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Determination of fat-soluble vitamins A, D2, D3, E and K3 by isotope dilution and LC-MS/MS instrument assembly

Keywords: water-soluble and water-insoluble vitamins, vitamers, coenzymes, cofactors, recommended daily intake (RDI), isotope dilution

1. SUMMARY

The purpose of our publication is the determination of the total amount (of natural origin and added) of fat-soluble vitamins A, D2, D3 and E in low amounts in foods (wheat flour, soft drinks, effervescent tablets) and dietary supplements using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method. The samples were diluted with isotope-labeled derivatives of the target components (vitamin A-d6, vitamin D2-d3, vitamin D3-d3, vitamin E-d6), and after extraction and saponification, they were purified by liquid-liquid extraction. After a solvent exchange, the concentration of the vitamins was determined on a C8 HPLC column using acidic mobile phases (0.1% formic acid in water/methanol) and LC-MS/MS technique. In dietary supplements, the analysis of the fat-soluble vitamin K3 may also be important, because the use of vitamin K3 is currently not approved in human formulations. During the determination of vitamin K3, saponification is not necessary, due to its structure, alkaline hydrolysis would lead to the decomposition of vitamin K3, so this component was analyzed by a method different from the one used for the other vitamins. LC-MS/MS analysis of small amounts of vitamin K3 is more complicated than that of other vitamins due to the low sensitivity of the MS instrument to vitamin K3. The determination of vitamin K3 was therefore carried out after chemical derivatization with L-cysteine as a derivatizing reagent, also with isotope dilution and LC-MS/MS technique. After intralaboratory validation, the methods were successfully used in domestic and international proficiency tests in infant formulas and liquid vitamin preparations.

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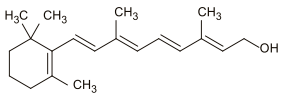
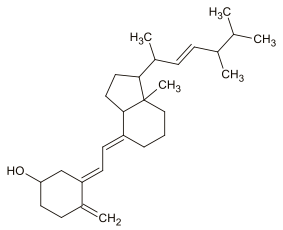
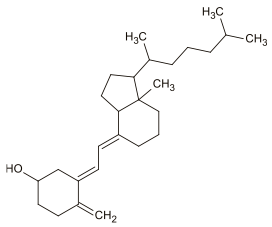
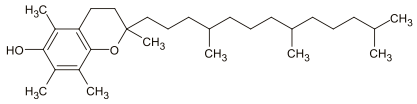
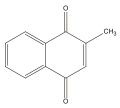
2. Introduction

Vitamins are organic molecules that are essential for the functioning of the human and animal body. They are necessary for the growth and maintenance of the cell population, for the proper functioning of certain organs, and for the maintenance of normal metabolism **[1]**. Vitamins are complex organic molecules whose structure and function in the body are very different from each other, so it is easiest to group them on the basis of their solubility. Based on this, water-soluble and fat-soluble vitamins are distinguished **[1]**. Vitamins B and C are water-soluble vitamins. These vitamins cannot be stored by the body for a long time, they are mostly excreted from the body in the urine, so it is necessary to replace them in the right amount every day. Vitamins A, D, E and K are fat-soluble vitamins (***Table 1***).

Table 1. Structure of the vitamins A, D2, D3, E and K3 investigated, their trivial names and most important physicochemical characteristics

Structural formula of the vitamin analyzed	Trivial name	Molecular mass g/mol	LogP
	Vitamin A (retinol)	265.5	5.7
	Vitamin D2 (calciferol)	396.7	7.5
	Vitamin D3 (cholecalciferol)		7.5
	Vitamin E (alpha-tocopherol)	430.7	12.2
	Vitamin K3 (menadione)	180.2	2.2

Unlike water-soluble vitamins, the body can store these vitamins for months. Vitamin intake can be accomplished through a varied diet.

We can distinguish between natural vitamins found in foods and synthetic vitamins added to foods. Unfortunately, the latter ones cannot be utilized in the same way as their natural counterparts, and they also reduce the utilization of other nutrients and put a strain on the kidneys. This is because the vitamins added to the samples are not accompanied by the enzymes, coenzymes and cofactors necessary for absorption, as opposed to the vitamins naturally occurring in foods. Based on Annex XIII of Regulation (EU) No 1169/2011 of the European Parliament and of the Council, the recommended daily vitamin and mineral intake reference values for adults are 800 µg/day for vitamin A, 5 µg/day for vitamin D, 12 mg/day for vitamin E and 75 µg/day for vitamin K **[2]**. Vitamin K3 is used in animal husbandry and is added to feed, but vitamin K3 cannot be mixed with food intended for human consumption and cannot occur in dietary supplements either **[3]**.

In the case of vitamin analysis, it is important to indicate whether it is the analysis of the vitamin added to the food or the determination of the total vitamin content. Naturally occurring vitamins B and vitamers are often present in the sample in a bound form, from which they can be released by hydrolysis or enzymatic sample preparation **[4]**. In the case of fat-soluble vitamins, however, sample preparation always includes a

saponification step, during which the vitamins are released from their bound form, thus making it possible to determine the total vitamin content **[5]**,**[6]**,**[7]**,**[8]**,**[9]**. In the current paper, the determination of fat-soluble vitamins is discussed.

According to the relevant standard, vitamin analysis must be carried out using a liquid chromatography (HPLC) method with an optical detector (HPLC-UV). These standards contain the determination of vitamins occurring in high concentrations (>mg/100 g). The analysis of vitamins occurring in lower concentrations (µg/100 g) requires a longer or more complicated sample preparation, during which a high degree of sample purification and enrichment is performed (e.g., using preparative HPLC) **[5]**,**[6]**,**[7]**,**[8]**,**[9]**, or we are forced to use a measurement technique that enables the selective determination of the target components even during the examination of complex matrices. One of these coupled techniques is liquid chromatography – tandem mass spectrometry (LC-MS/MS), which can be used with isotope dilution to determine the concentration of the tested compounds with high accuracy. In the course of isotope dilution, isotopically labeled (^{2}H, ^{13}C, ^{15}N, ^{18}O) analogs of the target components as internal standards (ISTD) are added to the samples and they are mixed homogeneously. These ISTDs compensate for the losses of target components during both sample preparation and instrumental analysis **[4]**. Our laboratory is committed to the use of isotope-labeled ISTDs, therefore we have developed an LC-MS/MS method for the determination of small amounts of fat-soluble vitamins, which contains all isotope-labeled analogs (deuterated compounds of vitamins). The purpose of our publication is to determine fat-soluble vitamins (A, D2, D3 and E) in wheat flour, soft drinks, effervescent tablets and dietary supplements using an LC-MS/MS instrument assembly, and also the validation and application of the method. Another goal was to develop an LC-MS/MS method for the determination of vitamin K3 in dietary supplements, during which chemical derivatization was attempted to achieve appropriate sensitivity.

3. Materials and methods

3.1. Materials and instruments used

Analytical grade standards of the vitamins (A, D2, D3, E and K3) and of the isotope-labeled analogs vitamin D2-d3, vitamin D3-d3, vitamin E-d6 and vitamin K3-d8, as well as L-cysteine, ascorbic acid, sodium hydroxide, Ascentis Express C8 (100 x 3 mm, 2.7 µm) HPLC column, HPLC grade solvents and formic acid were purchased from Sigma-Merck Kft. (Budapest, Hungary). Vitamin A-d6 was ordered from Cambridge Isotope Laboratories (Andover, MA, USA). Standards (with the exception of vitamin A) and internal standards vitamin E-d6 and vitamin K3-d8 were dissolved in ethyl alcohol so that their concentration was 1 mg/mL. The solutions were stored in a refrigerator at +4 °C for up to half a year. Labeled standards vitamin D2-d3 (100 µg/mL in methanol) and vitamin D3-d3 (1000 µg/mL in methanol) were obtained in a solution form. Vitamin A and vitamin A-d6 were dissolved in methanol containing 0.1% (m/v) butylated hydroxytoluene (BHT) (1 mg/mL) and stored at -18 °C for up to half a year. For calibration, a 10 µg/mL standard mixture (A, D2, D3, E) and a 10 µg/mL individual K3 standard solution were prepared in methanol and it was stored in a refrigerator at +4 °C for a maximum of 3 months. Of internal standards, an ISTD standard mixture of 20 µg/mL (vitamin A-d6, vitamin D2-d3, vitamin D3-d3, vitamin E-d6) and an individual ISTD standard solution of 10 µg/mL of vitamin K3-d8 were prepared in methanol and the were stored in a refrigerator at -18 °C for a maximum of 3 months.

For the LC-MS/MS studies, a Shimadzu Nexera UHPLC LC-30AD liquid chromatography system was used, which included a SIL-30AC autosampler, a CTO-20AC column thermostat and a CBM-20A communications bus module (Shimadzu Corporation, Kyoto, Japan). The triple quadrupole mass spectrometer coupled with the UHPLC was an AB Sciex 6500+ QTRAP with an IonDrive Turbo V Source ion source and a 6500 QTRAP instrument with a Turbo V Source ion source (the two systems were used alternately). The measuring software was Analyst (1.7.1) and the software used for quantification was MultiQuant (3.0.3) (Sciex; Warrington, Cheshire, UK).

The shaker used for extraction was a CAT S50 flask shaker (M. Zipperer GmbH, Ballrechten-Dottingen, Germany). A TurboVap II (Biotage, Uppsala, Sweden) type evaporator was used for the evaporation of the samples. Liquid vitamin dietary supplement proficiency testing samples and breakfast cereal quality control (QC) samples were ordered from FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, UK), while the infant formula proficiency testing sample was ordered from the National Food Chain Safety Office (NÉBIH, Budapest, Hungary).

3.2. Sample preparation for the determination of vitamins A, D2, D3 and E

The analyses of wheat flour, soft drinks, effervescent tablets and dietary supplements were performed. 1.00 g of a homogeneous sample was measured into a 60 mL glass tube and 50 µL of a 20 µg/mL ISTD solution (vitamin A-d6, vitamin D2-d3, vitamin D3-d3, vitamin E-d6) was pipetted onto it, then 20 mL of ethanol and 5 mL of distilled water were added. Following this, 0.5 g of ascorbic acid and 5 mL of 12.5 M sodium hydroxide solution were measured onto the sample. The sample was mixed at 60 °C on a magnetic stirrer for one and a

half hours. After the extraction/saponification, the sample was allowed to cool to room temperature and then 5 mL of distilled water and 5 mL of n-hexane was added. The sample was shaken for 1 hour (700 rpm), and then the liquid phases were allowed to separate for 10 minutes. 1.0 mL of the hexane phase was pipetted into a glass evaporating tube and it was evaporated to dryness at 40 °C under a stream of nitrogen. The sample residue was redissolved in 1.0 mL of methanol and it was filtered into an HPLC vial using a PTFE syringe filter (Gen-lab Kft., Budapest, Hungary). During sample preparation, there was a fivefold sample dilution.

3.3. LC-MS/MS method for the determination of vitamins A, D2, D3 and E

Vitamins were separated on a C8 HPLC column by linear and binary gradient elution (***Figure 1***). The aqueous mobile phase (eluent A) was 0.1% (v/v) formic acid in water, the organic mobile phase (eluent B) was 0.1% (v/v) formic acid in methanol. In the solvent gradient, the ratio of eluent B increased from 80% to 100% between 0 and 3 minutes, the ratio of eluent B was 100% between 3 and 10 minutes, the ratio of eluent B decreased to 80% between 10 and 10.1 minutes and it remained 80% until minute 14. The flow rate was 0.5 mL/min, analysis time was 14 minutes, injection volume was 5 µL, and the column thermostat temperature was 30 °C. MS/MS detection conditions are listed in ***Table 2***. The settings of the ion source were as follows: sheath gas: 45 units, gas 1 (nebulizer gas): 40 units, gas 2 (drying gas): 40 units, drying gas temperature: 350 °C, capillary voltage: +5,500 V.

Table 2. MRM ion transitions of vitamins A, D2, D3 and E and the corresponding voltage values. Ion transitions used for quantitative evaluation are marked in bold.

Parent ion (m/z)	Daughter ion (m/z)	Component	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Cell exit potential (V)
431.4	**165.1**	Vitamin E_1	120	9	40	15
431.4	137.1	Vitamin E_2	120	9	68	19
437.4	**171.1**	Vitamin E-d6_1	120	9	40	15
437.4	143.1	Vitamin E-d6_2	120	9	68	19
385.4	90.9	Vitamin D3_1	115	5	98	9
385.4	367.4	Vitamin D3_2	115	5	21	17
385.4	**259.2**	Vitamin D3_3	115	5	21	17
388.4	**90.9**	Vitamin D3-d3_1	150	10	46	11
388.4	259.2	Vitamin D3-d3_2	150	10	46	11
269.2	**93.1**	Vitamin A_1	100	11	35	5
269.2	157.2	Vitamin A_2	100	11	41	15
269.2	119.1	Vitamin A_3	100	11	31	5
275.2	**96.1**	Vitamin A-d6_1	100	11	35	5
275.2	122.1	Vitamin A-d6_2	100	11	31	5
397.2	**69.1**	Vitamin D2_1	96	10	51	8
397.2	91.1	Vitamin D2_2	96	10	83	8
400.2	**69.1**	Vitamin D2-d3_1	96	10	51	8
400.2	91.1	Vitamin D2-d3_2	96	10	83	8

3.4. Sample preparation for the determination of vitamin K3 in dietary supplements

1.00 g of a homogeneous sample was measured into a 60 mL glass tube and 100 µL of a 10 µg/mL vitamin K3-d8 ISTD solution was pipetted onto it, then 20 mL of ethanol and 5 mL of distilled water was added. The sample was extracted at room temperature for 1 hour (700 rpm), and then 15 mL of distilled water and 5 mL of n-hexane was added. The sample was shaken for 1 hour (700 rpm), and then the liquid phases were allowed to separate for 10 minutes. 1.0 mL of the hexane phase was pipetted into a glass evaporating tube and it was evaporated to dryness at 40 °C under a stream of nitrogen. The sample residue was redissolved in 0.5 mL of methanol and 0.5 mL of a freshly prepared 0.2% (v/v) solution of L-cysteine (1 mg/mL) with formic acid was added. After vortexing, the sample was allowed to stand at room temperature for half an hour until the reaction took place, and after another round of mixing, the sample was filtered into an HPLC vial using a hydrophilic PTFE syringe filter (Gen-lab Kft., Budapest, Hungary). During sample preparation, there was a fivefold sample dilution.

3.5. LC-MS/MS method for the determination of vitamin K3

After derivatization, vitamin K3 was separated on a C8 HPLC column by linear and binary gradient elution (***Figure 2***). The aqueous mobile phase (eluent A) was 0.1% (v/v) formic acid in water, the organic mobile phase (eluent B) was 0.1% (v/v) formic acid in methanol. In the solvent gradient, the ratio of eluent B was 20% between 0 and 1 minute, the ratio of eluent B increased from 20% to 70% between 1 and 5 minutes, the ratio of eluent B was 95% between 5.1 and 8 minutes, then the ratio of eluent B decreased to 20% at 8.1 minutes and it remained 20% until minute 12. The flow rate was 0.45 mL/min, analysis time was 12 minutes, injection volume was 10 µL, and the column thermostat temperature was 30 °C. MS/MS detection conditions are listed in ***Table 3***. The settings of the ion source were as follows: sheath gas: 45 units, gas 1 (nebulizer gas): 40 units, gas 2 (drying gas): 40 units, drying gas temperature: 350 °C, capillary voltage: +5,500 V.

Table 3. MRM ion transitions of derivatized vitamin K3 and the corresponding voltage values. Ion transitions used for quantitative evaluation are marked in bold.

Parent ion (m/z)	Daughter ion (m/z)	Component	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Cell exit potential (V)
294.1	**122.1**	Vitamin K3_1	51	10	13	14
294.1	173.1	Vitamin K3_2	51	10	21	20
294.1	105.1	Vitamin K3_3	51	10	47	12
294.1	205.1	Vitamin K3_4	51	10	17	24
294.1	77.1	Vitamin K3_5	51	10	77	34
294.1	115.1	Vitamin K3_6	51	10	67	12
301.1	**122.1**	Vitamin K3-d8_1	51	10	13	14
301.1	180.1	Vitamin K3-d8_2	51	10	21	20
301.1	109.1	Vitamin K3-d8_3	51	10	47	12
301.1	212.1	Vitamin K3-d8_4	51	10	17	24
301.1	120.1	Vitamin K3-d8_5	51	10	67	12

3.6. Optimizing ion transitions

1 µg/mL individual standard solutions diluted with 0.1% (v/v) formic acid in methanol were delivered from an infusion syringe to the mass spectrometer using a syringe pump, and with the help of the automatic optimization software, a minimum of 2 ion transitions were set for each component, except for vitamin K3. In the case of vitamin K3, 0.5 mL of the standard solution (10 µg/mL) was derivatized with 0.5 mL of L-cysteine solution and the derivative was optimized with 6 ion transitions in the mass spectrometer in order to find the transitions which the matrix compounds present in the sample do not possess within the retention time window of the K3 derivative.

3.7. Method validation

The determination of fat-soluble vitamins A, D2, D3 and E in wheat flour, soft drink, effervescent tablet and dietary supplement samples was validated by intralaboratory validation. The analytical performance characteristics examined were as follows: selectivity, identification (ion ratios), recovery at 0.5 and 5 mg/kg levels by the analysis of 10 parallel samples at each level, repeatability and reproducibility. The limit of quantification (LOQ) was determined from the signal-to-noise ratio. The determination of vitamin K3 in dietary supplements was validated on the basis of the same procedure at 0.1 and 1.0 mg/kg levels with 8 repetitions at each level. Calibration was checked by fitting a seven-point calibration curve where the points were 0.01 µg/mL, 0.05 µg/mL, 0.10 µg/mL, 0.50 µg/mL, 1.0 µg/mL, 5.0 µg/mL and 10.0 µg/mL. The concentration of ISTDs was 0.2 µg/mL.

4. Results and evaluation

4.1. LC-MS/MS method for the determination of fat-soluble vitamins

Due to their apolar nature, fat-soluble vitamins can be analyzed using atmospheric pressure chemical ionization (APCI) as an ion source during LC-MS measurements **[10]**. At the same time, the instrument used by us also ionized vitamins with high sensitivity using an electrospray ionization (ESI) source, so APCI was not necessary.

Following ion transition optimization, chromatographic separation was attempted on a C8 HPLC column, because vitamins A, D2, D3 and E, due to their lipophilic nature (***Table 1***), show too high retention on a C18 column. In addition, many samples contain large amounts of natural and/or added beta-carotene, whose hydrophobicity is even greater, so it can only be eluted from a C18 column after a long wash. The retention of vitamins was significantly reduced on the C8 column compared to that exhibited on a C18 column (***Figure 1***). The use of eluents with an acidic pH was chosen because of the positive ionization mode.

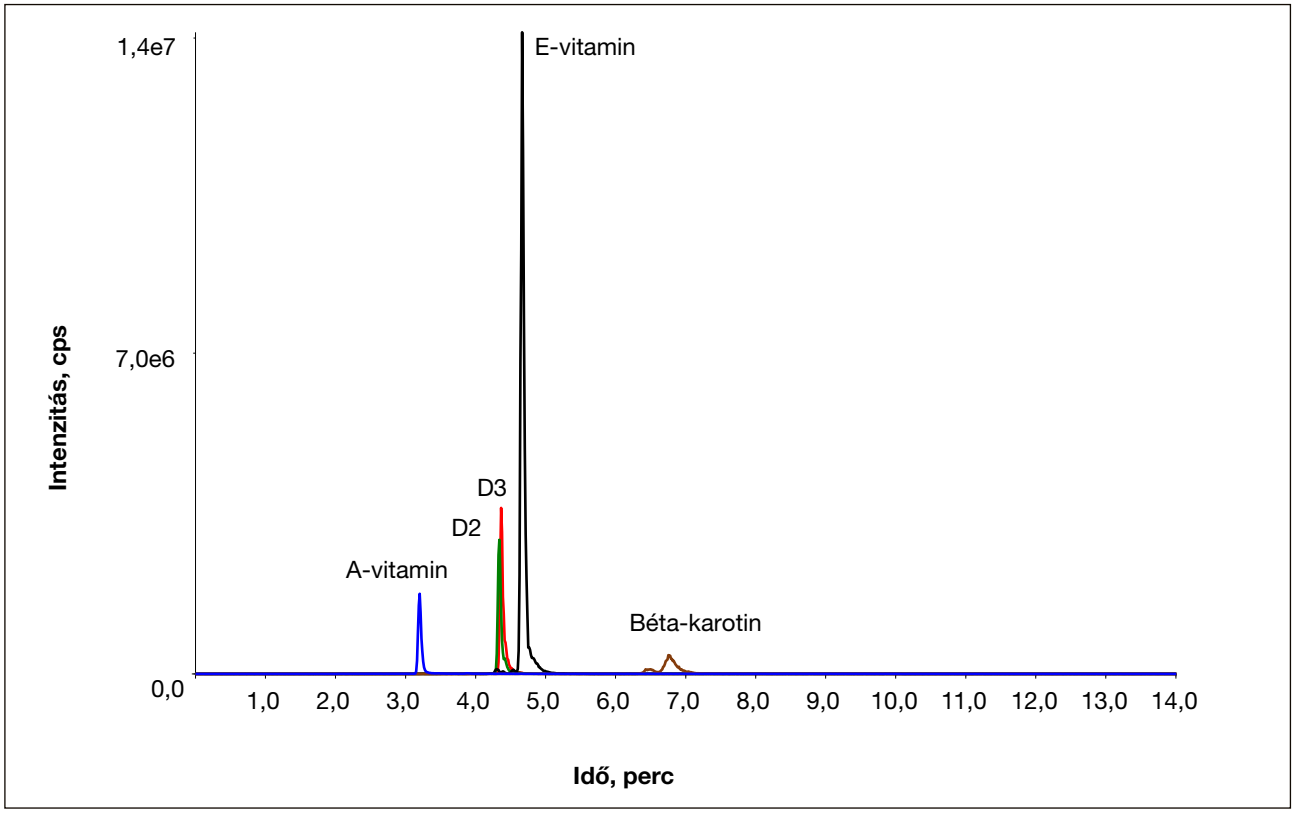
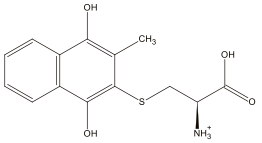
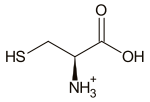


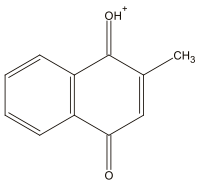
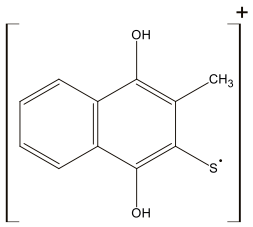
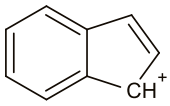
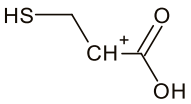
Figure 1. Separation of vitamins A, D2, D3 and E (1 µg/mL) on a C8 HPLC column.

Compared to vitamins K1 and K2, vitamin K3 is difficult to ionize in its native form, so it is hard to analyze with LC-MS. Yuan et al. recommended the chemical derivatization of vitamin K3, after which this vitamin can be detected with sufficient sensitivity using an MS instrument **[11]**. The derivatization applied by us is based on the method of Yuan et al., in which vitamin K3 is reacted with cysteamine under identical conditions, during which a Michael addition reaction takes place **[11]**. We performed the reaction not with cysteamine, but with L-cysteine. After the introduction of cysteine, the hydrophobicity of the derivative is much lower than that of native vitamin K3 (***Table 4***) and thus its retention on the C8 column is also reduced (***Figure 2***).

Table 4. Parent ion (m/z 294.1) and daughter ions of derivatized vitamin K3 recorded with an LC-ESI(+)-MS/MS instrument assembly.

Parent ion/daughter ion	m/z
	294.1
	122.1

The table is continued in the nex page

Parent ion/daughter ion	m/z
	173.1
	205.1
	115.1
	105.1

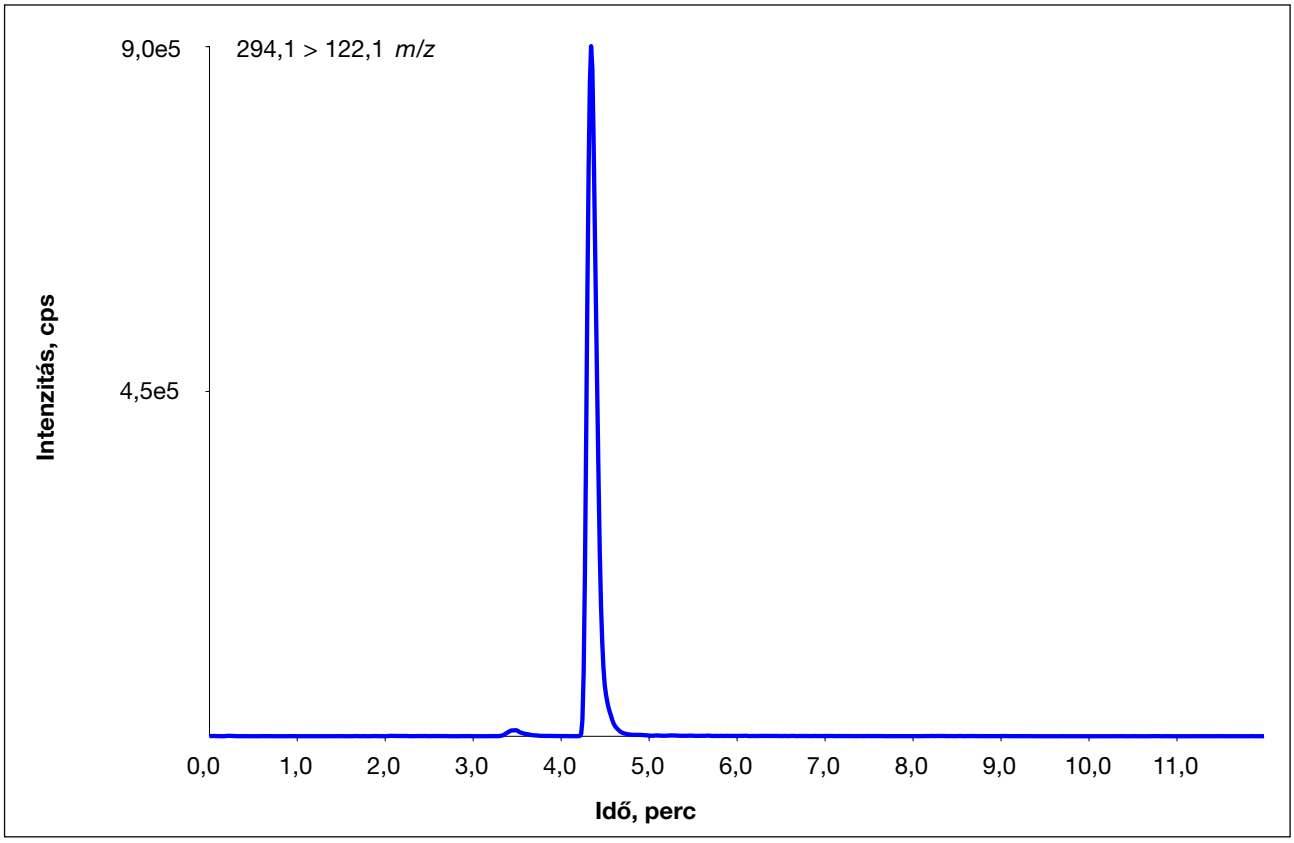


Figure 2. Separation of derivatized vitamin K3 (1 µg/mL) on a C8 HPLC column.

Completion of the derivatization reaction between vitamin K3 and L-cysteine was confirmed by recording a mass spectrum. Based on what was described in ***Section 3.4.,*** a 5 µg/mL derivatized solution was prepared and the mass spectrum of the derivative was recorded in Q1 scan mode, scanning the 200–400 *m/z* range (***Figure 3***). The $[M+H]^+$ monoisotopic mass of the quasi molecular ion (protonated molecule) of the assumed derivative is 294.1 Da, the signal of which appears in the spectrum (***Figure 3***).

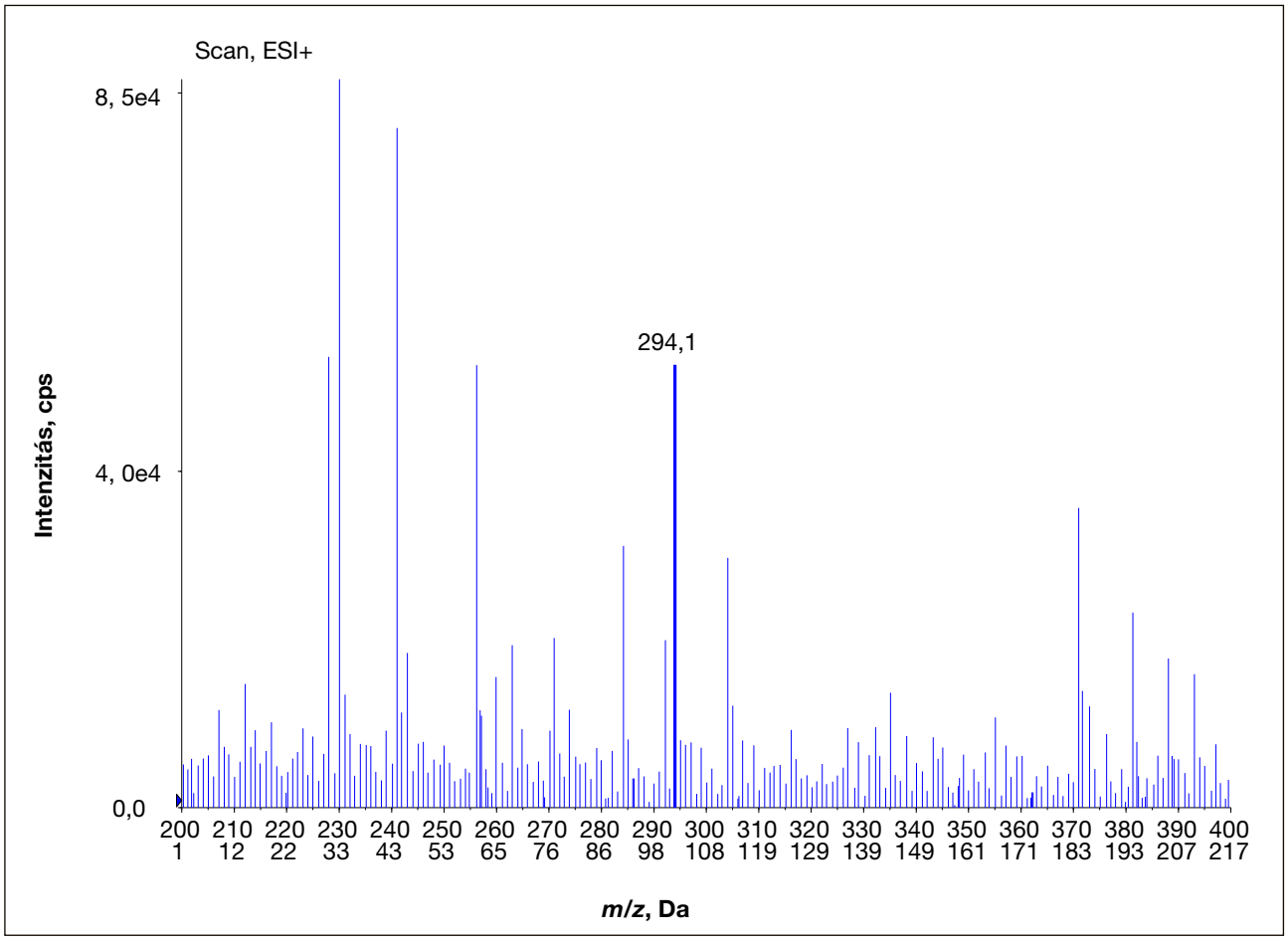


Figure 3. Mass spectrum of derivatized vitamin K3 (5 µg/mL).

Therefore, the reaction presumably also took place with L-cysteine, which was confirmed by recording the product ion spectrum (***Figure 4***).

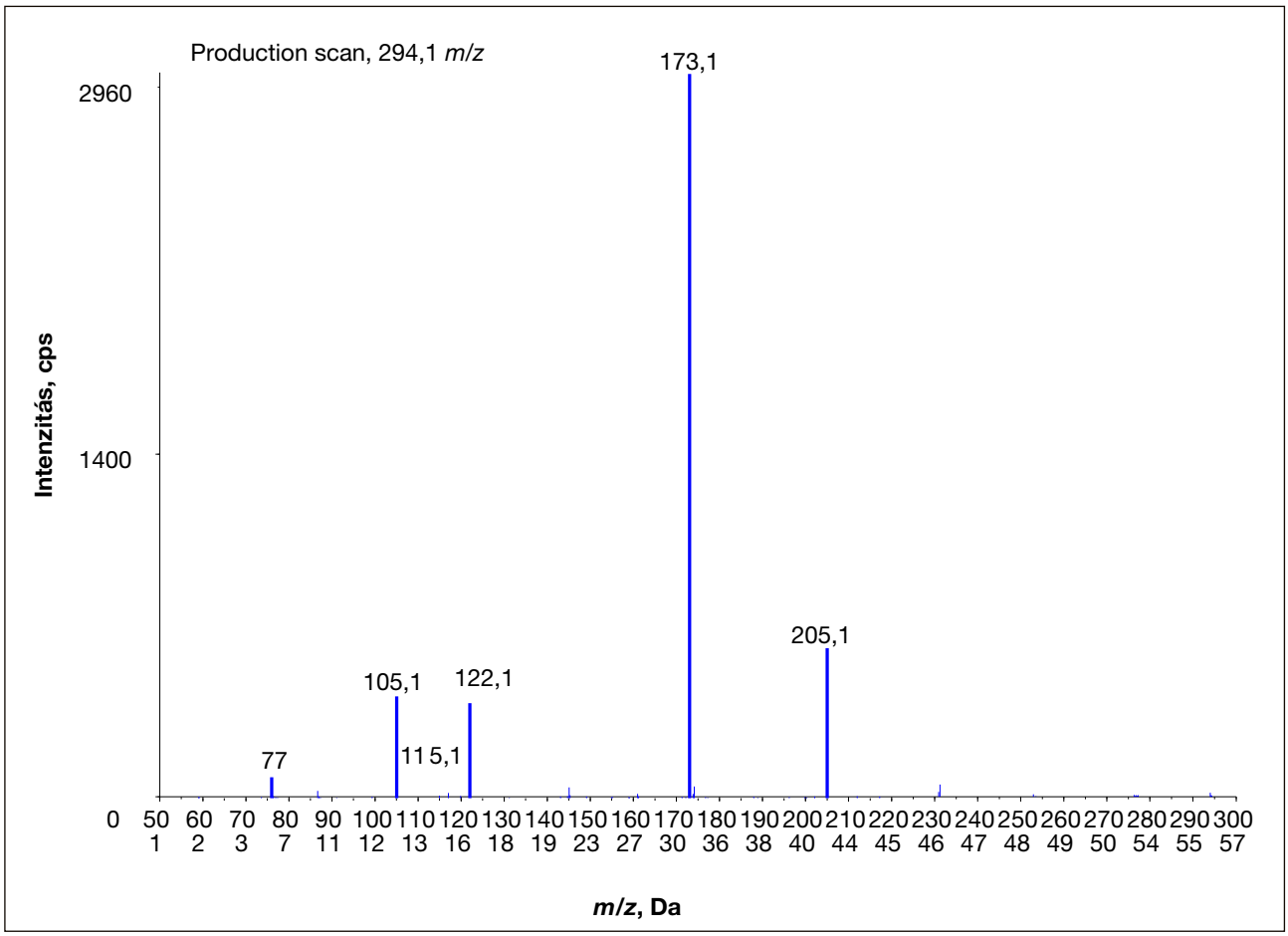


Figure 4. Product ion spectrum of derivatized vitamin K3 (5 µg/mL).

In the product ion spectrum, the *m/z* 294.1 ion was fragmented with a collision energy of 15 V, the fragments are listed in ***Table 4***. *m/z* 115.1 and *m/z* 205.1 ions clearly belong to vitamin K3, confirming the structures of vitamin K3 fragments reported by Yuan et al. **[11]**. The *m/z* 173.1 fragment corresponds to the protonated molecule of vitamin K3, while the *m/z* 122.1 fragment is the protonated molecule of L-cysteine.

4.2. Method validation, proficiency testing

During the validation of the methods, there were no interfering signals in the blank samples within the retention window of the target components and the ion ratios of the target components detected in the samples were the same as the ion ratios calculated for the calibration solutions, thus the condition for MS/MS identification was met. Calibration was linear between 0.01 and 1.0 µg/mL concentration, above which (1.0–10.0 µg/mL) the curve became quadratic in nature. Relative recovery values corrected with the ISTD fulfilled the 80-120% criterion and the precision values (RSD%) did not exceed 10% (***Tables 5-9***).

Table 5. Reproducibility analysis of vitamins A, D2, D3 and E in wheat flour at 0.5 and 5.0 mg/kg levels.

0.5 mg/kg	**Vitamin A**	**Vitamin D2**	**Vitamin D3**	**Vitamin E**
Mean (mg/kg)	0.504	0.511	0.509	0.476
S (mg/kg)	0.008	0.008	0.016	0.062
RSD%	1.63	1.64	3.11	13.0
Recovery%	101	102	102	95.2
5.0 mg/kg	**Vitamin A**	**Vitamin D2**	**Vitamin D3**	**Vitamin E**
Mean (mg/kg)	5.12	5.21	5.05	5.16
S (mg/kg)	0.237	0.008	0.113	0.174
RSD%	4.63	1.64	2.24	3.38
Recovery%	102	102	101	103

Table 6. Reproducibility analysis of vitamins A, D2, D3 and E in soft drinks at 0.5 and 5.0 mg/kg levels.

0.5 mg/kg	**Vitamin A**	**Vitamin D2**	**Vitamin D3**	**Vitamin E**
Mean (mg/kg)	0.504	0.505	0.508	0.531
S (mg/kg)	0.013	0.010	0.013	0.054
RSD%	2.55	2.03	2.49	10.1
Recovery%	101	101	102	106
5.0 mg/kg	**Vitamin A**	**Vitamin D2**	**Vitamin D3**	**Vitamin E**
Mean (mg/kg)	5.07	5.05	5.07	5.30
S (mg/kg)	0.107	0.068	0.092	0.085
RSD%	2.11	1.35	1.81	1.61
Recovery%	101	101	101	106

Table 7. Reproducibility analysis of vitamins A, D2, D3 and E in effervescent tablets at 0.5 and 5.0 mg/kg levels.

0.5 mg/kg	**Vitamin A**	**Vitamin D2**	**Vitamin D3**	**Vitamin E**
Mean (mg/kg)	0.514	0.509	0.493	0.526
S (mg/kg)	0.015	0.007	0.013	0.012
RSD%	3.01	1.46	2.59	2.19
Recovery%	103	102	98.5	105
5.0 mg/kg	**Vitamin A**	**Vitamin D2**	**Vitamin D3**	**Vitamin E**
Mean (mg/kg)	5.20	5.22	5.49	5.68
S (mg/kg)	0.214	0.184	0.217	0.135
RSD%	3.98	3.43	3.87	2.31
Recovery%	104	104	110	114

Table 8. Reproducibility analysis of vitamins A, D2, D3 and E in dietary supplements at 0.5 and 5.0 mg/kg levels.

0.5 mg/kg	**Vitamin A**	**Vitamin D2**	**Vitamin D3**	**Vitamin E**
Mean (mg/kg)	0.520	0.521	0.505	0.515
S (mg/kg)	0.024	0.019	0.012	0.039
RSD%	4.58	3.58	2.30	7.54
Recovery%	104	104	101	103
5.0 mg/kg	**Vitamin A**	**Vitamin D2**	**Vitamin D3**	**Vitamin E**
Mean (mg/kg)	5.33	5.63	5.49	4.87
S (mg/kg)	0.322	0.387	0.211	0.134
RSD%	5.98	6.80	3.87	2.67
Recovery%	107	113	110	97.5

Table 9. Reproducibility analysis of vitamin K3 in dietary supplements at 0.1 and 1.0 mg/kg levels.

01 mg/kg	**Vitamin K3**	**1.0 mg/kg**	**Vitamin K3**
Mean (mg/kg)	0.0931	Mean (mg/kg)	1.083
S (mg/kg)	0.0041	S (mg/kg)	0.0835
Recovery%	93.1	Recovery%	108
RSD%	4.40	RSD%	7.71

The limit of quantification (LOQ) was defined as the lower calibration point, which corresponds to 0.05 mg/kg due to the fivefold dilution of the sample. The LOQ could be further reduced by a lower sample dilution or by increasing the injection volume. The accuracy of the method was verified by participation in domestic and international proficiency tests. In the program organized by NÉBIH, infant formula contained vitamins A and E; the values assigned to the sample for vitamins A and E were 0.495 and 13.6 mg/100 g. The values detected by us were 0.465 and 13.6 mg/100 g, corresponding to Z-scores of -0.3 and 0.0. The condition for a successful proficiency test is $-2 \leq Z \leq 2$. Organized by FAPAS, the second proficiency test sample was a liquid vitamin dietary supplement in which the vitamin D3 content was analyzed and a vitamin D3 concentration of 0.206 mg/100 g was detected. The target value was 0.211 mg/100 g, for which the calculated Z-score is -0.2, so it was acceptable. Our proficiency test results are summarized in ***Table 10***.

Table 10. Proficiency testing results.

Matrix	Component	Measured value (mg/100 g)	Value assigned to the sample (mg/100 g)	Z-score value	Evaluation
Infant formula	Vitamin A	0.465	0.495	-0.3	Acceptable
	Vitamin E	13.6	13.6	0.0	Acceptable
Liquid vitamin dietary supplement	Vitamin D3	0.206	0.211	-0.2	Acceptable

5. Conclusions

The goal of this paper was to develop a new LC-MS/MS method for the determination of fat-soluble vitamins in food and dietary supplement samples. By combining the analysis with isotope dilution, it was possible to develop a method with great accuracy and high precision, which was validated within the laboratory and was successfully applied in domestic and international proficiency tests.

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