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COST-EFFECTIVE DETERMINATION OF TOTAL ASCORBIC ACID CONTENT AND ANTIOXIDANT ACTIVITY. A FOOD CHEMISTRY CASE STUDY

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Abstract

This study investigates the cost-effective and reliable determination of total ascorbic acid (AA) content, as well as antioxidant activity, from a range of 32 alcoholic and non-alcoholic beverages. 5 cold-pressed oil samples and 2 types of honey were also considered. Among the iodometric methods, the back-titration of unreacted iodine with thiosulfate yielded the best results for AA content. The method proved simple, fast, reliable, and accurate, with a recovery rate of ascorbic acid exceeding 99% in 2 standardized commercial pharmaceutical products. Total antioxidant activity determination was based on the reaction of antioxidant species with ABTS, followed by a photometric assessment of the reaction's extent. Results, expressed in terms of AA content, were good and reliable, yet in some cases less precise and accurate. Most beverages exhibited higher antioxidant activity than their respective AA content, likely influenced by other antioxidant compounds such as flavonoids and anthocyanins present in fruit-based drinks. Both methods proved useful in demonstrating the nutritional value of various easy to consume beverages, oils and honey.

Keywords*: total ascorbic acid content, antioxidant activity, food samples, iodometry, photometry.*

1. Introduction

1.1. The importance of vitamin C for the human body

Vitamin C (L-ascorbic acid) is an essential nutrient for the human body. Its importance was first recognized by the Hungarian scientist Albert Szent-Györgyi. Widely known under the generic name of ascorbic acid (AA), it has been the subject of continuous research due to its key role for the healthy physiological functioning of the human body [1, 2].

Vitamin C is crucial for the immune system (innate or adaptive), preventing and treating many respiratory and systemic infections [3]. It helps maintain blood pressure levels and has been linked to the prevention of cancer and cardiovascular diseases [1, 4]. It also has a positive effect on diabetes, common cold, stroke and anaemia recovery [5]. Recent research has revealed its role in the regulation of gene transcription and cell signalling pathways [3].

The human body is not able to produce the minimum necessary daily amounts of 110 mg for men and 78 mg vitamin C for women [1]. Thus, its intake must be ensured by means of external sources, such as suitable food or dietary supplements. Otherwise, its lack can lead to scurvy or other serious, even fatal diseases [3]. Vitamin C deficiency is a common problem in certain areas of the world, especially in low- and middle-income countries, but it is not uncommon among high-income regions either [6]. Since the absorption of vitamin C in the digestive system is of about 80% from a daily intake of 100 mg, a vitamin C rich diet is of utmost importance, even though the human body cannot absorb/store more than 2.5g (due to its partial oxidation to two metabolites: the active dehydroascorbic acid and the inactive oxalic acid) [4, 7].

The main natural sources of vitamin C are fruits (such as berries, papaya, kiwi, citrus fruits, peaches, apples or pears), vegetables (such as Brussels sprouts, cauliflower, cabbage or sweet pepper), some herbs (such as parsley or sorrel), and their respective juices. All can be incorporated into a rapid paced lifestyle [1, 4].

Some environmental factors, such as exposure to air, light or heat, improper storage conditions or various food processing procedures, may reduce the AA content of these products [5, 8]. Therefore, the market nowadays offers numerous vitamin C enriched fruit drinks and/or supplements [9, 10]. Ascorbic acid is identified in food chemistry as additive E300, being also often used either as a preservative and/or an antioxidant.

Hence, correct information about the nutrient and bioactive content of these ascorbic acid sources is essential in understanding their health benefits. As a result, there is abundant scientific literature providing detailed information about the methods to detect and assess the total AA content of both natural and synthetic Vitamin C sources.

1.2. Methods of ascorbic acid determination

The structural chemical formula of Vitamin C - the biologicaly active L-enantiomer of ascorbic acid - is presented in Figure 1. Its content in various samples is usually expressed as total ascorbic acid concentration. The latter corresponds to the racemic mixture of L and D enantiomers.

Numerous methods have been put forward for the quantitative determination of total AA content. A classical and inexpensive approach is based on titration with oxidizing solutions. Other techniques involve color reactions (for example with 2,6-dichlorophenol-indophenol), UV-VIS spectrophotometry, high performance liquid chromatography, capillary electrophoresis, chemiluminescence, fluorimetry, amperometry and various enzymatic methods, all of which present their respective advantages and disadvantages [1, 11, 12, 13].

Titrimetric methods are advantageous when analyzing a large number of samples, due to their simplicity, cost-effectiveness and speed. However, in the case of colored samples, end-point detection may be difficult. High-performance liquid chromatography is the most accurate method for the quantification of ascorbic acid, but it requires highly qualified laboratory expertise and expensive equipment. Electrophoresis and chemiluminescence are relatively cheaper alternatives, but their sensitivity is lower. Fluorimetry and voltammetry provide fast results, yet require special instrumentation and precise calibration [1, 14].

Among all the listed procedures, the simplest and most cost-effective is titration. It is also both precise and accurate, since it generates results which are comparable to the more sophisticated spectrophotometric and chromatographic methods. If it is carried out automatically, and/or on fresh samples, air induced oxidation of the ascorbic acid in the samples can be avoided or at least minimized [10].

The AA content is often correlated with the antioxidant capacity of a certain food sample. Since AA is a reducing agent, it can interact with all kinds of oxidizing species or free radicals [15]. In the food industry 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) is mostly used to assess the total antioxidant capacity of various products.

One purpose of this work was to determine the total ascorbic acid content of various beverages present in the Romanian market. These belonged to the following categories: cold-pressed and sugar preserved syrups, freshly squeezed fruit juices, carbonated soft drinks, pasteurized juices, and alcoholic beverages. Some honey and oil samples were also considered.

Another goal was to express the total antioxidant content of these products as well as correlate it with their AA concentration.

2. Experiment description

2.1. Reagents, equipment, and samples

Analytical grade purity reagents were used from commercial sources (Merck KgaA, Darmstadt, Germany). Deionized water (HydroPure 300, MultiLab, Bucharest, Romania) was used to prepare

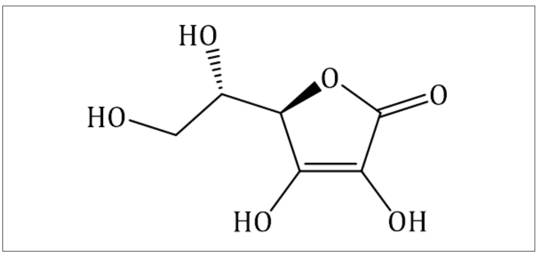


Fig. 1. *The structural formula of vitamin C.*

the following working solutions: 10^{-3} mol/L ABTS, 10^{-2} mol/L $Na_2S_2O_3$, 10^{-2} mol/L KI, 1.05×10^{-2} mol/L KIO_3, 0.5 mol/L HCl, 4.73×10^{-3} mol/L I_2/KI, and 2% starch, respectively. Ascorbic acid working solutions of various concentrations, as well as the sodium thiosulfate solutions were freshly prepared before each set of experiments.

Standard laboratory glassware and ustensils (Labbox, Labware, Bucharest, Romania) were used during the experiments: beakers, flasks, Schelbach line burettes, and adjustable volume micropipettes, respectively. A KERN laboratory scale (SC. Driatheli Group SRL, official partner of Kern, Győröd, Romania) and a VIS V-1100 single-channel spectrophotometer (DLAB, AMEX-lab, Bucharest, Romania) were also employed. The latter was operated by means of a dedicated software (M.Wave Professional). Photometric measurements were carried out in a glass cuvette with an optical path length of 1 cm. Statistical processing of obtained data was carried out with the dedicated tools of Microsoft Office Excel.

A total of 41 food samples were examined. These can be classified into 5 categories, as illustrated in **Figure 2.** The samples were collected from the Romanian market, from both commercial and individual producers, as follows: 9 cold-pressed fruit syrups (with added sugar for preservation), 6 pasteurized vegetable or fruit juices, 3 carbonated soft drinks, 3 freshly squeezed fruit juices, 11 alcoholic beverages, 5 cold-pressed oils, and 2 types of honey, respectively.

Two commercially available pharmaceutical products with standardized and declared ascorbic acid content were used to validate the employed methods: Aspirin Plus C (240 mg AA content) and Redoxon (1000 mg AA content) effervescent tablets (both manufactured by Bayer Bitterfeld GmbH, Greppin, Germany).

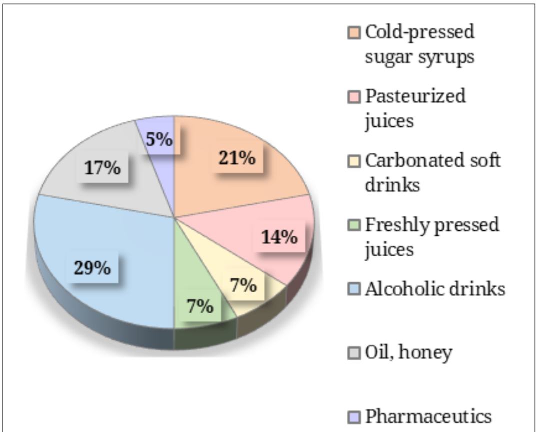


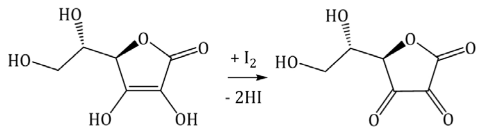
Fig. 2. *Categories of tested food samples.*

2.2. Experimental methods

Determination of total ascorbic acid content was carried out by means of titrimetric methods. These are based on the oxidation of ascorbic acid to dehydroascorbic acid by iodine - see reaction (1). The latter (I_2) can be added to the sample directly for „Iodometry 1", or can be generated in situ by means of the iodide/iodate (I^-/IO_3^-) redox couple in acidic media – see reaction (3), for „Iodometry 2".

Determination of total antioxidant capacity is based on the ability of the antioxidants in the sample to react with/ discolour the greenish-blue ABTS. The extent of this process is monitored photometrically at 735 nm.

2.2.1. Titrimetric determination of total ascorbic acid content



(1)

$$S_2O_3^{2-} + I_2 \longrightarrow 2\,I + S_4O_6^{2-} \qquad (2)$$

Iodometry 1: The procedure starts with addition of excess iodine to the liquid sample. Because of the excess I_2, the sample will preserve/ exhibit the characteristic brown/ brownish color of iodine. The ascorbic acid present in the sample reduces iodine to iodide. The remaining iodine is further (back-)titrated with sodium thiosulfate in the presence of starch – see reaction (2) **[14, 15]**. During titration the sample will turn pale yellow, then blue-indigo when a few drops of starch are added. The end-point is reached when the sample regains its initial color, or turns colorless (in case of synthetic AA colorless solutions). The advantage of this method lies in managing to oxidize the entire ascorbic acid amount of the sample in the desired way (by iodine), not in other reaction paths (such as by the oxygen dissolved from air into the liquid sample). In addition, the method is extremely simple and cost-effective.

Iodometry 2: Another indirect titrimetric method involves in situ iodine generation. Potassium iodide (KI), hydrochloric acid (HCl) and starch are added to the liquid sample. Further, it is titrated with potassium iodate (KIO_3). This will generate iodine (I_2) according to reaction (3), which instantly reacts with the AA in the sample – see reaction (1). As long as AA is still present, the sample's color remains unchanged. The end-point is reached when all AA is consumed and I_2 starts accumulating, hence changing the color of the sample (for example from colorless to dark blue). Because iodine is generated during the advance of titration, reaction (1) may be competition with the dissolved oxygen oxidation of AA. This may affect the results [7, 14].

$$IO_3^- + I^- + 6\,H^+ \longrightarrow I_2 + 3\,H_2O \qquad (3)$$

Liquid food (including oil) samples were analysed as such, by using aliquotes of 5 to 50 mL volume. For honey, 2g were dissolved in 10 mL of water, and the resulting aqueous solution was subjected to analysis. The pharmaceutical tablets were dissolved in water in 250 mL volumetric flasks, and the resulting solution was titrated. All titrations were performed in triplicate.

Calculus of results considered the sample and titrant volumes, as well as the concentrations of working solutions. Results were expressed in mg/L of ascorbic acid, except for honey, where they were given as mg/100g product.

2.2.2. Photometric determination of total antioxidant capacity

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) is a highly water soluble compound [16]. It exhibits a greenish-blue color, with an absorbance maximum at 735 nm (see also Figure 8). When added to a sample, some of it will react with reducing species present in the latter ([17, 18]), hence the absorbance value (color intensity) will drop. The difference in the absorbances is correlated with the total antioxidant activity via a calibration line. In the presented case, this was obtained by means of reacting the same amount of ABTS with various amounts of AA, according to the reaction scheme presented in Figure 3.

The method required freshly prepared/ diluted ascorbic acid solutions, as well as multiple and up to 5000 fold dilution of the employed liquid food samples.

Calibration as well sample measurements were caried out in triplicate. Calculus of results considered the sample volumes, their dilution coefficients, and a calibration line of 735 nm absorbance difference versus ascorbic acid concentration. Results were given in mg/L equivalent of ascorbic acid.

3. Results and discussion

3.1. End-point detection in titrimetric methods

In both employed iodometric methods, end-point detection is based on color changes. If a colorless sample or a synthetic AA solution are used, a switch from indigo-blue to colorless is observed for „Iodometry 1". Figure 4 illustrates the sequence of color changes during the classical titration of an aqueous iodine solution (A) with thiosulfate. First it turns yellow (B); after adding a few drops of starch, the color changes to deep blue (C); then further turns colorless (D) when titration is complete.

On the other hand, analysis of colored liquid food samples (such as fruit juices) requires for correct end-point detection a color comparison with the initial color of the sample. Figure 5. presents a similar to Figure 4 sequence, but for a red beetroot juice (B). When mixed with iodine (A) and starch it generates intense dark colors as in flasks (C) and (D). The end-point (E) of titration is reached when the initial juice's color (B) is obtained.

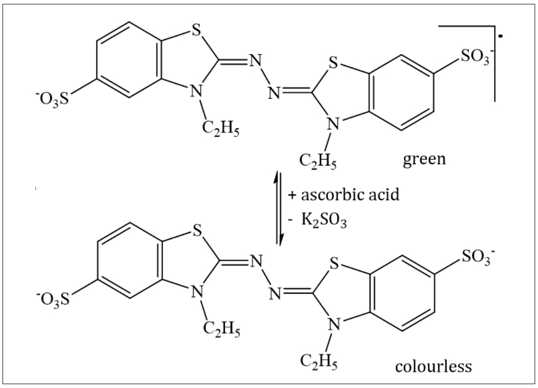


Fig. 3. *The reaction scheme between the ABTS radical and ascorbic acid.* [19]

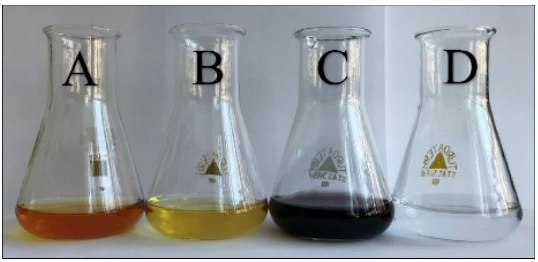


Fig. 4. *End-point detection. Color change sequence for the titration of iodine with thiosulfate - „Iodometry 1".*

For „Iodometry 2”, a synthetic AA solution will turn from colorless to deep blue when the end-point of KIO_3 titration is reached. The method works well for colorless or light colored samples. Yet in the case of red, blue or intensely colored food samples, end-point detection proves to be difficult, due to almost no, or just slight, color changes.

Figure 6 illustrates the trials for the titration of a homemade red wine. It is obvious that the sample itself (left) has almost the same color as the one which was considered at titration end-point (right). Another disadvantage of this method is the fact that although these titrations are carried out in acidic media, the corresponding chemical reactions are fairly slow, and hence, color development at room temperature takes sometimes up to 8 minutes.

As a result, „Iodometry 1” has given more reliable and reproductible results. However, both approaches were tested against 2 commercial pharmaceutical products with known AA contents. The results are summarized in Table 1.

The small values of deviations prove that both titrimetric methods generate precise data, yet not necessarely accurate in the case of „Iodometry 2” and the Redoxon tablet (probably because of its intense orange color, thus incorrect end-point detection). The Aspirin Plus C tablet generates a colorless aqueous solution, hence end-point detection is not hindered.

On the other hand, recoveries were very good for both analysed products in case of „Iodometry 1”. Hence, the latter proves to be precise, accurate and thus was chosen for the total ascorbic content determination in all considered samples.

3.2. Total ascorbic acid content of analysed samples

Total ascorbic acid content was determined for all 41 food samples presented in Section 2.1, by means of „Iodometry 1” described in Section 2.2.1 Figure 7 illustrates the results expressed as C_{AA} in mg/L. Each color corresponds to a certain class of samples.

The freshly squeezed juices (IV) have the highest ascorbic acid content, of around 500 mg/L. This is in line with literature data reporting that orange juice retains its bioactive compounds during storage. The vitamin C content of fresh orange juice is comparable with that of commercial ones, because of adequate modern industrial processing techniques [8].

Fig. 5. *End-point detection. Color change sequence for the back-titration of iodine with thiosulfate in a red color beetroot juice - „Iodometry 1”.*

Fig. 6. *End-point detection. Illustration of the similar colors of home made red wine sample (left) and its titrated correspondent (right) - „Iodometry 2”.*

Table 1. *Values returned during validation of employed experimental methods.*

Method	Redoxon	Aspirin Plus C
	Recovered (%)	
Iodometry 1	99.99 ± 0.73	99.43 ± 3.53
Iodometry 2	74.71 ± 0.33	99.00 ± 0.00
Photometry	113.97 ± 32.56	96.69 ± 14.11

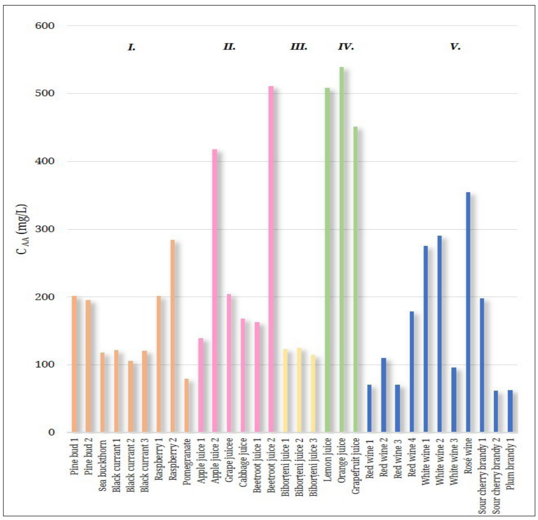


Fig. 7. *Total ascorbic acid content for: I. Cold-pressed sugar preserved syrups; II. Pasteurized juices; III. Carbonated soft drinks; IV. Freshly squeezed fruit juices; V. Alcoholic beverages.*

Among the cold-pressed sugar preserved syrups (class I), raspberry contains the most AA. It is followed by pine buds, blackcurrant and sea buckthorn, respectively. Within the pasteurized juice category (II), beetroot and apple juice prove best. In contrast, grape and cabbage juice show less AA content, yet still comparable with that of sample category I.

The AA present in carbonated soft drinks (III) is usually added during the manufacturing process. For example the label of the elder-lemon flavored „Biborţeni" beverage indicates 200 mg/L AA. Yet, recoveries are of approximately 65%.

Some alcoholic beverages (V) prove to be richer in AA than juices of class I (for example a few wines, especially the Rosé). One of the cherry brandies also has elevated AA content, probably because it was prepared by cold maceration of very ripe fruits. In contrast, preparation of the plum brandy requires distillation; hence, the ascorbic acid in the fruits breaks down when exposed to elevated temperatures.

It ought to be emphasized that data in Figure 7 characterize this particular set of samples. Hence, fluctuations of C_{AA} values, as well as reversed orders are also possible, depending on the species, season and place of harvest of the fruits and vegetables. However, the data prove that besides freshly squeezed juices, pasteurized products and cold-pressed syrups are also a valuable source of vitamin C for the modern human diet.

Among the liquid food samples subjected to AA content determination, were 5 oils obtained by cold-pressing. For these, the „Iodometry 1" method was employed as usual. Therefore, the results should be interpreted differently. The oils do not mix with the aqueous reagent solutions. Yet, because ascorbic acid is water soluble, it is extracted to some extent from the oily into the aqueous phase (mixtures were ultrasonicated for 10 minutes). Hence, results in Table 2 represent only partial values of each oil sample's AA content, yet still prove these to be good dietary sources of vitamin C (values are comparable or higher than the best ones in Figure 7).

Table2. *Partial ascorbic acid content of cold-pressed oils.*

Sample	C_{AA} (mg/L)
Walnut oil	535.6
Poppy seed oil	667.7
Grape seed oil	858.5
Sesame oil	271.5
Pumpkin seed oil	584.2

Table 3 shows the results for two types of honey: one already crystallized because of prolonged storage, the other fresh and liquid. Their aqueous solutions were subjected to „Iodometry 1" and the obtained values of C_{AA} showed no significant difference. Hence, even during a year long storage, honey still retains its vitamin C content intact.

Table 3. *Ascorbic acid content of honey samples*

Sample	C_{AA} (mg/100g)
Honey 1 (solid, crystallized during long storage	42.9±18.0
Honey 2 (fresh, in liquid form)	65.3±11.8

Data above are in agreement with other findings for the local maket: AA in acacia honey from Romania ranges between 77-99 mg/100g [20], and the mixed flower honey from Transylvania contains about 61 mg/100g AA [21]. Another study [22] mentions that honey usually has an ascorbic acid content between 0.34±0.00 and 75.8±0.41 mg/100g. Thus, results presented in Table 3 are realistic, consistent with other reported data, and prove once more that the selected method is effective in ascorbic acid content determination of various food samples, as long as they can be brought in a liquid aqueous form.

3.3. Total antioxidant activity of analysed samples

Figure 8 illustrates the molecular absorption spectrum of ABTS in aqueous solution – see curve 1. It exhibits a maximum at 735 nm. At this wavelegnth its molar absortivity coefficient, of 7484±753 L/mol.cm, was determined from the slope of an experimentally obtained Lambert-Beer line (triplicate measurements of 5 ABTS concentrations; correlation coefficient of 0.9945).

$$\Delta A_{735} = (1.57 \pm 1.48) \cdot 10^{-2} + (29642 \pm 2754.18) \cdot [AA] \quad (4)$$

Curve 1 was registered for a 5×10^{-5} mol/L ABTS concentration, whereas curve 2 corresponds to the same, but also contains 3×10^{-6} mol/L AA. Because of the reaction presented in Figure 3 a stoechiometric amount of ABTS is consumed, and the absorbance of the mixture drops. Hence, by using various AA contents (up to 7.5×10^{-6} mol/L), calibration line (4) was obtained from differences such as that between curves 1 and 2. This expresses antioxidant activity in terms of ascorbic acid concentrations, and as such, these ought to have higher values than the corresponding total AA contents of the respective samples.

Calibration line (4) has a correlation coefficient of 0.9834, and is based on triplicates of 7 measurement series, each at a different AA molar concentration, yet constant 5×10^{-5} mol/L ABTS.

In the case of a liquid food sample, the same amount of ABTS is added, and the 735 nm absorbance value is compared to that of curve 1. The samples have to be diluted so that their spectra lies under that of curve 1. **Figure 8** shows an example in curve 3, for a 500 fold diluted blackcurrant syrup. The antioxidant capacity is then expressed from the absorbance differences (ΔA_{735}) between curves 1 and 3, by means of equation (4). The same dilution of blackcurrant syrup is proven to have negligable absorbance values – see curve 4. Hence curve 3 is due, similarly to curve 2, only to the remanent ABTS content.

This method was applied to the aqueous solutions of Redoxon and Aspirin Plus C tablets. Results are presented in the last row of **Table 1.** The good recovery value of AA for Aspirin Plus C is in agreement with the fact that its only antioxidant species is ascorbic acid. On the other hand, in the case of the Redoxon tablet, recoveries exceeded 100%. This finding is in line with literature data **[23]**, and probably due to the presence of other antioxidant species in the formulation.

Figure 9 illustrates in greenish-blue the results of such determinations, expressed as mg/L AA, for 11 randomly chosen beverages from among the considered samples described in Section 2.1.

It can be observed that, as expected, the total antioxidant activity is usually higher than the ascorbic acid content illustrated by the orange color. Thus, it is not simply due to it, further proving that such beverages might be valuable sources of nutrients. The fruits and vegetables from which they are prepared also contain various flavonoids and anthocyanins with desired antioxidant proprieties.

Results in **Figure 9** are presented with their respective error bars. It is obvious that the employed titrimetric method is precise, accurate and reproducible, since relative standard deviation (RSD) values based on three distinct measurements range from 0.66% to 9.89%. On the other hand, the antioxidant activity values show poorer RSD values, within 10.51% and 37.88%. This lower precision might be due either to error propagation during the several consecutive but necessary dilution steps, or to the color (meaning non-zero 735 nm absorbance) of some samples.

4. Conclusions

In the context of rapid paced modern life, easy and sufficient Vitamin C uptake is of high importance for the human diet. Hence, its fast, reliable, and cost-effective determination from all kinds of food samples is an ongoing challenge.

Thus, a total of 41 food samples, mainly easy to consume beverages, were subjected to total ascorbic acid content determination. Two simple and cost-effective titrimetric methods were employed, both relying on the oxidation of AA by iodine. Yet only the one using back-titration of excess I_2 from

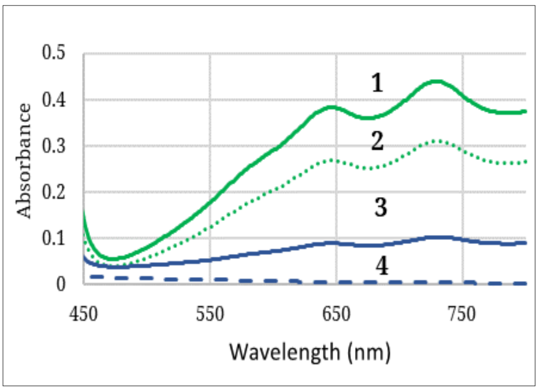


Fig. 8. *Molar absorbance spectra recorded for: 1. a $5x10^{-5}$ mol/L ABTS aqueous solution; 2. solution 1 containing $3x10^{-6}$ mol/L AA; 3. a 500 fold diluted blackcurrant syrup containing $5x10^{-5}$ mol/L ABTS; 4. a 500 fold diluted blackcurrant syrup.*

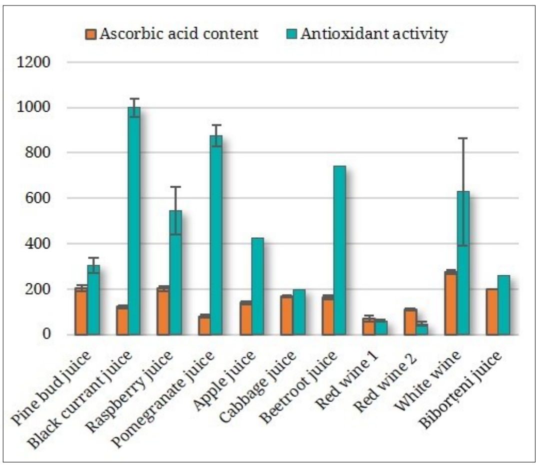


Fig. 9. *Comparison between total ascorbic acid content and antioxidant activity. Both are expressed in mg/L AA.*

the sample has proved to enable reliable and reproducible end-point detection. Results agreed with other reports, for example those referring to freshly squeezed orange juice, or to honey. For various cold-pressed oils only partial results could be obtained, but even so the high nutritional value of these has been proven.

For some of the beverages, total antioxidant activity expressed as ascorbic acid content, has been determined by means of a simple photometric method. Even if not as precise as the above-mentioned titrimetric technique, the procedure showed good results and proved once more the nutritional value of some analyzed products.

Both experimental techniques were validated by good recoveries from 2 commercially available pharmaceutic products with known ascorbic acid concentrations.

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