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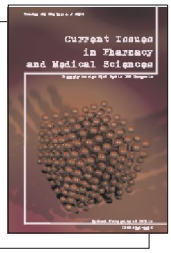

A comparison of vitamin C content determination by chromatographic and spectrophotometric methods according to standard PN-A-04019:1998

ARTUR MAZUREK*, MARZENA WŁODARCZYK-STASIAK

Department of Analysis and Evaluation of Food Quality, University of Life Sciences, Lublin, Poland

ABSTRACT

Vitamin C performs many important functions in the human organism. Besides, it is used in the therapy and prevention of many diseases, which is why the methods applied to determine it should ensure the correct results. The present paper compares the results of vitamin C determination by means of the spectrophotometric method in agreement with the binding standard PN-A-04019:1998 and the proposed method of the reversed-phase high-performance liquid chromatography with spectrophotometric detection. The comparison was made using the method of calculating the proportion of mean results and the uncertainty of its determination. The analysis of a sample of the certified reference material BCR-43 was conducted. It was shown that because the content of dehydroascorbic acid was not considered, the result obtained by the standardized spectrophotometric method differs in a statistically significant way from the certified value, as opposed to the result obtained by the chromatographic method. Results of determining vitamin C content in blackcurrant, apple and grapefruit juices by means of the two methods indicate statistically significant differences. Compatible results were obtained only for orange juice. This is caused by insufficient selectivity of the spectrophotometric method of vitamin C determination. The suggested chromatographic method is more selective than the spectrophotometric method and it enables to consider the content of dehydroascorbic acid. That is why it should be recommended for routine determinations of vitamin C content.

Keywords: vitamin C, ascorbic acid, dehydroascorbic acid, methods of determination

INTRODUCTION

The importance of vitamin C in keeping human organism healthy has been widely documented. Vitamin C takes part in enzyme activation, oxidative stress decrease and immunological reaction. It prevents infections of respiratory tracts and limits the risk of cardiovascular diseases and some cancers. It is also necessary in collagen synthesis and biosynthesis of some hormones. Instability of vitamin C combined with a relatively poor intestinal absorption decreases its physiological availability. Problems with maintaining high concentrations of vitamin C in plasma may have serious health consequences, especially in the appearance and development of degenerative diseases, which are largely caused by oxidative damage [10]. Many experts believe that the recommended consumption of vitamin C ranging from 45 to 120 mg/day (depending on the group of population) is too low for its optimal effect [17]. The human organism is not able to produce it by itself; hence, the only source of vitamin C is a diet. Approximately, 90% of the total consumption comes from plant products. Vitamin C includes ascorbic acid (AA) and dehydroascorbic acid (DHA). Both forms occur in all plants; however, their content changes very widely, both between different plants and between the species [6].

The optimal method to determine vitamin C should include the measurement of its total content (VitC), which is a sum of ascorbic acid and dehydroascorbic acid content. AA and DHA present very different physicochemical properties, which is why their simultaneous and direct analysis is a complex analytical problem. A direct measurement of ascorbic acid content, on the basis of its physicochemical properties, can be conducted by means of a wide variety of techniques, as opposed to the measurement of dehydroascorbic acid [3]. Due to problems with determining dehydroascorbic acid, its derivatization takes place (table 1), which usually consists of reduction to ascorbic acid, through adding a reducing agent to the

Corresponding author
* Department of Analysis and Evaluation of Food Quality, University of Life Sciences, Skromna 8, 20-704 Lublin, Poland
e-mail: artur.mazurek@up.lublin.pl

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sample. Next, the measurement of ascorbic acid content is performed, which is a sum of its initial content and the content emerging as a result of the reduction, which leads to the determination of the total content of vitamin C. The content of dehydroascorbic acid is determined through an additional measurement of ascorbic acid content in the sample without the stage of reduction, and the obtained result is subtracted from the total content of vitamin C (differential method) [12].

Table 1. Ways of derivatization of dehydroascorbic acid and the techniques connected with them

Ways of derivatization	Determination techniques
Reduction to ascorbic acid	All concerning ascorbic acid
Reaction with ortophenylenodiamin as a result of which chinoxaline derivative is produced	Fluorimetry Liquid chromatography with fluorimetric detection Polarography
Reaction with methanol as a result of which hydrazone is produced	Spectrophotometry
Reaction with dinitrophenylhydrazine as a result of which hydrazone is produced	Spectrophotometry

In 2003, the European Committee for Standardization introduced standard EN 14130:2003 "Foodstuffs. Determination of vitamin C by HPLC", which also had the status of a Polish standard. The applied ion pair chromatography is widely used to analyze vitamin C due to increased retention of ascorbic acid and dehydroascorbic acid and better separation of analytes from the sample matrix [14]. In 2010 the European Committee for Standardization withdrew standard EN 14130:2003 without replacing it [4]. The reason was that unreliable results were obtained because of the reduction of dehydroascorbic acid by means of cysteine at pH of about 7, which is not optimal for the stability of ascorbic acid. Additionally, there were problems connected with the long time of the column stabilization and an increase of pressure in the chromatographic system. At present, the only standard of vitamin C determination binding in Poland is PN-A-04019:1998, which was implemented on the basis of ISO standard 6557/2:1984. In the procedure of performance, two methods using 2,6-dichlorophenolindophenol of determining vitamin C content are provided: the titration method applied for colourless and poorly coloured products, and the spectrophotometric method for strongly coloured products. High-performance liquid chromatography ensures higher selectivity and sensitivity of measurements than spectrophotometric techniques so it is the most preferred analytical method used in studying vitamin C.

The purpose of the research was to compare the results of vitamin C determination by the spectrophotometric technique according to PN-A-04019:1998 with the results obtained using reversed-phase high-performance liquid chromatography with spectrophotometric detection (HPLC-DAD).

MATERIALS AND METHODS

*Materials.*The studies used grapefruit, orange and apple juices, black currant nectar and the certified reference material BCR-43. The juice and the nectar were purchased in a local shop. Only the label of the apple juice contained information on its fortification with vitamin C.

Ascorbic acid, 2,6-dichlorophenolindophenol and tris(2-carboxyethyl)phosphine were purchased in Sigma-Aldrich (USA), metaphosphoric acid was purchased in Merck (Germany), while ortho-phosphoric acid and xylene – in POCH S.A. (Poland).

All analyses were made in six repetitions and the temperature of approximately 23±2°C was kept during all stages of the analyses. The results are provided in mg of the analyte per 100 g of the product.

Spectrophotometric method of the determination of vitamin C content. Determination was conducted according to a standardized method PN-A-04019:1998 "Foodstuffs – Determination of vitamin C content", consisting of quantitative oxidation in the acid environment of ascorbic acid to dehydroascorbic acid by means of the excess of 2,6-dichlorophenolindophenol, and then extraction of the excess of the dye by means of xylene and the spectrophotometric determination of the excess of the dye with the wave length of 500 nm on a spectrophotometer Jasco V-630 (Japan).

Chromatographic method of the determination of vitamin C content. Reversed-phase HPLC-DAD method according to the methodology described by Mazurek and Pankiewicz [13] was used to analyze the total content of vitamin C, ascorbic acid and dehydroascorbic acid with an application of the differential method. A spectrophotometric detector with a diode matrix was used to detect the analytes. The separation was conducted in the conditions of isocratic elution. In the first stage, the content of ascorbic acid was determined in the sample; next quantitative reduction of dehydroascorbic acid was conducted by means of tris(2-carboxyethyl)phosphine and the total content of vitamin C was determined. The content of dehydroascorbic acid was calculated subtracting the initial content of ascorbic acid from the total content of vitamin C. The concentration of ascorbic acid in the extract was determined from an equation of the calibration curve made on the basis of the results of the analyses of standard solutions. Identification of ascorbic acid was established on the basis of the retention time and UV spectrum of the standard substance. A liquid chromatograph produced by Varian was used. It was equipped with a detector with a diode array (model 335), an isocratic pump (model 210), an injection valve 7725i (Rheodyne, USA), a column thermostat and a chromatographic column Gemini 150 x 4.6 mm (3µm C18) connected to a precolumn Gemini C18 4x3 mm (Phenomenex).

Correctness. Correctness of the analytical method was determined by conducting the analysis of a sample of the certified reference material and comparing the value of the measurement with the certified value. The comparison was made according to the Application Note No. 1 developed by the Institute for Reference Materials and Measurements [11]. For this purpose, the absolute difference between the mean measured value and the certified value was established according to the following formula:

$$\Delta m = |X_m - X_{CRM}|$$

where:
Δm – absolute difference between mean measured value and certified value
X_m – mean measured value
X_{CRM} – certified value.

Next, the value of expanded uncertainty of difference was determined calculated on the basis of the value of uncertainty for comparable values.

$$U_\Delta = k\sqrt{u_m^2 + u_{CRM}^2}$$

where:
U_Δ – expanded uncertainty of difference between result and certified value
u_{CRM} – uncertainty of certified value
k – coverage factor of 2 (for confidence interval of about 95%)
u_m – standard uncertainty connected with reproductibility of measurements determined on the basis of dependence

$$u_m = \frac{s}{\sqrt{n}}$$

where:
s – standard deviation of measurements
n – number of independent determinations

If $\Delta m \leq U_\Delta$, then there is no statistically significant difference between the result of the measurement and the certified value.

A comparison of results obtained by means of the chromatographic method and the standardized method was made using the method of calculating the relation of the mean results and uncertainty of their determination [9]. It consists of calculating the proportion P of mean values of the results of determinations and uncertainty U for so determined value P. The deduction is the following: if value 1 is contained within the interval of the calculated proportion P ± uncertainty of its determination (P – U, P + U), then it should be inferred that the compared mean values do not differ from each other in a statistically significant way. Values P and U were calculated using the following formulas:

$$P = \frac{X_{HPLC}}{X_{SPE}}$$

$$U = k\frac{\sqrt{s_{SPE}^2 + s_{HPLC}^2}}{\left(\frac{X_{SPE} + X_{HPLC}}{2}\right)}$$

where:
X_{SPE} – mean concentration determined by the spectrophotometric method
X_{HPLC} – mean concentration determined by the chromatographic method
k – coverage factor of 2 (for confidence interval of about 95%)
s_{SPE} – standard deviation of results obtained by the spectrophotometric method
s_{HPLC} – standard deviation of results obtained by the chromatographic method

RESULTS AND DISCUSSION

Correctness of the obtained results of determinations was studied using the analysis of the sample of the certified reference material BCR 431 and a comparison of the obtained mean value with the certified value, which is a sum of ascorbic acid and dehydroascorbic acid. Table 2 presents the values on the basis of which the correctness of the analytical method was determined. They point to a lack of statistically significant differences in the case of the result obtained by the chromatographic method. The result obtained by the spectrophotometric method differs from the certified value in a statistically significant way, which attests to its incorrectness. Spectrophotometric methods, owing to their availability and simplicity of analyses, are widely used in the analysis of vitamin C; however, neither of them is sufficiently specific [2]. The presence of certain substances in the sample matrix can cause interferences. Therefore, it is necessary to remove interferential substances from the matrix of the sample by means of masking substances or initial separation, which is not always effective. The standard of vitamin C determination binding in Poland, namely PN-A-04019:1998, does not make it possible to determine the total content of vitamin C (a sum of AA and DHA) in the sample, but only the content of ascorbic acid since dehydroascorbic acid does not react with 2,6-dichlorophenolindophenol. Its serious drawback is also the fact that other reducing substances present in the sample can reduce 2,6-dichlorophenolindophenol and increase the obtained results of analyses. The content of ascorbic acid in the certified reference material BCR 431, determined by the chro-

Table 2. Necessary data to establish the correctness of the analytical method

BCR-431							
X_{CRM}	u_{CRM}		X_m	u_m	Δm	U_Δ	$\Delta m \leq U_\Delta$
(mg/100g)	(mg/100g)		(mg/100g)	(mg/100g)	(mg/100g)	(mg/100g)	
483	9.8	HPLC-DAD	477.6	10.2	5.4	21.16	yes
		PN-A-04019	438	7.6	45	24.8	no

matographic method, is 403.8±9.8 mg/100g and it is clearly lower than that which is determined by the spectrophotometric method 438.0±18.6 mg/100g, which is caused by the presence of reducing compounds increasing the result of the spectrophotometric determination. The chromatographic method is characterized by a decisively greater specificity than the spectrophotometric method because the qualitative analysis of ascorbic acid is made on the basis of comparing the retention time and UV spectrum of the standard of this acid.

Table 3 contains a comparison of results of analyses of ascorbic acid obtained by the chromatographic method and by the spectrophotometric reference method in agreement with PN-A-04019:1998. The obtained results show statistically significant differences for black currant nectar, apple juice and grapefruit juice, while in the case of orange juice compatible results were obtained. This is most probably caused by the occurrence, in various degrees, of interfering compounds in the analyzed products in the spectrophotometric determination of vitamin C. The greatest difference was observed for black currant nectar, which is confirmed in other studies [5]. A clear advantage of the chromatographic method is the possibility of determining the total content of vitamin C and dehydroascorbic acid (table 3), which is not possible using the spectrophotometric method. Besides problems with direct determination of dehydroascorbic acid, accepting this method as a standardized method followed from the view that the content of this acid, especially in fresh products, was clearly lower than the content of ascorbic acid. Therefore, the fact of not considering dehydroascorbic acid in the determination was supposed to lead to negligible results [18]. Recent research shows that the method of the determination of vitamin C content which does not consider dehydroascorbic acid leads to big errors in determinations, especially in stored fruit and vegetables [13]. This is caused by an increase in the content of dehydroascorbic acid and its proportion in the total content of vitamin C while storing. This was especially well visible in the analysis of certified reference material BCR 431, where the high content of dehydroascorbic acid determined by the chromatographic method (73.8±7.5 mg/100g) led to the result of determination obtained by the spectrophotometric method which was not in agreement with the certified value. In the studies by Danielczuk [5] conducted by the spectrophotometric method in agreement with ISO 6557/2, 1984, and therefore, with the Polish Standard PN-A-04019 was used as a reference method in comparison with the enzymatic method. The methods were compared using a regression function analysis of the results obtained in parallel trials. Results of those studies point to incompatible results of the analysis of ascorbic acid content in currant juice obtained by means of both methods, which indicates their non-equivalence. Hernández [8] conducted the analysis of vitamin C content in tropical fruit using the method of high-performance liquid chromatography, which he compared with the results obtained by means of a standardized titration method with 2,6-dichlorophenolindophenol [1], which is equivalent to the titration method described in the Polish Standard PN-A-04019. Those studies found out the equivalence of the results obtained by the two analytical methods, which could be caused by a lower content of interfering substances in a given matrix of samples during the determination of vitamin C by the titration method.

Table 3. A comparison of results of analyses obtained by the chromatographic method (HPLC-DAD) and the spectrophotometric reference method (PN-A-04019)

Sample	Analyte	HPLC-DAD		PN-A-04019		P	U	Agreement
		x_{HPLC} (mg/100g)	s_{HPLC} (mg/100g)	x_{SPE} (mg/100g)	s_{SPE} (mg/100g)			
Apple juice	AA	9.16	0.24	11.01	0.77	0.83	0.16	no
	VitC	13.90	0.48	–	–	–	–	–
	DHA	4.74	0.46	–	–	–	–	–
Black currant nectar	AA	15.72	0.46	24.44	0.87	0.64	0.10	no
	VitC	18.66	0.42	–	–	–	–	–
	DHA	2.94	0.28	–	–	–	–	–
Orange juice	AA	23.24	0.80	25.20	0.67	0.92	0.09	yes
	VitC	24.82	0.72	–	–	–	–	–
	DHA	1.59	0.22	–	–	–	–	–
Grapefruit juice	AA	28.05	0.41	31.95	0.8	0.88	0.06	no
	VitC	31.40	0.76	–	–	–	–	–
	DHA	3.35	0.60	–	–	–	–	–

CONCLUSIONS

Officially published papers describe different recommended methods of determining vitamin C. A considerable part of them enables only the determination of ascorbic acid [13]. Most frequently, titration and fluorometric methods are used. It can be ascertained that the officially recommended methods do not catch up with the current progress in determining vitamin C. According to the present state of knowledge, the optimal method to be suggested is the method of high-performance liquid chromatography connected with a corona charged aerosol detector (Corona CAD) [15] or a tandem mass spectrometer

[7] since it is only these methods that allow for a simultanous direct detection of ascorbic acid and dehydroascorbic acid, which enables maximum simplification of the state of preparing the sample for the analysis. Their main fault, however, is the necessity to use a specific detector that is rarely encountered in laboratories. Hence, these methods are not widely spread. With the purpose of using a spectrophotometric detector most frequently used in liquid chromatography to determine the total content of vitamin C, the method suggested here performed a reduction of dehydroascorbic acid by means of tris(2-carboxyethyl)phosphine, which is now the most optimal reductor in the environment of the extraction with pH of about 2 [12,16]. That enabled to determine the total content of vitamin C through the measurement of ascorbic acid (which is a sum of ascorbic acid contained in the sample before the step of reduction and that which was produced as a result of the reduction of dehydroascorbic acid) using a spectrophotometric detector. Besides, the chromatographic method is less time-consuming and more selective than the spectrophotometric method and it should be recommended for routine determinations of vitamin C content.

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