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VALIDATION OF A RAPID METHOD FOR SIMULTANEOUS DETERMINATION OF VITAMINS A AND E IN MILK USING HPLC

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Key words: all-trans-retinol, α-tocopherol, milk, HPLC

A method for the simultaneous determination of vitamin A (all-trans-retinol) and vitamin E (α-tocopherol) in milk using the HPLC technique with UV detection (324 nm, vitamin A) and fluorescent detection (Ex295 nm/Em350 nm, vitamin E) was validated. A reverse phase LiChroCART™ 250-4 Superspher™ 100 RP-18 column was used for chromatographic separation. A mixture of methanol and H_2O (96.5:3.5 v/v) was used as the eluent (1 mL/min). The analyses were performed after spectrophotometric standardization of standard ethanol solutions, and the results were corrected by recovery. The residual coefficients of variation for the regression equation y=ax were 4.7% (vitamin A) and 8.3% (vitamin E), with $r^2 > 0.998$ (for both vitamins). The limit of quantitation was 0.02 μg/mL and 0.3 μg/mL, repeatability and reproducibility 14% and 12.5%, and uncertainty of the method 20.7% and 18.8% for vitamins A and E, respectively. Vitamin recovery from milk was 51.6-75.1% (vitamin A) and 70.1-87.8% (vitamin E). The results of the reference material analyses (CRM 421, SRM 2383) concur with the certified reference values. The analytical method described is precise, accurate, fast and inexpensive.

INTRODUCTION

High-performance liquid chromatography (HPLC) is the main technique used for determination of vitamins A and E, and these vitamins are most often determined in separate analyses. A method for determination of vitamin A (all-trans-retinol) and vitamin E (α-tocopherol) in animal tissue during one chromatographic analysis (HPLC) was described and validated by Gąsior & Pieszka [2006]. The present study describes a method for the simultaneous determination of all--trans-retinol and α-tocopherol in milk and characterizes the method in terms of parameters such as calibration linearity, limit of quantitation, repeatability, reproducibility, uncertainty and recovery. One of the validation elements is to use this method to analyse reference materials and confirm method reliability by comparing the results obtained with reference values. The newly developed methods would be characterized by little use of organic solvents employed during preparation of samples for analysis, unlike traditional techniques [AOAC, 1990].

MATERIAL AND METHODS

Reagents, equipment, conditions for analysis and materials studied. The solvents used were methanol, ethyl acetate and *n*-hexane with gradient grade for HPLC by Merck (Darmstadt, Germany). The reagents used were 96% ethyl alcohol by Chempur (Piekary Śl., Poland), L(+) ascorbic acid by Merck (Darmstadt, Germany) and analytical grade KOH and NaCl by POCH (Gliwice, Poland). The vitamin standards used were all-trans-retinol (vitamin A) and α-tocopherol (vitamin E) by Sigma-Aldrich-Fluka (St. Louis, USA). Gaseous nitrogen and deionized water (H_2O, Millipore, Vienna, Austria) were used for the analyses. A Merck-Hitachi HPLC system (Darmstadt, Germany), equipped with an L-7100 pump, L-7250 autosampler (40 μL injection), FL L-7485 fluorescent detector (for α-tocopherol determinations, λ Ex=295 nm, λ Em=350 nm) and L-7420 UV-VIS detector (for all-trans-retinol determinations, λ=324 nm) was used. Chromatographic separation was carried out on a LiChroCART™ 250-4 Superspher™ 100 RP-18 column, 4 μ (Merck, Darmstadt, Germany). The eluent used was methanol+H_2O (96.5:3.5 v/v, 1 mL/min). Data were integrated using HSM D-7000 LaChrom software by Merck-Hitachi (Darmstadt, Germany) and converted using Excel software. Standards were standardized using a Beckman DU640 spectrophotometer (Fullerton, USA). An ultrasonic cleaner (eluent degassing before analysis), a water bath and a vortex mixer (Reax Control Heidolph, Germany) were used for the analyses and preparation of samples.

A total of 26 different samples of goat's milk were used for validation study and determination of vitamin content. The samples were stored in polypropylene dishes at about -19°C until analysis. The method was validated using two reference materials. The first material was milk powder CRM

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421 [Finglas *et al.*, 1997], for which the certified vitamin E content was 99 ± 6 μg/g. The second reference material was a mixture of meat and vegetables [SRM 2383, 2002], for which the certified vitamin contents were 0.8 ± 0.15 μg/g (vitamin A) and 25 ± 3.3 μg/g (vitamin E). The samples of the CRM 421 reference material were taken for analyses in liquid form, *i.e.* after they were dissolved 10 times in H_2O. Seven samples of the SRM 2383 reference material and 4 samples of the CRM 421 reference material were analysed in replicate together with analysis of recovery. The results were compared with certified values of vitamin A (SRM 2383) and E contents (CRM 421 and SRM 2383) determined for this material.

Preparation of standard solutions and samples and making analyses. Standard ethanol solutions of all-trans--retinol (WA) and α-tocopherol (WE), with concentrations of 3 μg/mL and 62.4 μg/mL, respectively, were made separately. This was followed by spectrophotometric measurements of these solutions at the following wavelengths: 325 nm (all--trans-retinol) and 292 nm (α-tocopherol). Activity (%) was calculated by comparing the extinction values measured with the reference extinction values of 0.1835 for all-trans--retinol concentration of 1 μg/mL (325-326 nm, Sigma product information: [www.sigmaaldrich.com]) and 7.58 for α-tocopherol concentration of 1000 μg/mL (292 nm, [Eitenmiller & Landen, 1999]). Concentrated WA and WE standard solutions were stored at 2-8°C in vials wrapped in aluminium foil. WA and WE standard solutions were used to make the basic WAE standard of both vitamins at concentrations of 1.2 μg for vitamin A/100 μL and 31.2 μg for vitamin E/100 μL. These values were corrected using activity determined during the standardization of standard solutions. External standards were prepared by the dilution of the WAE standard to the range of concentrations adjusted to the vitamin content of the material studied.

The samples were measured or weighed (0.5 mL milk or 0.5 g SRM 2383) into vials (12 mL). A portion of 1 mL of saturated solution of ascorbic acid in ethanol, 2 mL KOH (60% m/v), 100 μL NaCl (1%, m/v) and 100 μL ethanol were added. Vials were sealed and shaken and their contents were saponified in a water bath (70(C, 60 min). After cooling, 4.5 mL 1% (m/v) NaCl solution was added, followed by extraction of 3 mL ethyl acetate and hexane mixture (1:9; v/v). After cooling and separation of layers, 1.5 mL of the supernatant was collected into chromatographic vials and evaporated to dryness under nitrogen in a water bath (40(C). The remainder was diluted in 400 μL ethanol. This solution was placed on a chromatographic column. The recovery was determined by preparing the samples supplemented with the standard, as above, adding 100 μL WAE instead of 100 μL ethanol. A blank sample was prepared in the same way as the analysed sample, by replacing its volume with an equivalent volume of H_2O.

To determine the level of vitamins A and E (all-trans-retinol and α-tocopherol) in the samples of milk and reference materials, standardization and correction of the standard concentrations was carried out, the external standard of the vitamin mixture was prepared, and a blank sample and the samples tested were prepared. This was followed by chromatographic analyses of the external standard, blank sample and the sample tested. For the analysis of recovery, additional samples with added standard were prepared and their analyses were performed in replicate. The vitamin content of the materials tested was determined with regard to the external standard and taking the blank sample and recoveries into account.

Validation. Over the 6 months, a mixture of vitamins was prepared four times in ethanol with concentrations of 1.17--1.40 μg/mL (vitamin A) and 34.75–37.44 μg/mL (vitamin E). Further solutions were obtained by 2-fold dilution with ethanol of the previous solution with the higher vitamin concentration, to produce standard solutions ranging from approx. 0.019 to approx. 1.40 μg all-trans-retinol, and from approx. 0.543 μg to approx. 37.44 μg α-tocopherol in 1 mL ethanol. The y=ax calibration curve was deleted from the data obtained. The limit of quantitation (*LOQ*) was calculated from the formula [Dobecki, 1998] $LOQ = n \times SD_0 / a$, where $n = 3$, SD_0 is the standard deviation of the peak area of a given vitamin in a blank sample, and a is the sensitivity of determination defined as the ratio of the peak area of a given vitamin to its level in the standard. The limit of quantitation was determined in terms of 1 mL milk.

Repeatability was defined as being not lower than the pooled coefficient of variation for determinations of 26 samples of goat's milk and 4 samples of CRM 421 reference milk ($k=30$) in replicate ($n=2$), carried out on successive days by the same laboratory worker. The pooled coefficient of variation (*CVkn*) was calculated from the formula

$$CVkn = \sqrt{\frac{\sum_k CVn^2}{k}}$$

where: k – number of samples; *CVn* – coefficient of variation ($n=2$) calculated from the formula:

$$CVn = 100 \times \frac{SD}{Xav}$$

SD – standard deviation for two measurements of a given sample; *Xav* – average from two measurements of a given sample.

Reproducibility was defined as being not lower than the pooled coefficient of variation for determinations of 7 samples of goat's milk ($k=7$) in replicate ($n=2$), carried out on one day and analogous determinations of the same samples on the second day. To determine reproducibility, consecutive pairs of determinations from both days were combined, using analogous formulas as for the repeatability calculation.

The uncertainty of the sample analysis (*Us*) was defined as the maximum value of repeatability and reproducibility and multiplied by 1.41, which corresponds to double the coefficient of variation of the arithmetic mean ($2 \times CVav$) from two measurements of a given sample ($n=2$; $p \leq 0.05$). The uncertainty of the standardization (*Ust*) was defined similarly as *Us*, but repeatability and reproducibility expressed as the pooled coefficient of variation was calculated based on the

determinations of 15 standard samples in replicate ($k=15$, $n=2$). The uncertainty of recovery (U_R) was defined as double the coefficient of variation of the arithmetic mean from measurement of 8 milk samples (CRM 421). The uncertainty of the method (U) accounts for the uncertainty of sample determination and uncertainty of the standard and recovery. It was calculated according to the Gauss law of propagation using the formula $U=(Us^2+Ust^2+U_R^2)^{1/2}$.

RESULTS AND DISCUSSION

The calibrations performed based on the standard solutions were characterized by good linearity, as confirmed by the high coefficients of correlation ($r^2>0.998$) and relatively low residual coefficients of variation (4.7% for vitamin A and 8.3% for vitamin E). The sufficiently low limit of quantitation, determined based on the method sensitivity, makes it possible to analyse samples with a low level of all-trans-retinol (above 0.02 μg/mL) and α-tocopherol (above 0.3 μg/mL).

The coefficient of variation (*CV* or *CVkn*) can be used to characterize method repeatability and reproducibility. The values of the coefficients of variation for the determined substances depend, among other things, on the content of these substances in the sample, the type of material determined and its homogeneity, and the errors the coefficients contain. These can include errors of determination by the apparatus itself (2-5%), but can also include other errors, *e.g*. errors at any stage of sample preparation (1.5%–7.3%; [PN ISO 14565, 2002; PN-EN ISO 6867, 2002; Barbas *et al.*, 1997; Pieszka *et al.*, 2002]) and errors of determination at different laboratories that use different methods [Dobecki, 1998], different laboratory assistants or different determination times (2.6% – 15% [Gąsior & Pieszka, 2006; PN ISO 14565, 2002; PN-EN ISO 6867, 2002; Barbas *et al.*, 1997]). The coefficients of variation obtained in the present study for the determinations of vitamins A and E (14% for vitamin A and 12.5% for vitamin E) were similar to the corresponding coefficients of variation obtained in the studies mentioned above.

The uncertainties were calculated on the basis of principles described in the Eurachem Guide [Ellison *et al.*, 2000]. The greatest effect on the uncertainty of measurement is exerted by the repeatability of the method and related errors of analysis such as weighing, hydrolysis, pipetting and transfer errors, errors due to inaccuracy of the measuring equipment used during sample preparation, and error in chromatographic determination. It is worth noting at this point that repeatability expressed as the pooled coefficient of variation CV_{kn} comprises the above errors only for chromatographic determinations of solutions derived from two parallel weighed samples prepared for analysis [Gąsior *et al.*, 2005].

If routine analysis of the sample is performed in replicate ($n=2$), the error of the mean value of these replicates is described by the coefficient of variation of the arithmetic mean *CVav,* equivalent to standard uncertainty u_x ($p\leq0.32$), which can be described by the following equation:

$$CVav = \frac{CVkn}{\sqrt{n}} = \frac{CVkn}{\sqrt{2}} = 0.707 \times CVkn$$

The extended uncertainty ($p\leq0.05$), designated as $U_{0.05}$, is described by the formula:

$$U_{0.05} = 2 \times CVav \approx 1.41 \times CVkn$$

The extended uncertainty, calculated in this way, is the uncertainty of sample analysis (*Us*).

This uncertainty for the level of vitamins A and E was 19.7% and 17.6%, respectively. Other factors of uncertainty, connected with the error of the standardization (*Ust,* 4.1%) and recovery (U_R, 5.0%), like *Us*, have some effect on the total method uncertainty. The uncertainty of the method (U, $p\leq0.05$) calculated in the studies described here was 20.7% (vitamin A) and 18.8% (vitamin E). This includes all stages of sample preparation, errors due to inaccuracy of the measuring equipment used during sample preparation and chromatographic analysis (*Us*), and the error of the standardization (*Ust*) and recovery (U_R). The uncertainty, together with the result treated as a mean of measurements, is of practical importance during the interpretation of the result and determines the range of tolerance (%) in which the actual value of the determination result should have a probability of 95% ($p\leq0.05$). The uncertainty determined in the present study expresses the error of analyses performed in one laboratory. It should be controlled at each sample analysis by checking repeatability and can be greater for those analyses which fail to meet the criterion of repeatability despite repeated tests.

The values for vitamin recovery from the samples of reference milk were 75.1% (vitamin A) and 87.8% (vitamin E). The mean recovery from the other milk samples was 51.6% and 70.1%, respectively. The recovery values and the observations made during sample preparation indicate that vitamin recovery clearly depends on the partition coefficient of vitamin concentration (Nernst partition law) between phases formed at extraction during sample preparation, and this, in turn, depends on the type of material analysed, including, most probably, the content of fat. By way of comparison, vitamin recovery values obtained in other studies [Qian *et al.*, 1998; Barbas *et al.*, 1997; Ake *et al.*, 1998; Gąsior & Pieszka, 2006] ranged form 62% to 102% (vitamin A) and from 76% to 112% (vitamin E).

The mean levels (μg/g) of vitamin E in the analysed samples of CRM 421 and SRM 2383 reference material were 95 (reference value range of 93-105) and 23.33 (reference value range of 21.7-28.3), respectively. The level (μg/g) of vitamin A determined in SRM 2383 was 0.87 (reference value range of 0.65-0.95). The vitamin levels found in the reference materials are within certified ranges (Figure 1), which shows the good compatibility and correctness of the analyses performed using the method described. Sample chromatograms for the determinations of all-trans-retinol (UV 324 nm) and α-tocopherol (FL: Ex295 nm, Em350 nm) in milk are given in Figures 2 and 3. The level of vitamins A and E (μg/mL) in the 26 samples of goat's milk was 0.86±0.32 (SD) and 1.00±0.29 (SD), respectively. The values obtained in the present study are comparable with approximate tabular values [Kunachowicz *et al.*, 2002].

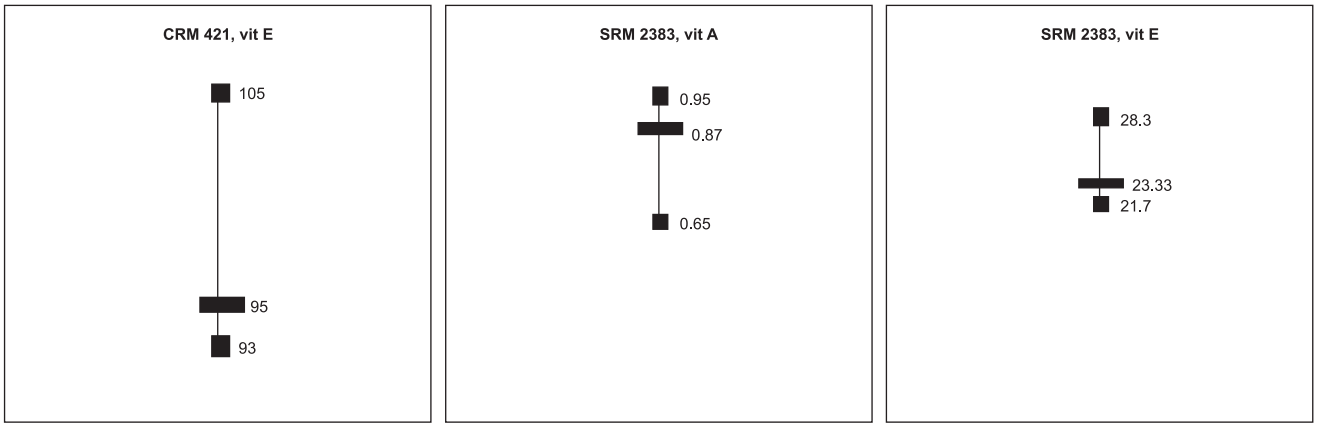

FIGURE 1. Determined levels of vitamins A and E in CRM 421 and SRM 2383 reference materials and ranges of certified reference values

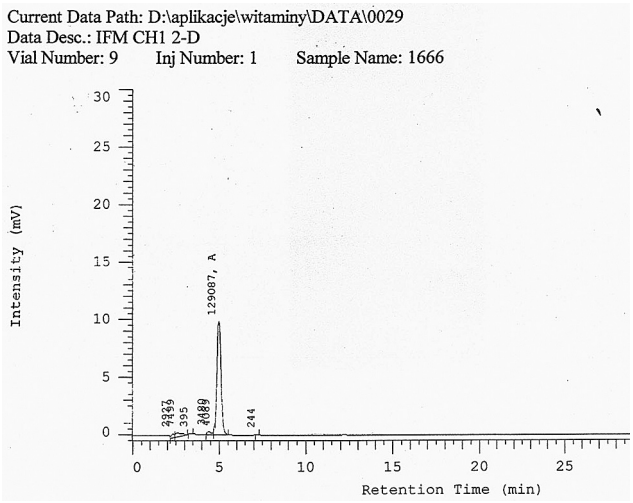

FIGURE 2. HPLC chromatogram for determination of all-trans-retinol (vit. A) in milk.

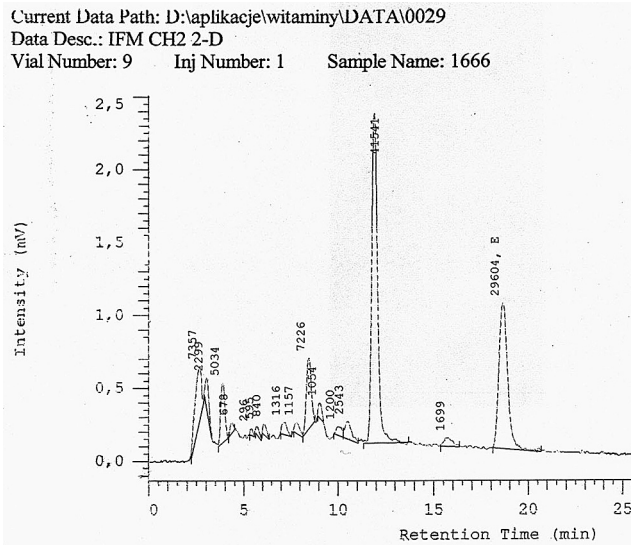

FIGURE 3. HPLC chromatogram for determination of α-tocopherol (vit. E) in milk.

CONCLUSIONS

The results of the validation procedure indicate that the proposed method is precise, sufficiently accurate, fast and inexpensive. The presented analytical method, which is based on the HPLC technique and covers recovery tests for a concrete matrix of the analysed material, is suitable for determining the vitamin A and E content of milk.

REFERENCES

1. Ake M., Fabre H., Malan A.K., Mandrou B., Column liquid chromatography determination of vitamins A and E in powdered milk and local flour: a validation procedure. J. Chromatogr., 1998, 826, 183-189.
2. AOAC, Official Methods of Analysis. Association of Official Analytical Chemists., 15th Edition, 1990, Washington DC, pp. 1071-1074.
3. Barbas C., Castro M., Bonet B., Viana M., Herrera E., Simultaneous determination of vitamins A and E in rat tissues by high-performance liquid chromatography. J. Chromatogr., 1997, 778, 415-420.
4. Dobecki M., Zapewnienie jakości analiz chemicznych. 1998, Instytut Medycyny Pracy im. prof. dra med. J. Nofera Press, Poland, Łódź, pp. 60, 95-96 (in Polish).
5. Eitenmiller R.R., Landen W.O., Vitamin analysis for the health and food sciences. 1999, CRC Press Inc., Boca Raton, USA, p. 116.
6. Ellison S.L.R., Rosslein M., Williams A. (Ed), Quantifying Uncertainty in Analytical Measurement., 2000. Eurachem/Citac Guide.
7. Finglas P. M., Berg H. van den, Froidmont-Görtz I., The Certification of the mass fractions of vitamins in three reference materials: margarine (CRM 122), milk powder (CRM 421) and lyophilized Brussels sprouts powder (CRM 431) – vitamins A, D3 & E in CRM 122; C, D3, E & Niacin in CRM 421, and C & Niacin in CRM 431., 1997, Luxembourg, Off. for Off. Publ. of the Europ. Communities (Report EUR 18039 EN).
8. Gąsior R., Pieszka M., Evaluation of vitamins A and E level in meat by HPLC. Anim. Sci., 2006, 1, Suppl. October 2006, 88--89.

9. Gąsior R., Ślusarczyk K., Szczypuła M., Validation of a method for determining amino acids in acid hydrolysates of feeds. Ann. Anim. Sci., 2005, 5, 1, 181-197.
10. Kunachowicz H., Nadolna I., Iwanow K., Przygoda B., Wartość odżywcza wybranych produktów spożywczych i typowych potraw. 2002, PZWL Warszawa (in Polish).
11. Pieszka M., Gąsior R., Barowicz T., Evaluation of HPLC method for the rapid and simple determination of α-tocopherol acetate in feed premixes. J. Anim. Feed Sci., 2002, 11, 527-536.
12. PN-EN ISO 14565:2002. Animal feeding stuffs-Determination of vitamin A content-Method using high-performance liquid chromatography (in Polish).
13. PN-EN ISO 6867:2002. Pasze. Animal feeding stuffs-Determination of vitamin E content-Method using high-performance liquid chromatography (in Polish).
14. Qian H., Sheng M., Simultaneous determination of fat-soluble vitamins A, D and E and pro-vitamin D2 in animal feeds by one-step extraction and high-performance liquid chromatography analysis. J. Chromatogr., 1998, 825, 127-133.
15. Standard Reference Material 2383, Certificate of Analysis, 2002, National Institute of Standards & Technology, Gaithersburg, MD 20899.
16. Sigma Product Information: [www.sigmaaldrich.com], retinol all trans, R-7632.

WALIDACJA SZYBKIEJ METODY JEDNOCZESNEGO OZNACZANIA WITAMIN A ORAZ E W MLEKU TECHNIKĄ HPLC

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Zwalidowano metodę jednoczesnego oznaczania witaminy A (all-trans-retinol) i witaminy E (α-tokoferol) w mleku techniką HPLC z detekcją UV (324 nm, witamina A) i fluoroscencyjną (Ex295 nm/Em350 nm, witamina E). Do rozdziału chromatograficznego użyto kolumny LiChroCART™ 250-4 Superspher™ 100 RP-18. Jako eluent (1 mL/min) zastosowano mieszaninę metanolu z H_2O (96,5:3,5 v/v). Alkoholowe roztwory próbek analizowano po uprzednim zmydleniu witamin (KOH, 60%, m/v, 70°C, 60 minut) w środowisku etanolu, wysoleniu, ekstrakcji (octan etylu i heksan, 1:9, v/v) górnej warstwy mieszaniny, odparowaniu pod azotem i rozpuszczeniu pozostałości w etanolu. Wyniki analiz korygowano o wcześniej wyznaczony odzysk (51,6-75,1%, witamina A i 70,1-87,8%, witamina E). Resztowe współczynniki zmienności równania regresji y=ax wynosiły 4,7% (witamina A) i 8,3% (witamina E), a $r^2 > 0,998$ (dla obu witamin), granica oznaczenia ilościowego 0,02 μg/mL i 0,3 μg/mL, powtarzalność i odtwarzalność 14% i 12,5%, a niepewność metody 20,7% i 18,8%, odpowiednio dla witaminy A i E. Wiarygodność metody potwierdzono na materiałach referencyjnych (CRM 421 i SRM 2383). Opisana metoda analityczna, jest precyzyjna, dokładna, szybka i tania.