

## MODIFICATIONS OF SPECTROPHOTOMETRIC METHODS FOR ANTIOXIDATIVE VITAMINS DETERMINATION CONVENIENT IN ANALYTIC PRACTICE\*

Maciej Rutkowski, Krzysztof Grzegorzczuk  
Medical University of Łódź

**Abstract.** Although there are numerous methods for quantitative determination of antioxidative vitamins: C, E, and A, there are no methods favourable for the broadly understood analytic practice – they have various faults and limitations or require expensive apparatus. Therefore, we have elaborated modifications of valuable spectrophotometric methods for determination each of those vitamins, which originally could not be applied for laboratory practice from various regards. They are based on: for vitamin C – colour reaction with periodically prepared phosphotungstate reagent; for vitamin E – colour reaction with batophenanthroline,  $\text{FeCl}_3$  and  $\text{H}_3\text{PO}_4$ ; for vitamin A – spectrophotometric measurement of extracts of tested samples. Control tests showed complete correctness of analytic parameters obtained with those modifications with preservation of advantages of the original methods. Therefore they can be successfully implemented to the routine clinical analyses'. The elaborated modifications can also be used for determination of the a/m vitamins in foodstuffs, for example: in juices, milk and homogenates or extracts of solid food.

**Key words:** antioxidative vitamins, spectrophotometry, food, biological samples

### INTRODUCTION

In nutritional sciences and medicine, there is much interest in the analytics of vitamins C, E and A because, besides having vitamin activity, they are also characterized by antioxidative action and are therefore defined as antioxidative vitamins. By neutralising reactive oxygen species and free oxygen radicals in the organism, these vitamins play

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Corresponding author – Adres do korespondencji: Dr Maciej Rutkowski, Chair of Clinical Chemistry and Biochemistry of Medical University, Hallera 1, 90-647 Łódź, Poland, e-mail: marut@neostrada.pl

a significant role in prevention of numerous metabolic degenerative diseases [Przeciwnieniaczne... 2007]. As food is the main source of the vitamins for humans, determination of their content in food products seems to be an important element of nutritive value evaluation of those products [Kompendium... 2006]. Clinical tests which are performed on the biological material collected from patients are equally important, for they allow to recognize deficiency of those vitamins and to introduce some changes to the everyday diet and/or apply supplementation with pharmaceutical vitamin preparations.

However, performing antioxidative vitamin determinations is a difficult analytical task. This is largely due to instability of these compounds which are susceptible to atmospheric oxygen and other oxidative agents, as well as to heat, light, high and low pH values, and the presence of transient metal ions [Kołodziejczyk 2004]. Choosing appropriate analytical methods which would be sufficiently sensitive, accurate, selective, and simultaneously not excessively time- and work-consuming, being also adjusted to small sample volume, available for routine analysis and financially affordable (cost of apparatus and reagents) can also be troublesome. Although numerous methods for determination of vitamins C, E and A are available: titrometric or – currently most frequently used – instrumental: electrochemical, fluorometric, spectrophotometric and chromatographic [Moszczyński and Pyć 1999], still they are burdened with various faults and limitations. Reports frequently fail to describe important methodological details, which can compel own individual interpretations and inhibit successful implementation of those methods into the analytical practice.

The authors previously elaborated their own modifications of few known spectrophotometric methods for determination of: vitamin C – acc. to Kyaw [1978], vitamin E – acc. to Tsen [1961] and vitamin A – acc. to Bessey et al. [1946]. These methods despite that they are selective, fast and easy to perform, as well as applicable to the semi-micro scale tests and cheap, are not suitable for general implementation due to the reasons given in the Discussion. Introduction of complements, corrections and changes to those methods have led to rise of modifications, convenient for the broadly understood analytical practice.

Although the above modifications have been developed for determination of antioxidative vitamins in blood, they have also been applied for assays in other biological samples including the highly acidic gastric juice and also tissues [e.g. Rutkowski et al. 1999, 2002]. The authors desire to propose the possibility of application of these modifications in food analytics.

## MATERIAL AND METHODS

### Material

Comestible or biological liquid, homogenate (or extract) of a food sample or tissue.

### Equipment

A centrifuge and, depending on the vitamin determined: a test-tubes shaker, a water bath, an UV lamp 250-300 nm, a VIS or UV spectrophotometer, glass or quartz cuvettes 1-1.5 ml.

## Reagents

**Determination of vitamin C:** phosphotungstate reagent (PR) – prepared periodically, as it's used up (suspension of 150 g sodium tungstate *molybdenium-free* and 60 g sodium hydrogen phosphate anhydrous in 240 ml deionized (DI) water, mix with heating to dissolve and add slowly 145 ml 3.7 M sulphuric acid (VI); heat the solution for 2 hours with reflux condenser not allowing it to boiling; after cooling the solution down, adjust pH to 1.0 adding dropwise concentrated sulphuric acid (VI) – the reagent should be light greenish-yellow, a darker one is useless); 56.8  $\mu\text{M}$  vitamin C (L-ascorbic acid) standard solution made with use 50 mM solution of oxalic acid as a solvent.

**Determination of vitamin E:** anhydrous ethanol; xylene (mixture of isomers); 6.02 mM solution of batophenanthroline\* (stable for three weeks in a fridge), 0.98 mM solution of anhydrous iron chloride (III) (stable for one week in a fridge) and 40 mM solution of crystalline orthophosphoric acid – all in anhydrous ethanol; 23.2  $\mu\text{M}$  standard solution of vitamin E (as substance Trolox\* – in DI water, or as  $\alpha$ -tocopherol – in anhydrous ethanol).

**Determination of vitamin A:** xylene (a/a); 1 M solution of potassium hydroxide in 90% ethanol.

## Performing of determinations

Analyse fresh samples, working on the stand protected against the direct raining light.

**Determination of vitamin C** [Rutkowski et al. 1998]:

- measure 1 ml of the analysed liquid into the centrifugal test-tube, add 1 ml of the PR, mix thoroughly and leave in a room temperature for 30 minutes
- centrifuge the tube (7000 $\times$ g, 10 minutes), and collect the whole of the separated supernatant with a pipette – the supernatant is a **test sample** for spectrophotometric measurements
- prepare the **standard sample** as above (using 1 ml of the standard solution instead of the analysed liquid), without centrifugation
- measure the absorbance of the test sample  $A_x$  and of the standard sample  $A_s$  at 700 nm against the mixture PR : 50 mM solution of oxalic acid = 1:1 (v/v) as a reference sample
- calculate concentration  $c_x$  of vitamin C ( $\mu\text{M}$ ) in the analysed liquid, using the formula:

$$c_x = \frac{A_x}{A_s} \cdot c_s$$

where:

$c_s$  – concentration of the standard solution.

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\***Batophenanthroline** is a commonly used colloquial name for 4,7-diphenyl-1,10-phenanthroline. **Trolox** is a synthetic, water soluble analogue of vitamin E (precisely of  $\alpha$ -tocopherol – it is predominating form), constituting the 6-hydroxy-2,5,7,8-tetramethylchromano-2-carboxylic acid.

**Determination of vitamin E** [Rutkowski et al. 2005]:

- measure 0.5 ml of the analysed fluid into the test-tube I (centrifugal) with a tight stopper, add 0.5 ml of anhydrous ethanol and shake vigorously the plugged test tube for 1 minute
- add 3 ml xylene, plug the test tube and shake vigorously for another 1 minute
- centrifuge the tube to separate the extract (1500×g, 10 minutes); simultaneously measure 0.25 ml solution of batophenanthroline into a usual test-tube II
- collect 1.5 ml of the extract (upper layer), transfer to the test-tube II and mix the content
- add 0.25 ml of FeCl<sub>3</sub> solution to the test tube II, mix, add 0.25 ml of H<sub>3</sub>PO<sub>4</sub> solution and mix again – this way a **test sample** is obtained for spectrophotometric measurements
- prepare the **standard sample** (0.5 ml of the standard solution instead of the analysed liquid): using Trolox – prepare as the test sample, using α-tocopherol – add 0.5 ml of DI water instead of anhydrous ethanol at the beginning of the analysis; do not centrifuge this sample
- measure absorbance of the test sample A<sub>x</sub> and of the standard sample A<sub>s</sub> at 539 nm against the blank test (preparation – as the test sample but using water instead of the analysed liquid)
- calculate concentration c<sub>x</sub> of vitamin E (μM) in the analysed liquid, using the a/a presented formula.

**Determination of vitamin A** [Rutkowski et al. 2006]:

- measure 1 ml of the analysed liquid to the test-tube I (centrifugal) with a tight stopper and add 1 ml of the KOH solution, plug the tube and shake vigorously for 1 minute
- heat the tube in a water bath (60°C, 20 minutes), then cool it down in cold water
- add 1 ml of xylene, plug the tube and shake vigorously again for 1 minute
- centrifuge the tube (1500×g, 10 minutes), collect the whole of the separated extract (upper layer) and transfer it to the test tube II made of “soft” (sodium) glass
- measure the absorbance A<sub>1</sub> of the obtained extract at 335 nm against xylene
- irradiate the extract in the test tube II to the UV light for 30 minutes, then measure the absorbance A<sub>2</sub>
- calculate the concentration c<sub>x</sub> of vitamin A (μM) in the analysed liquid, using the formula:

$$c_x = (A_1 - A_2) \cdot 22.23$$

where:

22.23 – multiplier received on basis of the absorption coefficient of 1% solution of vitamin A (as the retinol form) in xylene at 335 nm in a measuring cuvette about thickness = 1 cm.

## RESULTS

Our own study over modifying of the selected methods for quantitative determination of vitamins C, E and A, providing them the usefulness for the analytical practice, consisted of three stages presented in detail in Discussion.

In the first stage, keeping without changes the analytic bases it was executed on – the equipment and procedural modifications – resulting from requirements of routine analyses’ – in performing ways of determinations definite in original methods. The result of this stage of investigations was conferment to the modified methods of a technically useful form for the analytical practice.

The second stage of the study involved modifications of the analytical bases for individual methods with regard to the results obtained in the first stage in order to avoid any existing descriptive gaps, inconsistencies and experimentally found defects as well as to adapt the method by Tsen [1961] which was designed solely for determining vitamin E in solutions – for determination in biological material. The joint results of both the first and second stage became own modifications of the selected original methods, which the control of analytical correctness was subjected in the third stage of the study.

Control tests were made according to performance of determinations in biological liquids. A series of standard solutions – in bovine serum as a solvent – of vitamin C and water-soluble forms of vitamins E and A were used. Individual series included concentrations within the physiological range for those vitamins ( $\mu\text{M}$ ): for vitamin C (as L-ascorbic acid): 14.2, 28.4, 56.8, 85.2, 113.6, for vitamin E (as substance Trolox) 5.8, 11.6, 23.2, 34.8, 46.4, for vitamin A (as a complex of retinol acetate and cyclodextrane) 0.465, 0.93, 1.86, 2.79, 3.72.

The solutions were subject to the procedures according to the developed algorithms of determinations and final absorbance measurements yielded sets of points determining analytical curves in the A,c coordinate system which are presented in Figure 1. Based on the curves we found as follows:

- fulfilment of the Lambert-Beer law within the specified range of concentrations with the resulting complete linearity of curves and their passing through the point 0
- high accuracy of the modified methods which was determined by achievement of the curves correlation coefficients almost even to 1
- very good sensitivity of all the methods (detection limit of app.  $0.05 \mu\text{M}$ ) resulting from large angles of inclination of the curves
- no effect of the serum proteins on the a/m curves suggesting their complete denaturation during the analytical works and lack of interference from other reducers present in the serum.

Appropriate precision of the modified methods was confirmed by recovery tests, mean values of which, were obtained from three times repeated tests and were only slightly different than 100%. Additionally, high precision of the methods was confirmed by their repeatability and reproducibility. The parameters were tested for 5 consecutive days: concentrations of vitamins C, E and A in serum solutions stored at  $-80^\circ\text{C}$  (nominal concentrations: 51.3, 21.6, 1.34  $\mu\text{M}$ , respectively) were determined. Intra-serial coefficient of variation was calculated based on the mean concentrations obtained each day ( $n = 10$ ) and ranged from 0.7% to 3.2%. Inter-serial coefficient of variation calculated based on the individual daily means ( $n = 5$ ) was even 4.3-5.2%.

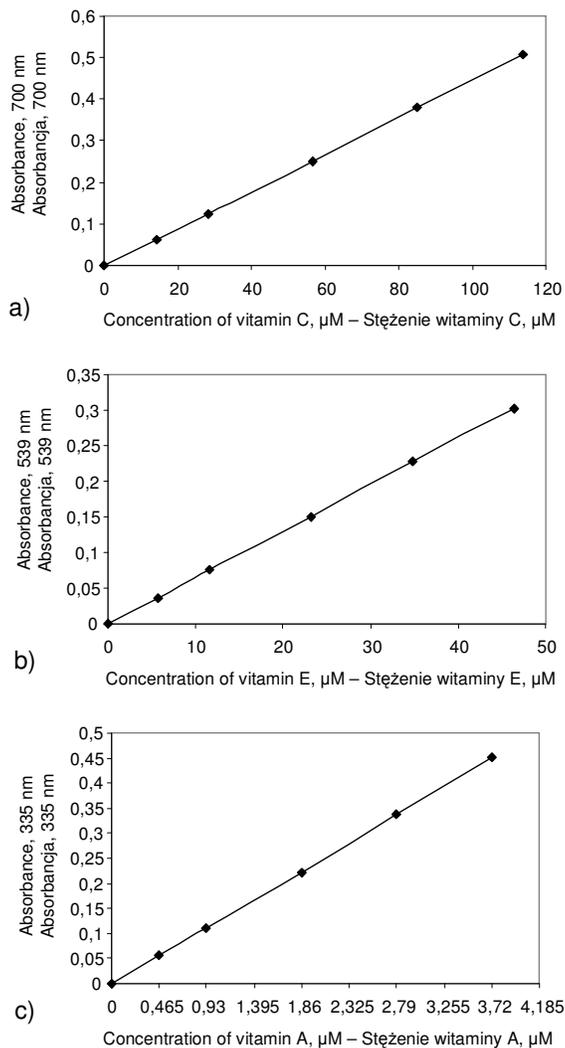


Fig. 1. Analytical curves for the elaborated modifications of spectrophotometric methods for antioxidative vitamins determination: a) vitamin C (using standard solutions of L-ascorbic acid), b) vitamin E (using standard solutions of Trolox – hydrophilic analogue of  $\alpha$ -tocopherol), c) vitamin A (using standard solutions of hydrophilic complex of retinol acetate with cyclodextrane); in all the a/m cases bovine serum was used as a solvent

Rys. 1. Krzywe analityczne dla opracowanych modyfikacji spektrofotometrycznych metod oznaczania witamin antyoksydacyjnych: a) witamina C (z użyciem roztworów wzorcowych kwasu L-askorbinowego), b) witamina E (z użyciem roztworów wzorcowych Troloxu – hydrofilowego analogu  $\alpha$ -tokoferolu), c) witamina A (z użyciem roztworów wzorcowych hydrofilowego kompleksu octanu retinolu z cyklodekstranem); rozpuszczalnikiem użytych roztworów była surowica bydlęca

Values of analytical parameters for the developed modifications of all methods which were obtained during the validation studies discussed above, are presented in the Table 1. The authors of the original methods did not perform similar studies, any comparative analysis is therefore impossible.

Table 1. Analytical parameters of the authors' modifications of spectrophotometric methods for antioxidative vitamins determination

Tabela 1. Parametry analityczne własnych modyfikacji spektrofotometrycznych metod oznaczania witamin antyoksydacyjnych

Modification of determination method of vitamin Modyfikacja metody oznaczania witaminy	Correlation coefficient of analytical curve Współczynnik korelacji krzywej analitycznej	Detection limit Granica wykrywalności μM	Recovery factor Współczynnik odzysku %	Coefficient of variation, % Współczynnik zmienności, %	
				intra-serial wewnątrzseryjny	inter-serial międzyseryjny
C	0.999	0.05	99.0-101.2	0.7-0.9	5.2
E	0.998	0.04	98.8-100.5	0.8-2.7	4.8
A	0.999	0.06	98.7-100.2	0.9-3.2	4.3

## DISCUSSION

To be able to determine vitamins A, E and C in biological material in a precise, fast and inexpensive way spectrophotometry was chosen. Despite availability of numerous instrumental methods, spectrophotometry seems to be most frequently used in analytical laboratories. This is due to a good precision of the method, availability of spectrophotometers as not very expensive apparatuses and usage of easily accessible, because relatively cheap reagents. As a result, spectrophotometric methods are widely used, among others in food analytics for determination of many natural compounds, including vitamins.

Numerous spectrophotometric methods allow quantitative determination of the reduced form of vitamin C (L-ascorbic acid) or, so called, the total vitamin C – a sum of the reduced and oxidised form (dehydro-L-ascorbic acid). A popular method using 2,4-dinitrophenylhydrazine [Roe and Kuether 1943] is used for determination of the total vitamin C. The method, however is not sufficiently accurate, its results being overstated due to the presence of 2,3-dioxo-L-gulonic acid (biologically inactive metabolite of the vitamin C) in the tested samples, besides it also requires 3-hour incubation at 37°C and addition of concentrated H<sub>2</sub>SO<sub>4</sub> to the samples. Another well known method which uses aqueous solutions of 2,2'-bipyridyl, FeCl<sub>3</sub> and H<sub>3</sub>PO<sub>4</sub> [Sullivan and Clarke 1955], allows selective determinations of the reduced vitamin C, but is characterized by low sensitivity, slow course of the reaction (1 hour) and turbidity of the tested samples which requires their final centrifugation. Various spectrophotometric adaptations of the Tillmans' titration method using 2,6-dichlorophenolindophenol (for example [Omaye et al. 1979]) are similarly applied. They are easy to perform but not entirely selective and require separate measurements of absorbance for the resulting colour and following its removal.

Vitamin E can be spectrophotometrically determined with two popular methods concerning the so called, total vitamin E which is a mixture of four tocopherols and four tocotrienols. According to the first one [Emmerie and Engel 1938] vitamin E is ex-

tracted with petroleum benzine from the tested samples and the extracts are exposed to ethanol solutions of 2,2'-bipyridyl and  $\text{FeCl}_3$ . The resulting colour allows for absorbance measurement but is unstable and lasts only for 30 seconds; selectivity and sensitivity of the determination low, as well. In the other method [Hashim and Schuttringer 1966] those drawbacks have been avoided by substituting 2,2'-bipyridyl with batophenanthroline and introducing  $\text{H}_3\text{PO}_4$  to increase stability of the colour. Sample extraction with heptane was also adopted in this method. A later modification [Desai and Machlin 1985] differs in the concentrations and volume of reagent solutions and exchanges heptane as the extracting agent for less flammable xylene. Our attempts to implement the latter method showed that the resulting colour is too dark, and absorbance readings fluctuate within the range of  $\pm 0.002$ . Marked instability of the resulting colour which was found for the modified version, makes reliable results impossible to obtain.

There are two known spectrophotometric methods for determination of vitamin A which are used for extracts. The most widely used one [Carr and Price 1926] is based on the use of  $\text{SbCl}_3$  solution in chloroform acting upon the chloroform-based extract of the vitamin A. The method is characterized by a high sensitivity but also by carotenoid interference (analytical corrections are required) with the resulting colour extremely unstable (app. 10 seconds). Moreover,  $\text{SbCl}_3$  is susceptible to traces of moisture and shows corrosive properties. Vitamin A can also be determined using the method of direct spectrophotometric measurement in UV [Parrish 1977]. The test sample mixed with ethanol and aqueous solution of KOH is heated under nitrogen until boiled for 30 minutes for deproteinization and hydrolysis of the vitamin A esters (mainly retinol acetate), for their absorption maxima are different from those of the free retinol in UV. The hydrolysate is extracted with ethyl ether, washed with water and the separated extract is evaporated under nitrogen till dry. The residue dissolved in isopropanol is measured for absorbance at several wavelengths. However, carotenoids and other polyenes with absorption peaks close to that of retinol interfere with vitamin A.

It should also be noted that in case of quantitative determination of vitamin A using the last method, the results are obtained calculationally, following substitution of the measured absorbance values to two formulas. In case of all the other methods of determination of antioxidative vitamins which have been discussed, the results are read from standard curves (as it is known the preparation is time- and work-consuming task). All but the last of the presented methods requires also some additional work and time for deproteinization at the test samples – using trichloroacetic acid which can act destructively on the determined vitamins (those susceptible to, among others, low pH values – see Introduction) as it is a strong acid.

Numerous drawbacks of the popular methods for determination of antioxidative vitamins that have been shown here drew our interest to less known spectrophotometric methods, which – according to their authors – are free from analytical faults.

We decided to introduce a selective and fast method for determination of the reduced form of vitamin C, using a periodically prepared PR [Kyaw 1978]. This reagent becomes reduced by the L-ascorbic acid which is contained in the sample and produces the tungsten blue, absorbance of which is measured. The PR denaturates also proteins contained in the sample, eliminating thus the necessity of protein removal as a separate step. The method of selective determination of vitamin E (total) [Tsen 1961] which was chosen, has the same background as the previously discussed batophenanthroline method with its modification, but uses different concentrations and ratios of reagent solutions. Despite the fact that the method was developed for analysis of vitamin E in solutions, correct results of the trials (using the solutions of  $\alpha$ -tocopherol in ethanol)

encouraged us to adapt it to performing determinations in biological liquids. For the determination of vitamin A, we have decided to apply a method of direct spectrophotometric measurement in UV [Bessey et al. 1946], which is based on another principles than the method by Parrish [1977]. The test sample is protein-depleted and hydrolysed with the KOH solution in ethanol, which allows more gentle and shorter heating (60°C, 20 minutes) without the necessity to use the nitrogen atmosphere. The kerosene-xylene mixture in which KOH does not dissolve, is used for extraction which makes rinsing with water redundant. Besides, due to the mixture low volatility the procedure does not require any change of the solvent or any evaporation associated with this process. The extract absorbance measurements (at a single wavelength) are combined with removal of interference and include as follows: measurement I of the sum absorbance of vitamin A and the interfering substances, vitamin A liquidation with UV light, measurement II of the interfering substances absorbance, calculation of the determined vitamin A absorbance being a difference between the results of measurements I and II. The computational result of the totally selective determination is obtained from one simple formula.

The works on implementation of vitamin C determination method according to Kyaw [1978] showed however, that the phosphotungstate reagent which is prepared according to the original description, is useless. Descriptive gaps and methodological inconsistencies which were found exclude the possibility of applying the presented analytical procedure as a guideline for determinations. Lack of a detailed description of the analytical procedure put a severe obstacle in the process of adaptation of the method by Tsen [1961] to determinations carried out in biological fluids. Besides, the adaptation should concern – according to the subject of the study – the analysis of vitamin E solutions. It also seems impossible to introduce a method for determination of vitamin A according to Bessey et al. [1946] in a simple way because the method requires using high-purity kerosene which is not easily accessible, 20 × 0.3 cm test-tubes with an unusual way of centrifugation of the post-extraction mixtures (using an electric drill with a special head; following centrifugation the author cracked the pre-cut tubes at the border of the organic and aqueous phase and collected extracts with a pipette), a specially constructed UV lamp for irradiation of extracts and non-typical narrow spectrophotometric cuvettes.

Our modification of the method for determination of vitamin C [Kyaw 1978] avoids a troublesome transfer of post-reaction mixtures from usual test-tubes to the centrifugal test-tubes and introduces a laboratory treatment of the analysed fluids in these last. Centrifugation parameters were changed to receive some more compact sediments which facilitates separation and collection of supernatants. Procedures which were found missing or described in an incomplete form were completed. Changes in the way of the phosphotungsten reagent preparation were of the largest significance for the modification.  $\text{Na}_2\text{WO}_4$  *molybdene-free* was used (Mo content  $\approx 0.001\%$ ),  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  undergoing hydration to 7-, and 12-hydrate upon storage and mentioned in the original method was replaced by the anhydrous salt and the 3.7 M solution of  $\text{H}_2\text{SO}_4$  was introduced instead of the mixture 15:5  $\%_v$  of water and  $\text{H}_2\text{SO}_4$  ( $d = 1.84$ ). The reagent was prepared using the DI water so that the water was free from traces of heavy metals and the reagent was protected from boiling during the 2-hour heating period. An easy method of pH adjustment to the required value of 1.0 was adopted.

To develop an analytic procedure which would be adapted to performing determinations in biological liquids was the key issue while modifying the method for determination of vitamin E [Tsen 1961]. We agreed to carry out the processing in centrifugal test-tubes, from the denaturation with ethanol as it does not introduce any strange ions, does not change pH and does not destroy the susceptible vitamin E. The sample is also par-

tially extracted, which was supplemented with extraction with a non-polar xylene. A shaker was used for mixing the content of the test tubes during protein removal and extraction. Original concentrations of reagent solutions were preserved, but it was impossible to apply the original volume ratios between them and the analysed liquids, for vitamin E which is present in those liquids was diluted during protein removal and extraction by ethanol and xylene – before the colour reaction took place. The whole volume ratios were corrected to achieve vitamin E concentration equal to the original method. To ensure stability of the produced colour reaction, all the applied reagents were used in anhydrous form including ethanol. Instead of the original small table which was used for a simplified calculation of concentrations based on the measured absorbance, a formula was applied to precisely calculate concentrations of the vitamin determined.

In the modification of the method for determination of vitamin A [Bessey et al. 1946] easily accessible equipment was introduced to the analyses: centrifugal test-tubes – for processing and centrifugation of the analysed liquids, a test-tubes shaker – for mixing of the tubes content during deproteinization with hydrolysis and extraction, a typical laboratory centrifuge, a popular analytical UV lamp (“Emita”, with a 6 W burner and the Wood filter; Famed, Łódź) and standard semi-microcuvettes. Comparative tests of extraction with the kerosene-xylene mixture and with xylene alone, based on the absorption spectra in UV and recovery tests, allowed removal of the unavailable high-purity kerosene and the use of xylene solely for the determination purposes. Bessey et al. [1946] did not prove the necessity of using kerosene for the extraction of the analysed samples. He provided just a theoretical comparison of four solvents: petroleum benzin, toluene, xylene and kerosene, evaluating thus their ability to dissolve vitamin A and their volatility – and he chose the last two as a 1:1 mixture. It must be noted, that the commercially available kerosene is available as “pure” and “pharmacopoeial” grade of purity and its further purification is a laborious task lasting for several days. Having xylene introduced as the sole extracting agent in our own studies, the time period of the extract exposition to UV was optimised and the value of the coefficient present in the calculation formula was corrected.

Based on the absorption spectra the wavelength used for absorbance measurements was corrected for the two latter methods (original: 534 nm for vitamin E, 328 nm for vitamin A), which was caused by the applied modifications and because of using old-fashioned, less precise spectrophotometers by the original authors (we used the Lambda 14P apparatus from Perkin-Elmer). To facilitate the analytical procedures, algorithms for determinations have been developed for all modified methods. They are presented in Materials and methods.

The presented own modifications of the selected methods for spectrophotometric determination of antioxidative vitamins, besides having very good analytical parameters (see: Results) and being fully usable for routine analysis, ensured preservation of the advantages of the original methods: adaptation for semi-micro scale determinations, and their low costs and easy, fast laboratory performance. That allowed implementation of the above mentioned modifications into the clinical practice, and numerous scientific studies, where they were successfully used not only for analyses performed in blood, but also in other biological samples. In the authors’ opinion they can be further used for determination of vitamins C, E and A in food products, which – from the analytical point of view – are also the analytes of the biological origin, for example: in juices (following their discolouration with activated coal), milk and solid products (their homogenates or extracts). Although equipmentally advanced instrumental techniques predominate in food analyses, spectrophotometric method is also used and its usability could be increased by methods which are suggested in this study.

## CONCLUSIONS

1. The developed modifications of methods for determination of antioxidative vitamins allowed to avoid substantial faults associated with the original methods and to preserve all of their advantages being thus, in contrast to the previous methods, fully applicable for routine determinations.

2. All those modifications are characterized by selectivity of determinations, high precision, very good sensitivity and accuracy; they are fast, easy to perform, cheap and made with an easily accessible equipment.

3. Beneficial effects of implementation of the above mentioned modifications for determinations in various biological samples which are performed for clinical and scientific purposes, provide a chance for using them in food analytics.

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### **DOGODNE DLA PRAKTYKI ANALITYCZNEJ MODYFIKACJE SPEKTROFOTOMETRYCZNYCH METOD OZNACZANIA WITAMIN ANTYOKSYDACYJNYCH**

**Streszczenie.** Mimo istnienia wielu metod ilościowego oznaczania witamin antyoksydacyjnych: C, E i A, brak jest metod dogodnych dla szeroko rozumianej praktyki analitycznej – mają różne wady i ograniczenia lub wymagają kosztownej aparatury. Opracowano więc własne modyfikacje wartościowych spektrofotometrycznych metod oznaczania każdej z tych witamin, z różnych względów nienadających się w postaci oryginalnej do powszechnego laboratoryjnego wprowadzenia. Ich podstawą są: dla witaminy C – barwna reakcja z wykonywanym okresowo odczynnikiem fosforowolframianowym, dla witaminy E – barwna reakcja z batofenantroliną,  $\text{FeCl}_3$  i  $\text{H}_3\text{PO}_4$ , dla witaminy A – pomiar spektrofotometryczny ekstraktów badanych próbek. Badania kontrolne wykazały pełną poprawność parametrów analitycznych tych modyfikacji, z zachowaniem zalet oryginalnych metod, co pozwoliło wprowadzić je z powodzeniem w rutynowych analizach klinicznych. Opracowane modyfikacje mogą być także stosowane do oznaczania w.w. witamin w artykułach spożywczych, np. sokach, mleku i homogenatach lub ekstraktach próbek stałej żywności.

**Słowa kluczowe:** witaminy antyoksydacyjne, spektrofotometria, żywność, próbki biologiczne

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