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Spectrophotometric method for fast quantification of ascorbic acid and dehydroascorbic acid in simple matrix for kinetics measurements

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Abbreviated title

Spectrometric quantification of ascorbic and dehydroascorbic acids

Abstract

A simple, rapid and reliable method was developed for quantifying ascorbic (AA) and dehydroascorbic (DHAA) acids and validated in 20 mM malate buffer (pH 3.8). It consists in a spectrophotometric measurement of AA, either directly on the solution added with metaphosphoric acid or after reduction of DHAA into AA by dithiothreitol. This method was developed with real time measurement of reactions kinetics in bulk reactors in mind, and was checked in terms of linearity, limits of detection and quantification, fidelity and accuracy. The linearity was found satisfactory on the range of 0-6.95 mM with limits of detection and quantification of 0.236 mM and 0.467 mM, respectively. The method was found acceptable in terms of fidelity and accuracy with a coefficient of variation for repeatability and reproducibility below 6% for AA and below 15% for DHAA, and with a recovery range of 97-102% for AA and 88-112% for DHAA.

Key words: analytical method, validation, near-the-line fast measurement, food, vitamin C.

Highlights

- A simple method is proposed for ascorbic and dehydroascorbic acids quantification.
- The method is based on a direct spectrophotometric measurement of AA.
- The method has low requirements on lab equipment and is hence inexpensive.
- The method is validated (linearity, fidelity and accuracy) for 0 to 6.95 mM of AA.
- Limits of detection and quantification are equals to 0.236 and 0.467 mM.

Chemical compounds studied in this article

Ascorbic acid (PubChem CID: 54670067); Dehydroascorbic acid (PubChem CID: 440667).

1. Introduction

Vitamin C is a nutrient essential for human diet with beneficial effects on health due to its primary defensive function of free-radical scavenger of reactive oxygen and nitrogen species. This vitamin is mainly found in fruit (e.g. citrus fruit, kiwis, mangos, red berries contain between 20 and 50 mg of vitamin C in 100 g of product) and vegetables (e.g. parsley, peperroni, cabbage, broccoli and tomato contain between 40 and 200 mg of vitamin C per 100 g of product) (Amiot-Carlin, Caillavet, Causse, Combris, Dallongeville, Padilla, Renard, & Soler, 2007; Liu, Heying, & Tanumihardjo, 2012; Dominguez-Perles, Mena, Garcia-Viguera, & Moreno, 2013; Park, Im, Ham, Kang, Park, Namiesnik, Leontowicz, Leontowicz, Trakhtenberg, & Gorinstein, 2015). The term vitamin C refers to compounds exhibiting the same full or partial biological activity as *L*-ascorbic acid (AA). It includes natural compounds such as AA itself, but also synthetic compounds such as *D*-isoascorbic acid (also known as erythorbic acid) or ascorbyl palmitate (fat-soluble form of the vitamin C) (Ball, 2006; Eitenmiller, Ye, & Landen, 2008). The dehydroascorbic acid (DHAA), which is the oxidized form of AA, exhibits *in vivo* a full vitamin C activity as it can be reduced to its AA form in the human body. Depending on the authors it can be explicitly

included in the vitamin C group of compounds (Eitenmiller et al., 2008) or not (Ball, 2006) even if the definition of vitamin C remains the same for both authors. It is therefore important to measure both AA and DHAA for evaluating the total vitamin C content in a product (Barret & Lloyd, 2012). AA is also often added as an antioxidant and anti-browning aid for processed food products containing fruit and vegetables, either for enrichment or fortification, or for protecting other nutrients (like phenolic compounds) from oxidation. In many studies, due to its high sensitiveness to applied conditions, it is used as an indicator in the perspective of estimating the retention of nutrients during processing of food. In fact, AA and DHAA are highly unstable at high temperature (Laing, Schlueter, & Labuza, 1978; Lin & Agacollo, 1979; Rojas & Gerschenson, 1997, 2001; Yuan & Chen, 1998; Burdurlu, Koca, & Karadeniz, 2006; Oey, Verlinde, Hendricks, & Van Loey, 2006), high pH (Rogers & Yacomeni, 1971; Wilson, Beezer, & Mitchell 1995), light (Solomon & Svanberg, 1995), in presence of oxygen (Solomon & Svanberg, 1995; Garcia-Torres, Ponagnadla, Rouseff, Goodrich-Schneider, & Reyes-De-Corcuera, 2009; Van Bree, Baetens, Samapundo, Devlieghere, Laleman, Vandekinderen, Nosedá, Xhaferi, De Baets, & De Meulenaer, 2012), and of traces of transition metals or of enzymes (Serpen & Gökmen, 2007).

Several papers reviewed the methods proposed for AA and/or DHAA quantification in food products. Many methods have been developed: titrimetry, photometry, polarometry, amperometry, thin layer chromatography, gas chromatography, and HPLC (Hasselmann & Diop, 1983; Arya, Mahajan, & Jain, 2000; Ball, 2006). Recently, Pisoschi, Pop, Serban and Fafaneata (2014) also reviewed the potentialities and analytical performances of electrochemical methods for AA assessment in various media. The AOAC method for vitamin preparations and juices is a titrimetric method based on the reduction of the pink 2,6-dichlorophenolindophenol (DCPIP) with AA in an acid solution. It is a rapid, precise and low cost method, that can also be miniaturized on microplates prior to absorbance detection at 505 nm (Pénicaud, Peyron, Bohuon, Gontard, & Guillard, 2010), but interference with substances reacting with DCPIP can occur and DHAA does not participate to the redox reaction (Ball, 2006). Arya, Mahajan and Jain (2001) described the spectrophotometric methods for the determination of vitamin C in different substrates. All the methods they cited involved vitamin C reaction with a chemical compound either to produce or to consume a molecule easily measurable by spectrophotometry. A classical approach is based on the oxidation of AA to DHAA and its reaction with 2,4-dinitrophenylhydrazine (DNPH) to form osazone of diketogulonic acid, which yields a stable brown color with sulfuric acid with an absorption at 540 nm proportional to the total quantity of AA and DHAA (Ball, 2006). Direct spectrophotometry is rarely used for the measurement in food extracts because of the spectral interferences with many substances. Chromatographic methods are the only ones that make it possible to separate AA from interfering substances present in food. AA can be detected directly by either absorbance or electrochemical detectors, or by mass spectrometry. In order to perform both AA and DHAA quantification using UV detection, the reduction of DHAA must be inserted in the method (Odriozola-Serrano, Hernadez-Jover, & Martin-Belloso, 2007; Takayanagi, Nishiuchi, Ousaka, Oshima, & Motomizu, 2009) or DHAA must be derivatized using 4,5-dimethyl-1,2-phenylenediamine prior to fluorescence detection (Novakova, Solich, & Solichova, 2008). HPLC methods are highly selective and generally more sensitive than spectrophotometric or titration methods. The current state of HPLC methods for simultaneous determination of AA and DHAA in food have been extensively reviewed by Novakova et al. (2008) and Spinola, Llorent-Martinez and Castilho (2014). UHPLC (ultra-high performance liquid chromatography) has also been recently applied to the analysis of vitamin C in foods, with shorter analysis times and much lower solvent consumption (Spinola, Mendes, Camara, & Castilho, 2013; Klimczak & Gliszczynska-Swiglo, 2015). For his part, Deutsch (2000) focused on DHAA and reviewed most quantification methods including crystallographic, infrared and ultraviolet studies, nuclear magnetic resonance, mass spectrometry, electrochemical detection, and separation methods. LC-MS methods remain especially promising because of its short analysis time, as well as high sensitivity and selectivity. Szultka, Buszewska-Forajta, Kaliszan and Buszewski (2014) demonstrated the feasibility of this technique for the determination of AA (with limit of quantification < 0.5 ng/mL) and the study of AA degradation products.

Direct UV spectrophotometry remains nevertheless an interesting option for rapid and inexpensive quantification of both AA and DHAA in solutions. AA has its absorption maximum in the range of 244-265 nm depending of the pH. DHAA in solution absorbs UV light well at 185 nm, but has little absorbance above 220 nm (Deutsch, 2000). Reduction must therefore be performed prior to measurement. Dithiothreitol (DTT) is the most common reducing agent (Esteve, Farré, Frigola, & Garcia-Cantabella, 1997; Novaka et al, 2008; Takayanagi et al., 2009), but tris[2carboxylethyl]phosphine hydrochloride (TCEP), dimercaptopropanol (Odriozola-Serrano et al., 2007; Chebrolu, Jayaprakasha, Yoo, Jifon, & Patil, 2012), or cysteine (Herrero-Martinez, Simo-Alfonso, Deltoro, Calatayud, & Ramis-Ramos, 1998;

Chiari, Nesi, Carrea, & Righetti, 1993) may serve as alternatives. Odriozola-Serrano et al. (2007) obtained significantly better recovery values in fruit using DTT as reducing agent when compared to dimercaptopropanol. The content of DHAA is then calculated by subtracting the initial AA content from the total AA content after the conversion. This method has the disadvantage that reducing agents could interfere with the measurement and decrease the sensitivity of the method. However DHAA reduction is rapid (< 10 min), and thus makes it possible a rapid quantification of DHAA.

The reliability of a method is the prerequisite for a correct interpretation of analyses. All analytical methods require proper optimization and validation to ensure their suitability for further applications and to yield reliable results. Statistical methods must be used for providing acceptance criteria on the key elements necessary for validation: linearity, selectivity, accuracy, bias, precision, and lower limits of detection and quantification (Feinberg & Laurentie, 2010; Spinola et al, 2014).

The aim of this work is to validate a method for the determination of AA and DHAA in buffer solutions by direct spectrophotometric absorbance using a proven and robust methodology proposed by Feinberg and Laurentie (2010). This method must be reliable, but as rapid and simple as possible in order to be used for measuring real time kinetics of AA and DHAA contents versus different heating and oxygenation conditions.

2. Materials and methods

2.1. Reagents

Ascorbic acid crystalline (AA) (99%), dehydroascorbic acid (DHAA) (99%), DL-dithiothreitol (DTT) (99%), and ethyl acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Malic acid, metaphosphoric acid, tris(hydroxymethyl) aminomethane, and sodium phosphate monobasic dihydrate were purchased from VWR Prolabo chemicals (Luther Worth, England). Sodium hydroxide (97%) and sodium phosphate dibasic dihydrate were purchased from Carlo Erba (Val de Reuil, France). Water was purified through a Milli-Q (Millipore, Bedford, MA, USA) water system and used for all solutions preparations and dilutions.

2.2. Equipment

Measurements were performed on a UV-vis scanning spectrophotometer (SPECORD210 Plus, Analytik Jena AG, Jena, Germany) in the 200 to 300 nm wavelength range, with a slot of 1 nm, a reading speed of 10 nm/s, and an integration time of 0.1 s.

2.3. Ascorbic and dehydroascorbic acids determination

All AA and DHAA solutions were prepared in 20 mM malate buffer (pH 3.8) just before being used. Each solution was prepared by weighting independently the required amount of AA and/or DHAA powder. The other solutions used for these determinations were newly prepared for each serial of measurements.

2.3.1. Ascorbic acid determination

Ascorbic acid solutions were diluted to 1/50 (v/v) in an aqueous solution of 3% metaphosphoric acid (w/v) in order to avoid further oxidation. Chebrolu et al. (2012) recently confirmed that AA was stable up to 48 h in metaphosphoric acid extracts at room temperature. The absorbance of the mixture was recorded against a 20 mM malate buffer diluted 1/50 in metaphosphoric acid, and read at 243 nm, wavelength for which AA exhibited the maximum absorbance (Fig. 1). The absorbance value was then converted into AA concentration using a calibration curve obtained the same day.

2.3.2. Dehydroascorbic acid determination

The method is based on the reduction of DHAA into AA, so as to use the same methodology as for the analysis of native AA. The reduction method consisted in mixing 0.5 mL of sample with 0.5 mL of 30 mM DTT solution prepared in 0.1 mM phosphate buffer (pH 7.5) and 1 mL of 0.2 M tris HCl buffer (pH 8.2). The mixture was homogenized using a vortex, then placed in an ice bath, and kept dark for 30 min. After 30 min of reaction, DTT in excess was eliminated by three successive extractions with ethyl acetate immiscible with malate buffer (stirring by rotation for 1 min each time to promote contact between the phases), and AA concentration was determined as previously described. The wavelength exhibiting the maximum absorbance was the same as for pure AA and the absorbance value was not affected by the added reagents, demonstrating a good selectivity (Fig.1). The value read at 243 nm (A_{total}) was then converted into total AA content using the calibration curve. A first DHAA content, called $[DHAA]_{bc}$ (before correction), was determined by calculating the difference between the total AA content and the initial AA

content measured on the sample prior to reduction. A correction (see below) was then applied to obtain the true DHAA content, called [DHAA].

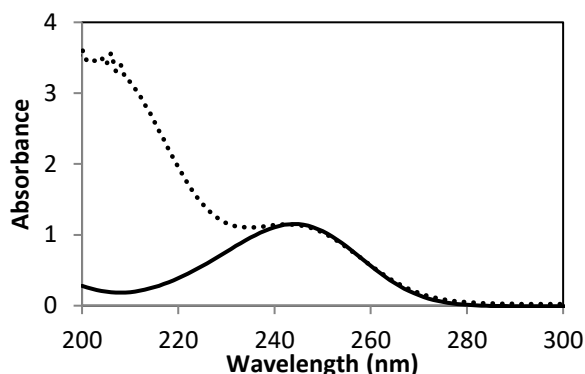


Fig. 1. Absorbance spectra recorded on AA (—) and DHAA converted into AA (- -) solutions prepared in 20 mM malate buffer diluted at 1/50 in a 3% metaphosphoric acid solution (w/v).

2.4. Calibration and validation plans

The calibration and validation plans for the quantification of AA and DHAA contents were based on guidelines proposed by Feinberg and Laurentie (2010), defining the number of experiments necessary to achieve meaningful statistics (Table 1).

Table 1. Calibration and validation plans used for the quantification of AA and DHAA contents

	Calibration plan (Number of experiments)		Validation plan (Number of experiments)		
	AA	AA	DHAA		
			TC* = 5.6	TC = 4	TC = 1.5
I = series (new set of experiments)	4	4	3	3	3
K = levels of concentration	5	4	7	5	5
J = replication par level	3	3	3	3	3
Total number of experiments	60	48	63	45	45

*TC = Total content in AA and DHAA (mM)

2.5. Tools for method validation

The reliability of the method was validated with regards to its linearity, limits of detection, fidelity, and accuracy.

2.5.1. Linearity

In the present method, AA content ([AA]) is deduced from an indirect measurement (absorbance recorded at 243 nm: $A_{243 \text{ nm}}$) requiring a calibration. As AA contents are supposed to vary in the range of 0 - 5.6 mM in further experiments, linearity was checked over a larger range of 0 - 6.95 mM. Five standard solutions evenly distributed over the concentration range (*i.e.* 0, 0.58, 2.37, 4.63, and 6.95 mM, respectively) were used in 12 repetitions (4 series of 3 replicates). The linearity was expressed using Eq.1

$$A_{243 \text{ nm}} = a_1 \times [\text{AA}] + a_0 \quad (1)$$

where a_1 was the fitted slope, also called sensitivity, and a_0 the intercept.

The linear model was fitted using the method of least-squares, and the adequacy of the fit was tested using a procedure of analysis of variances (ANOVA) (Feinberg & Laurentie, 2010).

Experimental Fisher values (F -values) were compared to their tabulated values ($F_{\alpha; DoF1; DoF2}$) for the selected significance level (α) and for 2 degrees of freedom, $DoF1$ for the linear regression and $DoF2$ for residual errors. In our case, α was set either to 0.01, $DoF1 = 1$ (for the linearity test) or $p-2$ (for the

nonlinearity test), and $DoF2=N-p$ (with N the total number of measurements and p the number of levels (or tested solutions)). F -values were calculated from the analysis of variances (Feinberg & Laurentie, 2010). Linearity was tested by comparing the experimental Fischer value for linearity (F) to its tabulated value. For $F_1 > F_{0.01;1;N-p}$, the calculated regression can be accepted. On the same way, non-linearity was tested by comparing the experimental Fischer value for non-linearity (F_{nl}) to its tabulated value. For $F_{nl} \leq F_{0.01;p-2;N-p}$, the linear regression gives an accurate description on the whole range of content values. If $F_{nl} > F_{0.01;p-2;N-p}$, the calibration curve is not linear over the selected range and its size must be reconsidered.

2.5.2. Limit of detection / limit of quantification

The limit of detection (LOD) is the lowest concentration likely to be reliably distinguished from a blank and at which detection is feasible. The limit of quantification (LOQ) is the lowest concentration at which the product can not only be reliably detected, but measured with a sufficient and/or defined degree of confidence. LOD and LOQ at a 95% confidence level were calculated from the parameters a_1 and a_0 (Eq. 1) obtained by the method of least squares as follows

$$LOD = \frac{a_0 + 3s_{a0}}{a_1} \quad (2)$$

$$LOQ = \frac{a_0 + 3s_{a0}}{a_1} \quad (3)$$

where s_{a0} is the standard deviation of the blank signal (the intercept).

2.5.3. Fidelity

The fidelity of the method is characterized by the repeatability (r) and the reproducibility (R). The repeatability is the maximum deviation at a 95% confidence level between two results obtained during a single analytical run (same experimenter, same set of solutions, and same day). The reproducibility is the maximum deviation at a 95% confidence level between two within-laboratory measurements obtained at different times (different days) and that may involve different analysts, equipment, or reagents. An analysis of variance for a single random effect model was used to determine the repeatability and reproducibility. The coefficients of variation for repeatability (CV_r) and for reproducibility (CV_R) were calculated as the ratios of standard deviation of replicates to mean of replicates, according to Feinberg and Laurentie (2010).

2.5.4. Accuracy

The accuracy of a method characterizes the difference between the experimentally measured and true values, and is expressed in the form of an absolute bias (i.e. difference between the theoretical and the measured mean concentration) and a percent recovery (ratio of the measured concentration to the theoretical one $\times 100$). A method can be considered accurate if the recovery is in the range of $100 \pm 15\%$ (Shah *et al.*, 1992).

2.5.5. Correction factor

A correction factor can be applied if a systematic bias is observed on the measured values. The methodology consists in expressing first the linear correlation between the measured and the reference values. If the slope is not equal to 1 as expected, it will correspond to an average percent recovery, and the intercept will correspond to a systematic bias. Two correction factors can then be calculated: $1/\text{slope}$, used to correct the unsatisfactory percent recovery, and the intercept, used to correct the systematic bias. The concentration can be calculated as

$$[X]_{\text{final}} = \left([X]_{\text{bc}} \times \frac{1}{m} \right) - \text{Int} \quad (4)$$

where $[X]_{\text{bc}}$ is the concentration measured (before correction), m the slope of the linear dependence between the actual concentrations measured and the reference values, and Int the intercept of the new regression line according to concentrations corrected with m versus reference values.

3. Results and discussion

3.1. Calibration of ascorbic acid concentration from absorbance measured at 243 nm

The repetitions of absorbance (4 serials with 3 repetitions) measured for each of the 5 levels of concentration were plotted against the average value of AA content for each level, as calculated from actual mass (reference). A linear regression analysis was applied, and the adequacy of the linear regression model was tested. Results obtained for the linearity test, the equation calculated for the calibration line, and the limits of detection and quantification are given in Table 2. Considering these statistical results, the fitted linear regression model is acceptable (linearity test), and the model is linear over the full range 0 - 0.695 mM (nonlinearity test). As shown in Table 2, *LOD* is 0.236 mM, and *LOQ* is 0.467 mM.

Table 2. Statistical results to assess the adequacy of the linear regression model for the calibration curve

Source of variation	Sum of squares	Degrees of freedom	Variance	F calculated	F tabulated ($p = 0.01$)	Conclusion
Linearity	7.235	1	7.23497	93852	7.770	Satisfactory
Nonlinearity	0.0002	3	0.00007	0.879	4.675	Satisfactory
Residual	0.0019	25	0.00008			
Total	7.2371	29				

Calibration curve	
Model	
Slope (a_1)	0.2135
Intercept (a_0)	0.0292
R^2	0.9956
s	0.0071

Limits of detection and quantification.	
Limit of detection (<i>LOD</i> , in mM)	0.236
Limit of quantification (<i>LOQ</i> , in mM)	0.467

3.2. Validation of the quantification of ascorbic acid

The ascorbic acid concentrations obtained for the validation plan were calculated from the absorbance by applying the calibration lines made during the same series (data not shown, available as supplementary material 1).

These experimental results made it possible to calculate the fidelity and the accuracy of the method for each level of concentration (Table 3). This method is characterized by coefficients of variation (*CV*) for repeatability and for reproducibility below 3%, except for the lower concentration of 1.162 mM for which *CV* values of 5.72% and 8.36% were calculated for repeatability and reproducibility, respectively. However the method was considered validated in terms of fidelity on the whole range because the precision around the mean values never exceeded 10% of variation. The method was also considered accurate because all the values obtained were close to the reference value, with a maximal absolute bias of -0.08 mM and a mean percent of recovery of 97.5%. Nevertheless all the biases were slightly negative, indicating that the true values were slightly underestimated by this measurement method.

A new serial of experiments was performed in order to check the validity of the calibration model on different analyses carried out by different experimenters. Results showed that the established calibration parameters made it possible to calculate AA concentrations adequately as the calculated concentrations were linearly correlated to the reference concentrations with a slope of 1.011 and an intercept of -0.127 ($R^2 = 0.987$; $n = 29$), *i.e.* very close to the bisector.

Table 3. Values calculated for evaluating the precision of ascorbic acid analysis

Levels	Average [AA] from weighted mass (mM)	Average measured [AA] (mM)	Fidelity		Accuracy	
			CV _r (%)	CV _R (%)	Absolute Bias	Recovery (%)
A	1.16 ± 0.01	1.10 ± 0.09	5.72	8.36	-0.05	95.4
B	2.91 ± 0.01	2.83 ± 0.07	2.18	2.40	-0.08	97.3
C	4.05 ± 0.01	3.99 ± 0.06	1.26	1.49	-0.06	98.3
D	5.86 ± 0.04	5.80 ± 0.1	0.90	1.90	-0.06	99.0

3.3. Validation of the quantification of dehydroascorbic acid

3.3.1. Correction of the DHAA concentration as a function of the total absorbance measured at 243 nm

Series of measurements were made on different solutions containing mixtures of AA and DHAA in different proportions, and for 3 levels of total content in AA + DHAA. Results on Fig. 2A show that the direct application of the calibration line fitted for AA to absorbance values measured on the solutions before and after reduction of DHAA did not make it possible to calculate, by difference, the correct concentrations in DHAA, if compared to the mass weighted for the preparation of the solutions. An average percent recovery significantly different from 100%, and a systematic bias depending on the total content in AA + DHAA were observed. The methodology proposed by Feinberg and Laurentie (2010) for correcting the values (Eq. 4) was applied in order to obtain a unique regression model for calculating DHAA contents whatever the total content in AA + DHAA in the range of 0 - 5.6 mM. Results plotted on Fig. 2A show that the fitted slopes of the linear regression models calculated for each series corresponding to a total content in AA + DHAA were relatively close. A new linear regression model was built taking as unique slope, for all concentrations, the mean value of the 3 previous slopes ($\bar{m} = 0.7524$) (Fig. 2B).

In order to increase the percent recovery and have a slope of regression line equal to 1, calculated concentrations of DHAA were corrected by multiplying the data by the inverse of this average slope, resulting in modified intercepts for each regression line (Fig. 2C). These new intercept values varied with the total content in AA + DHAA, and therefore with the total absorbance measured after reduction (A_{total}). A new regression model was built in order to be able to predict the intercept value (Int) as a function of A_{total} (Eq. 5) with a very good determination coefficient ($R^2 = 0.9921$).

$$Int = 1.014 \times A_{total} + 0.1529 \quad (5)$$

It could be noticed that all these correction steps have a minimal impact on the adequacy between the experimental data and the values given by the linear models. On Fig. 2A, R^2 coefficients were equal to 0.9721, 0.9889 and 0.9735 for concentrations of 1.5, 4.0 and 5.7 mM, respectively. However, R^2 values could not be determined for the subsequent steps. The adequacy can also be characterized by calculating the relative root mean square error ($RMSE$) between the experimental values and those calculated thanks to the different linear regressions. The calculated $RMSE$ did not evolve much, increasing between Fig. 2A and Fig. 2C from 7.4 to 7.7% at 1.5 mM, from 4.7 to 5.3% at 4.0 mM, and from 7.5 to 8.0% at 5.7 mM, hence confirming the negligible impact of the corrections steps on the adequacy of the models.

Finally, Fig. 2D shows the agreement between the corrected values for DHAA content and the direct proportionality line. The equation for correcting DHAA concentration value is determined from the equation 1 as follows:

$$[DHA] = \left([DHA]_{bc} \times \frac{1}{\bar{m}} \right) - Int = \left([DHA]_{bc} \times \frac{1}{0.7524} \right) - (1.014 \times A_{total} + 0.1529) \quad (6)$$

where $[DHA]_{bc}$ is the concentration of DHAA before correction, determined by difference between the total AA content and the AA content measured on the sample prior to reduction, and $[DHA]$ is the final value of concentration after correction taking the total content in AA + DHAA into account.

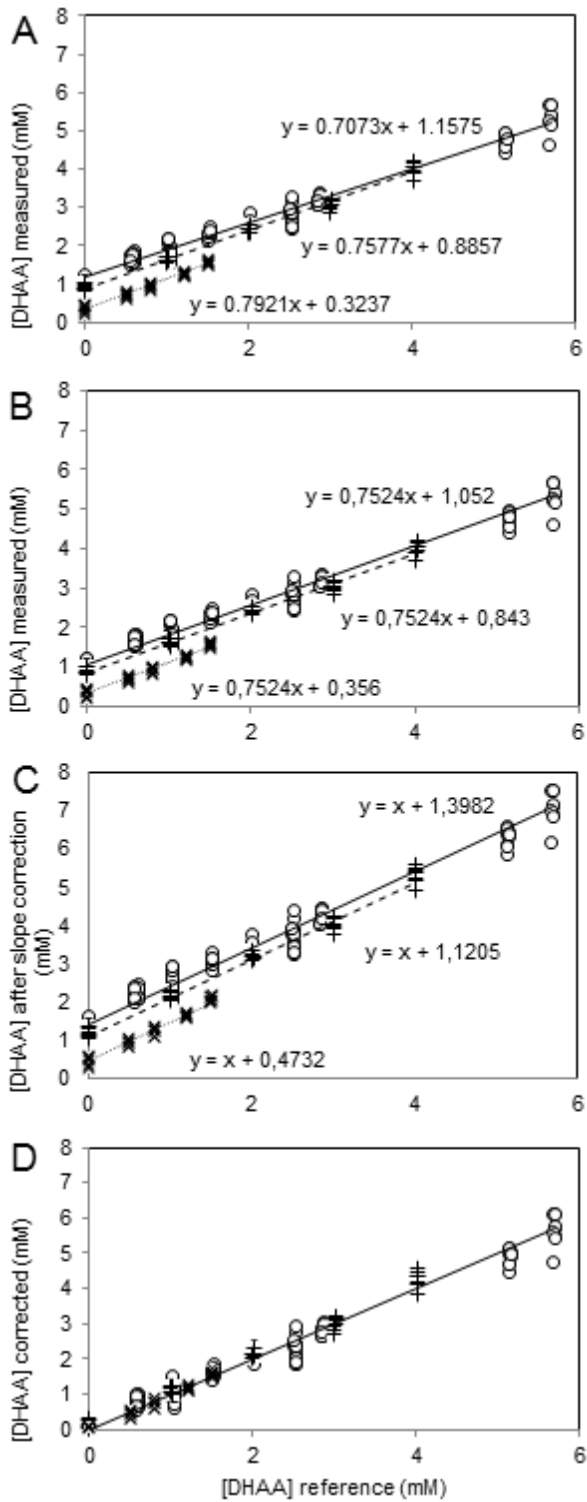


Fig. 2. Correction of DHAA content values as a function of the total concentration in AA + DHAA: (A) raw values from absorbance measured at 243 nm when applying the calibration regression model calculated for AA; (B) linear regression models fitted with the same unique slope for all total concentrations in AA + DHAA; (C) linear regression models corrected in order to have a slope equal to 1; (D) DHAA values corrected by the intercept calculated as a function of the total absorbance (A_{total}). "□" for [AA + DHAA] = 5.6 mM; "+" for [AA + DHAA] = 4 mM; "x" for [AA + DHAA] = 1.5 mM.

3.3.2. Validation plan

All the experiments planned to validate the quantification of DHAA content (data not shown, available as supplementary material 2) were done and Table 4 shows the values characterizing the fidelity of the method. All the coefficients of variation are below 15%, except for repeatability at 1.02 mM ($CV_r = 16.12\%$), and for reproducibility at concentrations in the range of 0 - 1.02 mM ($12 < CV_R < 21\%$). It is a classical observation that repeatability and reproducibility are lower for concentrations near the LOQ, because the reducing agents tend to interfere with the measurement (Moeslinger, Brunner, & Spieckermeann, 1994). All the values obtained are close to the true value, with a maximal absolute bias in the range of 0.01 - 0.28 mM (in absolute value) and a percent recovery of $100 \pm 13\%$, except for a concentration level of 0.6 mM. The method was therefore considered accurate.

The validity of the correction factor was checked as previously described for AA measurements. The representation of calculated concentrations versus reference concentrations gave a linear correlation, with a slope of 1.010 and an intercept of -0.140 ($R^2 = 0.996$; $n = 9$), very close to the bisector.

Table 4. Values calculated for evaluating the precision of dehydroascorbic acid analysis

Levels	Average [DHAA] calculated from weighed mass (mM)	Average measured [DHAA] (mM)	Fidelity		Accuracy	
			CV_r (%)	CV_R (%)	Absolute Bias	Recovery (%)
A	0	0.04 ± 0.13	-	-	0.04	-
B	0.504 ± 0.002	0.44 ± 0.08	9.27	21.38	-0.06	88.2
C	0.594 ± 0.021	0.68 ± 0.18	11.11	17.95	0.19	131.6
D	0.804 ± 0.002	0.75 ± 0.10	5.70	15.3	-0.06	93.1
E	1.018 ± 0.012	1.15 ± 0.18	16.12	17.08	0.13	112.7
F	1.210 ± 0.003	1.17 ± 0.05	3.28	11.98	-0.04	96.7
G	1.521 ± 0.018	1.61 ± 0.14	6.09	6.90	0.08	105.2
H	2.014 ± 0.009	2.08 ± 0.13	6.03	6.51	0.07	103.5
I	2.521 ± 0.009	2.26 ± 0.31	7.42	14.13	-0.26	89.7
J	2.862 ± 0.009	2.80 ± 0.17	1.81	6.79	-0.06	97.9
K	3.009 ± 0.002	3.00 ± 0.17	2.77	6.23	0.01	100.2
L	4.022 ± 0.006	4.24 ± 0.21	3.42	5.44	0.22	105.5
M	5.154 ± 0.009	4.87 ± 0.24	1.82	6.79	-0.28	94.6
N	5.713 ± 0.046	5.67 ± 0.44	8.21	7.73	-0.04	99.3

4. Conclusion

Both methods for measuring AA and DHAA contents were acceptable in terms of fidelity and accuracy, with a percent recovery varying between 97 and 102 % for AA, and between 88 and 112% for DHAA. The coefficient of variation for repeatability and reproducibility were good and below 6% for AA. The CV values for DHAA analysis were significantly higher, due to interferences with chemicals produced during the reduction by DTT prior to spectrophotometric recording. Repeatability and reproducibility could nevertheless be considered acceptable with coefficients of variation below 15% in the range of 0 - 5.67 mM for repeatability and of 0.8 - 5.67 mM for reproducibility, respectively. Both methods made it possible a fast and reliable quantification of AA content (in about 5 min) and of DHAA content (in about 35 min, including the 30 min of waiting time for the reduction of DHAA in AA) in aqueous solutions, and are therefore totally appropriate for following reaction kinetics on buffer solutions in agitated reactors.

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Supplementary Material 1

Measured and predicted values for the validation of AA content analysis

Levels	Series	[AA] from weighted mass (mM)	[AA] from absorbance measured at 243 nm (triplicates) (mM)		
			1	2	3
A	1	1.167	1.16	1.19	0.98
	2	1.145	1.24	1.20	1.17
	3	1.170	0.98	1.05	1.02
	4	1.167	1.07	1.14	1.1
B	1	2.907	2.82	2.93	2.93
	2	2.907	2.88	2.78	2.74
	3	2.918	2.77	2.88	2.8
	4	2.907	2.76	2.86	2.79
C	1	4.065	4.06	4.04	3.97
	2	4.043	4.02	4.04	3.97
	3	4.043	3.94	4.01	4.06
	4	4.065	3.86	3.93	3.98
D	1	5.882	5.74	5.73	5.73
	2	5.803	5.84	5.77	5.68
	3	5.871	5.88	5.98	5.98
	4	5.882	5.70	5.79	5.80

Supplementary Material 2

Experimental measured data and predicted values for the validation of DHAA content analysis

Levels	Series	Concentrations calculated from weighted mass (mM)		[AA] from absorbance measured at 243 nm (n=3) (mM)	[DHAA] from absorbance measured at 243 nm and corrected (mM)		
		[AA]	[DHAA]		1	2	3
A	1	5.695	0	5.58	-0.04	0.10	-0.03
	2	5.689	0	5.63	0.15	0.18	-0.03
	3	5.695	0	5.63	-0.20	-0.10	-0.05
B	4	1.003	0.502	0.98	0.34	0.35	0.34
	5	1.091	0.505	1.07	0.54	0.44	0.46
	7	1.003	0.504	0.98	0.56	0.47	0.49
C	1	5.127	0.574	5.13	0.50	0.56	0.41
	2	5.127	0.580	5.06	0.73	0.79	0.71
	3	5.119	0.570	5.16	0.70	0.32	0.44
	10	5.133	0.609	5.30	1.01	0.90	0.93
	11	5.139	0.620	5.39	0.68	0.79	0.68
	12	5.139	0.609	5.39	0.71	0.69	0.64
D	4	0.702	0.802	0.66	0.62	0.63	0.63
	5	0.705	0.806	0.75	0.86	0.79	0.75
	6	0.702	0.805	0.67	0.87	0.80	0.79
E	1	4.713	1.034	4.76	0.84	1.29	1.25
	2	4.701	1.028	4.66	0.68	1.15	1.34
	3	4.702	1.022	4.68	0.85	1.24	1.36
	7	3.009	1.008	2.96	0.98	1.04	1.02
	8	3.018	1.011	3.06	1.07	1.20	1.21
	9	3.012	1.002	2.99	1.07	1.20	1.21
F	4	0.301	1.205	0.28	1.09	1.14	1.13
	5	0.308	1.207	0.36	1.23	1.16	1.13
	6	0.308	1.210	0.28	1.26	1.20	1.19
G	1	4.190	1.552	4.22	1.50	1.70	1.66
	2	4.201	1.528	4.20	1.41	1.56	1.70
	3	4.182	1.522	4.12	1.66	1.86	1.73
	4	0	1.502	-0.04	1.48	1.64	1.63
	5	0	1.507	0.02	1.56	1.52	1.52
	6	0	1.516	-0.02	1.64	1.57	1.71
H	1	3.742	2.010	3.72	2.06	1.95	1.73
	2	3.690	2.010	3.82	1.93	1.84	2.31
	3	3.690	2.022	3.65	1.96	1.91	2.06
	7	2.033	2.027	1.97	2.06	2.00	2.13
	8	2.021	2.004	2.09	2.13	2.15	1.99
	9	2.004	2.010	2.00	2.29	2.29	2.14
I	1	3.291	2.510	3.35	2.10	2.09	2.33
	2	3.180	2.527	3.24	2.42	2.76	2.95
	3	3.191	2.510	3.27	2.34	2.47	2.50
	10	3.219	2.527	3.28	2.42	2.01	2.31
	11	3.219	2.527	3.29	2.12	2.33	2.03
	12	3.219	2.527	3.30	1.83	1.84	1.89
J	1	2.884	2.855	2.92	2.68	2.63	2.63
	2	2.862	2.872	2.93	3.10	3.06	3.04
	3	2.884	2.860	3.03	2.75	2.93	2.81
K	7	1.016	3.007	1.05	2.71	2.83	2.90
	8	1.014	3.010	1.06	3.15	3.13	3.15
	9	1.011	3.010	1.05	2.97	3.18	3.11
L	7	0	4.015	0.15	3.82	4.11	4.12
	8	0	4.026	0.06	4.33	4.53	4.32
	9	0	4.026	0.06	4.17	4.44	4.34
M	1	0.591	5.146	0.64	4.69	5.00	4.96
	2	0.591	5.152	0.64	5.51	5.28	5.47
	3	0.573	5.163	0.80	5.36	5.23	5.30
N	1	0	5.766	0.16	5.70	5.76	5.44
	2	0	5.686	0.13	4.73	5.57	6.11
	3	0	5.686	0.07	5.48	6.09	6.11