates between 99.3% and 101.0%. The limit of detection and quantification were determined to be 4 µg/mL and 12 µg/mL, respectively. Application of the method to both pure choline and commercial multicomponent dietary supplements showed that drying choline prior to analysis is necessary for reliable results. The method provided consistent quantification of choline in supplements, with measured amounts closely matching label claims, and the largest deviation observed was approximately 7 milligrams. This RP-HPLC method is suitable for routine quality control of choline in dietary supplement formulations.

1 Introduction

Choline is an essential nutrient that plays a fundamental role in human health, contributing to the structure and function of cell membranes, lipid metabolism, and the synthesis of the neurotransmitter acetylcholine [1]. Adequate choline intake is critical for normal cognitive development, liver function, and the regulation of homocysteine metabolism, with deficiency linked to a variety of adverse health outcomes, including neurological and hepatic disorders. Although choline can be synthesized endogenously, dietary sources such as eggs, meats, and dairy products are necessary to meet physiological requirements, and supplementation is often recommended to ensure adequate intake, particularly in populations at risk of deficiency [2, 3].

With the increasing popularity of dietary supplements containing choline, ensuring the quality and accuracy of these products has become a pressing concern for both public health and regulatory agencies [4]. Analytical determination of choline in complex supplement matrices poses significant challenges due to its high polarity, hygroscopicity, and the presence of various excipients and active ingredients that may interfere with accurate quantification. Reliable measurement methods are therefore essential for quality control and to verify label claims in commercial choline supplements [5].

High-performance liquid chromatography (HPLC) is widely recognized as a robust and sensitive technique for the separation and quantification of choline and its derivatives in both raw materials and finished products [6, 7]. The development of a reliable HPLC method for choline analysis requires careful optimization of chromatographic conditions, including the selection of stationary and mobile phases, detection wavelength, and sample preparation protocols, to ensure specificity and reproducibility in complex matrices. Furthermore, method validation according to International Council for Harmonisation (ICH) guidelines is necessary to demonstrate the method's suitability for its intended purpose, including assessments of specificity, linearity, accuracy, precision, and sensitivity [8-11].

In this study, we address these analytical challenges by developing and validating an RP-HPLC method specifically designed for the separation and quantitative determination of choline in dietary supplements. The method was systematically optimized and validated in accordance with ICH guidelines, and subsequently applied to the analysis of both pure choline and commercial supplement formulations, providing a reliable tool for routine quality control and regulatory assessment of choline-containing products.

2 Materials and methods

Chromatographic separation and identification were performed using an UltiMate Dionex 3000 SD liquid chromatograph (Thermo Fisher Scientific Inc) with a UV-DAD detector. Data processing was carried out using Chromeleon 7.2 SR3 software. The substances used for the preparation of the mobile phase were Acetonitrile (Merck KGaA, Germany), distilled water

(obtained using the Adrona Crystal 7 system), Triethylamine and Orthophosphoric acid. Other solvents and reagents, prepared and meeting the purity requirements of the European Pharmacopoeia 10.0 were Lactic acid, Tetrabutylammonium hydroxide, Ammonium hydroxide and Formic acid. A standardized choline (certified purity > 99%) was bought from Sigma-Aldrich (Steinheim, Germany). The combined dietary supplements containing choline were from the brands Naturepharma (Livuron Hepa caps Exp.date 01.2024, Batch number S-0101; Livuron forte caps Exp.date 09.2024, Batch number S-0309), produced by Naturpharma Musachevo, Elin Pelin, Sofia District, Bulgaria and Nature Product Pharma® (Optihepan caps Exp.date 12.2023, Batch number 249699), produced by Natur Product Bulgaria, Sofia, Bulgaria.

2.1 Preparation of Stock Solution for Analysis

Exactly 0.0010 g of choline substance is weighed on an analytical balance, then quantitatively transferred into a 10 mL volumetric flask and brought to the mark with the mobile phase acetonitrile : water = 75:25 v/v ($CH_3CN:H_2O$ = 75:25), which is also a suitable solvent. The final concentration is 100 µg/mL.

2.2 Preparation of Solution for Validation

For validation of the analytical procedure, 5 solutions with increasing concentrations (50, 75, 100, 125, 150 µg/mL) were prepared. For this purpose, the required amount of concentrated solution was measured and then diluted to a volume of 10 mL with the mobile phase.

2.3 Preparation of the Mobile Phase

The mobile phase is prepared volumetrically by mixing acetonitrile and distilled water in a ratio of 75:25, with water added up to the mark. The mobile phase is filtered through a filter with a pore size of 0.45 microns.

2.4 Sample Preparation and Preparation of Test Solutions from Dietary Supplements

2.4.1 Preparation of Choline Solution from the Combined Product Livuron Hepa Caps

The contents of 1 capsule (0.6827 g), containing 83 mg choline, are weighed on an analytical balance. From this, 0.2056 g, corresponding to 25 mg choline, is measured and diluted with distilled water to 25 mL in a volumetric flask. The solution is first filtered through a paper filter, then through a membrane filter (0.22 µm). 1 mL of the resulting filtrate is pipetted and transferred into a 10 mL volumetric flask, and the volume is brought up with the mobile phase. The concentration is 100 µg/mL. Analysis is performed after solid-liquid extraction.

2.4.2 Preparation of Choline Solution from the Combined Product Livuron Forte Caps

The contents of 1 capsule (0.6566 g), containing 83 mg choline, are weighed on an analytical balance. From this, 0.1978 g, corresponding to 25 mg choline, is measured and diluted with distilled water to 25 mL in a volumetric flask. The solution is first filtered through a paper filter, then through a membrane filter (0.22 µm). 1 mL of the resulting filtrate is pipetted and transferred into a 10 mL volumetric flask, and the volume is brought up with the mobile phase. The concentration is 100 µg/mL. Analysis is performed after solid-liquid extraction.

2.4.3 Preparation of Choline Solution from the Combined Product Optihepan

The contents of 1 capsule (0.3113 g), containing 24.69 mg choline (60 mg choline bitartrate), are weighed on an analytical balance. From this, 0.1260 g, corresponding to 10 mg choline, is measured and diluted with distilled water to 10 mL in a volumetric flask. The solution is first filtered through a paper filter, then through a membrane filter (0.22 µm). 1 mL of the resulting filtrate is pipetted and transferred into a 10 mL volumetric flask, and the volume is brought up with the mobile phase. The concentration is 100 µg/mL. Analysis is performed after solid-liquid extraction.

2.4.4 Sample Preparation

Sample preparation was performed by solid-liquid extraction using a Thermo Scientific SolEx SCX SPE Cartridge, and the experiment was conducted under vacuum with the operating mode presented in Table 1.

Table 1. Solid-liquid extraction.

Eluent	Stage
5 mL of 5% ammonium hydroxide in methanol	conditioning
10 mL distilled water	conditioning
5 minutes	drying
5 ml 1% formic acid in water	equilibration
5 mL	sample loading
5 ml 1% formic acid in water	washing
5 ml 1% formic acid in methanol	washing
5% ammonium hydroxide in methanol	elution

2.4.5 Chromatographic System

The chromatographic determination was conducted with the parameters listed in the Table 2.

Table 2. Chromatographic system parameters.

System parameter	
Column	Purospher C18 column (4.6 x 250mm, 5µm[1])
Mobile phase	CH_3CN: H_2O (75:25)
Flow rate	0,8 mL/min[2]
Injection volume	20 µL[3]
Wavelength	205 nm[4]
Temperature	25 ° C

[1]**µm** – **micrometres**; [2]mL/min – millilitre per minute; [3]µL – microliter; [1]nm – nanometre;

3 Results and discussion

Choline is an important nutrient, often referred to as a vitamin B group of nutrients. It plays a crucial role in physiology of humans by maintaining the structures of cell membranes and involved as a precursor in the biosynthesis of some neurotransmitters like acetylcholine. This compound is essential for maintenance of liver and brain functions and health.

As characteristic for all vitamin type of molecules most of the required choline is introduced into the body through the diet and only a small portion is biosynthesized. The most common dietary sources of choline are eggs, meat, poultry and some vegetables like broccoli and cauliflower, as well as beans, nuts, seeds, and whole grains.

Choline supplementation may improve memory and cognitive function, may reduce the risk of preeclampsia and plays an important role in maintaining cardiovascular health. On the other hand, choline deficiency can lead to liver and muscle damage, and potentially contribute to cardiovascular disease and neurological issues.

Except by diet, choline is available as a supplement, either alone or in combination with other nutrients like B vitamins. The recommended daily intake of choline varies by age and sex, with adults typically needing 425-550 mg per day. High doses of choline supplements may cause side effects like sweating, fishy body odour, and gastrointestinal issues. Thus the information on the proper amount of choline contained in the corresponding supplement is of high importance to maintaining the normal physiological functions of the body and human health.

3.1 Development of the Chromatographic Method

To develop the target RP-HPLC method, the influence of the factors listed below on the chromatographic behavior of the analyte was investigated. Changes in these parameters mainly affect the retention time of the analyzed substance and the symmetry of the chromatographic peaks.

3.2 Selection of Stationary Phase

Based on literature data regarding choline, its physicochemical properties, and experimental data, it was determined that the Purospher C18 column (4.6 x 250 mm, 5 µm) is the most suitable for this study, as it allows for the application of the method for the determination of choline in dietary supplements.

3.3 Selection of Mobile Phase

An important parameter determining the applicability of the chromatographic procedure as an analytical method is the ability to achieve good separation and obtain symmetric peaks. The composition of the mobile phase is crucial for this. To achieve optimal conditions, the composition of the mobile phase was varied: initially, a phase with orthophosphoric acid in water, adjusted to pH 6 with triethylamine, was used for choline determination. This buffer proved unsuitable due to poor peak shape. It was replaced with a non-buffered phase: acetonitrile:water in a 75:25 v/v ratio, which led to significant improvement in separation and was found to be the most suitable for this analysis.

3.4 Selection of Wavelength

To determine the appropriate wavelength for detecting the analyte choline, both alone and in mixtures, a UV-Vis analysis of standard choline solutions was performed. The obtained spectrum indicated that the most suitable wavelength for choline determination is 205–208 nm, which was used in subsequent measurements.

3.5 Selection of Solvent

Choline is a quaternary amine, soluble in water, so distilled water was initially used as the solvent. However, it was found that this solution did not exhibit good chromatographic behavior. Methanol was then tested, but as it absorbs in the region of choline's absorption maximum, this also resulted in poor chromatographic performance. This led to the use of the already selected mobile phase for analysis, namely acetonitrile:water in a 75:25 v/v ratio, which significantly improved separation and provided satisfactory peak shapes.

3.6 Method Validation

Method validation involves a series of tests aimed at proving that the analytical methods used are suitable for their intended purpose. The developed RP-HPLC method was validated according to ICH Q2, examining the validation characteristics of specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and system suitability.

3.7 System Suitability

System suitability evaluates the robustness of the analytical method using appropriate parameters. The symmetry factor was chosen to assess column performance. The peak asymmetry for choline was close to 0.98, indicating a symmetric peak shape. The high number of theoretical plates (24 961) demonstrated the high efficiency of the column.

3.8 Specificity

Specificity is defined as the ability of the analytical method to provide results that are not affected by the presence of other substances expected in the test solution, such as impurities, degradation products, excipients, etc. It was found that the solvent absorption does not interfere with choline absorption. Therefore, the method was found to be specific. The chromatogram of the mobile phase is shown in Fig. 1.

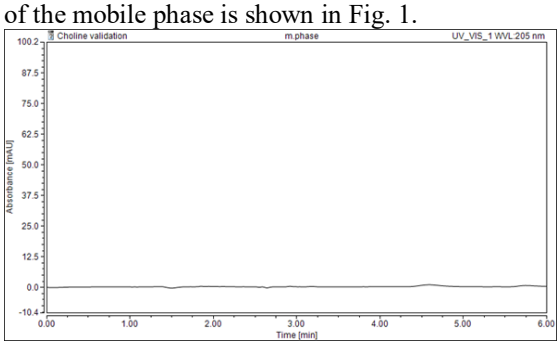

Fig. 1. Chromatogram of the mobile phase.

3.9 Accuracy

Accuracy is the closeness of agreement between the actual and accepted reference value. The accuracy of the developed method was assessed by calculating the mean percentage recovery of choline at three different concentration levels (50%, 100%, 150%). Three samples at each concentration were injected. The recovery rate ranged from 99.3% to 101.0% (Table 3), indicating that the method is accurate.

Table 3. Data for the accuracy evaluation of the proposed HPLC method.

Compound	Concentration level, %	Amount added (µg/mL[5])	amount found (µg/mL[5])	Recovery rate %
Choline	50	50	49.85	99.7
			50.09	100.2
			46.85	99.7
	100	100	99.77	100.8
			99.32	99.3
			100.98	101.0
	150	150	150.09	100.1
			149.64	99.8
			149.37	99.6

[5]µg/mL – microgram per millilitre

3.10 Linearity

Linearity is defined as the ability to obtain (within a given range) results that are linearly dependent on the concentration of the analyzed substance. To assess linearity, five standard solutions with concentrations of 50, 75, 100, 125, and 150% were prepared. The results were subjected to linear regression analysis, and Fig. 2 shows the dependence of the peak area of the selected compound on concentration.

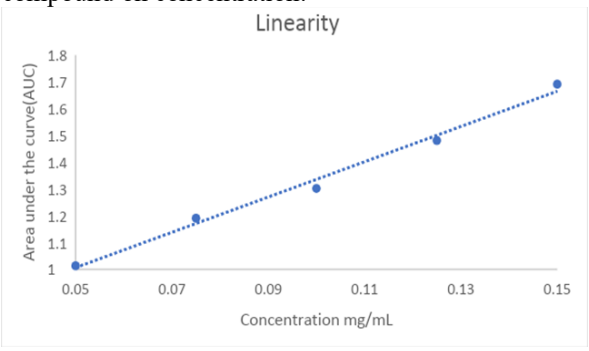

Fig. 2. Linearity of choline in water:acetonitrile (75:25).

The correlation coefficient (R^2) (Table 4) of the calibration curve was greater than 0.9900, confirming linearity in the range 50–150 µg/mL.

Table 4. Parameters for determining linearity.

Linear equation	Correlation coefficient
y= 6,584x+0,6772	0,9904

3.11 Precision

Precision is defined as the closeness of agreement between a series of measurements obtained from the same homogeneous sample under specified conditions. Precision is considered at three levels: repeatability, intermediate precision, and reproducibility. The precision of an analytical procedure is expressed as the mean, standard deviation, and relative standard deviation of a series of measurements, which were determined for the specific chromatographic procedure. For this purpose, six samples with a concentration of 100 µg/mL were analyzed, and the relative standard deviation of the peak areas was calculated (Table 5).

Table 5. Evaluation of the repeatability of the developed method.

Sample 100 µg/mL[5]	Area under the curve
1	7.702

2	7.726
3	7.750
4	7.737
5	7.715
6	7.721
Mean value	7.725167
Standard deviation	0.016822
Relative standard deviation	0.217751

[5]µg/mL – microgram per millilitre

3.12 Sensitivity

The limit of detection (LOD) is defined as the lowest detectable concentration of a standard solution.

The limit of quantification (LOQ) is a measure of the lowest amount that can be determined with acceptable precision and accuracy.

LOD and LOQ were also evaluated using linear regression, linearity, and accuracy. LOD (4 µg/mL) and LOQ (12 µg/mL) were calculated using the following equations:

$$"LOD"=3.3\times S_a/b \quad (1)$$

$$"LOQ"=10\times S_a/b \quad (2)$$

3.13 Application of the Method for Substance Analysis

After successful validation of the choline substance as described above, it was found during analysis that choline is highly hygroscopic. Poorly dried pure choline led to deviations in chromatographic behavior (Fig. 3).

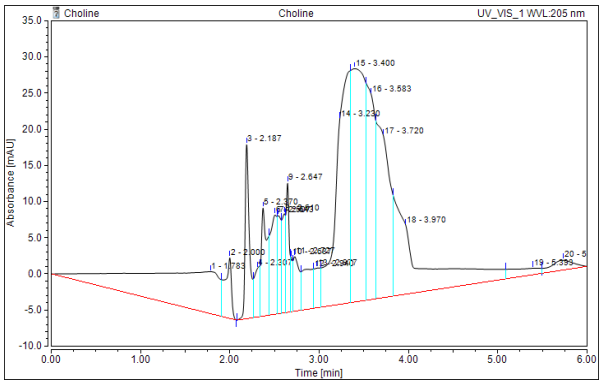

Fig. 3. Chromatogram of choline substance.

This necessitated its preliminary drying and subsequent preparation of standard solutions immediately before analysis, due to its instability. Drying was performed in a Sartorius oven at a constant temperature of 100°C (Fig. 4).

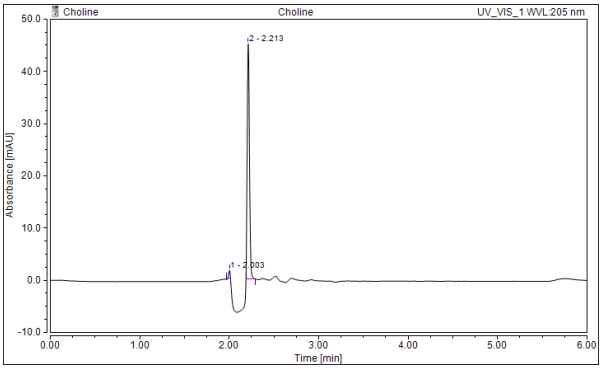

Fig. 4. Chromatogram of choline substance after drying.

3.14 Application of the Method for Analysis of Dietary Supplements

The method used for the analysis of pure choline was applied for its identification and quantitative determination in dietary supplements. The initial approach, which included weighing the sample, dissolving, filtering the solution, subsequent centrifugation, preparation of a standard solution, and analysis, did not achieve good separation of choline in the dietary supplements tested (Fig. 5).

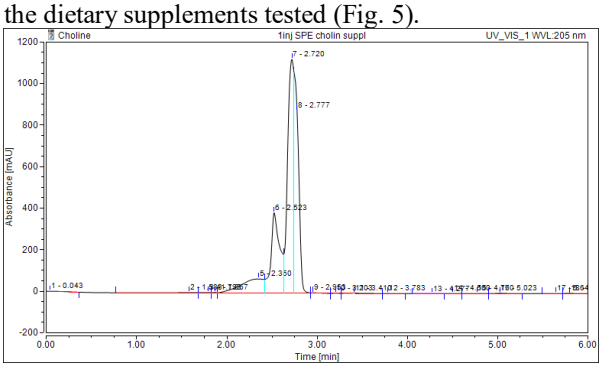

Fig. 5. Chromatogram of classical sample preparation of choline in dietary supplements.

This required a change in the working method, incorporating solid-liquid extraction (SLE) as sample preparation. Initially, working solutions from the respective dietary supplements were prepared as follows: weighing samples of the supplements, dissolving in water, filtering sequentially through paper and syringe filters (0.22 µm), and diluting with the mobile phase water:acetonitrile in a 75:25 v/v ratio. The SLE included the following steps: wetting first with 0.1 M lactic acid, then with distilled water, followed by application of the dietary supplement solution, with a final washing step using water and 0.1 M tetrabutylammonium hydroxide. For the analysis, a Thermo Scientific SolEx SCX SPE Cartridge was used, and the experiment was carried out under vacuum. The final solution obtained was analyzed, but the data showed unsatisfactory separation, so the SLE solutions were modified. For conditioning, first 5 mL of 5% ammonium hydroxide in methanol was used, followed by washing with 10 mL distilled water. After 5 minutes of drying, equilibration with 5 mL of 1% formic acid in

water was performed, and the sample was loaded. For washing, 5 mL of 1% formic acid in water and formic acid in methanol were used sequentially. For elution of the analyte, 5% ammonium hydroxide in methanol was used. The final solution obtained was chromatographed, and the results showed good separation of choline in the mixture when using solid-liquid extraction, with the expected retention time and a well-defined peak Fig. 6, Fig. 7 and Fig. 8, for each marketed supplement.

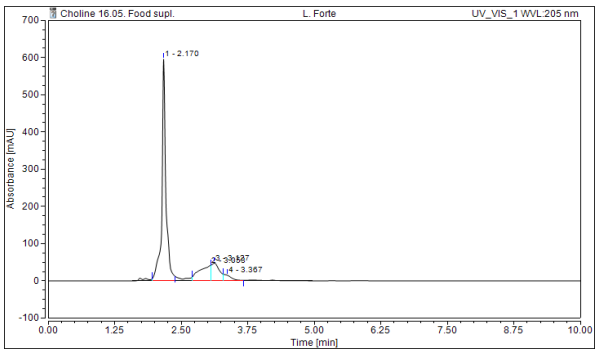

Fig. 6. Chromatogram of choline in dietary supplement Livuron Forte.

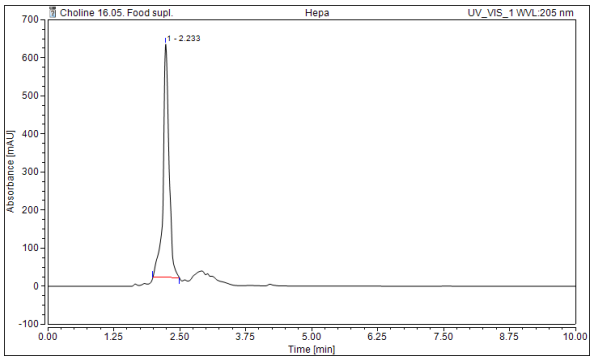

Fig. 7. Chromatogram of choline in dietary supplement Livuron Hepa.

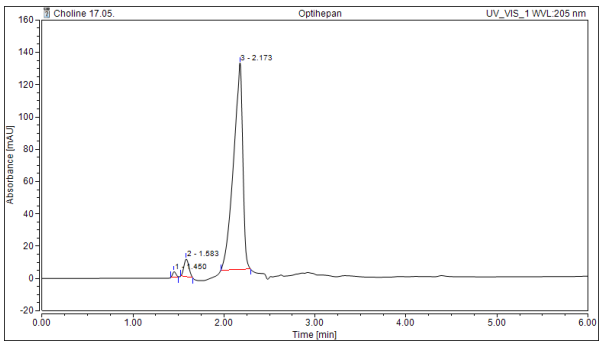

Fig. 8. Chromatogram of choline in dietary supplement Optihepan.

Using the data from the analyses of dietary supplements by the external standard method, the concentration of choline in all three products was calculated. The results show agreement between the declared and found amounts, with the first supplement showing the greatest deviation of about 7 milligrams (Table 6).

Table 6. Results from the analysis of choline in dietary supplements.

Dietary supplement	Labeled amount (mg[6])	Found (mg[6])
Livuron Forte	83	76±1.027 mg
Livuron Hepa	83	81±0.953 mg
Optihepan	60	63±2.166 mg

[6]mg – milligrams

4 Conclusions

An RP-HPLC method for the separation and determination of choline was developed and rigorously validated in accordance with ICH requirements, demonstrating specificity, linearity, accuracy, precision, and sensitivity. The method was successfully applied to the analysis of choline both as a pure substance and in multicomponent dietary supplements. It was established that drying choline immediately prior to analysis is necessary for reliable results. When applied to dietary supplements, the method produced results consistent with the amounts declared on product labels, with the greatest observed deviation being approximately 7 milligrams in the first supplement analysed.

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Conflict of 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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