Optimization and Validation of a UV–HPLC Method for Vitamin C Determination in Strawberries (Fragaria ananassa *Duch.*), Using Experimental Designs

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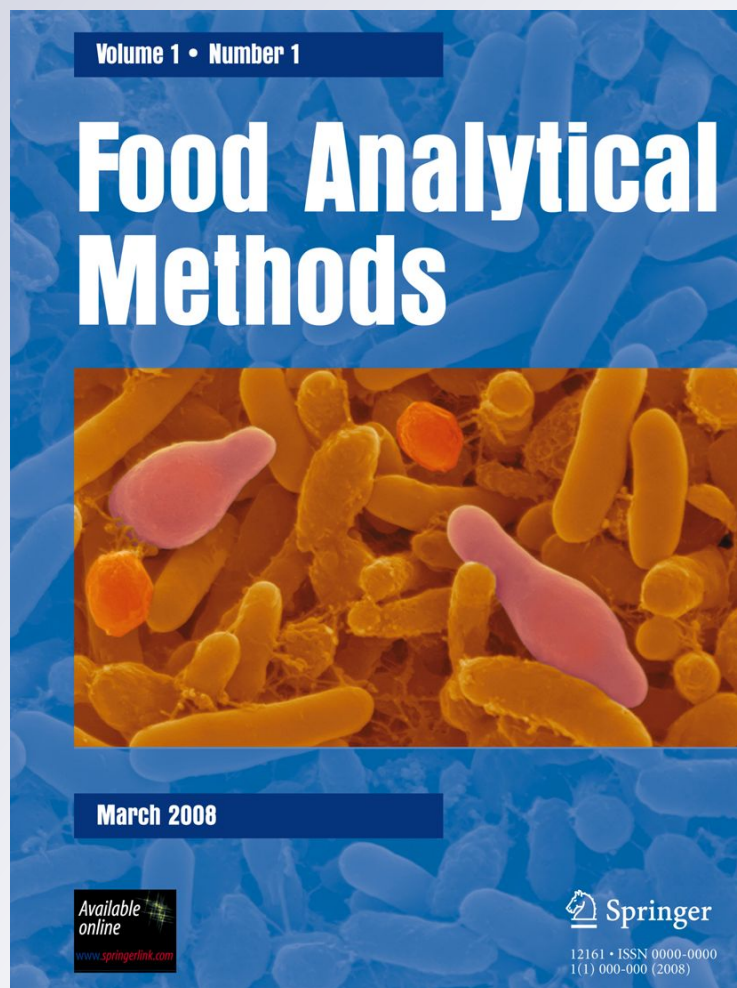María S. Cámara · Daniel R. Güemes ·
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Abstract Vitamin C or total ascorbic acid (TAA) in fruits can be assumed as ascorbic acid (AA) plus dehydroascorbic acid (DHA) content. The aim of this work was to optimize and validate, using experimental designs, a new high-performance liquid chromatographic method for vitamin C determination in strawberries. The mobile phase (MP) consisted of a 0.03 M sodium acetate/acetic acid buffer, 5% methanol. For optimization, a Box–Behnken design was used (three factors at three levels: (a) pH of MP, 3.8–5.8; (b) wavelength, 240–270 nm; and (c) flow rate, 0.5–1.2 ml min^{-1}). Responses were: AA and TAA areas, peak widths, and retention times. A global optimization was performed using the Derringer desirability function, and a value of 0.84 was reached for the combination of design factors: A=5.8, B=251 nm, and C=1.15 ml min^{-1}. Method validation, using AA standard solutions, included: linearity study, limits of detection and quantification, and calibration and analytical sensitivity quantifications. Precision and accuracy were studied in strawberry extracts. The coefficients of variation (percent) were: AA, 1.5%; TAA, 1.8%, and DHA, 4.9%. Accuracy was evaluated with AA standard spiked in 30–150% range of the expected amount of analyte in real samples. The joint confidence elliptical region test and t test were employed for the study of the difference between recoveries (percent) and the ideal 100%. The robustness was analyzed using a fractional factorial design (3^{4-2}), and an AA recovery study after slight changes in operative variables was performed. The results indicate that the optimized method was linear, sensible, precise, accurate, and robust.

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Introduction

Ascorbic acid, commonly known as vitamin C, is a powerful natural antioxidant present in a wide range of foods and beverages (Lopes et al. 2006). As humans cannot synthesize it, fruits and vegetables are their major dietary sources (Nováková et al. 2008). In general, fruits are considered the best dietary food sources, with blackcurrant being especially rich at 200 mg 100 g^{-1}, strawberry at 40–60 mg 100 g^{-1}, and citrus fruits at 30–50 mg 100 g^{-1}. However, not all fruits contain such levels, apples, pears, and plums

represent only a very modest content of vitamin C (3–5 mg 100 g^{-1}) (Davey et al. 2000).

L-Ascorbic acid (AA), the main biologically active form of vitamin C, is reversible-oxidized to form L-dehydroascorbic acid (DHA). This last form also exhibits biological activity, but further oxidation generates diketogulonic acid, which has no biological function, and the reaction is no longer reversible (Hernández et al. 2006). Therefore, vitamin C named as total ascorbic acid (TAA) in fruits can be assumed as the sum of AA and DHA contents, and consequently, the analysis of both species can be considered of utmost importance for the evaluation of total vitamin activity in fruits. Furthermore, due to its antioxidant properties, vitamin C is related to the maintenance of the organism oxidation–reduction potential (Maia et al. 2007). As a potent antioxidant, AA has different properties such as the ability to eliminate different reactive oxygen species and to keep vitamin E in the reduced state, playing a role in stress resistance. It also acts as a cofactor of many enzymes (by keeping metal ions in the reduced state) and seems to be the substrate for oxalate and tartrate biosynthesis (Davey et al. 2000; Hernández et al. 2006; Maia et al. 2007).

There are many analytical methods for ascorbic and dehydroascorbic acid determinations in food matrices. High-performance liquid chromatography (HPLC) methods are the preferred ones because they are faster and more effective than spectrophotometric, titration, or enzymatic methods, and they do not usually need derivatization (Nováková et al. 2008). There are Association of Official Agricultural Chemists (AOAC) official methods for vitamin C determination in vitamin preparations and juices, but they present many technical limitations. The AOAC's official method, based on the titration of AA with 2,6-dichloroindophenol in acidic solution, is not applicable to all matrices, and only AA can be determined. Hence, the method is adequate only when concentration of DHA is low. Additionally, substances naturally present in fruits such as tannins, betannins, sulfhydril compounds, Cu (II), Fe(II), Mn(II), and Co(II) are oxidized by the dye Maia et al. 2007; AOAC 1995; Arya et al. 2000). Furthermore, the final point is difficult to detect because of the color of strawberry extracts. The microfluorometric AOAC method allows measuring TAA, but it needs a final step of derivatization with o-phenylendiamine to produce a fluorophor (AOAC 1995).

In previous literature, AA and DHA are determined by HPLC utilizing a wide range of chromatographic conditions. When employing conventional reverse phase HPLC, mobile phase pH ranges from 2.1 to 7.8, and UV detection covers from 210 to 278 nm (Iwasa 2000; Odriozola-Serrano et al. 2007; Gibbons et al. 2001). As reagents to reduce DHA to AA, DL-1,4-dithiothreitol or 2,3-dimercapto-1-propanol are commonly used (Odriozola-Serrano et al. 2007; Sánchez-Mata et al. 2000; Cordenunsi et al. 2005; da Silva Pinto et al. 2008). This operation can recuperate any loss caused by oxidation during procedure and allows the determination of

dehydroascorbic acid content as the difference between total ascorbic acid content after reduction and ascorbic acid content.

Regarding the determination of ascorbic and dehydroascorbic acid in fruits, Odriozola-Serrano et al. (2007) and da Silva Pinto et al. (2008) employ lower salt concentration mobile phases which is advisable to avoid problems in HPLC systems but a pH around 2.5 (Odriozola-Serrano et al. 2007; da Silva Pinto et al. 2008). Since this pH value is close to the limit value for the stability of silica columns (which will be used in the present work), it would be necessary to develop a method with a lower salt concentration at an appropriate pH value in order to maintain column stability. Acidic conditions are emphasized in the AA sample preparation extraction; therefore, a compromise solution between vitamin C determination and the HPLC column stability would be necessary in the election of the mobile phase pH.

HPLC methodology must be validated according to well-recognized analytical parameters established by official compendiums. Linearity, limits of detection and quantification, accuracy, precision, and robustness must be experimentally obtained (Maia et al. 2007). However, the analytical procedures for obtaining these statistical parameters are not very common in analytical food method development.

The aim of this work was to optimize and validate, using experimental designs, a new analytic strategy for the determination of vitamin C in strawberries by UV–HPLC.

Experimental

Apparatus and Software

Liquid chromatography (LC)/ultraviolet (UV) analysis was performed on a Konik KNK-500-A series liquid chromatograph, coupled to a variable wavelength detector (UVIS 200 Konik Instruments, Barcelona, Spain). Samples were injected into the chromatographic system through a manual injector equipped with a 20-μL loop. Design Expert v.7 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for the optimization of the chromatographic conditions (data analysis and desirability function determination). The statistical analysis of the validation data was performed with Statgraphics Centurion XV 15.2.06 (Statpoint Technologies, Inc., Warrenton, VA, USA) and Matlab 7.6.0.324 (Math Works, Natick, MA, USA).

Reagents

Sodium acetate trihydrate, potassium phosphate dibasic pro-analysis, glacial acetic acid, and L-(+) ascorbic acid were acquired from Laboratorios Cicarelli (Reagents S.A., San Lorenzo, Santa Fe, Argentina); DL-dithiothreitol (DTT) from Sigma-Aldrich, Inc., (St. Louis, MO, USA); and

metaphosphoric acid from Merck KGaA (Damnstadt, Germany). Distilled water was purified using an Easy Pure II RF System (Barnstead International, IA, USA), and this purified water was used for preparing all the solutions.

HPLC Conditions

Separations were achieved in a reversed phase column Phenomenex Gemini 5 μ C18 110A attached to a Phenomenex guardcolumn (Phenomenex Inc., CA, USA) at room temperature (25 °C). The mobile phase, under isocratic conditions, consisted of a 0.03 M sodium acetate/acetic acid buffer, 5 % methanol. Mobile phase pH was adjusted according to the design optimization conditions at 3.8, 4.8, and 5.8 as presented in Table 1. Mobile phases were filtered through a 0.45-μm nylon membrane filter and degassed under vacuum.

Stock Standard Solution

A stock standard solution of ascorbic acid (4.0 gL^{-1}) was prepared in an extracting solution of metaphosphoric acid (30 gL^{-1}) and acetic acid (80 gL^{-1}), according to AOAC (1995). For doing this solution, 0.1 g of ascorbic acid powder, oven-dried for 1 h at 105 °C, was dissolved in 25 mL of the extracting solution, protected from light and stored at 4 °C until use.

Strawberry Sample Preparation

Fully ripe strawberries (*Fragaria ananassa* Duch., cv. Festival) were obtained from the market in Santa Fe, Argentina. Fruits were washed with tap water, dried on absorbent paper, and after that calyxes and peduncles were separated. Then, a representative sample (approximately 500 g) was crushed, thoroughly homogenized, and stored at −80 °C until analysis. For the extraction, 5 g of homogenized strawberries were added to 25 mL of extracting solution. The mixture was homogenized for 1 min, sonicated in an ultrasonic bath for 15 min, and then centrifuged at 12.000×g for 20 min at 4 °C. The supernatant was separated, and 1 mL of it was diluted with mobile phase to achieve a final volume of 6 mL, then filtered through a 0.45-μm Millipore membrane and injected in the HPLC system for quantifying the content of ascorbic acid (AA) of the samples. For total ascorbic acid (TAA) quantification, 1 mL of supernatant was added with 0.2 mL of DTT solution (5 mg L^{-1} DTT prepared in 2.58 M potassium phosphate dibasic) (Odriozola-Serrano et al. 2007). The mixture was kept in the dark at room temperature for about 2 h. Then it was made-up to volume with mobile phase to achieve a final volume of 6 mL, filtered through a 0.45-μm Millipore membrane, and injected into the HPLC system. Samples were protected from light and were manipulated in an ice bath to avoid losses by oxidation. Dehydroascorbic acid (DHA) content was calculated as the difference between the total and ascorbic acid content (Sánchez-Mata et al. 2000).

Optimization Procedure

A Box–Behnken design using three factors at three levels (coded levels: −1, 0, +1) was used for optimization of ascorbic acid determination by HPLC. It consisted of 15 experiments (chromatographic runs) under different conditions (Table 1).

Table 1 Experimental data for AA and TAA areas, retention times, and peak widths in the design conditions

pH Mobile phase	Flow rate (ml min^{-1})	Wavelength (nm)	AA area	TAA area	AA retention time (min)	TAA retention time (min)	AA peak width (sec)	TAA peak width (sec)
4.8	0.85	255	507.5	593.1	3.5	3.5	29.3	30.8
4.8	0.85	255	573.4	547.0	3.6	3.5	25.4	31.1
4.8	0.85	255	569.3	544.1	3.5	3.5	26.5	34.0
4.8	0.50	270	441.5	395.9	6.0	5.9	34.6	42.1
4.8	0.50	240	640.9	634.7	6.0	5.9	37.6	45.6
5.8	0.50	255	1,098.4	1,132.9	5.4	5.4	39.0	40.9
5.8	1.20	255	457.4	451.7	2.3	2.2	17.8	19.4
3.8	1.20	255	140.8	139.6	3.1	3.1	13.5	16.9
3.8	0.50	255	345.4	355.1	7.4	7.4	28.5	37.4
4.8	1.20	270	176.8	155.6	2.5	2.5	16.5	17.5
3.8	0.85	240	340.4	344.0	4.4	4.4	21.9	25.4
5.8	0.85	270	306.3	328.4	3.2	3.2	18.9	22.0
4.8	1.20	240	276.9	272.8	2.5	2.4	17.9	20.6
3.8	0.85	270	62.1	56.5	4.4	4.4	13.8	17.0
5.8	0.85	240	426.4	437.5	3.2	3.2	20.9	23.5

Experimental runs were performed in random order

The factors involved were pH of the mobile phase (uncoded levels 3.8, 4.8, and 5.8); flow rate (uncoded levels 0.5, 0.85, and 1.2 mL min^{-1}); and wavelength (uncoded levels 240, 255, and 270 nm). The responses were chromatographic area, retention time, and peak width for ascorbic acid and total ascorbic acid. Ascorbic acid samples were a dilution of the stock standard solution (as it was described in the section "Stock Standard Solution") made-up with extracting solution to obtain a concentration of 0.08 gL^{-1}. Then, a dilution with mobile phase was performed, obtaining a final concentration of 0.016 gL^{-1}. To quantify the total ascorbic acid in samples, a reduction with a solution of DTT (5 mg L^{-1}) was performed. The reduction conditions were 1.2 mL DTT in 4 mL of 0.2 gL^{-1} ascorbic acid solution prepared since the stock standard solution; kept in the dark for 2 h, diluted with extracting solution to 0.08 gL^{-1}; and finally with mobile phase to achieve 0.016 gL^{-1}. These solutions were protected from light and were manipulated in an ice bath.

Validation Procedure

The optimized HPLC method was validated for the determination of ascorbic and total ascorbic acid in strawberries. Linearity, limits of detection and quantification, and calibration and analytical sensitivity were calculated through standard ascorbic acid solutions. Precision, accuracy, and robustness were assessed using strawberry samples. All the experiments were performed in the optimized conditions: mobile phase pH (0.03 M sodium acetate/acetic acid buffer, 5% methanol, pH=5.8); flow rate (1.15 mL min^{-1}), wavelength (251 nm), and room temperature (25 °C) under isocratic conditions.

Linearity and Other Figures of Merit

Adequate amounts of standard solutions of ascorbic and total ascorbic acid (0.08 gL^{-1}) prepared in triplicates as it was previously described, were diluted with mobile phase to obtain solutions with the following concentrations: 0.004, 0.008, 0.012, 0.016 and 0.020 gL^{-1} covering the range from 50.0% to 150.0% of the expected concentration of ascorbic acid in strawberry samples. Integrated peak areas were plotted against AA and TAA concentrations and were used for the linearity test, determination of detection and quantification limits, and calibration and analytical sensibility.

Precision

Six independent strawberry samples, prepared as described in the "Strawberry Sample Preparation," were analyzed through the full analytical method in order to know the repeatability or intra-day variation method.

Accuracy

Portions of 5 g of homogenized strawberries, processed in the same manner as indicated in the sample preparation, were spiked with appropriate volumes of the stock standard solution of ascorbic acid in order to achieve solutions of concentration of ascorbic and total ascorbic acid which were analogous to those employed in linearity tests. The ascorbic acid amounts spiked were between 30% and 150% of the expected amount of analyte in strawberry samples (triplicates).

Robustness

Robustness was studied employing a 3^{4-2} fractionary factorial design in nine chromatographic runs (Bianchini et al. 2009; Massart et al. 1997). The factor levels of this design were fixed slightly up and down the optimized conditions previously determined. They were pH of the mobile phase, 5.7–5.9; absorption wavelength, 250–252 nm; flow rate, 1.10–1.20 mL min^{-1}, and the percentage of methanol in the mobile phase, 3–7%. The proposed response was the recovery percentage of AA and TAA after strawberry sample spiked at
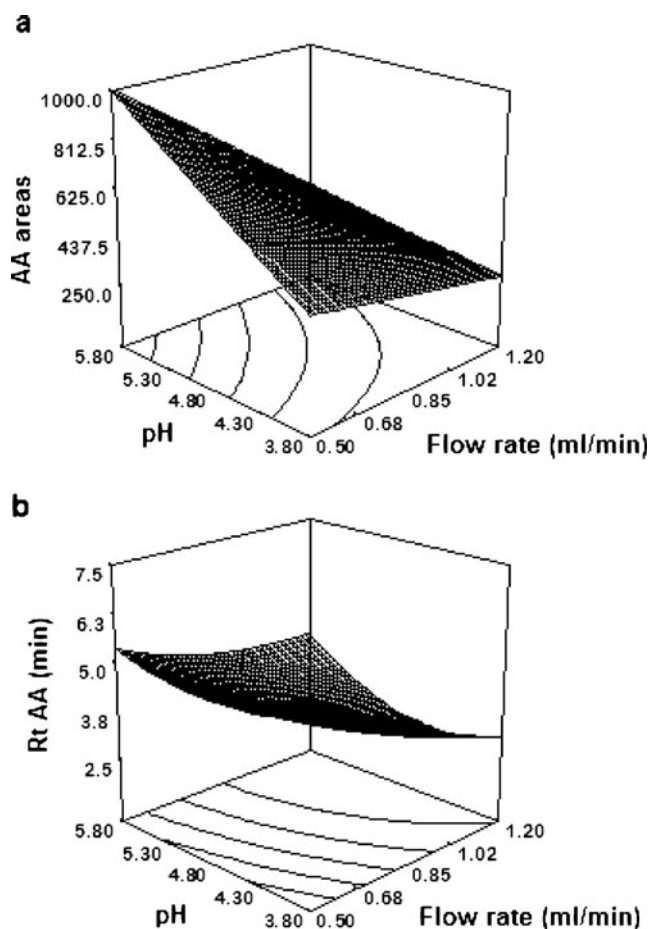

Fig. 1 Effect of pH mobile phase and flow rate **a** on the AA area and **b** on the AA retention time (Rt AA) at a wavelength=255 nm

90% of the AA expected amount. The sample processing was similar as indicated in the determination of accuracy.

Results and Discussion

Optimization of the HPLC Method for the Determination of Ascorbic and Total Ascorbic Acid Using Response Surface Methodology

Response Surface Methodology (RSM) uses second order polynomial models to describe the responses of the chromatographic variables in the experimental region. The first step in the application of the experimental design for optimization of chromatographic conditions is the selection of most critical factors and its appropriate ranges for the analytical technique and equipment to be used. This was based in preliminary research in literature, in previous experiments, and in known instrumental limitations. Regarding the last topic, the HPLC system used in this study does not provide a diode-array detector; therefore, it was a good option to know the optimal wavelength to choose this variable as a design factor. Box–Behnken designs are a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs. All design points are located at the center of the edges of a hypercube and are all situated on the surface of a sphere (Ferreira et al. 2007). Furthermore, three replicates in the central point were included in order to estimate the experimental error.

The experimental data are shown in Table 1. It was observed that AA areas increased with the increase of the pH of the mobile phase and also with the decrease of the flow rate (Fig. 1a). Higher mobile phase pHs and flow rates produced lower retention times for AA (Fig. 1b), and an
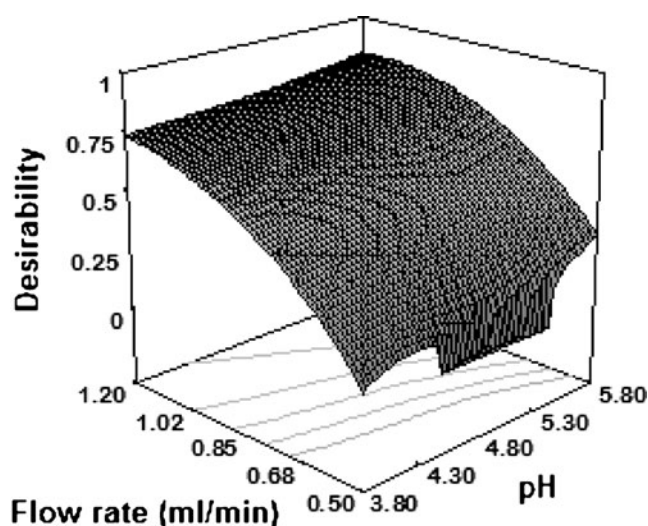

Fig. 2 Derringer's overall desirability obtained for simultaneous optimization (at a wavelength=251 nm) of all responses (AA and AAT areas, retention times, and peak widths)
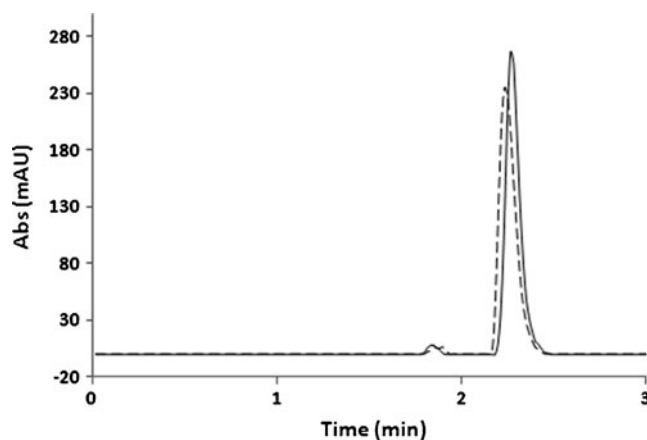

Fig. 3 Typical ascorbic acid (*dashed line*) and total ascorbic acid (*continuous line*) chromatogram in a pH mobile phase of 5.8 and 251-nm and 1.15-mL min^{-1} flow rate

increase in flow rate with decreased mobile phase pHs produced narrower peak widths (not shown). A similar behavior was observed for TAA (not shown). The wavelength factor was a significant factor in the AA and TAA areas and peak width responses. A maximum for the AA and TAA areas were observed in approximately 255 nm in the experimental region (figure not shown). However, when a more exhaustive analysis of the corresponding RSM models obtained for each response was performed, factors (such as mobile phase pH and flow rate) affected the responses differently. This situation emphasizes the need to perform active multifactor optimization of the chromatographic separation. Therefore, Derringer's desirability function (overall desirability) was used for optimization of AA and TAA responses: areas, retention times, and peak widths. The overall desirability was reached maximizing the areas and minimizing retention times and peak widths. The closeness of the overall desirability to the unity indicates the degree of matching of the combined criteria to the global optimum (Ferreira et al. 2007).

Initially, Derringer's desirability was calculated separately for AA (overall desirability=0.83) and TAA (overall

Table 2 Statistical analysis for linearity of the calibration curves of AA and TAA

Item	Results	
	AA	TAA
F test for homoscedasticity evaluation	1.50 (19)[a]	2.51 (19)[b]
F test for linearity evaluation	1.00 (2.89)[c]	2.66 (2.89)[d]
Calibration sensibility	34.9	35.4
Calibration line	$y=34.9x-46.8$	$y=35.4x-45.2$
R^2	0.996	0.998

The values in parentheses correspond to critical values at $\alpha=0.05$ and the degrees of freedom: [a=b] ($\nu_1=2$, $\nu_2=2$); [c=d] ($\nu_1=13$, $\nu_2=10$)

Table 3 Precision (repeatability or intra-day variation) of the method with strawberries samples ($n=6$)

Analyte	Concentration (mg 100 g^{-1})[a]	R.S.D. (%)
AA	40.0±0.6	1.5
DHA[b]	12.3±0.9	4.9
TAA	51.3±0.6	1.8

[a] Mean value ± standard deviation

[b] Quantified indirectly by TAA and AA difference

desirability=0.86). These optimization procedures resulted in wavelengths slightly different for AA and TAA analysis. A wavelength of 253 nm was determined to be the optimum for the analysis of AA, while 249 nm was the recommended one for TAA. The predicted optimal mobile phase pH and flow rate for AA and TAA showed to be the same (mobile phase pH=5.8 and 1.15 mL min^{-1}).

The wavelength difference can be explained as a small change produced in the pH sample after the DTT addition. Ascorbic acid is a compound which absorbs in the ultraviolet spectrum in the range 244–265 nm, and its absorption depends on the pH of the solution (Nováková et al. 2008; Tannenbaum 1982).

Finally, the desirability for the six responses (AA and TAA areas, retention times, and peak widths) was determined simultaneously, and it was attained a maximum overall desirability of 0.84 when mobile phase pH was equal to 5.8; flow rate 1.15 mL min^{-1}, and wavelength 251 nm (Fig. 2).

In Fig. 3, a typical chromatogram in the optimal conditions is shown; in this situation, a retention time of 2.4 minutes was observed for ascorbic acid with or without DTT reduction (AA and TAA analysis, respectively).

Validation of the HPLC Method for the Determination of Ascorbic and Total Ascorbic Acid in Strawberries

Linearity

Linearity was evaluated for AA and TAA procedure using standard solutions prepared as described in the section "Linearity and Other Figures of Merit." The data

homoscedasticity and linearity were analyzed through an F test, according to Danzer and Currie (1998). The difference between the observed and critical value of F was not significant for both ascorbic acid calibration lines (Table 2). The peak areas (y) were plotted against ascorbic acid concentrations (x), and a least-squares analysis was performed. The coefficients of the linear model for AA and TAA are shown in the Table 2. Linearity tests have not exceeded the tabulated values (Table 2), therefore the linearity relationship between the areas (dependent variable), and analytes concentration (independent variable) can be assumed for AA and TAA procedure, within the tested concentration range. Moreover, a normal distribution of the residual plots was observed (data not shown).

Detection and Quantification Limits

Limit of detection (LOD) can be defined as the minimum concentration reliably detectable for the method and the limit of quantification (LOQ) is the minimum concentration reliably measurable for it. The LOD and the LOQ were calculated by the equations (Olivieri and Goicoechea 2007):

$$LOD = 3.3\,S_0 \tag{1}$$

$$LOQ = 10\,S_0 \tag{2}$$

S_0 is the standard deviation of the predicted analyte concentration in a blank sample which depends on the noise level in the response data, slope of the calibration curve, and analyte concentration in the calibration samples (Olivieri and Goicoechea 2007; Boque et al. 2002; Muñoz de la Peña et al. 2003).

The magnitudes of both limits are closely linked to the sensitivity of the method. The values of LOD obtained for the ascorbic and total ascorbic acid were 1.2 10^{-3} and 8.8 10^{-4} g L^{-1}, respectively and the LOQ valued were 3.4 10^{-3} g L^{-1} for ascorbic acid and 2.5 10^{-3} g L^{-1} for total ascorbic acid. The fact that LOQ values determined in the present experiment were lower than all the values of ascorbic acid analyzed in strawberry samples is a clear evidence of the validity of the method.

Table 4 Recovery experimental results

	Added AA (mg 100 g^{-1})	Found AA[a] (mg 100 g^{-1})	Found TAA[a] (mg 100 g^{-1})	Recovery AA[a] (%)	Recovery TAA[a] (%)
	12	12.1±0.8	12.6±1.1	100.6±6.2	104.8±9.1
	24	23.0±1.4	24.0±0.9	95.9±5.7	100.0±3.5
AA ascorbic acid, TAA total ascorbic acid	36	34.2±0.9	35.0±1.8	95.1±2.4	97.3±4.9
	48	44.3±1.2	45.3±0.2	92.3±2.5	94.3±0.4
[a] Mean value ± standard deviation ($n=3$)	60	57.8±0.7	58.8±0.3	96.3±1.2	98.0±0.4

Fig. 4 EJCR test results for ascorbic acid (**a**) and total ascorbic acid (**b**), *ellipses* show ideal points (1, 0)
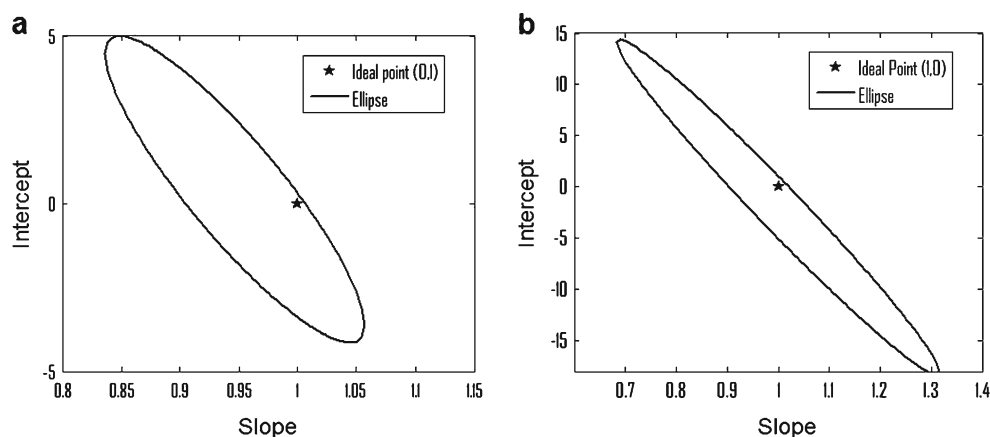
Calibration and Analytical Sensitivity

The calibration sensibility (SEN) is equal to the slope of the calibration line and indicates the variation of response produced by a unit change in analyte concentration. The slope of the total ascorbic acid calibration line was higher than the ascorbic acid one (Table 2); indeed it could be considered that the TAA detection is slightly more sensitive than the AA detection.

The analytical sensitivity (γ) (Eq. 3) is defined as the relation between the SEN and the instrumental noise (S_y); the last one can be estimated for the standard deviation of the residues of the linear regression (Muñoz de la Peña et al. 2003).

$$\gamma = \frac{\text{SEN}}{S_{y/x}} \qquad (3)$$

The γ parameter is best interpreted in terms of its inverse (γ^{-1}). The γ^{-1} values of ascorbic and total ascorbic acid were $4.1\,10^{-4}$ and $3.0\,10^{-4}$ g L^{-1}, respectively. As the inverse of the analytical sensitive indicates the lowest concentration difference that is noticeable throughout implementation of the method, these results indicate a better sensibility in the TAA determination.

Precision

The precision of the method was studied in terms of its repeatability or intra-day variation. For performing it, six independent strawberry samples were analyzed through the full analytical method. In Table 3, the AA, TAA, and DHA mean content of strawberries analyzed is shown; the repeatability was expressed as the relative standard deviation (R.S. D. (percent). These results are in agreement with the maximum acceptable value (R.S.D. (percent)=5.3) recommended by the Association of Official Analytical Chemists for analytes representing in the sample between 0.1% and 0.01% (AOAC 1998). Hence, a good repeatability was observed even for DHA; the latter result is in accordance

with Sánchez-Mata et al. (2000), who proposed the calculation of DHA in green beans by subtracting the initial AA content from the TAA after DTT conversion (Sánchez-Mata et al. 2000). The latter method is conventionally called the subtraction approach and from the point of view of increasing sensitivity and selectivity for DHA is better than UV directly detection (Sánchez-Mata et al. 2000). However, Odriozola-Serrano et al. (2007) did not acquire good results when DHA was thus calculated in strawberries, obtaining R. S.D. (percent) between 11.5% and 30.8% (Odriozola-Serrano et al. 2007). In relation with these results, we can conclude that a good sampling, homogenization, and maintaining AA stability in solution after extraction of the plant material are necessary for successful results. Ascorbic acid is stable when dry but very unstable in solutions. There are lots of factors that negatively affect their stability, including natural and UV light, increasing pH and temperature, and the presence of oxygen or metal ions (Nováková et al. 2008). Therefore, is necessary to carefully keep the influence of these variables to a minimum throughout the complete procedure in order to obtain good precision in ascorbic acid analysis.

Table 5 Experimental results of the design of method robustness

pH Mobile phase	Wavelength (nm)	Flow rate (ml min^{-1})	Organic modifier (methanol %)	TAA recovery (%)
5.7	250	1.10	3	96.0
5.7	251	1.15	5	94.4
5.7	252	1.20	7	90.1
5.8	250	1.15	7	91.2
5.8	251	1.20	3	97.1
5.8	252	1.10	5	100.4
5.9	250	1.20	5	90.1
5.9	251	1.10	7	96.9
5.8	251	1.15	5	98.0

Runs were performed in random order

Accuracy

Accuracy of the method was evaluated with AA standard-spiked strawberry samples prepared in triplicates in 30%, 60%, 90%, 120%, and 150% of the expected amount of analyte in real samples (approximately 40–60 mg AA per 100 g). Concentrations were estimated using calibration lines obtained for AA and TAA, and then the calculated concentrations were compared with the real spiked ones. Table 4 shows results including the recovery percentage for AA and TAA. Accuracy was studied through the joint confidence elliptical region elliptical joint confidence region (EJCR) test, which allows investigating whether the point (1, 0) is included in the joint confidence elliptical region of the slope and the intercept. In this situation, the intercept may be considered to be zero and the slope to be unity, which leads to the recovery, which can be considered as 100% (González et al. 1999). Figure 4a and b show the ellipses obtained for the AA and TAA recovery tests. As can be seen the optimal point is included in both, therefore a good accuracy have been reached with the proposed methodology.

In addition, the global recovery was calculated for the 15 determinations of ascorbic and total ascorbic acid, and the corresponding mean values were 98.1% and 98.6%. A Student's t test was applied, which allows concluding if significant difference exists between the recovery value obtained and the ideal 100%. Experimental t values (1.30 for AA and 0.88 for TAA) were compared with a tabulated $t_{(\nu = 14; \alpha = 0.05)} = 2.145$. These results allow concluding that good accuracy was attained in strawberry AA and TAA determination.

Robustness

The robustness of an analytical method indicates the reliability method degree after common and slight changes in the operative variables. The AA and TAA recovery values obtained were analyzed through a multifactorial ANOVA test, and the results indicate that the impact of the variations was within acceptable limits. AA recovery percentage was not influenced for none of the factor variations ($p>0.05$) (data not shown), but TAA was affected by the flow rate variation ($p<0.05$). The recovery results for TAA are presented in Table 5. The mean recovery was 95±4%, which clearly indicates the robustness of the present method.

Conclusions

A convenient and rapid UV–HPLC method for the quantification of AA, DHA, and TAA in strawberries was developed using an experimental design and was simultaneously optimized through Derringer's desirability function. Optimal conditions of the wavelength absorption, flow rate, and mobile phase pH were obtained. An exhaustive method validation was achieved, and it showed that the proposed method is linear in the studied concentration range, sensible, precise, accurate, and robust. The additional contribution of this work is to present a complete study in the optimization and validation of a strawberry analysis method providing more reliable and traceable results by the analytical laboratories.

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