COMPARATIVE STUDY OF THE ENZYMATIC METHOD FOR DETERMINATION OF VITAMIN C WITH ROUTINE METHODS ACCORDING TO ISO

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One of the main indices of the nutritional values of fruit and vegetable products as well as the correctness of the technological process is the content of vitamin C.

The two routine (according to ISO) methods for the determination of vitamin C, the titrimetric and the spectrophotometric methods, currently in use in industrial Polish laboratories are often not sensitive enough or are time-consuming (especially the second method). That is why the enzymatic method described by the Boehringer Mannheim R-Biopharm company was employed for the determination of vitamin C. Using the analytic methods for routine purpose requires their validation. The purpose of this paper was a comparison of the results of determination of vitamin C by the enzymatic method with the results of these routine methods (titrimetric and spectrophotometric). The determinations were made on three groups of fruit and vegetable juices. The methods were compared by an analysis of regression function of results obtained in parallel experiments. In the conclusion the agreement of results obtained by enzymatic method with the results of the titrimetric method are presented. It gives an opportunity to employ the enzymatic method for vitamin C determination in analytical and industrial laboratories.

INTRODUCTION

Vitamin C is necessary in a proper human diet. It performs a number of important functions. Its content influences the redox level of human system, takes part in the biosyntesis of collagen, carnitine, catecholamines, metabolism of L-tyrosine and influences metal ions absorption and secretion and lipids metabolism [Wartanowicz & Ziemlański, 1992].

On request of the *Association of the Industry of Juices and Nectars from Fruits and Vegetables* of the European Union (*AIJN*), The Technical Committee Experts established a Code of Practice [AIJN, 2001] – which includes analytical methods for the evaluation of fruit and vegetable juices.

As a reference method for the determination of vitamin C, high performance liquid chromatography (HPLC) method is proposed in this study, which is also suggested by International Federation of Fruit Juice Producers – IFFJP (another name: International Fruit Juice Union – IFU) [IFFJP, 1985]. Another international organization The Association of Official Chemists – AOAC, as a reference method for the determination of vitamin C, proposes the fluorescence spectrometry (or microfluorometry) method, based on the reaction of dehydroascorbic acid with o-phenylenediamine [AOAC, 1990].

The International Standardization Organization (ISO) has proposed two routine analytical methods with the use of 2,6-dichlorophenolindophenol for the determination of vitamin C in fruits, vegetables and derived products, namely: the titrimetric method applied to light-coloured and colourless products [ISO 6557/2, 1984], and the spectrophotometric method applied to dark products.

The fluorescence spectrometry method mentioned above is also proposed as a reference by ISO [ISO 6557/1, 1986].

Other automatic methods are also used in the determinations of L-ascorbic acid: for example, with the application of capillary zone electrophoresis [Liao *et al.*, 2000] and polarography [Kajita & Senda, 1972].

According to our laboratory practice for fruit and vegetable products, the proposed routine methods of ISO included in Polish Standards are often not sensitive enough. The spectrophotometric method is also time-consuming and automatic methods need expensive apparatus.

Methods for industrial laboratories must be accurate, rapid, easy, without requiring expensive apparatus and use ecologically safe reagents at a reasonable cost. The enzymatic methods are now often applied in the Member States of the European Union countries for the determination of many nutrients in fruit and vegetable products [Horubała, 1994]. Many enzymatic methods have been published as standards and placed in official sets of analytical methods (*AIJN*, *AOAC*, *IFU*) in almost all countries of European Community. The enzymatic methods by Boehringer-Mainheim R-Biopharm GmbH are currently proposed in the *Code of Practice of AIJN* for the determination in juices

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of such sugars as: glucose, fructose, sucrose, sorbitol and starch and such acids as: citric and D-isocitric acid, D- and, L-malic acid and lactic acid [AIJN, 2001].

Using the analytic methods for routine purpose requires their validation. The matter of this article is a designation of the utility of the enzymatic method of vitamin C determination in juices established by the Boehringer-Mainnheim group [Henniger, 1997], with special regard to equivalence and correlation of results in comparison to ISO methods.

MATERIALS AND METHODS

Twenty three samples of sauerkraut juices, 20 samples of orange juices and 20 samples of blackcurrant juices were purchased in local shops. Three parallel determinations of vitamin C content by the titrimetric [ISO 6557/2, 1984], spectrophotometric [ISO 6557/2, 1984] and enzymatic methods were made for each sample.

Analysis by the enzymatic method of Boehringer [Henniger, 1997] is performed in two ways. The extent of reduction of tetrazolium salt MTT [3-(4,5-dimethylthia-zolyl-2)-2,5-diphenyltetrazolium bromide] to formazan by vitamin C, in the presence of the electron carrier PMS (5-methylphenazinium methosulfate) at pH 3.5 is used to determine the total vitamin C content. Only the L-ascorbate fraction, as a part of all reducing substances present in the sample, is oxidatively removed by ascorbate oxidase (AAO) in the presence of oxygen for the specific determination of L-ascorbic acid, in a blank sample determination. The L-ascorbic acid content is calculated according to Lambert and Beer law from the absorbance difference of MTT and MTT-formazan before and after reaction, at $\lambda=578$ nm.

Determinations in this experiment were conducted using a commercially available test-combination kit (catalogue No. 409677).

The vitamin C content determined by titrimetric and spectrophotometric [ISO 6557/2, 1984] methods using 2,6-dichlorophenolindophenol is actually a sum of ascorbic acid content and the content of other reducing compounds in the sample. An additional control test using formaldehyde, in order to remove compounds reducing 2,6-dichlorophenolindophenol different from L-ascorbic acid, may be performed in the spectrophotometric method. In these methods, as vitamin C was determined as the sum of contents of L-ascorbic acid and other reducing compounds issued from the total reduction of 2,6-dichlorofenolindophenol.

This approximation is often used in industrial practice: in this work it was applied for systematic reasons.

Similarly, in the enzymatic method, vitamin C content was issued from the total reduction of MTT. The specific determination of L-ascorbic acid was conducted in a blank sample determination.

STATISTICAL METHODS

An analysis of equivalence of the results of two measurement methods: enzymatic and each one of ISO method was made. The following model of linear regression was employed [Eckschlager, 1974; Seber, 1997]:

$$Y = aX + b + \varepsilon \tag{1}$$

where: X – is the result obtained by the first method; Y – is the result of the second method; a and b – unknown parameters; ε – a random error and X and Y are normally distributed variables.

In the case of equivalence of methods, there should be a=1 and b=0 and the methods being compared are called equivalent if X=Y for all investigated samples.

Hence, the verification of hypothesis was first taken:

$$H_0: a=1, b=0 \tag{2}$$

If $(X_1, Y_1), ...,(X_n, Y_n)$ are *n* samples of parallel results obtained by methods 1 and 2 respectively, a test of H_0 may be derived on the basis of a general theory of testing linear hypothesis in linear models [Kajita & Senda, 1972].

We used the following statistical testing (H_0: a=1, b=0) [Kajita & Senda, 1972]:

$$F_{emp} = \frac{(\hat{a}-1)^2 \sum_{i=1}^{n} X_i^2 + 2(\hat{a}-1)\hat{b}\sum_{i=1}^{n} X_i + n\hat{b}^2}{\sum_{i=1}^{n}(Y_i - (\hat{a}X_i + \hat{b}))^2} \cdot \frac{n-2}{2} \tag{3}$$

where LSE estimators of *a* and *b* are:

$$\hat{a} = \frac{\text{cov}(X,Y)}{\text{var}\,X} \qquad \hat{b} = \bar{Y} - \hat{a}\bar{X} \tag{4}$$

If the H_0 is true, then the test statistic has *F* (Snedecor) distribution with (2, *n*–2) degrees of freedom. For the calculated value F_{emp} of test statistic the *p-value* is calculated (p-value means probability occurring in the observed test, when the tested hypothesis is true).

$$p = P\{ F_{2, n-2} > F_{emp}\} \tag{5}$$

If *p* is less than the given significance level α then the hypothesis is rejected.

The strength of relation between results may be described by the correlation coefficient of Pearson:

$$r = \frac{\text{cov}(X,Y)}{\sqrt{\text{var}X}\sqrt{\text{var}Y}} \tag{6}$$

There are two main reasons for rejecting (H_0: a=1, b=0): a # 1 or b # 0. In the next step, two hypotheses will be tested:

$$H_a: a=1 \text{ and } H_b: b=0 \tag{7}$$

The verification of the above hypothesis was done on the basis of appropriate confidence intervals for regression coefficients. The inference will be standard: if 1 is in the confidence interval for *a*, then hypothesis H_a will not be rejected, otherwise the hypothesis is rejected. Similarly, if zero is in the confidence interval for *b*, then the hypothesis H_b is not rejected. Four possible situations are described in Table 1.

First situation. Parameters a # 1 and b # 0 are accepted. It shows that the investigated methods are not consistent. Here, a systematic error has occurred (*b*) as well as a systematic loss (a<1) of a part of the vitamin C. If for example a=0.7, then the second method (Y) shows only 70% of results of the first method (X).

TABLE 1. Possible situations for tested hypotheses.

A possible situation	H_a rejected	H_a not rejected
H_b rejected	Situation 1 (a≠1 and b≠0)	Situation 2 (a=1 and b≠0)
H_b not rejected	Situation 3 (a≠1 and b = 0)	Situation 4 (a=1 and b=0)

Second situation. Here a=1 and b # 0 are applied. It suggests that the considered methods give shifted results. The value of a shift equals the systematic error μ. The difference between the results is always the same, so it implies that at least one of the methods shows a systematic error. The value of a systematic error may be estimated by a standard confidence interval for mean difference. Let μ be the mean difference between results of methods and $Z_i = Y_i - X_i$ be the difference between i-th results. The confidence interval for μ (at the 1–α level) is of the form:

$$(\overline{Z} - t_{\alpha, n-1} S/\sqrt{n}, \ \overline{Z} + t_{\alpha, n-1} S/\sqrt{n}) \qquad (8)$$

where: $\overline{Z}$ – the average of results; $S = \sqrt{1/n-1 \sum_{(Z_i - \overline{Z})^2}}$ – a sample standard deviation; $t_{\alpha, n-1}$ – critical value of a t distribution with (n–1) df.

Third situation. (a # 1 and b=0). This situation is similar to the second one. The only difference is the lack of a systematic error.

Fourth situation. (H_0: a=1, b=0). Here, both tested hypotheses are not rejected. It suggests that for some contents of vitamin C, the results of both methods coincide and for others they do not. To solve the problem, some additional determinations are needed.

RESULTS AND DISCUSSION

The statistical analysis was performed using an Excel Microsoft Office spreadsheet. In Table 2, the names applied

TABLE 2. The designation of variables.

No.	Designation	Sample – method – vitamin C or KA
1	**S-T-C**	sauerkraut juices – the titrimetric method – – vitamin C
2	**S-E-A**	sauerkraut juices – the enzymatic method – – L-ascorbic acid
3	**S-E-C**	sauerkraut juices – the enzymatic method – – vitamin C
4	**O-T-C**	orange juices – the titrimetric method – – vitamin C
5	**O-E-A**	orange juices – the enzymatic method – – L-ascorbic acid
6	**O-E-C**	orange juices – the enzymatic method – – vitamin C
7	**B-S-C**	blackcurrant juices – the spectrophotometric method – vitamin C
8	**B-S-A**	blackcurrant juices – the spectrophotometric method – L-ascorbic acid
9	**B-E-A**	blackcurrant juices – the enzymatic method – – L-ascorbic acid

to variables are given. Because the methodology of the ISO methods and the enzymatic method are quite different, a comparison of L-ascorbic acid and vitamin C contents was also made for: vitamin C by the ISO titrimetric method and L-ascorbic acid by the enzymatic method [orange juices; situation 2 (Figure 5) and sauerkraut juices; situation 3 (Figure 7)]; as well as for vitamin C by the ISO spectrophotometric method and L-ascorbic acid by the enzymatic method [blackcurrant juices; situation 2 (Figure 6)].

Numerical results were illustrated in Figures 1–7. There are shown observed points and a matching regression line (a tram line) with an appropriate confidence limit. Additionally, in each figure there is a line of full agreement of results, *i.e.* the line Y=X (an inconsecutive line).

All results of statistical analysis are given below figures. The first two columns contain the names of variables compared: independent X and dependent Y. Consecutive columns contain estimated regression coefficients with appropriate 95% confidence intervals. In the r column sample correlation coefficients are given. The last two columns are connected with the verification of a full agreement hypothesis: H_0: a=1, b=0: Column $F_{emp.}$ contains the value of the F statistic and the column p-value contains the appropriate significance level.
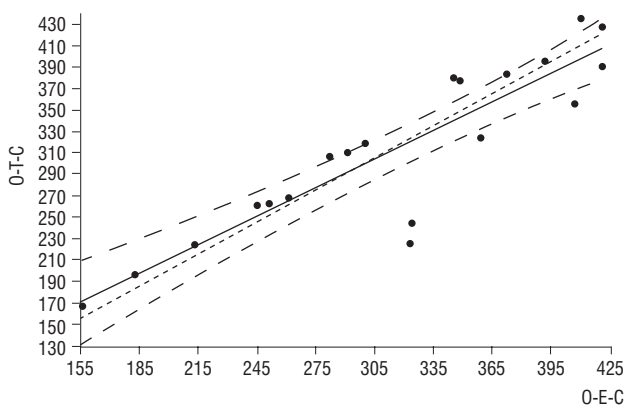
Equivalent methods

Titrimetric and enzymatic methods of vitamin C determination in sauerkraut and orange juices may be considered as equivalent methods. Here, p – *values* for hypothesis H_0; a=1, b=0 are 0.2075 and 0.5458, respectively, and they are greater than the significance level 0.05 (Figures 1 and 2).

The correlation coefficients r were high and statistically significant (α=0.05; n=20): 0.8926 for vitamin C determinations in orange juices (Figure 2) and 0.9084 for vitamin C determinations in sauerkraut juices (Figure 1).
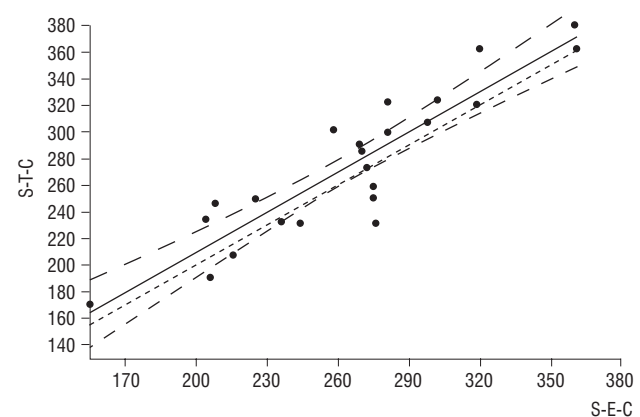
X (mg/L)	Y (mg/L)	a	b (mg/L)	r	F_{emp}	p
			Equivalent methods			
S-E-C	S-T-C	1.0057 (0.7956; 1.2158)	7.6115 (-49.1485; 64.3715)	0.9084	1.6964	0.2075

FIGURE 1. Equivalent methods: vitamin C determined by titrimetric method in sauerkraut juices (S-T-C) *vs.* vitamin C determined by enzymatic method in these juices (S-E-C).
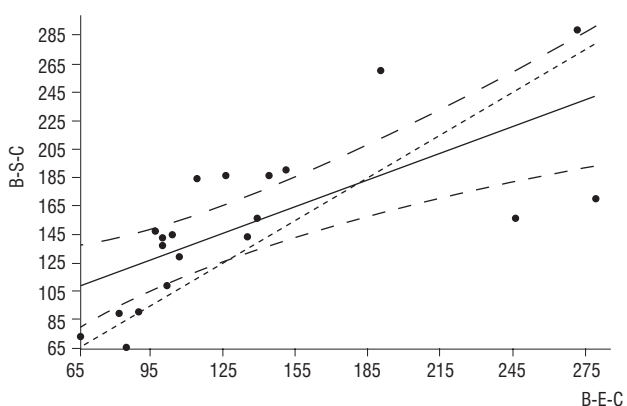
X (mg/L)	Y (mg/L)	a	b (mg/L)	r	F_{emp}	p
			Equivalent methods			
O-E-C	O-T-C	0.8878 (0.6657; 1.1098)	32.4708 (-39.6076; 104.5491)	0.8926	0.6263	0.5458

FIGURE 2. Equivalent methods: vitamin C determined by titrimetric method in orange juices (O-T-C) *vs.* vitamin C determined by enzymatic method in these juices (O-E-C).
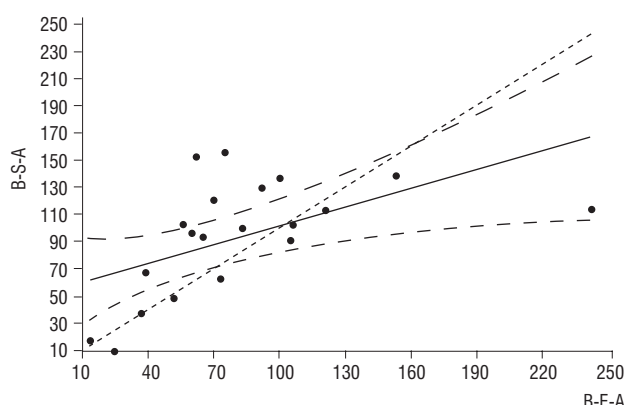
Not equivalent methods

Situation 1. The enzymatic and spectrophotometric methods applied to blackcurrant juices show the greatest differences. For vitamin C content estimation in these juices, for both these methods, both hypotheses H_a and H_b are rejected. The results of enzymatic method show about 62% of vitamin C content given by the spectrophotometric method, simultaneously overstating each determination by about 66.4 mg/L (Figure 3). The same situation is for KA determined by these methods in blackcurrant juices (Figure 4). Comparatively wide intervals of confidence indices for the regression function suggest that the matrices of the analytical material (blackcurrant juices) had a different influence on the results of the analysis.

X (mg/L)	Y (mg/L)	a	b (mg/L)	r	F_{emp}	p
			Not equivalent methods – situation 1:			
B-E-C	B-S-C	0.6293 (0.313; 0.9455)	66.4001 (19.1605; 113.6397)	0.7018	4.5231	0.0256

FIGURE 3. Not equivalent methods – situation 1: vitamin C determined by spectrophotometric method in blackcurrant juices (B-S-C) *vs.* vitamin C determined by enzymatic method in these juices (B-E-C).
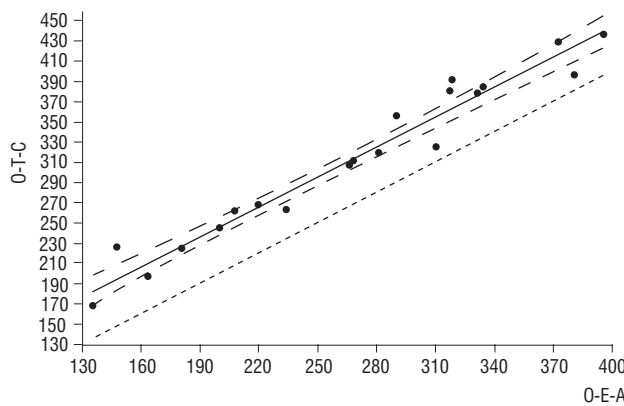

X (mg/L)	Y (mg/L)	a	b (mg/L)	r	F_{emp}	p
			Not equivalent methods – situation 1			
B-E-A	B-S-A	0.4533 (0.094; 0.8125)	56.5078 (22.3038; 90.71180	0.5299	6.1193	0.0094

FIGURE 4. Not equivalent methods – situation 1: KA determined by spectrophotometric method in blackcurrant juices (B-S-A) *vs.* KA determined by enzymatic method in these juices (B-E-A).

The correlation coefficients *r* were low enough and statistically significant (α=0.05; n=20): 0.7018 for vitamin C determinations (Figure 3) and 0.5299 for KA determinations (Figure 4).

Situation 2. The next group of results is connected with the second situation. The lower results of L-ascorbic acid content obtained by the enzymatic method to the results of vitamin C obtained by the titrimetric method were noted for orange juices (Figure 5) and when comparing L-ascorbic acid by the enzymatic method with vitamin C by the spectrophotometric method in blackcurrant juices (Figure 6). This feature of enzymatic method *vs.* titrimetric method has been noted in literature [Henniger, 1997]. It appears that

X (mg/L)	Y (mg/L)	a	b (mg/L)	r	F_{emp}	p
			Not equivalent methods – situation 2			
O-E-A	O-T-A	0.9850 (0.8799; 1.0902)	48.6534 (19.3839; 77.923)	0.9775	68.0559	0.0000

FIGURE 5. Not equivalent methods – situation 2: vitamin C determined by titrimetric method in orange juices (O-T-C) *vs.* KA determined by enzymatic method in these juices (O-E-A).
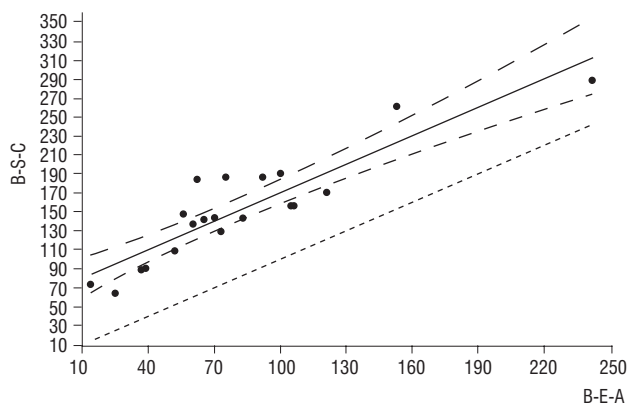
X (mg/L)	Y (mg/L)	a	b (mg/L)	r	F_{emp}	p
Not equivalent methods – situation 2						
B-E-A	B-S-C	1.0011 (0.7628; 1.2395)	70.6075 (47.9162; 93.2988)	0.9013	80.1602	0.0000

FIGURE 6. Not equivalent methods – situation 2: vitamin C determined by spectrophotometric method in blackcurrant juices (B-S-C) *vs.* KA determined by enzymatic method in these juices (B-E-A).

X (mg/L)	Y (mg/L)	a	b (mg/L)	r	F_{emp}	p
Not equivalent methods – situation 3						
S-E-A	S-T-C	0.8303 (0.7393; 0.9212)	-8.6533 (-34.1409; 16.8342)	0.9721	199.9623	0.0000

FIGURE 7. Not equivalent methods – situation 3: vitamin C determined by titrimetric method in sauerkraut juices (S-T-C) *vs.* KA determined by enzymatic method in these juices (S-E-A).
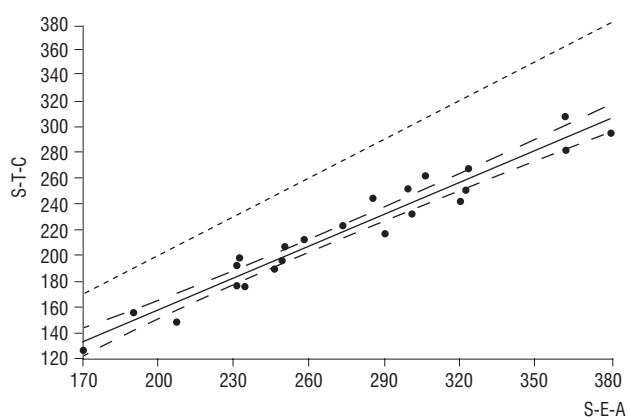
a lowering of the results by the enzymatic method exists also to the spectrophotometric method. It was stated on the basis of the results that the proportion of L-ascorbic acid content to vitamin C content is stable and (F_{emp}) is equal to about 68% (Figure 5) for orange juices and 80% (Figure 6) for blackcurrant juices. It agrees with reality, although the determinations were conducted by different methods, L--ascorbic acid is only one of the vitamin C components. The tested samples of orange and blackcurrant juices contained significant quantities of reducing compounds other than vitamin C. For these methods (Figures 5 and 6), hypothesis H_a was not rejected, but hypothesis H_b was rejected. In such a situation, the relative systematic error was estimated in the way described below. The values of these errors μ are given in Table 3.

TABLE 3. Relative systematic error μ.

X	Y	μ
O-E-A	O-T-C	(36.8315; 52.4685)
B-S-A	B-S-C	(42.6189; 74.8811)
B-E-A	B-S-C	(59.3248; 82.0752)

The correlation coefficients *r* for vitamin C and KA determinations were high and statistically significant ($\alpha=0.05$; n=20): 0.9775 for orange juices (Figure 5) and 0.9013 for blackcurrant juices (Figure 6). The correlation coefficient testifies to the strength of the relationship of methods but not of an equivalence. If these coefficients are high, the obtained regression function may be treated as the conversion equation of one method results to the results of the second method.

Situation 3. The sauerkraut juices behaved unexpectedly different from orange juices during a comparison of the same variants of the methods. The results of the titrimetric method of vitamin C determination and the enzymatic method of L-ascorbic acid determination produce situation 3. Much more discordant results were obtained by these methods in the sauerkraut juices (Figure 7) than described above (situation 2).

Situation 4 did not exist during an analysis of the results obtained.

CONCLUSIONS

1. Enzymatic and ISO titrimetric methods of vitamin C determination used for orange and sauerkraut juices were equivalent methods. Hypothesis (H=a=: a=1 and H_b: b=0) at a significance level of 0.05 were not rejected.
2. Enzymatic and ISO spectrophotometric methods of vitamin C and L-ascorbic acid determination used for blackcurrant juices were not equivalent methods. Hypotheses (H_a: a=1 and H_b: b=0) at a significance level of 0.05 were rejected.

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PORÓWNANIE EMZYMATYCZNEJ METODY OZNACZANIA ZAWARTOŚCI WITAMINY C Z RUTYNOWYMI METODAMI WG ISO

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Jednym z głównych wskaźników świadczących o wartości odżywczej produktów owocowo-warzywnych, jak również prawidłowości przebiegu procesu technologicznego jest zawartość witaminy C.

Stosowane obecnie w laboratoriach przemysłowych, zalecane przez Międzynarodową Organizację Normalizacyjną (ISO), dwie rutynowe metody oznaczania witaminy C: miareczkowa i spektrofotometryczna, są często zbyt mało czułe lub, w przypadku drugiej z nich, zbyt pracochłonne. Dlatego zaproponowano enzymatyczną metodę oznaczania witaminy C, opracowaną przez niemiecką firmę Boehringer Mannheim GmbH Biochemica. Stosowanie metod analitycznych do celów rutynowych wymaga przeprowadzenia ich walidacji. W tym celu przeprowadzono analizę porównawczą wyników ozanczeń witaminy C metodą enzymatyczną z wynikami uzyskanymi metodami rutynowymi: miareczkową i spektrofotometryczną, Oznaczenia zostały wykonane na trzech grupach soków owocowych i warzywnych. Porównanie metod przeprowadzono metodą analizy funkcji regresji wyników uzyskiwanych w próbach równoległych. W efekcie stwierdzono zgodność wyników uzyskiwanych metodą enzymatyczną z wynikami metody miareczkowej, co daje możliwość stosowania metody enzymatycznej w laboratoriach analitycznych, w tym przemysłowych.